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Proteome Analysis of the Plant Pathogenic Fungus *Monilinia laxa* Showing Host Specificity

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Summary

Brown rot fungus *Monilinia laxa* (Aderh. & Ruhl.) Honey is an important plant pathogen in stone and pome fruits in Europe. We applied a proteomic approach in a study of *M. laxa* isolates obtained from apples and apricots in order to show the host specifity of the isolates and to analyse differentially expressed proteins in terms of host specifity, fungal pathogenicity and identification of candidate proteins for diagnostic marker development. Extracted mycelium proteins were separated by 2-D electrophoresis (2-DE) and visualized by Coomassie staining in a non-linear pH range of 3–11 and M_r of 14–116 kDa. We set up a 2-DE reference map of *M. laxa*, resolving up to 800 protein spots, and used it for image analysis. The average technical coefficient of variance (13 %) demonstrated a high reproducibility of protein extraction and 2-D polyacrylamide gel electrophoresis (2-DE PAGE), and the average biological coefficient of variance (23 %) enabled differential proteomic analysis of the isolates. Multivariate statistical analysis (principal component analysis) discriminated isolates from two different hosts, providing new data that support the existence of a *M. laxa* specialized form f. sp. *mali*, which infects only apples. A total of 50 differentially expressed proteins were further analyzed by LC-MS/MS, yielding 41 positive identifications. The identified mycelial proteins were functionally classified into 6 groups: amino acid and protein metabolism, energy production, carbohydrate metabolism, stress response, fatty acid metabolism and other proteins. Some proteins expressed only in apple isolates have been described as virulence factors in other fungi. The acetolactate synthase was almost 11-fold more abundant in apple-specific isolates than in apricot isolates and it might be implicated in *M. laxa* host specificity. Ten proteins identified only in apple isolates are potential candidates for the development of *M. laxa* host-specific diagnostic markers.

Key words: proteomics, fungi, two-dimensional electrophoresis

Introduction

Proteomic methods, including two-dimensional electrophoresis (2-DE), multi-dimensional liquid chromatography (LC), mass spectrometry (MS) and other derived methodological versions, allow separation, quantification and identification of thousands of proteins in complex mixtures. Global proteome analysis has become an important approach in gene expression analysis, particularly for organisms with limited genomic resources, since database similarity search allows a high success rate of protein identification.

Pathogenic fungi are major plant pathogens, which cause significant losses in economically important crops. A better understanding of fungal biology, pathogenicity and plant-fungus interactions is needed for efficient and sustainable disease management. It can be obtained by employing various methodological approaches, one of them being proteomics. In phytopathogenic fungi, proteomic approaches have been applied in order to find

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differentially expressed proteins due to altered growth conditions or development stages as well as for the identification of proteins of fungal mycelia (*1–7*), secretome (*4,8,9*) and subproteomes (*10–13*). Mycelial proteomes of *Botrytis cinerea* (*3*) and *Sclerotinia sclerotiorum* (*4*) were characterized by the creation of 2-DE reference maps and protein identification. In addition to descriptive proteomics, a comparative approach is a valuable tool allowing the detection of changes in protein abundance between different states. In corn smut fungus *Ustilago maydis*, proteins involved in the transition from budding to filamentous growth, which marks the switch to a pathogenic form, were identified (*5*). Proteomics has also been used successfully in the analysis of metabolic pathways such as mycotoxin synthesis by *Fusarium graminearum* (*6*) and cellulose degradation by *B. cinerea* (*7*). Secreted proteins are of particular interest in phytopathogenic fungi due to their importance in plant-pathogen interactions. Analyses of proteins secreted by *F. graminearum* during growth in different media and during infection of wheat heads revealed proteins that could be important in the interaction (*8*). Comparative proteomics of fungal species and strains has contributed to the understanding of the fungal infection process and host range (*2,14,15*), pathogenicity and identification of candidate virulence proteins (*16–18*).

In the present work, a proteomic study of one of the brown rot fungi, *Monilinia laxa* (Aderhold & Ruhland) Honey and its specialized form *M. laxa* (Aderh. & Ruhl.) Honey f. sp. *mali* Wormald sensu Harrison was performed.

Brown rot, caused by three *Monilinia* species, is one of the most important diseases in stone and pome fruits and is expressed as blossom, shoot and twig blight early in the season, and as fruit rot during ripening and in the postharvest period (*19,20*). Brown rot pathogens infect a wide range of Rosaceae species, although they show a certain level of host preference. *M. laxa* is a blossom and twig as well as fruit pathogen, mainly infecting stone fruits, but it can be also found on apples, pears and other Pomoideae spp. (*20*). *M. laxa* f. sp. *mali* is a specialized form of *M. laxa* that is found in apple orchards and only infects apple trees. *M. laxa* f. sp. *mali* causes blossom wilt, primarily in Europe, although the pathogen is rarely observed and not well studied (*21*). However, withering of apple shoots and spreading throughout apple production areas can occasionally be found and sometimes easily mistaken for the disease symptoms caused by outbreaks of *Erwinia amylovora* (*22*). In such cases, unambiguous and rapid diagnosis of *M. laxa* f. sp. *mali* might avoid the application of eradicative disease control measures against fire blight (*E. amylovora*).

The existence of the biological specialization of apple isolates and f. sp. *mali* taxonomic classification has recently been supported by the analysis of pome and stone fruits for *M. laxa* isolates, using amplified fragment length polymorphism (AFLP) markers (*23*). In order to characterize differences between apple-specific and other *M. laxa* isolates further, we employed a proteomic methodology.

The main aims of this study are to establish a *M. laxa* mycelial proteome reference 2-DE map and to identify differences in the protein profile between apricot isolates and apple isolates showing host specificity. A proteomic approach using 2-DE gel electrophoresis for protein separation, mass spectrometry for protein identification and bioinformatic tools for data analysis were employed. Over 800 mycelial proteins were detected on the 2-DE map, revealing differences in the presence/absence of proteins, as well as in the protein expression level between the two groups of isolates, enabling confirmation of the taxonomic classification of apple isolates. The proteins detected only in apple *M. laxa* isolates could be further used for the development of molecular diagnostic markers and some of the identified differentially expressed proteins might provide an initial insight into the molecular mechanisms of the host specificity or virulence of the pathogens.

Materials and Methods

Strains and culture conditions

Three different isolates of *Monilinia laxa* f. sp. *mali* MLX0657, MLX0658 and MLX0659 from apples and three different isolates of *Monilinia laxa*, MLX0623, MLX0624 and MLX0631 from apricots were included in the analysis. The isolates were collected from various collection sites in Slovenia and are described in detail, including taxonomic classification, in our previous work (*23*). Strains were cultivated in the dark on potato dextrose agar (PDA, Biolife Italiana, Milan, Italy) for 14 days at 20 °C. Five mycelial plugs were cut from the edge of the colony and used to inoculate 150 mL of malt extract broth (Merck KGgA, Darmstadt, Germany) in Erlenmeyer flasks. Each isolate was grown in triplicate (3 flasks), representing biological replications. Liquid fungal cultures were grown on a rotary shaker at 25 °C and 125 rpm in the dark for 3 days. For long-term storage, strains were grown on PDA slants and stored at 4 °C.

Protein extraction

Mycelial biomass was collected from each individual flask by filtration, washed with 0.9 % NaCl, weighed and frozen in liquid nitrogen. Frozen mycelia were ground to a fine powder with a pestle in a pre-cooled mortar. The mycelium was directly dissolved in cooled extraction buffer (7 M urea, 2 M thiourea, 4 % 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.8 % immobilized pH gradient (IPG) buffer 3–11 NL, 20 mM 1,4-dithiothreitol (DTT), 20 mM Tris) in a ratio of 0.5 mL of buffer per 1 g of mycelium. The mixture was then sonicated for 10 s and centrifuged at 13 000×*g* for 20 min at 4 °C. The protein concentration in the supernatant was determined with a 2-D Quant Kit (GE Healthcare, Little Chalfont, UK).

2-D electrophoresis and image analysis

Precipitation of proteins was carried out by the addition of three volumes of ice-cold acetone with 13.3 % trichloroacetic acid (TCA) and 0.3 % DTT overnight. After 15 min of centrifugation at 20 000×*g* and 4 °C, the supernatant was removed and the pellet was rinsed twice in ice-cold acetone containing 0.3 % DTT. The suspension was centrifuged again and the pellet was air dried for 5 min at room temperature. Samples were dissolved

in rehydration buffer consisting of 7 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % IPG buffer 3–11 NL, 20 mM DTT and 0.002 % Bromophenol Blue. Dissolving was aided by sonification in an ultrasonic bath for 15 min, followed by 1-hour incubation at room temperature. After centrifugation at 16 000×*g* for 5 min, the supernatant was collected and applied by rehydration loading onto IPG strips of 24 cm in length, covering a non-linear pH range of pH=3–11 (GE Healthcare). The IPG strips in an Immobiline Dry Strip Reswelling Tray (GE Healthcare) were covered by fluid oil (Dry Strip Fluid, GE Healthcare), balanced horizontally and left overnight (approx. 13 h) at room temperature. The rehydrated strips were then subjected to isoelectric focusing (IEF), which was carried out at 15 °C on an IPGPhor3 apparatus (GE Healthcare) by employing the following protocol: 300 V for 1 h, 500 V for 1 h, gradient to 1000 V in 1 h, gradient to 10 000 V in 3 h, 10 000 V for 2.2 h, total of 40 kVh. The electrode wicks on the cathode side were soaked with 3 mg/mL of DTT to reduce streaking in the basic area. After focusing, the strips were stored at –80 °C for later use. Prior to SDS-PAGE, the IPG strips were equilibrated in SDS equilibration buffer (75 mM Tris-HCl, pH=8.8, 6 M urea, 30 % glycerol, 2 % SDS) containing 1 % DTT, by gentle shaking for 15 min, followed by another 15 min in equilibration buffer containing 2.5 % iodoacetamide. The strips were then loaded onto vertical 12.5 % polyacrylamide gels, sealed with agarose solution and run on an Ettan DALTSix unit (GE Healthcare) at 10 mA/gel for 1 h, followed by 40 mA/gel until the Bromophenol Blue front reached the bottom of the gel. For each individual extract, electrophoresis was repeated three times, resulting in three technical repetitions. After electrophoresis, the gels were fixed in 50 % ethanol and 3 % phosphoric acid overnight, washed three times for 20 min in ddH_2O, pre-incubated in 34 % methanol, 3 % phosphoric acid and 17 % ammonium sulphate for 1 h, Coomassie Brilliant Blue G-250 was then added (0.35 g per 1 L of solution) and left to stain for 5 days, with constant shaking. The gels were then washed with ddH_2O and scanned on an ImageScanner III (GE Healthcare).

Images were analyzed by ImageMaster Platinum 2D v. 6.0 software (GE Healthcare) for spot detection, spot matching and interclass analysis. Spots were detected automatically and edited manually on each image, using various control tools such as 3-D view. Spots were quantified using the volume criterion (the volume of each spot was divided by the total volume of all spots in the gel and the result was expressed in %). Gel matching was performed automatically using two landmarks, and then matches were checked and corrected manually. After spot detection and matching, triplicate gels of the same sample (technical replicates) were combined in synthetic gels to generate three gels (biological replicates) of each isolate. Technical and biological variation was calculated using normalized spot volumes of spots present on all gels. For technical variation, coefficients of variance (CV) were calculated within each group of technical replicates and average values across individual isolates and overall average were then determined. CVs of biological variation were calculated using synthetic gels representing biological replicates. Histograms for the matched spots on the gels were also visually inspected for reproducibility.

Statistical analysis of normalized spot volumes was performed using Student's *t*-test ($p<0.05$). Spot volume data were subjected to principal component analysis (PCA) using GenStat software (VSN International Ltd, Hemel Hempstead, UK) to assess both reproducibility within replicates and the level of difference between isolates from apple and apricot. Calculations were made for differential spots determined by the *t*-test at $p<0.05$ and showing 2-fold protein expression increase or decrease.

In-gel digestion

Protein spots of interest were automatically excised using Ettan Spot Picker (GE Healthcare) from replicate gels and transferred to 96-well plates. Proteins in the gel pieces were digested with modified trypsin (sequencing grade; Promega, Madison, WI, USA) by using a ProGest robotic digestion workstation (Genomic Solutions Ltd, Cambridgeshire, UK). The protocol consisted first of reducing the proteins by submerging the gel pieces in DTT (60 °C, 20 min), followed by an alkylation step with iodoacetamide (25 °C, 10 min) and the proteins were then digested with trypsin (37 °C, 8 h). The resulting tryptic peptide extracts were dried by rotary evaporation using an SC110 Speedvac (Savant Instruments, Hyderabad, India) and dissolved in 0.1 % formic acid solution for mass spectrometric analysis.

LC-MS/MS analysis and database searching

Peptides from each sample were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Inc., Billerica, MA, USA) coupled to an UltiMate 3000 LC System (Dionex, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). Peptide samples were loaded for separation on a monolithic capillary column (200 μm i.d.×5 cm; Dionex) and eluted with a linear gradient from 97 % eluent A (3 % acetonitrile in water containing 0.05 % formic acid), 3 % eluent B (80 % acetonitrile in water containing 0.04 % formic acid) to 55 % eluent A, 45 % eluent B for 12 min at a flow rate of 2.5 μL/min.

Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300–1500 m/z (data were the average of three scans) and up to three precursor ions were selected from the MS scan range of 100–2200 m/z. Precursors were actively excluded within a 1-minute window and all singly charged ions were excluded.

Peptide peaks were detected and deconvoluted automatically using data analysis software (Bruker Daltonics Inc.). The Mascot (Matrix Science Ltd, London, UK) search engine was used for database searching. Mass lists in the form of Mascot generic files were created automatically and used as the input for Mascot MS/MS ion search of the NCBInr database using the Matrix Science web server with the following parameters: NCBInr database with a taxonomy parameter set to Other Fungi, trypsin with one missed cleavage permitted, fixed modification of C (carbamidomethyl), variable modification of M (oxidation), peptide tolerance ±1.0 Da, MS/MS tolerance ±0.5 Da and peptide charge to 2+ and 3+.

Results and Discussion

2-DE, image analysis, analytical and biological variability

The primary aim of this study was to establish a mycelial proteome reference 2-DE map for *M. laxa.* Three isolates of *M. laxa* collected from apples (MLX0657, MLX 0658 and MLX0659) and three isolates collected from apricots (MLX0623, MLX0624 and MLX0631) were included. All isolates were grown in rich culture medium and proteins were extracted from the harvested mycelia. For 2-DE separation, 450 μg of proteins were loaded by rehydration onto 24 cm long strips of non-linear pH=3–11 and the gels were stained with Coomassie Brilliant Blue G-250. Three biological replicates of each isolate and three technical replicates of each sample were included in the experiment (a total of 54 gels). More than 800 spots were resolved and detected by digital image analysis and visual confirmation (Fig. 1). In order to quantify the variances associated with the 2-DE experiments and the differential protein expression between culture batches from all six strains, both analytical and biological variability were calculated. Spot volume data from 252 matched spots present on all 27 gels in each group were used for estimating technical and biological variation. Coefficients of variance (CV) were calculated for each protein spot and then averaged to give a cumulative CV given as the average CV of the analytical or biological dataset. In technical replicates, 93 and 92 % of the spots showed CVs below 30 % in the apricot and apple groups, respectively (Fig. 2). The overall average technical CV for both groups was low (13 %) and showed high reproducibility of protein extraction and 2-DE PAGE analysis, which was achieved by uniform conditions and standard protocols. This analytical variance was similar to that reported from other fungal mycelial extracts (*3*,*17*). Biological variations were calculated comparing the synthetic gels generated from technical replicates. CVs below 30 % showed 78 and 76 % of spots in the apricot and apple groups, respectively. The overall average biological CV was 23 %, which is within the reported values (*24*) and indicates that the 2-DE images are acceptable for differential analysis of the isolates.

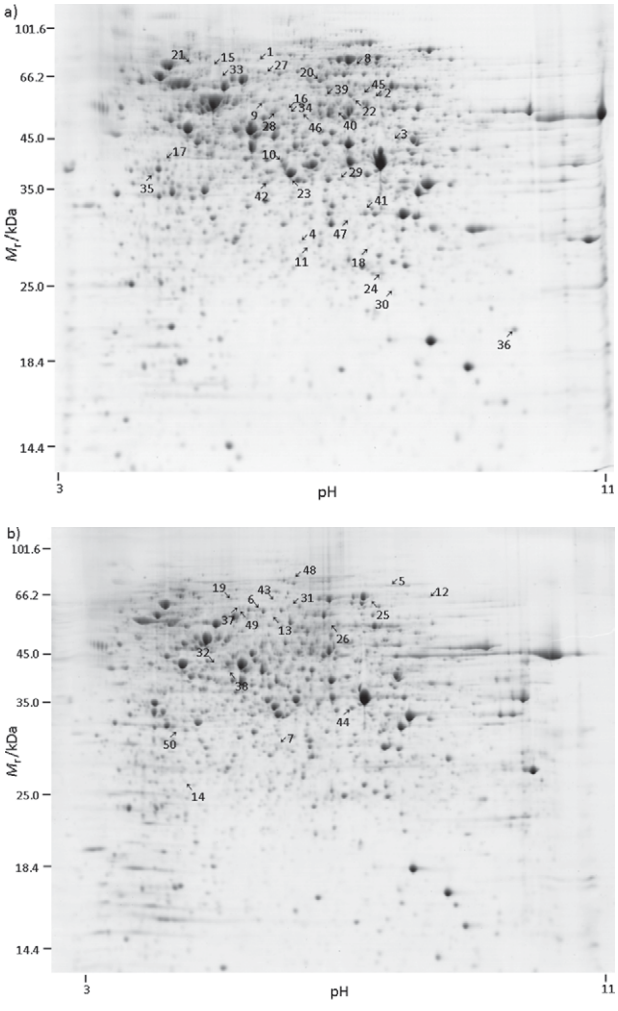

Fig. 1. Representative image of 2-DE PAGE gels of proteins isolated from *M. laxa* mycelia from apple (a) and apricot (b) groups. Proteins (450 μg) were separated on 24-cm, non-linear pH= 3–11 gradient IPG strips and 12.5 % polyacrylamide gels, and visualized by Coomassie Brilliant Blue G-250. Spots analyzed by MS/MS are indicated by arrows and numbers that correspond to the identified proteins summarized in Table 1

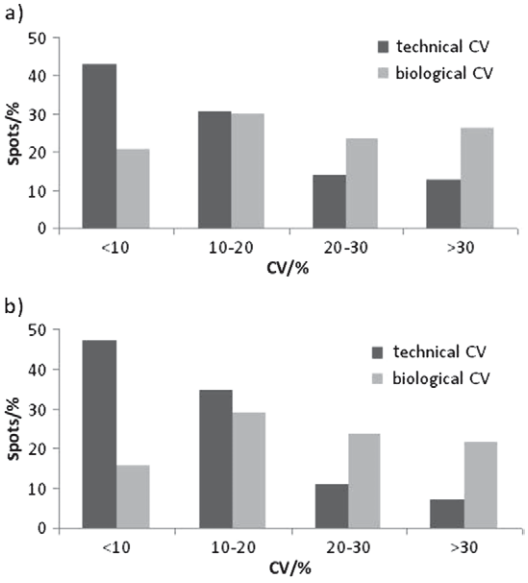

Fig. 2. Distribution of coefficients of variance (CV) in the apricot (a) and apple (b) isolates

Differential expression analysis and principal component analysis

The second aim of the study was to confirm through proteomics the difference between the isolates from apples and apricots, which had previously been shown on a genetic level by AFLP analysis (*23*). The 2-DE patterns of isolates from the two groups were highly similar, with some difference in the presence/absence of spots, but with significant differences in normalized spot volumes. Multivariate statistical method principal component analysis (PCA) was applied in order to assess the power of 2-DE in discriminating the isolates according to their host (Fig. 3). As input data, we used normalized spot volumes of 123 spots that were differentially expressed by means of

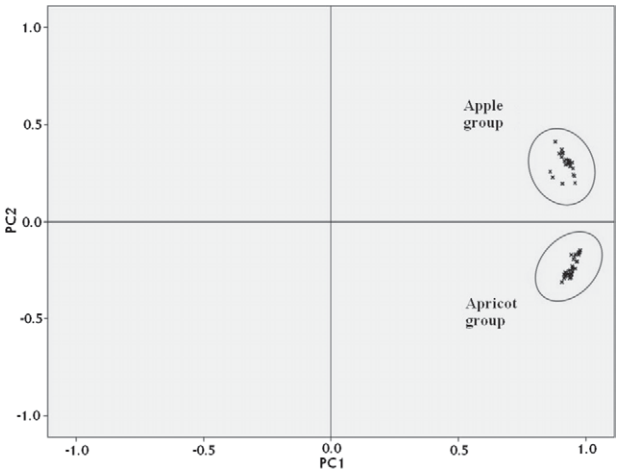

Fig. 3. Score plot of principal component analysis (PCA)

Student's *t*-test ($p<0.05$) and present on all 54 gels, since one of the limitations of PCA analysis is that it cannot handle missing values (*25*). PCA separated the two groups well according to their isolation origin. PC1 explained 87 % of variance but did not account for the grouping and discrimination of samples. Most of the variability, therefore, could not be linked to the isolates but rather to random effects of technical (protein extraction, electrophoresis, staining, *etc*.) or biological (fungal growth) origin. The low percentage of variability associated with the host specificity of isolates is also a consequence of excluding isolate-specific spots from the calculation due to missing values.

PC2 explained 7 % of variation and we were able to discriminate the isolates into two groups: those isolated from apples and those isolated from apricots. This observation supports the clustering of *M. laxa* isolates from different hosts obtained by AFLP analysis (*23*) and confirmed the differentiation of isolates from apple and other hosts.

LC-MS/MS analysis

Comparative analysis was performed between the two groups of *M. laxa* isolates in order to identify the differentially expressed proteins that could provide an initial insight into the molecular mechanisms of host specificity or virulence of the fungus. However, it is important to stress that some proteins are expressed only on interaction with the host and are not constitutively expressed during cultivation in artificial media. Consequently, such proteins could not be detected in this study. Analysis of a total of 836 spots by Student's *t*-test ($p<0.05$) selected 140 differentially expressed spots. Among 140 spots, 92 showed 2-fold protein abundance. Of the 92 spots, a total of 50 spots with the highest reproducibility were selected for the LC-MS/MS analysis. Mascot similarity search identified 41 positive hits with more than one significant peptide (Table 1). Some proteins appeared in gels as more than one spot with the same apparent molecular mass but with different pI values and abundance, presumably due to post-translational modifications or isoenzyme variation. Some spots were identified as a mix of proteins, which has also been previously reported (*3*,*17*,*26*). Identified mycelial proteins are mostly part of different metabolic processes and were functionally clas-

Table 1. Identified proteins from different isolates of *M. laxa*

N^a	Putative protein identity	pI[b]	M_r[c]	Organism[d]	Acc. no.[e]	Sign. pept.[f]	Total score[g]	Apple/ apricot fold diff.[h]
2	GTP cyclohydrolase II	6.02	58157	*Botryotinia fuckeliana*	CCD51450	8	686	1.4
3	carbamoyl-phosphate synthase, small subunit	6.65	50200	*Botryotinia fuckeliana*	XP_001561199	2	297	–1.1
4	6-phosphogluconolactonase	5.57	28335	*Sclerotinia sclerotiorum*	XP_001591742	6	375	1.6
5	elongation factor 2	6.36	93801	*Botryotinia fuckeliana*	CCD46147	12	1338	1.8
6	NADH dehydrogenase, subunit G	5.83	81926	*Nectria haematococca*	XP_003051572	5	360	1.2
	glycyl-tRNA synthetase	5.52	74994	*Sclerotinia sclerotiorum*	XP_001598549	2	225	
7	mitochondrial processing peptidase β subunit	5.94	52779	*Botryotinia fuckeliana*	XP_001559775	2	205	7
8	anthranilate synthase	5.71	82890	*Sclerotinia sclerotiorum*	XP_001587317	4	269	2.4
9	UDP-N-acetylglucosamine pyrophosphorylase	5.53	56848	*Botryotinia fuckeliana*	XP_001550341	9	956	2.5
10	V-type (H+)-ATPase V1, B subunit	5.76	57545	*Sclerotinia sclerotiorum*	XP_001588235	2	287	apple only
	xylitol dehydrogenase	5.94	38429	*Botryotinia fuckeliana*	XP_001552084	2	287	
	shikimate 5-dehydrogenase	5.34	36307	*Botryotinia fuckeliana*	XP_001560215	2	126	
12	phosphofructokinase	6.56	87308	*Sclerotinia sclerotiorum*	XP_001587610	7	797	2.4
13	protein disulfide isomerase (PDI)	5.24	54733	*Botryotinia fuckeliana*	XP_001548398	2	193	2.1
14	heat shock protein 70	5.25	67261	*Chaetomium globosum*	XP_001220896	4	315	–1.1
	predicted phosphatase/phosphohexomutase	5.10	27328	*Sclerotinia sclerotiorum*	XP_001598632	3	168	
16	heat shock protein SSB	5.33	67095	*Sclerotinia sclerotiorum*	XP_001592724	9	703	1.3
	eukaryotic glutathione synthetase	5.52	56778	*Sclerotinia sclerotiorum*	XP_001591994	3	368	
	V-type (H+)-ATPase V1, B subunit	5.76	57545	*Sclerotinia sclerotiorum*	XP_001588235	3	343	
	trehalose-6-phosphate synthase	5.53	59364	*Sclerotinia sclerotiorum*	XP_001586818	3	307	
17	dienelactone hydrolase and related enzymes	4.76	35782	*Botryotinia fuckeliana*	XP_001548143	3	215	1.7
18	tetrahydrofolate dehydrogenase/cyclohydrolase	6.31	101372	*Sclerotinia sclerotiorum*	XP_001595805	2	218	1.3
19	eukaryotic translation initiation factor 3 subunit 7 (eIF-3)	5.28	64278	*Sclerotinia sclerotiorum*	XP_001585078	7	696	1.7

Table 1. – continued

N[a]	Putative protein identity	pI[b]	M_r[c]	Organism[d]	Acc. no.[e]	Sign. pept.[f]	Total score[g]	Apple/ apricot fold diff.[h]
20	acetolactate synthase catalytic subunit	5.95	63131	*Sclerotinia sclerotiorum*	XP_001590157	2	244	10.8
	Ras-like GTPase family/type 1 glutamine amidotransferase	6.05	56911	*Sclerotinia sclerotiorum*	XP_001589075	2	172	
22	phosphoglucose isomerase	6.47	52006	*Botryotinia fuckeliana*	CCD44021	6	551	apple only
23	thiamine biosynthesis protein Nmt1	5.85	35171	*Glarea lozoyensis*	EHK97636	5	355	10.3
	M28 Zn-peptidase	5.33	43328	*Botryotinia fuckeliana*	CCD52560	2	227	
	eukaryotic translation initiation factor 3	5.70	37594	*Sclerotinia sclerotiorum*	XP_001595804	2	210	
24	V-type (H+)-ATPase V1, B subunit	5.76	57545	*Sclerotinia sclerotiorum*	XP_001588235	5	449	apple only
25	aconitate hydratase, mitochondrial	6.26	85676	*Sclerotinia sclerotiorum*	XP_001587807	4	537	2.3
26	aspartate-tRNA ligase	5.93	63163	*Botryotinia fuckeliana*	CCD48962	4	535	4.3
27	NADH-ubiquinone oxidoreductase 78 kDa subunit, mitochondrial	6.33	81521	*Sclerotinia sclerotiorum*	XP_001597573	7	717	apple only
28	protoporphyrinogen oxidase	5.61	57624	*Botryotinia fuckeliana*	XP_001552022	5	542	1.1
	pyruvate kinase	5.95	57517	*Grosmannia clavigera*	EFX05805	4	451	
	aldehyde dehydrogenase	5.06	49020	*Sclerotinia sclerotiorum*	XP_001585014	3	290	
	heat shock 70 kDa protein	5.00	59934	*Botryotinia fuckeliana*	XP_001555459	2	152	
	glutathione S-transferase	5.41	47020	*Botryotinia fuckeliana*	XP_001592794	2	123	
29	crotonase/enoyl-coenzyme A (CoA) hydratase	9.04	35335	*Sclerotinia sclerotiorum*	XP_001586894	4	443	2.7
31	AAA ATPase	5.85	84974	*Sclerotinia sclerotiorum*	XP_001592469	6	507	3.9
	ABC transporter/elongation factor 3 (EF-3)	6.17	69982	*Sclerotinia sclerotiorum*	XP_001592060	5	442	
	tryptophan synthase	5.93	78674	*Botryotinia fuckeliana*	CCD56562	4	350	
32	enolase	5.32	47188	*Sclerotinia sclerotiorum*	XP_001594265	18	1383	1.7
33	molecular chaperone DnaK	5.80	73018	*Sclerotinia sclerotiorum*	XP_001589111	12	1144	1.7
	phenylalanyl-tRNA synthetase β chain	5.26	69692	*Botryotinia fuckeliana*	XP_001551363	2	293	
34	acetylglutamate kinase	8.61	97650	*Botryotinia fuckeliana*	XP_001554591	2	272	apple only
	transketolase	5.46	67259	*Botryotinia fuckeliana*	XP_001553108	2	219	
	aldehyde dehydrogenase	5.77	53909	*Botryotinia fuckeliana*	XP_001554714	2	158	
35	anion-transporting ATPase (ArsA ATPase)	4.83	37889	*Botryotinia fuckeliana*	XP_001552593	5	510	1.8
	thiazole biosynthetic enzyme Thi4	5.13	35118	*Botryotinia fuckeliana*	XP_001549602	5	471	
	aspartate aminotransferase	5.03	44722	*Botryotinia fuckeliana*	XP_001559632	2	205	
36	peptidyl-prolyl *cis-trans* isomerase B	5.58	32132	*Sclerotinia sclerotiorum*	XP_001587974	5	245	apple only
	GatB domain-containing protein	9.02	22766	*Botryotinia fuckeliana*	XP_001551463	2	115	
37	Sec1 family protein	5.51	77526	*Sclerotinia sclerotiorum*	XP_001593526	4	651	–1.5
38	mitochondrial processing peptidase β subunit	5.94	52779	*Botryotinia fuckeliana*	XP_001559775	8	782	–1.3
	eukaryotic peptide chain release factor subunit 1	5.34	48919	*Botryotinia fuckeliana*	CCD44867	10	684	
	glutathione S-transferase	5.41	47020	*Botryotinia fuckeliana*	XP_001560910	2	388	
	Hsp70 protein	5.07	79453	*Sclerotinia sclerotiorum*	XP_001597692	2	179	
39	T-complex protein 1, γ subunit	5.92	59271	*Sclerotinia sclerotiorum*	XP_001592815	14	1274	apple only
40	6-phosphogluconate dehydrogenase	5.89	55018	*Botryotinia fuckeliana*	XP_001558673	3	260	apple only
42	arginase	5.67	35878	*Sclerotinia sclerotiorum*	XP_001588035	3	214	apple only
	eukaryotic initiation factor 4A	5.14	45008	*Sclerotinia sclerotiorum*	XP_001594651	2	188	
	thiamine biosynthesis protein Nmt1	5.95	38591	*Sclerotinia sclerotiorum*	XP_001588250	3	170	
43	ABC transporter/elongation factor 3 (EF-3)	6.40	70539	*Glarea lozoyensis*	EHK98087	3	255	1.2
	tryptophan synthase	6.26	62949	*Botryotinia fuckeliana*	XP_001557197	2	241	
45	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	6.05	86413	*Botryotinia fuckeliana*	XP_001549321	6	533	apple only
46	NADP-specific glutamate dehydrogenase	5.81	49152	*Sclerotinia sclerotiorum*	XP_001590355	3	370	apple only
	succinate dehydrogenase subunit A	6.72	54602	*Botryotinia fuckeliana*	CCD47494	3	243	
	dihydrolipoyl dehydrogenase, mitochondrial	6.66	54672	*Botryotinia fuckeliana*	XP_001550603	2	201	
	DnaJ-class molecular chaperone	5.77	46112	*Botryotinia fuckeliana*	XP_001549243	2	191	
	aldehyde dehydrogenase	5.77	53909	*Botryotinia fuckeliana*	XP_001554714	2	134	
48	GH31/α-glucosidase	5.64	109375	*Sclerotinia sclerotiorum*	XP_001597484	3	417	4.1
50	pyruvate decarboxylase	5.66	63052	*Botryotinia fuckeliana*	XP_001550574	5	266	1.5

[a]spot numbers 1–50 correspond to 2-DE gel (Fig. 1), [b]theoretical value of pI, [c]theoretical value of M_r, [d]organism with the highest peptide score hit, [e]NCBI accession number, [f]number of statistically significant matched peptides, [g]Mascot protein score, [h]fold difference in protein quantity between apple and apricot groups of isolates (negative numbers indicate down-regulation)

sified into 6 groups: proteins that take part in amino acid and protein metabolism are most abundantly represented (29 identified proteins), 6 identified proteins are involved in energy production, 10 proteins participate in carbohydrate metabolism, 9 proteins are classified as a group of stress response-associated proteins, 2 proteins are part of fatty acid metabolism; and some other identified proteins were not classified into any significant group (Fig. 4).

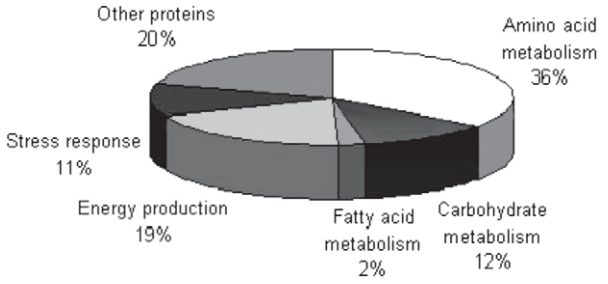

Fig. 4. Classification of *M. laxa* mycelial proteins into different functional categories based on their putative function

Most of the classified proteins were more abundant or detected only in isolates from apple, indicating several differences in metabolism. The following proteins (Table 1) were identified only in apple isolates: V-type (H+)-ATPase V1, B subunit; phosphoglucose isomerase; mitochondrial NADH-ubiquinone oxidoreductase, 78-kDa subunit; acetylglutamate kinase; peptidyl-prolyl *cis-trans* isomerase B; T-complex protein 1, γ subunit; 6-phosphogluconate dehydrogenase; arginase; 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase and NADP-specific glutamate dehydrogenase. However, the relation between these proteins and host specificity remains unclear. In most cases, host specificity is associated with the production of host-specific toxins, as for example toxin Ptr ToxA of wheat pathogen *Pyrenophora tritici-repentis* (*27*). Another possible suggested determinant of host specificity is misregulation of biosynthetic pathways (*28*). An example of such misregulation includes acetolactate synthase, an enzyme encoded by a nuclear gene, whose product is targeted to mitochondria (*29*). The enzyme catalyzes the first common step in biosynthesis of the branched chain amino acids leucine, isoleucine and valine. The enzyme activity is inhibited by various branched amino acids, depending on the regulatory subunits, so it has been suggested that a pathogen possessing a single acetolactate synthase inhibitable by valine would be limited to hosts either low in free valine or high in leucine and isoleucine. In our experiment, acetolactate synthase was identified in a spot that was almost 11-fold more abundant in apple-specific isolates, indicating a possible involvement of pathway misregulation in *M. laxa* host specificity.

Some of the other identified proteins have been shown to have a role in virulence in both plant and animal pathogens. Disruption of the phosphoglucose isomerase gene in the rice pathogen *Xanthomonas oryzae* led to virulence attenuation (*30*). Phosphogluconate dehydrogenase was shown to be involved in the adhesion of the animal bacterial pathogen *Streptococcus suis* to host cells (*31*). Peptidyl prolyl *cis-trans* isomerase was found to be virulence determinant in the rice pathogen *Magnaporthe grisea*, since knock-out mutants were impaired in virulence-associated functions, such as penetration peg formation and appresorium turgor generation (*32*). Molecular chaperones are also important virulence factors, by allowing the pathogens to overcome a hostile environment resulting from host responses such as oxidative burst (*33*).

In addition, the identified apple-specific proteins can serve as candidate molecules to be converted into DNA-based or cDNA-based markers and tested for *M. laxa* f. sp. *mali* diagnostics using a PCR or RT-PCR approach.

Conclusion

A proteomic approach was used in the study of brown rot fungus *M. laxa* from apple and apricot isolates in order to differentiate two types of isolates on the proteome level, which might contribute to an understanding of the processes of fungal host specialization and pathogenicity, as well as to searching for candidate proteins suitable for the development of diagnostic marker(s).

We first established a 2-DE reference map of mycelial proteins, resolving up to 800 protein spots. The average technical and biological coefficients of variance in both apricot and apple groups showed that the analysis was highly reproducible and enabled proteomic differential study of the isolates. Multivariate statistical analysis (PCA) was applied, which discriminated the isolates from the two different hosts, providing new confirmation of the existence of a *M. laxa* specialized form f. sp. *mali.* Of a total of 50 differentially expressed spots, 41 proteins were successfully identified by LC-MS/MS and were classified into 6 functional groups. Ten proteins were found only in apple isolates and some of them have been implicated in fungal pathogenicity. One protein, which was almost 11-fold more abundant in apple-specific isolates, suggests a possible involvement in *M. laxa* host specificity. The relation among the identified differentially expressed proteins, host specificity and pathogenicity remains unclear, though, and needs further study. However, the analysis provided 10 candidate proteins that can be used for the development of diagnostic markers for the studied pathogens.

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