Putative Rust Fungal Effector Proteins in Infected Bean and Soybean Leaves

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ABSTRACT

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The plant-pathogenic fungi *Uromyces appendiculatus* and *Phakopsora pachyrhizi* cause debilitating rust diseases on common bean and soybean. These rust fungi secrete effector proteins that allow them to infect plants, but their effector repertoires are not understood. The discovery of rust fungus effectors may eventually help guide decisions and actions that mitigate crop production loss. Therefore, we used mass spectrometry to identify thousands of proteins in infected beans and soybeans and in germinated fungal spores. The comparative analysis between the two helped differentiate a set of 24 *U. appendiculatus* proteins targeted for

secretion that were specifically found in infected beans and a set of 34 *U. appendiculatus* proteins targeted for secretion that were found in germinated spores and infected beans. The proteins specific to infected beans included family 26 and family 76 glycoside hydrolases that may contribute to degrading plant cell walls. There were also several types of proteins with structural motifs that may aid in stabilizing the specialized fungal haustorium cell that interfaces the plant cell membrane during infection. There were 16 *P. pachyrhizi* proteins targeted for secretion that were found in infected soybeans, and many of these proteins resembled the *U. appendiculatus* proteins found in infected beans, which implies that these proteins are important to rust fungal pathology in general. This data set provides insight to the biochemical mechanisms that rust fungi use to overcome plant immune systems and to parasitize cells.

Obligate biotrophic rust fungi evolve as quickly as their hosts. This feature, inherent to their fitness, is problematic to agriculture where time and time again rust fungi overcome plant disease resistance genes (Johnson 1961). Rust fungal evolutionary attributes include both sexual and asexual life cycles, alternative or secondary host habitats, and the production of a diverse set of molecules that subvert plant immune systems and tilt plant biochemistry to favor fungal survival. Although scientists have fought rusts at the forefronts of life cycle stages and alternate host ranges (Roelfs 1982), the notion of circumventing the biochemical processes by which rusts overcome plant immunity captures the imaginations of many contemporary scientists these days.

The basic model for how rust fungi infect plants is an amalgam of what is known for rusts and other biotrophic and hemibiotrophic basidiomycete, ascomycete, and oomycete plant pathogens. Common to all cases is the observation that the pathogens secrete proteins to attach to leaves, to modulate cellular exterior surfaces, and to disable, disrupt, or evade host surveillance systems. For example, spores of the broad bean rust *Uromyces viciae-fabae* and wheat stem rust *Puccinia graminis* secrete proteins to anchor the spores to the leaf cuticle (Deising et al. 1992; Nirmala et al. 2011). The tubes that emerge from germinated spores, known as germings, also secrete proteins as they migrate across a leaf surface (Epstein et al. 1987). Germings then form appressoria that generate pressure to allow the fungus to enter the host apoplastic space by going through the stomata or by penetrating the epidermis directly

(Edwards and Bonde 2011; Hoch et al. 1987). Either way, it is likely that proteins are secreted at this stage too, given evidence in *Ustilago maydis* (corn smut) and *Colletotricum higginsianum* (anthracnose) (Kleemann et al. 2012; Lanver et al. 2014). Once inside the leaf, rust fungi secrete proteins that allow them to breach the walls of the cells they infect (Rauscher et al. 1995). Afterward they secrete proteins through their haustoria that interface plant cells (Kemen et al. 2005). Haustoria are the conduits for nutrient acquisition (Voegelé et al. 2001), but haustoria also allow rust fungi and other haustoria-producing microbes to pass proteins into a host to interrupt the host's immune system (Garnica et al. 2014).

An effector is a pathogen protein that contributes to a pathogen's virulence, and effectors can be secreted by rusts at any time during infection. The mechanism by which effectors are secreted from rust fungi is not understood, but we have tried to glean clues from *Phytophthora* spp. that also use haustoria to establish biotrophy. Downstream of a canonic amino-terminal signal that targets a *Phytophthora* effector for secretion is an Arg-X-Leu-Arg (RXLR) amino acid motif that allows the protein to be taken into the host (Whisson et al. 2007). Sequence surveys for this motif have identified hundreds of potential effectors in related *Phytophthora* spp. genomes (Jiang et al. 2008). The RXLR motif, however, has not been observed as readily in rust fungal proteins, and no other consensus motif has been identified that so easily distinguishes rust effectors or allows their entry into the host (Link et al. 2014; Puthoff et al. 2008; Rafiqi et al. 2010).

Few rust effectors have been characterized. Avirulence (Avr) proteins are a subclass of effectors defined by their elicitation of cognate resistance (R) proteins in plants (Flor 1971), and this straightforward definition has been used to describe a few flax rust secreted proteins as Avr effectors (Barrett et al. 2009; Catanzariti et al. 2006; Dodds et al. 2004). By contrast, the non-Avr effectors that by definition do not elicit R proteins are more difficult to identify. So far, evidence for gene expression in haustoria and protein transfer into plant cells are primary characteristics of non-Avr effector candidates. RTP from *U. fabae* was the first non-Avr

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rust fungal effector found to cross the haustorium into a plant cell (Kemen et al. 2005). RTP may be a cysteine protease inhibitor or may provide structural support for the haustorium (Kemen et al. 2013; Pretsch et al. 2013). The comparison of two rust fungal genomes revealed the prevalence of small, cysteine-rich, secretion signal-bearing proteins of unknown function which could comprise a set of non-Avr effectors (Duplessis et al. 2011). Four of these candidates from *Melampsora larici-populina* were shown to be expressed in infected plants and associate with infection hyphae and haustoria (Hacquard et al. 2012). Nonetheless, at least eight other *M. larici-populina* haustorium-expressed proteins without cysteine-rich regions have been found to exhibit subcellular localization and protein-protein interactions in cells of the nonhost *Nicotiana benthamiana* (Petre et al. 2015). Hence, in vivo evidence of RNA and protein accumulation, not sequence feature inference, is driving effector discovery for rusts (Sperschneider et al. 2015).

The hunt to characterize effector proteins remains vigorous for rust fungi like *U. appendiculatus*, which infects beans, and *Phakopsora pachyrhizi*, which infects beans, soybeans, and kudzu. These rusts reduce the productivity of a staple food for indigent peoples and raise the costs of feed and oil sources for industrialized nations (Araya et al. 2004) (Kelly et al. 2015). Over the last 10 years, we have used mass spectrometry to identify proteins from beans and soybeans and from their respective rust fungi with an overall goal of mitigating production losses from rust diseases. Our research has led to the discovery of common bean and soybean proteins associated with resistance responses to *U. appendiculatus* and *P. pachyrhizi* infections (Cooper et al. 2011, 2013; Lee et al. 2009). We also have characterized the protein composition of *U. appendiculatus* uredospores and the changes in protein accumulation during uredospore germination (Cooper et al. 2006, 2007). To perform this research, we generated organism-specific cDNA sequence information that allowed us to interpret the tandem mass spectra (Link et al. 2014; Puthoff et al. 2008; Thibivilliers et al. 2009), but we also mined these sequences to identify candidate rust fungal effectors with signals targeting them for secretion (Link et al. 2014). During that time the genome sequences for soybean and common bean were resolved (Schmutz et al. 2010, 2014). Thus, we now can perform mass spectrometry on infected leaves, differentiate between plant and rust fungus peptide tandem mass spectra, and validate the existence of candidate effectors. So far, we have identified 58 *U. appendiculatus* candidate effectors from infected common bean leaves and 16 *P. pachyrhizi* candidate effectors from infected soybean leaves. These findings extend our knowledge of rust fungal proteins expressed in plants during infection.

MATERIALS AND METHODS

Inoculations. Two primary leaves of 10-day-old *P. vulgaris* ‘Black Valentine’ plants were sprayed with a liquid suspension of uredospores of *U. appendiculatus* race 41 (Cooper et al. 2007; Lee et al. 2009). The plants were placed in an 18°C dew chamber for 24 h, and then moved to a 23°C growth chamber with fluorescent lighting. Inoculated leaves were harvested 6 days later, prior to the appearance of symptoms. One inoculated plant was kept as an indicator to ensure successful infection, which was measured as two to four pustules per leaf surface (square centimeter). Experiments in which indicator plants did not develop symptoms at 10 days after inoculation (DAI) or did not display desired infection density were abandoned. Three biological replicate experiments were performed at the USDA-ARS Soybean Genomics and Improvement Laboratory in Beltsville, MD.

The first trifoliate leaves on 3-week-old *Glycine max* ‘Williams 82’ were sprayed with a water suspension of uredospores of *P. pachyrhizi* clonal isolate Louisiana 4-1 (Cooper et al. 2011). The plants were placed in an 18°C dew chamber for 24 h, and the inoculated leaves were harvested 7 days later. Inocula were

adjusted to produce the highest possible density of pustules per trifoliate surface area. One inoculated plant was kept as an indicator to ensure successful infection. Experiments in which the indicator plants did not develop symptoms by 14 DAI or did not display desired infection density were abandoned. Three complete biological replicate experiments and subsequent protein preparations were performed at the USDA-ARS Foreign Disease-Weed Science Research Unit Biological Safety Level-3 Plant Pathogen Containment Facility at Ft. Detrick, MD, under the appropriate USDA-APHIS permit (Melching et al. 1983).

Protein preparation. Infected leaves were used to create soluble protein extracts (S30), extracts from crude membranes and organelles (P30), and extracts from cell walls (CW) (Lee and Cooper 2006; Lee et al. 2009). Leaves (4 g) were ground in liquid nitrogen and homogenized in a buffer (100 mM Tris-HCl, pH 8.5, 5 mM dithiothreitol [DTT], 1 mM ethylenediaminetetraacetic acid [EDTA], 1% plant proteinase inhibition mixture [Sigma-Aldrich, St. Louis, MO], and 1% dodecyl-beta-maltoside [DBM]) with a glass grinder. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was centrifuged at 30,000 × *g* for 1 h producing the P30 pellet and S30 supernatant. The S30 supernatant was filtered through a 0.45-μm low protein retention polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MS), and the protein was precipitated in 25% trichloroacetic acid (TCA) and acetone, washed in acetone and resolubilized in 8 M urea/100 mM Tris-HCl, pH 8.5. The P30 pellet was resuspended in homogenization buffer and triturated with a glass grinder. The mixture was incubated for 30 min with orbital shaking at 1,000 rpm and centrifuged at 1,400 × *g* for 15 min. The supernatant was retained and filtered through a 5 μm PVDF membrane. The protein was TCA/acetone precipitated and resuspended in 8 M urea/Tris-HCl, pH 8.5, plus 0.5% DBM followed by sonication in a water bath for 20 min. The CW pellet was created after grinding leaves in liquid nitrogen and buffer (8 M urea, 100 mM Tris-HCl, pH 8.5, 5 mM DTT, 1 mM EDTA, and 2% DBM) and then with a glass grinder. The mixture was centrifuged at 1,000 × *g* for 10 min and the pellet was washed four times in 100 mM Tris-HCl, pH 8.5, 5 mM DTT, and 1 mM EDTA. CW proteins were extracted from the pellet (in 8 M urea, 100 mM Tris-HCl, pH 8.5, 5 mM DTT, 1 mM EDTA, and 2% DBM) by incubating the mixture for 20 min at room temperature, vortexing three times with 3 min intervals and then centrifuging at 1,400 × *g* for 15 min at room temperature. The supernatant was retained and filtered through a 5 μm PVDF membrane.

Asexual uredospores (0.7 g) for *U. appendiculatus* race 41 were germinated in 4 ml of water in the presence of beta-ionone and Tween 20 in a glass dish in an 18°C dew chamber for 24 h (Cooper et al. 2007). The spores were examined by microscopy and collected if more than 50% had germinated. The germinated spores and the germination medium were brought to a final concentration of 100 mM Tris-HCl, pH 8.5, 5 mM DTT, 1 mM EDTA, and 1% proteinase inhibitor and were transferred to 2 ml culture tubes containing 2.8 μm ceramic beads. The contents were mixed in an Omni Bead Ruptor 24 (Omni International, Kennesaw, GA) for 75 s followed by three consecutive series of vortexing and ice incubation. The contents were centrifuged at 1,000 × *g* for 10 min and filtered through a 5 μm PVDF membrane, and the proteins were precipitated from the supernatant using TCA/acetone. Proteins were resolubilized in 8 M urea/100 mM Tris-HCl, pH 8.5.

Protein concentrations were estimated by bicinchoninic assay (Pierce, Rockford, IL). Protein (500 μg) from each fraction was reduced in tris(2-carboxyethyl)phosphine, carboxyamidomethylated with iodoacetamide, and digested with trypsin. The digested samples were desalted using solid phase extraction with SPEC-PLUS PT C18 columns (Varian, Lake Forest, CA).

Mass spectrometry. The peptide fractions were analyzed by biphasic liquid chromatography–tandem mass spectrometry, also known as MudPIT (Washburn et al. 2001; Wolters et al. 2001). The peptides were loaded off-line and then separated on-line on

homemade biphasic columns prepared from 365 μm (outer diameter) $\times$ 75 μm (inner diameter) fused silica with a 5- μm tip and packed with 9 cm of reverse phase C18 resin (Aqua, 5 μm , Phenomenex, Torrance, CA) followed by 4 cm of strong cation exchange resin (Luna, 5 μm , Phenomenex). A 12-step elution procedure consisting of stepwise increasing concentrations of salt solution followed by increasing gradients of organic mobile phase was performed (Florens and Washburn 2006). Solvent flow was 200 nL/min and was controlled with an Accela HPLC pump (Thermo Fisher Scientific, Waltham, MA) and a T-split junction where 2,100 V was applied (Florens and Washburn 2006). The eluent was electrosprayed directly into the orifice of an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) controlled by Xcalibur 2.0.7 software (Thermo Fisher Scientific). A parent-ion scan was performed in the Orbitrap over the range of 400 to 1,600 m/z at 30,000 resolution with 500,000 automatic gain control (AGC), 500 msec ion injection time, and 1 microscan. Lock-mass was enabled (Olsen et al. 2005). Data-dependent MS² was performed in the linear ion trap with 10,000 AGC and 100 msec ion injection times with 1 μscan . MS² was performed on the 10 most intense MS ions, with minimum signals of 1,000. MS² spectra were obtained using an isolation width of 2 m/z and normalized collision energy of 35%. Dynamic exclusion was used with repeat count of 2, 30 s repeat duration, a list of 500, list duration of 2 min, and exclusion mass width of ± 0.7 Da.

Peptide identification. MS² spectrum data files were extracted from the raw data with Bioworks 3.3.1 (Thermo Fisher Scientific) using the following parameters: 600 to 4,500 mass range, 0 group scan, 1 minimum group count, and 5 minimum ion counts. MS² spectra were searched with Mascot 2.5.1 (Perkins et al. 1999). Search parameters were for tryptic digests with one possible missed cleavage, fixed amino acid modification [+57, C], variable amino acid modification [+16, M], monoisotopic mass values, ± 10 ppm parent ion mass tolerance, ± 0.8 Da fragment ion mass tolerance and #¹³C=1 enabled. The searched databases included version 1.0 protein models of *P. vulgaris* (31,638 sequences; <https://phytozome.jgi.doe.gov/>) plus alignment-oriented (Cooper et al. 2007) translated protein sequences derived from contiguous, overlapping expressed sequence tags from *U. appendiculatus* (11,844 sequences; [Link et al. 2014]), and version 6.0 protein models for *G. max* ‘Williams 82’ (55,787 sequences; <https://phytozome.jgi.doe.gov/>) plus alignment-oriented translated protein sequences derived from contiguous, overlapping expressed sequence tags from *P. pachyrhizi* (10,393 records; [Link et al. 2014]).

Mascot output files were processed by PANORAMICS², a probability-based program that determines the likelihood that peptides are correctly assigned to proteins (Cooper et al. 2010; Feng et al. 2007). PANORAMICS² considered peptide matches made by Mascot and for each calculated the probability that a peptide generated an observed spectrum. Analysis was limited to peptides having Mascot Ions score-Identity score differences not less than negative 5. PANORAMICS² then calculated a probability that a protein was in a sample with respect to the selected database and the observed spectra. The probability that a protein was not identified by the peptide matches (false-positive rate) is 1 minus the calculated protein probability. Parsimony was achieved by grouping via protein probability calculations. For any protein group associated with multiple protein records, we chose the first record to represent the group, but retained the association to the other records. Peptide sequences reported here had Mascot expect values less than 0.05 (Supplementary Table S1).

RESULTS

Our objective was to identify *U. appendiculatus* candidate effectors in infected plants. Although proteins are secreted by rust fungi during numerous stages of infection, proteins secreted from haustoria are prime candidates for contributing to fungal virulence

and biotrophy. There were 715 *U. appendiculatus* candidate secreted proteins derived from cDNAs sequenced from purified haustoria that were our targets (Link et al. 2014; Puthoff et al. 2008).

We examined *U. appendiculatus* race 41 on *P. vulgaris* ‘Black Valentine’. We investigated a susceptible interaction rather than an avirulent/resistant interaction because we discovered that hypersensitive cell death and resistance severely limited the amount detectable fungal protein from avirulent strains (Lee et al. 2009). Therefore, the need to have detectable amounts of fungal protein drove our experimental decisions. At the same time, we also wanted to find haustorium proteins. Haustoria are produced within the first 24 h after inoculation, but it is difficult to find fungal proteins among plant proteins at that time (Lee et al. 2009). Hence, we sampled common bean leaves at 6 DAI knowing that we had missed the earliest steps of infection, but we still hoped to detect some important haustorial proteins secreted to maintain biotrophy.

Infected plant leaves were fractionated by differential centrifugation into crude cell wall (CW), crude organelle and membrane (P30), and soluble components (S30) to increase the numbers and types of detectable proteins. Mass spectrometry analysis of these fractions yielded tens of thousands of tandem mass spectra matching peptides encoded by haustorial cDNAs and the genome of common bean (Link et al. 2014; Schmutz et al. 2014). We made no attempt to identify peptides using genome information from other rusts because sequence orthology has been insufficient for identifying *U. appendiculatus* effectors (Link et al. 2014). Peptide-spectrum matches were used in a probability model to identify proteins (Feng et al. 2007). Some peptides mapped to both a plant protein and a fungal protein, but we ignored such ambiguous identifications and instead focused on the distinct matches to rust fungal proteins (>95% confidence).

We detected about 700 *U. appendiculatus* proteins among 2,500 total proteins in each P30 fraction, about 500 *U. appendiculatus* proteins among 1,800 total proteins in each S30 fraction, and about 200 *U. appendiculatus* proteins among 900 total proteins in each CW fraction. Thus, it appeared that about a quarter of all proteins we identified from infected beans were *U. appendiculatus* proteins. To better understand this proportion, we looked at the numbers of tandem mass spectra assigned to the proteins because the number of spectral observations for a protein (i.e., its summed spectral count) can correlate linearly with the amount of protein in a sample (Cooper et al. 2010). One bean protein that we expected to find in the S30 fraction was the cytosolic glutamine-dependent asparagine synthase 1 (Phvul.008G259900.1), and it had a spectral count of 126. Based on this marker, we defined highly abundant proteins as those having summed spectral counts greater than 100. By contrast, the *U. appendiculatus* asparagine synthase (Ua_contig04761) had a spectral count of 3, which means that this protein was at the lower end of detectability. But not all rust fungal proteins had low abundance. *U. appendiculatus* elongation factor 1- α (Ua_contig00283), one of the higher accumulating proteins in germlings (Cooper et al. 2007), had a summed spectral count of 125 in infected plants. Nevertheless, that was about 40 times less than the normalized spectral count for the highly abundant bean ribulose biphosphate carboxylase small chain 1A. Similar observations were seen in the P30 fractions enriched for organelles where the bean photosystem I light harvesting complex gene 3 (Phvul.008G289700.1) had a summed spectral count of 166, and the *U. appendiculatus* NADH-ubiquinone oxidoreductase (Ua_contig07133) had a summed spectral count of 25. These examples are provided to generally demonstrate the range of abundances for the plant and fungal proteins. We concluded that it was more difficult to detect fungal proteins compared with plant proteins, and there may have been many fungal proteins below our limits of detection. Nevertheless, we assigned significance to the fungal proteins that were detectable above a plant protein background with the thought that there may be an association between detectability, abundance, and pathology.

As mentioned earlier, there are multiple opportunities during the course of infection for rust fungi to secrete proteins. Therefore, we separately examined proteins isolated from purified, germinated race 41 spores and their germination water to help differentiate between *U. appendiculatus* proteins secreted from germplings prior to infection and proteins secreted during plant infection. The average number of germling proteins found was 1,300, and about 90 proteins overall matched the candidate effector list for *U. appendiculatus*. Consequently, we were able to distinguish two categories of secreted bean rust proteins: (i) those found more than once in infected beans, but not in any of five germling analyses, and (ii) those found more than once in infected beans and at least once in five germling analyses. We assembled separate sets of data to address the hypotheses that germplings secrete some of the same effectors that haustoria do but that haustoria secrete unique effectors too.

Overall, 58 *U. appendiculatus* proteins found in infected plants matched the list of candidate secreted proteins (Tables 1 and 2, Supplementary List S1). Twenty-four of these *U. appendiculatus* proteins were not found in germplings, which implies that they were unique to plant infections and may therefore be haustorial specific

(Table 1). All but one could be matched to orthologs found in other rusts, but half of these matched proteins with no discernible function, and they had some of the weakest identities to other rust fungi homologs. This suggests that these proteins of unknown function are undergoing diversification particular to *U. appendiculatus*, and such evidence is thought to support the hypothesis that these proteins uniquely allow rusts to infect their adapted hosts (Catanzariti et al. 2006; Hacquard et al. 2012). Meanwhile, the more recognizable proteins included two RTP homologs and a PIG15p homolog. These are positive findings for this experiment since RTP is transferred from the haustorium into the plant cell, and PIG15p, which is partly similar to trehalose 6-phosphate synthase (Tps), localizes to the extrahaustorial matrix (Kemen et al. 2005). In addition to these proteins, we found two family 76 and two family 26 glycoside hydrolases. One of each was found in the S30 fractions whereas the others were found in the P30 fractions. This result suggests that these proteins may have different subcellular activities during infection. The three most abundant proteins overall were a chitinase, the PIG15p homolog, and a protein of unknown function (Ua_contig15794). At the other end of the spectrum, INF56, the gene of which is expressed after germling differentiation (Xuei et al. 1992), a pectinesterase family protein,

TABLE 1. *Uromyces appendiculatus* proteins targeted for secretion and found among three infected leaf analyses (CW, P30, and S30 leaf fractions) but not in five germling analyses (germ reps)^a

Rust protein	Top BLAST hit	Identity to top hit	Protein description	Germ reps	CW spCT	P30 spCT	S30 spCT
Ua_contig00017	PSTG_05290 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	68%	Fungal phospholipase B-like	0	0,0,0	0,0,0	2,1,0
Ua_contig07374	PGTG_06328 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	42%	Unknown	0	0,0,0	0,0,0	8,2,0
Ua_contig01899	PGTG_14319 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	33%	Unknown	0	0,0,0	0,0,0	7,3,8
Ua_contig05026	VP01_655g11 [<i>Puccinia sorghi</i>]	58%	Family 26 glycoside hydrolase	0	0,0,0	0,0,0	6,17,2
Ua_HYP_05C_F_D01	PGTG_03566 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	66%	Family 76 glycoside hydrolase	0	0,0,0	0,0,0	10,10,8
Ua_contig01412	PGTG_03545 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	69%	Family 76 glycoside hydrolase	0	0,0,0	0,1,1	0,0,0
Ua_contig14096	PGTG_06491 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	33%	Unknown	0	0,0,0	5,2,0	0,0,0
Ua_contig06302	PSTG_01861 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	50%	Similar to rust transferred protein	0	0,0,0	0,5,5	0,0,0
Ua_contig01814	PGTG_14685 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	61%	Family 26 glycoside hydrolase	0	0,0,0	3,1,1	0,0,0
Ua_contig07178	PSTG_03323 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	53%	Unknown	0	0,0,0	0,5,0	1,0,0
Ua_contig15794	PSTG_04761 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	43%	Unknown	0	0,0,0	7,1,0	17,30,10
UAHYP_10C_F_B12	VP01_3828g2 [<i>Puccinia sorghi</i>]	40%	Unknown	0	0,2,0	0,0,0	0,0,0
Ua_contig00490	Zinc metalloprotease [<i>Streptococcus pneumoniae</i>]	30%	Unknown	0	0,2,3	0,0,0	0,0,0
Ua_contig11323	PSTG_08597 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	41%	Unknown	0	5,0,0	0,0,0	0,0,0
Ua_contig08770	PSTG_08364 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	82%	Aspartic peptidase	0	7,0,0	0,0,0	0,0,0
Ua_contig08587	PGTG_04481 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	51%	Unknown	0	34,9,0	0,0,0	0,0,0
UAHYP_01A_F_A02	PGTG_00407 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	78%	Unknown	0	1,0,0	0,1,0	0,0,0
Ua_contig07280	AAA34218.1 [<i>Uromyces appendiculatus</i>]	99%	Inf56	0	4,5,0	1,1,0	0,0,0
Ua_HYP_19A_R_H01	PSTG_04357 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	58%	Unknown	0	0,0,3	0,1,2	0,0,0
Ua_contig00019	PSTG_08958 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	52%	Unknown	0	0,3,0	2,0,4	0,0,0
Ua_contig06414	rust transferred protein [<i>Uromyces viciae-fabae</i>]	79%	Similar to rust transferred protein	0	10,0,0	0,2,2	0,0,0
Ua_contig05237	PSTG_14246 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	59%	Pectinesterase family	0	0,0,1	0,0,0	0,4,0
Ua_contig14349	PIG15p [<i>Uromyces viciae-fabae</i>]	35%	PIG15p-like, trehalose phosphatase family	0	1,0,0	6,5,3	32,33,21
Ua_contig05732	PSTG_08478 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	63%	Chitinase	0	2,0,0	4,7,10	10,22,2

^a The summed spectral count (spCT) is shown for each replicate fraction.

and a phospholipase B-like protein had only a few spectral counts and were not as readily detectable.

While some rust proteins appeared to uniquely accumulate in infected beans, there were 34 proteins found in both infected beans

and *U. appendiculatus* germings (Table 2). All of these proteins appeared to have homologs in other rust fungi, but seven had similarity to proteins with no discernible functions. As for the more recognizable proteins, three had peptidyl-prolyl cis-trans isomerase

TABLE 2. *Uromyces appendiculatus* proteins targeted for secretion and found among three infected leaf analyses (CW, P30, and S30 leaf fractions) and in germling analyses (germ reps)^a

Rust protein	Top BLAST hit	Identity to top hit	Protein description	Germ reps	CW spCT	P30 spCT	S30 spCT
Ua_contig01249	MELLADRAFT_123912 [<i>Melampsora larici-populina</i> 98AG31]	71%	FKBP-type peptidyl-prolyl cis-trans isomerase domain	3	0,0,0	0,0,0	2,4,0
Ua_HYP_19C_R_A06	Peptide-methionine (S)-S-oxide reductase [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	78%	Methionine sulfide reductase	3	0,0,0	0,0,0	13,6,7
Ua_contig00898	PSTG_01024 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	59%	Thioredoxin-like superfamily	3	0,0,0	1,3,0	0,0,0
Ua_contig01830	PSTG_03259 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	75%	Serine carboxypeptidase	3	0,0,0	2,0,2	0,0,0
Ua_HYP_16D_F_B01	VP01_2109g4 [<i>Puccinia sorghi</i>]	60%	Unknown	2	0,0,0	0,2,3	0,0,0
Ua_contig06749	PGTG_07001 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	84%	Unknown	1	0,0,0	0,7,7	0,0,0
Ua_contig05181	PGTG_00638 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	64%	Unknown	3	0,0,0	1,3,0	0,0,0
Ua_contig04622	PSTG_14038 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	72%	FAD/NAD-binding domain	1	0,0,0	0,1,5	0,0,0
Ua_contig00583	PGTG_00454 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	98%	Prohibitin-2	2	0,0,0	3,7,6	0,0,0
Ua_contig02338	VP01_1064g7 [<i>Puccinia sorghi</i>]	77%	Mitochondrial import receptor	1	0,0,0	3,6,8	0,0,0
Ua_HYP_18B_F_C09	PGTG_13504 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	78%	Unknown	2	0,0,0	4,8,5	0,0,0
Ua_contig08273	PGTG_14417 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	84%	Copper radical oxidase	1	0,0,0	3,7,3	0,0,0
Ua_contig05556	PSTG_04309 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	77%	Aspartic peptidase	3	0,0,0	2,5,3	0,0,0
Ua_contig01553	Hypothetical protein [<i>Puccinia striiformis</i> f. sp. <i>tritici</i>]	67%	Beta-Ig-H3/fasciclin domains	3	0,0,0	0,0,1	6,6,0
Ua_contig00456	PSTG_01749 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	81%	Peptidyl-prolyl cis-trans isomerase B	3	0,0,0	0,4,1	8,5,3
Ua_contig01113	PSTG_08709 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	66%	Unknown	2	0,0,0	0,1,0	9,8,4
Ua_contig01056	PSTG_01766 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	38%	Unknown	3	0,0,0	1,1,0	6,6,2
Ua_contig03019	PSTG_00066 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	93%	Karyopherin Kap95 nuclear import	3	0,0,0	0,5,4	15,5,10
Ua_contig10381	PSTG_01910 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	81%	Heat shock 90 family	1	0,0,0	1,0,3	6,18,2
Ua_contig06217	VP01_2148g3 [<i>Puccinia sorghi</i>]	82%	HSP70 actin superfamily	3	0,0,0	0,9,2	0,3,0
Ua_contig07202	PSTG_11377 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	62%	Glutathione-S-transferase family	3	0,0,0	4,1,1	8,10,2
Ua_contig01391	PGTG_14692 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	81%	Secreted subtilisin-like serine protease	3	0,0,0	7,3,6	11,12,5
Ua_contig01382	PGTG_08470 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	29%	Unknown	1	2,0,0	0,0,0	0,0,0
UAHYP_08C_F_A08	PGTG_07000 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	78%	Phosphatidylglycerol/ phosphatidylinositol transfer protein	4	7,0,0	0,0,0	0,0,0
Ua_HYP_25C_F_D02	PGTG_11656 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	64%	Ser-Thr-rich glycosyl-phosphatidylinositol-anchored membrane family	1	6,0,0	0,4,0	0,0,0
Ua_contig08119	PSTG_14392 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	92%	Peptidyl-prolyl cis-trans isomerase	2	1,0,0	1,0,0	0,0,0
Ua_HYP_06C_F_A11	PSTG_06116 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	60%	Disulfide isomerase	1	3,0,0	1,0,6	0,0,0
Ua_contig00065	Cytochrome P450 monooxygenase [<i>Puccinia sorghi</i>]	75%	Cytochrome P450	1	0,4,0	6,5,4	0,0,0
Ua_contig00105	PGTG_17753 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	81%	Fumarate reductase	3	0,1,0	3,2,3	0,2,0
Ua_contig08573	PSTG_14661 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	54%	Fasciclin domain cell adhesion	3	12,4,0	2,2,2	7,14,0
Ua_contig04950	PSTG_09382 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	79%	Family 5 glycoside hydrolase/ mannosidase	3	5,0,0	10,0,2	2,4,0
Ua_contig00403	VP01_2177g2 [<i>Puccinia sorghi</i>]	51%	Mycelial surface antigen	1	0,11,0	4,4,4	13,7,2
Ua_contig06995	PGTG_05714 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	62%	Chitinase	3	25,9,0	15,18,20	24,29,9
Ua_contig05359	Glucose-regulated protein [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	95%	Glucose regulated 70-kda heat shock bip-like	3	10,39,14	29,58,46	52,62,10

^a The summed spectral count (spCT) is shown for each replicate fraction.

domains, two had fasciclin domains, and seven had domains for sulfur and other oxidation/reduction activities. In addition, there were several peptidases and heat shock proteins. Similar proteins have previously been found in germinated *U. appendiculatus* spores (Cooper et al. 2007), but their orthologs have also been found in barley powdery mildew haustoria (Godfrey et al. 2009). Hence, these proteins may originate from haustoria, or from infection hyphae often copurified with haustoria, or both (Puthoff et al. 2008). On the other hand, a chitinase (different than the chitinase specific to infected beans), and a glucose-regulated 70 kDa heat shock protein, the two most abundant candidate effectors found, seem more likely to originate from infection hyphae than haustoria because of their prevalence in germlings (Cooper et al. 2007). Nevertheless, these proteins may be secreted throughout the fungal life cycle. For example, it is possible that peptidyl-prolyl cis-trans isomerases and heat shock proteins found in both germlings and infected plants may need to be secreted to help proteins fold or regain their tertiary conformations, a requirement for proteins that are secreted from cells or transported through haustoria in denatured forms (Wang and Heitman 2005).

To better understand the relevance of these proteins as candidate effectors for bean rust infection, we performed a comparative analysis with virulent *P. pachyrhizi* isolate Louisiana 4-1 on susceptible *G. max* 'Williams 82'. Infected soybean leaves were sampled a day later than beans to follow a schedule favorable to experimentation within the containment facility at Ft. Detrick. There were 319 *P. pachyrhizi* candidate secreted proteins that were our targets (Link et al. 2014; Puthoff et al. 2008), and we discovered 16 proteins from this list (Table 3; Supplementary List S1). This is fewer than the number found for *U. appendiculatus*. Possible explanations for the difference could be a smaller biomass after infection relative to *U. appendiculatus*, one more day of *P. pachyrhizi* development, a smaller candidate list, or differential sample handling (in restrictive containment conditions by a technician other than the one who processed the beans). Nine of the proteins, however, were similar to *U. appendiculatus* proteins found in bean leaves. Fumarate reductase, disulfide isomerase, aspartic peptidase, and glucose regulated 70-kDa heat shock protein have been found in *P. pachyrhizi* germlings (Luster et al. 2010) and orthologs for these proteins were found in *U. appendiculatus* germlings, which suggests that these proteins may be secreted during multiple stages of the soybean rust fungal life cycle. The chitinase, family 26 glycoside hydrolase and unknown protein Pp_contig04760 resembled orthologs found in *U. appendiculatus*-infected bean plants

but not found in germlings. Therefore these proteins may be uniquely secreted during soybean rust infections. In summary, we have found that orthologous fungal proteins appear in plants infected by rusts of two different genera, *Uromyces* and *Phakopsora*. These findings support the hypothesis that these putative secreted proteins are generally important to rust fungal pathology and may therefore be effectors.

DISCUSSION

The identification of rust fungal effector proteins is one of the more daunting tasks in plant pathology, mainly because rust fungi cannot be cultured in vitro and are therefore not tractable to traditional genetics approaches to create mutant alleles. Nevertheless, there has been recent progress in rust fungal genetic transformation that exploits host resistance for selection (Lawrence et al. 2010). It is also possible to perform reverse genetics assays by expressing fungal genes in plants to induce gene silencing back to the rust fungus (Panwar et al. 2013a, b). Each method may lead to better ways to determine which genes encode effectors. But until these methods are refined and adapted for each rust fungus and plant system, the best way of determining whether a rust fungal protein is an effector is to monitor its biochemical activity in plants. One classical approach is to determine if a rust fungal protein is an Avr that elicits hypersensitive disease resistance (Catanzariti et al. 2006, 2010; Dodds et al. 2004, 2006; Nirmala et al. 2011). Not all effectors, however, are Avr proteins, so other techniques are needed to deduce their roles in pathogenicity and virulence. Methods include monitoring the passage of proteins from the fungus into the plant, finding their locations in a plant cell, and discovering their associations with plant proteins, be it in natural hosts or experimental surrogates. Such methods have been successfully used to identify effectors by a process of trial and error (Hacquard et al. 2012; Kemen et al. 2005; Petre et al. 2015; Rafiqi et al. 2010; Upadhyaya et al. 2014). We, however, have taken a different approach. By performing mass spectrometry on infected plant leaves, we have found 58 *U. appendiculatus* and 16 *P. pachyrhizi* candidate effector proteins. The cDNAs for these proteins encode signals targeting them for secretion, and the comparison between the candidate proteins found in germinated spores and those from infected plants suggests that a subset of the proteins may be specific to infected plants. It is therefore possible that a subclass of proteins defined here is secreted from differentiated fungal cells found in infected plants. Such cells may

TABLE 3. *Phakopsora pachyrhizi* proteins targeted for secretion and found among three infected leaf analyses (CW, P30, and S30 leaf fractions)^a

Rust protein	Top BLAST hit	Identity to top hit	Protein description	CW spCT	P30 spCT	S30 spCT
Pp_contig01910	Ua_contig05026 (Table 1)	43%	Family 26 glycoside hydrolase	0,0,0	0,0,0	0,5,0
Pp_contig00784	Ua_HYP_08C_F_A08 (Table 2)	65%	Phosphatidylglycerol/phosphatidylinositol transfer protein	0,1,0	0,0,0	0,0,0
PpGI_contig537	VP01_673g2 [<i>Puccinia sorghi</i>]	43%	Unknown	0,2,0	0,0,0	0,0,0
Pp_contig00105	VP01_578g8 [<i>Puccinia sorghi</i>]	58%	Mitofilin domain	0,2,0	0,0,0	0,0,0
Pp_contig01721	PSTG_04482 [<i>Puccinia striiformis</i> f. sp. <i>tritici</i> PST-78]	36%	Unknown/sbr82	0,3,0	0,0,0	0,0,0
Pp_contig00888	Ua_contig00105 (Table 2)	85%	Fumarate reductase	0,5,0	0,0,0	0,0,0
Pp_contig06366	PGTG_13236 [<i>Puccinia graminis</i> f. sp. <i>tritici</i> CRL 75-36-700-3]	54%	Unknown	1,2,0	0,0,0	0,0,0
Pp_contig00934	MELLADRAFT_72534 [<i>Melampsora larici-populina</i> 98AG31]	81%	Calnexin	0,1,0	0,0,1	0,0,0
Pp_contig00374	VP01_816g3 [<i>Puccinia sorghi</i>]	63%	Phosphatidylethanolamine-binding domain	0,3,0	3,0,0	0,0,0
Pp_contig04760	Ua_contig14096 (Table 1)	39%	Unknown	0,2,0	3,2,2	0,0,0
PpGI_contig2186	No match	0	Longest ORF 56-139, length 84, frame +3	4,6,2	2,0,2	0,0,0
Pp_contig00004	Ua_contig05359 (Table 2)	89%	Glucose regulated 70-kda heat shock bip-like	10,10,0	2,8,13	0,0,0
PpGI_contig2344	Ua_contig00898 (Table 2)	31%	Disulfide isomerase	5,7,0	0,0,0	2,0,0
Pp_contig01414	Ua_HYP_06C_F_A11 (Table 2)	61%	Disulfide isomerase	21,5,4	9,10,3	7,0,5
Pp_contig02895	Ua_contig05556 (Table 2)	67%	Aspartic peptidase/sbr15	17,0,7	0,6,7	7,16,12
Pp_contig09994	Ua_contig05732 (Table 1)	47%	Chitinase	15,1,12	1,0,6	0,11,6

^a The summed spectral count (spCT) is shown for each replicate fraction.

include appressoria, substomatal vesicles, infection hyphae, haustorial mother cells, and haustoria.

Mass spectrometry has been used before to identify rust fungal proteins in plants. Song et al. (2011) isolated *P. tritici* infection structures from wheat 6 DAI and detected proteins targeted for secretion. These proteins included Tps, chitinases, glycosidases, peptidyl-prolyl cis-trans isomerases, disulfide isomerases, and several proteins of unknown function (Song et al. 2011). Our results generally overlap, which suggests that similar proteins from the evolutionarily related bean rust and soybean rust fungi are complicit to rust fungal pathology. For example, rust fungi must interface their haustorial membranes with plant cell membranes to establish biotrophy. Chitinases may allow the fungus to breach its own cell wall and expose the fungal membrane, while the family 26 and 76 glycoside hydrolases encompassing endo-1,4-beta-mannosidases and endo-alpha-mannanases, respectively, may help degrade plant cell walls or may modify proteins and small molecules linked to sugars.

The proteins that we have discovered may provide other insights into the mechanisms of fungal pathogenicity and biotrophy. Haustorium adhesion to a plant cell membrane may be accomplished with family 76 proteins (Kitagaki et al. 2002) and fasciclin domain proteins, which are also important for pathogenicity in ascomycete *Magnaporthe oryzae* (Bergstralh et al. 2015; Liu et al. 2009). The RTP homologs may play similar roles in stabilizing haustorial membrane architectures (Kemen et al. 2013). The heat-shock proteins and peptidyl-prolyl cis-trans isomerase domain proteins may also help stabilize this interface or stabilize proteins that are transported across, while the proteins with oxidation/reduction activity may manage free radical generation that occurs during infection (Egan et al. 2007). Serine and aspartic peptidases may also regulate this interface or be translocated into the plant cell where they have specific activities that disrupt a plant's immune system (Zhu et al. 2004). The P30 membrane-localized prohibitin-2, a protein known to associate with mitochondria in animals, may regulate cell proliferation (Merkwirth and Langer 2009).

One of the more intriguing findings is the prevalence of the PIG15p homolog that has amino acid identity to Tps. Tps catalyzes the synthesis of trehalose, which can be converted into glucose, but Tps may have an alternate function in pathology not directly related to trehalose synthesis (Foster et al. 2003). In *M. oryzae*, Tps1 senses plant glucose-6-phosphate and activates glucose-6-phosphate dehydrogenase which converts NADP to NADPH, activating genetic switches to regulate separate pathways for carbon and nitrogen metabolism and virulence (Fernandez and Wilson 2011). In essence, the sensing of plant glucose-6-phosphate may signal to the fungus that it has breached the plant cell wall and that it is time for the fungus to deploy effectors and to assimilate plant nutrients (Badaruddin et al. 2013; Fernandez and Wilson 2012). It is possible that a similar process is happening in *U. appendiculatus*. Whether such a process is regulated by the PIG15p homolog remains to be determined. Doubt is cast because the PIG15p homolog is 75% smaller than Tps1, exhibits similarity to the carboxyl end of Tps1, and encodes a secretion signal whereas Tps does not. Thus, the role of this homolog in trehalose synthesis and glucose signaling is speculative, but its role in pathology appears to be important given its secretion signal and its greater prevalence for detection in infected leaves compared with germinated spores.

Although this study revealed many candidate effector proteins, there were several limitations. First, the candidate sequence databases that were used for peptide-spectrum matching did not represent the entire genomes for *U. appendiculatus* race 41 and *P. pachyrhizi* clonal isolate Louisiana 4-1. The data also comprised sequences from *U. appendiculatus* race SWBR1 and *P. pachyrhizi* isolates Taiwan 72-1 and Thai1 (Link et al. 2014; Posada-Buitrago and Frederick 2005; Puthoff et al. 2008). Hence, it is possible that not all of the effector genes specific to our isolates were among the lists we screened and that some of the true race 41 and isolate Louisiana 4-1 proteins are

somewhat different from the protein sequences we used for spectrum matching. Second, we do not know if the putative secreted proteins we detected were actually secreted into the host, although we do know that they were expressed to high enough levels for detection during a period in which haustoria had formed. The detection of the RTP homologs, however, suggested we were close to target. Third, haustoria are not the only fungal cells that secrete effectors (Nirmala et al. 2011), but we believe that separate analyses of proteins from fungal germings helped differentiate between proteins formed before and after haustorial formation and that some of the proteins we detected are reasonable candidates for being secreted from haustoria.

Several groups of scientists have sequenced cDNAs from fungal haustoria to identify effectors while others have bioinformatically mined genes to find candidates (Catanzariti et al. 2006; Duplessis et al. 2011; Godfrey et al. 2010; Hacquard et al. 2012; Hahn and Mendgen 1997; Hahn et al. 1997; Jakupovic et al. 2006; Link et al. 2014; Posada-Buitrago and Frederick 2005; Puthoff et al. 2008). These approaches share the common deficiency of not informing whether a protein is actually produced by its message during infection. With that in mind, one advantage of tandem mass spectrometry is that it provides direct evidence of protein translation. Even though we have not been able to search for all effectors for *U. appendiculatus* and *P. pachyrhizi* because their complete genome reference sequences are not yet known, tandem mass spectra are static representations of peptides found, and these spectra can be interrogated again when genomic blueprints are better resolved. Thus, we believe these tandem mass spectral data sets will remain useful for many more years to come. In the meantime, we will begin to investigate in greater detail some of the proteins we have found. It may be possible to test their functions in hosts and nonhosts by transient expression (Petre et al. 2015; Wang et al. 2006) or by host-induced gene silencing (Panwar et al. 2013a, b).

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