

Proteomic response of *Moniliophthora perniciosa* exposed to pathogenesis-related protein-10 from *Theobroma cacao*

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ABSTRACT. TcPR-10, a member of the pathogenesis-related protein 10 family, was identified in EST library of interactions between *Theobroma cacao* and *Moniliophthora perniciosa*. TcPR-10 has been shown to have antifungal and ribonuclease activities *in vitro*. This study aimed to identify proteins that are differentially expressed in *M. perniciosa* in response to TcPR-10 through a proteomic analysis. The fungal hyphae were subjected to one of four treatments: control treatment or 30-, 60- or 120-min treatment with the TcPR-10 protein. Two-dimensional maps revealed 191 differentially expressed proteins, 55 of which were identified by mass spectrometry. The proteins identified in all treatments were divided into the following classes: cell metabolism,

stress response, zinc binding, phosphorylation mechanism, transport, autophagy, DNA repair, and oxidoreductases. The predominant class was stress-response proteins (29%), such as heat shock proteins; these proteins exhibited the highest expression levels relative to the control treatment and are known to trigger defense mechanisms against cytotoxic drugs as well as TcPR-10. Oxidoreductases (25%) were overexpressed in the control and in 30-min treatments but exhibited reduced expression at 120 min. These proteins are involved in the repair of damage caused by oxidative stress due to the contact with TcPR-10. Consistent with the antifungal activity of TcPR-10, several proteins identified were related to detoxification, autophagy or were involved in mechanisms for maintaining fungal homeostasis, such as ergosterol biosynthesis. These results show that the sensitivity of the fungus to TcPR-10 involves several biochemical routes, clarifying the possible modes of action of this antifungal protein.

Key words: Pathogenesis-related protein 10; Oxidoreductases; Differentially expressed proteins; Stress-response proteins

INTRODUCTION

Moniliophthora perniciosa (Basidiomycota, Agaricales, Marasmiaceae) is the causal agent of witches' broom disease (Aime and Phillips-Mora, 2005) in the cacao tree (*Theobroma cacao* L.). This disease is considered the most important cause of loss of cacao production in the South America and Caribbean, with important social, economical, and ecological impacts on the affected regions (Kilaru and Hasenstein, 2005; Scarpari et al., 2005). The life cycle of *M. perniciosa* is hemibiotrophic and is divided into i) biotrophic (parasitic) phase, characterized by monokaryotic hyphae that induce hyperplasia and hypertrophy of the plant tissue, loss of apical dominance, axillary shoot proliferation, and abnormal stems (green broom), and ii) necrotrophic (saprophytic) phase, characterized by dikaryotic hyphae containing clamp connections that correspond, at the plant level, to the necrosis and death of infected tissues distant from the primary infection site (dry broom). Basidiocarp production and spore formation occur on the surface of the necrotic tissues of the infected plant (Aime and Phillips-Mora, 2005).

The major damage caused by witches' broom disease has motivated several genomic and proteomic studies for a better understanding of the physiology of this pathogen and its mechanism of attack on cocoa. Gesteira et al. (2007) constructed cDNA libraries from two cacao cultivars, one resistant and one susceptible to *M. perniciosa*, and identified the TcPR-10 gene (pathogenesis-related protein 10 of *T. cacao*). TcPR-10 is a member of a family of acidic proteins (PR-10) that are found in some gymnosperms and angiosperms and that are responsive to intracellular defense processes (Islam et al., 2009; Xie et al., 2010). Members of the PR10 family have a p-loop motif, a highly conserved glycine-rich motif (GXGGXGXK; 47-55 amino acids) that is involved in ribonuclease activity (Lytle et al., 2009). Another feature of the PR10 family is the presence of phosphorylation sites that are characteristic of cAMP-dependent kinases (Bantignies et al., 2000). The presence of three common phosphorylation sites in PR-10 proteins suggests a general or specific RNase effect against exogenous RNA (Park et al., 2004). Recent reports identified DNase activity in PR-10 proteins in rice (Kim

et al., 2011) and grape (He et al., 2013), suggesting that this protein may act as a nuclease in programmed cell death.

In 2009, Pungartnik et al. reported the ribonuclease and antifungal activity of the heterologous protein TcPR-10 against *M. perniciosa* and *Saccharomyces cerevisiae*. The authors also showed an *in vitro* antifungal activity of TcPR-10 leading to the inhibition of *M. perniciosa* growth. Additionally, Menezes et al. (2012) showed that the allergenic potential of TcPR-10 may be diminished or abolished by the introduction of specific point mutations into the protein without disrupting its RNase activity. They showed that TcPR-10 has a strong RNase activity against *M. perniciosa* RNA, and that this response is both concentration- and time-dependent. Several PR-10 protein family members have been reported to hydrolyze (Liu et al., 2005; Yan et al., 2008) and this activity can be crucial during the attack of a pathogen, because it activates apoptotic processes in the pathogen to limit the invasion.

The ribonuclease properties and antifungal activity of PR-10 molecules are widely described in the literature. Park et al. (2004) identified CaPR-10, a protein expressed in the hot pepper (*Capsicum annuum*) that is known to inhibit the hyphal extension of *Phytophthora capsici*, possibly by inhibiting its translation activity. Pinto and Ricardo (1995) identified a group of inducible low molecular weight acidic proteins with N-terminal amino acid sequences similar to those of the PR-10 proteins in leaves from *Lupinus albus*. These proteins, PR-p16.5, PR-p16.5b, and PR-p16.5c, are induced upon infection by *Colletotrichum gloeosporioides*. Flores et al. (2002) described an antifungal PR-10 group protein, the 18-kDa storage protein ocatin in the oca (*Oxalis tuberosa*), an Andean tuber. *In vitro*, this protein inhibits the growth of several phytopathogenic fungi, including *Fusarium oxysporum* and *Rhizoctonia solani*.

The use of proteomic tools to assess the effects of antifungal agents has been successfully applied to provide a comprehensive examination of the changes occurring in the pathogen. Cagas et al. (2011) investigated the changes in the *Aspergillus fumigatus* proteome in response to the stress generated by coumarin, a known antifungal drug, and the possible mechanism of action by which the compound produces lethal effects against the fungus.

In this study, we used proteomic analyses to identify proteins that are differentially expressed in *M. perniciosa* hyphae treated with TcPR-10, or not treated (control), to elucidate the mechanisms that are directly or concurrently involved in the response of *M. perniciosa* to the antifungal compound TcPR-10. To our knowledge, this is the first study describing and analyzing proteins expressed by *M. perniciosa* in response to PR-10 from *T. cacao*.

MATERIAL AND METHODS

Obtaining the TcPR-10 protein

The *TcPR-10* gene (accession No. ES439858) was isolated from the *T. cacao* x *M. perniciosa* interaction cDNA library described by Gesteira et al. (2007). The gene was cloned into the expression vector pET28a, and the TcPR-10 recombinant protein was expressed in *Escherichia coli* BL21 (DE), as previously described by Pungartnik et al. (2009) and Menezes et al. (2012). A stock solution of recombinant TcPR-10 protein was prepared at a concentration of 30 mg/mL.

M. perniciosa growth conditions

M. perniciosa strain ALF553 cultures were grown as described by Filho et al. (2006).

Dikaryotic cultures were grown in CPD (2% glucose, 2% peptone) in liquid medium without agitation at 25°C for 5 to 7 days. *M. pernicioso* was obtained from the fungal collection of UESC, originally kept at Comissão Executiva do Plano da Lavoura Cacaueira.

Exposure of the fungus *M. pernicioso* to TcPR-10

The *M. pernicioso* hyphae were incubated with the recombinant TcPR-10 protein (final concentration of 3 µg/mL) for 30, 60, or 120 min as described by Filho et al. (2006). A culture that was not exposed to the protein was used as a control. After harvesting, the fungal hyphae were frozen in liquid N₂ and stored at -80°C. Three independent experiments were performed for each incubation time.

***M. pernicioso* protein extraction and quantification**

Protein was extracted from *M. pernicioso* according to Pirovani et al. (2008). Protein extracts were subjected to a second cleaning method for total protein purification as described by Meyer et al. (1988). The proteins were then quantified using the 2-D Quant kit according to manufacturer instructions (GE Healthcare, Milwaukee, WI, USA) and using bovine serum albumin as standard.

2-D gel electrophoresis

Isoelectric focusing was performed with 350 mg protein in a final volume of 250 µL rehydration solution (7 M urea, 2 M thiourea, 1% CHAPS, 100 mM DTT, 0.5% IPG buffer, pH 3-10, containing a pinch of bromophenol blue). Samples were applied to 13-cm IPG strips with a linear pH gradient from 3 to 10 (Amersham Biosciences, Immobiline DryStrip™). The strips were treated with equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) containing 1% DTT (dithiothreitol) and equilibration buffer containing 2.5% iodoacetamide. The second dimension of the electrophoresis was performed with a Hoefer SE 600 vertical Ruby (GE Healthcare) electrophoresis system at 15°C under 80 V/200 mA. The gels were fixed in a solution containing 40% ethanol and 10% acetic acid for 1 h and then stained using Coomassie brilliant blue G according to manufacturer instructions (Sigma).

2-D gel image analysis

Three 2-D gels were obtained for each incubation time, and the most representative gel was chosen as the reference for the analysis. The images of the 2-D gels were produced by the ImageScanner II (GE Healthcare) at a resolution of 600 dpi, and the digitized images were analyzed using the Image Master 2-D Platinum v.7.0 program (GE Healthcare) for spot detection, quantification and matching. After automated detection and matching, manual editing was performed for individual spots.

Gel groups were established according to the experimental design, and spot-normalized volume was used to select statistically significant different (fold-change, ANOVA, false-discovery rate) spots between the treatments analyzed in the experiment. The spot values were normalized using the following formula: intensity of each spot/total intensity, in which the total intensity refers to the sum of all spots belonging to the same gel. Internal molecular markers were used to determine the experimental isoelectric point (pI) and molecular weight for each protein of interest.

Significant differences between incubation times were determined for each spot. One-way ANOVA was applied to compare the percentage volume values of matched spots. A spot was considered to be differentially expressed if it exhibited a fold-change higher than 1.2. The ratio of the accumulation of proteins was quantified by Nano LC-ESI-Q-TOF performed with ImageMaster 7.0/GE, using the control treatment as reference.

Preparation of spots for mass spectrometry (MS)/MS

The differentially expressed spots were manually excised from the 2-D gel, and the gel pieces were destained in NH_4HCO_3 containing 50% acetonitrile (ACN). Gel fragments were dehydrated in ACN and dried in a SpeedVac (Eppendorf). Next, a 25-ng/ μL trypsin solution (Gold Promega) was added to the sample, which was then incubated at 4°C for 10 min to allow residual solution to be absorbed by the gel fragments. NH_4HCO_3 was then added to cover the pieces, and the samples were incubated at 37°C for 16 h. The supernatant was collected and transferred to a new tube. The gel fragments were washed twice with 50% (v/v) ACN and 0.1% (v/v) trifluoroacetic acid, and the supernatant was collected. The supernatants obtained in the last two steps were pooled and concentrated under vacuum to a volume of 20 μL .

MS analysis

MS was performed using NanoLC-ESI-Q-TOF Micro™ (Micromass, Waters, Milford, MA, USA). The peptides were separated by nano-chromatography using a reversed phase column (nanoAcquity UPLC - Waters) coupled to a mass spectrometer NanoLC-ESI-Q-TOF Micro™ (Micromass, Waters). Initially, the peptides were desalted using a Symmetry C18 pre-column (Waters) (5 μm , 180 μm in internal diameter x 20 mm in length) and an analytical column (1.7 μm BEH300, C18 100 μm x 100 mm). The flow rate was 0.6 $\mu\text{L}/\text{min}$ with 50 min/run, and 4 μL of each sample was injected. The peptides were separated using a gradient of 1% ACN and then ionized in a capillary at 3000 V, fragmented in positive ion mode, with a cut-off of a relative intensity of at least 10 counts; the three most intense ions (scans) were analyzed with collision energies ranging between 20 and 95 eV according to the mass/load (m/z) of peptide.

The MS data analysis was performed using the Masslynx 4.0 software (Waters-Micromass, Manchester, UK). Protein identification was performed using a Mascot server (Version 2.1.04, Matrix Science) and the *Magnaporthe grisea* (<http://www.broadinstitute.org/annotation/fungi/magnaporthe/>) database. The parameters for the MS data analysis were the following: i) only tryptic peptides with up to one missed cleavage site; ii) carbamidomethyl cysteine as a fixed modification; and iii) oxidized methionine as a variable modification.

RESULTS AND DISCUSSION

In the present study, 2-D gel analysis and MS were used to elucidate the adaptive responses of *M. perniciosa* to the antifungal agent TcPR-10. Proteins with different abundances at different exposure times were identified. The 2-D maps displayed a profile of proteins with molecular weights ranging from 3 to 201 kDa and pI values from 3 to 10. We observed that, in the control treatment, 69% of spots had a pI value between 3 and 7, and 31% had a pI value between 7 and 10; in the 30-min treatment gel, 61% of spots had a pI value between 3 and 7, and 39% had a pI value between 7 and 10; in the 60-min treatment gel, 62% of spots had a pI value between 3

and 7, and 38% had a pI value between 7 and 10; and in the 120-min treatment gel, 50% of spots had a pI value between 3 and 7, and 50% had a pI value between 7 and 10 (Figure 1).

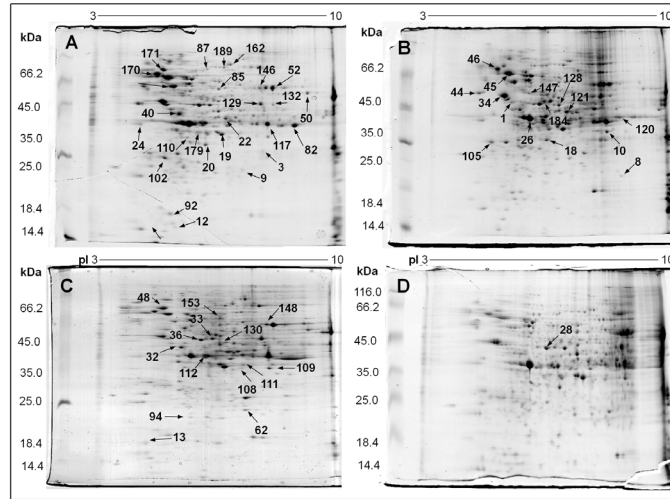


Figure 1. Protein profile on a 2-DE gel of the fungus *Moniliophthora perniciosa* treated with the antifungal protein TcPR-10 (3 $\mu\text{g}/\text{mL}$) obtained from cacao. **A.** Control (without TcPR-10); **B.** 30 min; **C.** 60 min; **D.** 120 min after exposure to TcPR-10. The gels were stained with colloidal Coomassie blue G-250 solution. Arrows indicate spots excised from the gel for differentially expressed proteins identified by mass spectrometry. pI = isoelectric point.

The number of protein spots from each treatment, the number of differentially expressed proteins between treatments, and the exclusive proteins are presented in a Venn diagram (Figure 2). A total of 461 differentially expressed proteins were analyzed by MS, but only 55 were identified (Tables 1 and 2; Figure 1).

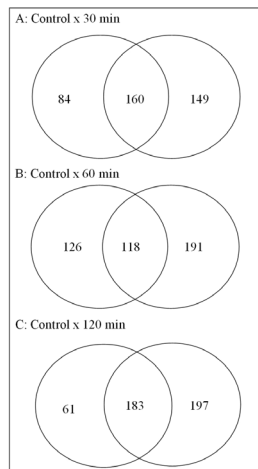


Figure 2. Venn diagram of the proteomic analysis of the fungus *Moniliophthora perniciosa* after exposure to TcPR-10 (3 $\mu\text{g}/\text{mL}$) or the control treatment (without TcPR-10) for 30, 60 and 120 min. The number of differentially expressed spots was determined using the 2-D gel image analysis software. The statistical analyses of the matched proteins and spots were performed using ANOVA.

Table 1. Differentially expressed (upregulated) proteins identified by mass spectrometry (NANO/ESI/Q-TOF).

Match ID	Gel	MW	pI	Accession No.	Protein	Biological process	Control x 30	Control x 60	Control x 120
18	B	28	6.8	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	178.397	0.770195	-0.703513
45	B	68	5.0	Q01877	Heat shock protein HSP1	Stress response	171.898	0.809091	-0.705884
92	A	17	5.3	P23301	Eukaryotic translation initiation factor 5A-1	Protein biosynthesis	153.074	0.760242	-0.519375
105	B	23	6.9	O42766	14-3-3 protein homolog	Stress response	133.195	0.863126	-0.845349
34	B	44	5.7	P00830	ATP synthase subunit beta	Transport	132.282	0.761055	-0.580592
1	B	45	5.4	P17967	Protein disulfide-isomerase 1	Cell redox homeostasis	123.172	0.770709	-0.629766
46	B	75	4.7	P16474	78-kDa glucose-regulated protein homolog	Stress response	112.712	0.671421	0.63484
44	B	62	4.4	Q52454	Cobalamin synthase	Zinc ion binding	109.902	-0.546875	0.594864
33	C	38	6.4	P32318	Thiazole synthase	Suicide enzyme; stress response and in DNA damage tolerance	102.195	0.830277	-0.801889
82	A	31	7.6	P61889	Malate dehydrogenase	Oxidoreductase	15.995	0.436343	-0.390386
22	A	34	6.8	P41338	Acetyl-CoA acetyltransferase	Ergosterol biosynthetic process	-0.392888	462.225	-0.388934
50	A	54	9.1	P07251	ATP synthase subunit alpha, mitochondrial	ATP catabolic process	-0.803737	243.439	-0.835913
129	A	42	7.2	P00560	Phosphoglycerate kinase	Phosphoprotein	-0.7911	193.703	-0.842342
130	A	42	6.9	P19358	S-adenosylmethionine synthase 2	One-carbon metabolism	0.827082	136.415	-0.794706
112	C	32	6.5	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	0.606736	166.442	-0.615852
153	C	64	6.5	P17820	Chaperone protein DnaK	Stress response	0.680602	117.191	-0.414151
8	B	23	8.5	P43773	ATP-dependent protease ATPase subunit HslU (heat shock)	Stress response	-0.74422	114.433	0.849442
147	B	58	6.7	P40530	Pyruvate kinase	Carbohydrate metabolism	0.763106	101.327	-0.61609
52	A	58	8.5	O6FM63	Autophagy-related protein 18	Autophagy	-0.924253	18.815	-0.951401
13	C	15	5.2	O74983	Ubiquitin-conjugating enzyme	Postreplication repair	-0.793504	0.982094	125.934
20	A	32	6.2	P15019	Transaldolase	Pentose-phosphate shunt (stress response)	-0.539107	0.812164	108.524

The numbering of the spots coincides with the numbering shown in Figure 1. The ratio of the accumulation of proteins quantified by NanoLC-ESI-Q-TOF was calculated using ImageMaster 7.0/GE using the control treatment as reference. pI = isoelectric point.

Table 2. Differentially expressed (downregulated) proteins identified by mass spectrometry (NANO/ESI/Q-TOF).

Match ID	Gel	MW	pI	Accession No.	Protein	Biological process	Control x 30	Control x 60	Control x 120
102	A	24	5.9	O74770	Probable phosphoketolase	Stress response	-111.257	-	-111.257
111	C	32	7.2	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	0.849117	0.928823	-100.518
62	C	21	6.9	Q9AAML4	GTP-binding nuclear protein	Autophagy	-0.419916	-	-0.419916
146	A	58	7.7	P39522	Dihydroxy-acid dehydratase, mitochondrial	Amino acid biosynthesis	-0.110582	-	-0.110582
171	A	125	5.1	P25694	Cell division control protein 48	Cell cycle; Autophagy	-0.247938	0.549927	-0.285886
3	A	26	7.8	P79071	60S ribosomal protein L6	Cytoplasmic translation	-0.349022	0.612684	0.600063
120	B	37	8.3	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	0.993355	0.915481	-0.795396
121	B	38	6.8	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	-0.742154	0.895575	-0.878184
109	C	30	8.0	P17505	Malate dehydrogenase, mitochondrial	Oxidoreductase	-0.754071	0.801155	-0.89945
148	C	58	7.9	I2DFD6	Catalase	Oxidoreductase	-0.382351	-	-0.382351
108	C	30	7.1	P00359	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	-0.307501	-	-0.307501
132	A	45	8.1	P38720	6-Phosphogluconate dehydrogenase, decarboxylating 1	Oxidoreductase	-0.348645	-	-0.348645
162	A	90	7.7	P82610	5-Methyltetrahydropteroyltri glutamate, homocysteine methyltransferase	Stress response	-0.553217	-	-0.553217
28	B	38	6.4	O9RA63	Chaperone protein ClpB	Stress response	-0.871045	-0.513589	0.871045
24	A	35	4.2	P02829	Heat shock protein 82	Stress response	0.7276	-0.957569	0.828066
170	A	124	4.8	P55737	Heat shock protein 90-2	Stress response	-0.311785	0.484568	-0.411449
85	A	72	6.7	Q01877	Heat shock protein HSS1	Stress response	-0.571641	0.982635	-0.982635
48	C	84	4.9	P54651	Heat shock cognate 90-kDa protein	Stress response	-0.577448	0.999935	-0.597114
87	A	100	6.0	O74225	Heat shock protein Hsp88	Stress response	-0.741642	-	-0.741642
184	B	48	6.4	P32318	Thiazole synthase	Stress response	-	-0.789057	-0.789057
12	A	16	5.8	P07280	40S ribosomal protein	Ribosome biogenesis	-0.391769	0.87409	0.704391
19	A	31	6.6	P36010	Nucleoside diphosphate kinase	Response to DNA damage stimulus	-0.721473	0.803665	-0.832114
94	A	20	5.6	P36010	Nucleoside diphosphate kinase Ndk1	Repair of UV radiation- and etoposide-induced DNA damage	-	-	-0.328555
9	A	24	7.8	P40303	Proteasome component PRE6	Proteasomal ubiquitin-dependent protein catabolic process; regulation of mitotic cell cycle	-0.681787	-0.681787	-
10	B	25	8.1	C6A011	UPP0107 protein TSIB_1943	Phosphorylation	-0.38004	-0.441773	0.757425
179	A	32	5.9	P40825	Alanine-tRNA ligase, mitochondrial	Protein biosynthesis	-	-0.327165	-0.327165
40	A	52	5.6	P16140	V-type proton ATPase subunit B	Phosphoprotein	-0.584401	0.873055	0.853564
36	C	45	6.1	P10507	Mitochondrial-processing peptidase subunit beta	Phosphoprotein	0.951766	0.832967	-0.656623
26	B	35	5.7	P64201	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	Protein biosynthesis	0.795523	0.928544	0.908495
32	C	42	5.5	Q10055	ATP-dependent RNA helicase fall	rRNA processing	0.618478	0.96849	-0.720532
128	B	41	6.5	P39954	Adenosylhomocysteinase	Phosphoprotein	0.607278	0.524697	-0.583788
117	A	36	7.0	P49089	Asparagine synthetase [glutamine-hydrolyzing] 1	Phosphoprotein	-0.543259	-	-0.543259
110	A	32	5.4	P38272	SWI5-dependent HO expression protein 3	Transport	-0.272577	-	-0.272577
189	A	97	6.9	Q8 MML5	Paxillin-B	Zinc ion binding	-	-0.584424	-0.584424

The numbering of the spots coincides with the numbering shown in Figure 1. The ratio of the accumulation of proteins quantified by NanoLC-ESI-Q-TOF was calculated using ImageMaster 7.0/GE using the control treatment as the reference. pI = isoelectric point.

30-min stress

At 30 min, certain proteins were overexpressed over 100 times compared to the control treatment, e.g., eukaryotic translation initiation factor 5A-1 (related to protein biosynthesis), protein disulfide-isomerase 1 (cell redox homeostasis), and thiazole synthase suicide enzyme (stress response and DNA damage tolerance).

Several PR-10 protein family members have been reported to hydrolyze RNA and this ribonuclease activity, when perceived by the cell, induces the synthesis of proteins required for the reduction of the associated damage. Thus, ATP synthase is mobilized to meet the ATP requirements of the cell, and cobalamin synthase is increased to provide a substrate for Krebs cycle activities (ATP synthase subunit beta, for transport; and cobalamin synthase, for zinc ion binding). Cobalamin, also known as vitamin B12, is used by mammalian cells in methylcobalamin and adenosylcobalamin, cofactors that are required for the methylation of homocysteine to methionine in the cytosol and for the conversion of methylmalonyl coenzyme A to succinyl coenzyme A in mitochondria, respectively (Zhao et al., 2013).

Under stress, the metabolism of the fungus *M. perniciosa* is strictly aerobic. Therefore, the preferred route of energy use is aerobic, using ergosterol derivatives that can be easily mobilized to the membrane (Huang and Tang, 2007). Glyceraldehyde-3-phosphate is oxidized to dihydroxyacetone phosphate by glyceraldehyde-3-phosphate dehydrogenase, and this reaction uses ATP and produces NADH (used directly in oxidative phosphorylation). Glyceraldehyde-3-phosphate dehydrogenase (spots 18, 102, 108, 111, 112, 121) is a key glycolytic enzyme that exists primarily in the cytoplasm (Hara et al., 2005), but when in the nucleus, it plays a role in gene transcription, DNA replication, DNA repair, and nuclear RNA export. Glycerol is a substrate for glycolysis, but during the aerobic production of ATP, it is considered a non-fermentable carbon source (Nevoigt and Stahl, 1997). The enzyme 6-phosphogluconate dehydrogenase (6PGDH; decarboxylating) (spot 132) catalyzes the rate-limiting NADPH-producing step in the pentose phosphate pathway. 6PGDH plays an important role in the tolerance against oxidative stress (Izawa et al., 1998).

We noted that once the cells perceive the damage, primarily involving oxidative stress, antioxidant defenses are activated (stress response proteins, heat shock protein HSS1, 78-kDa glucose-regulated protein homolog, and 14-3-3 protein homolog). One striking feature of the 14-3-3 proteins is their ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. This variety of interacting proteins allows 14-3-3 to play important roles in a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptosis, and cell cycle control (Fu et al., 2000).

Heat shock proteins (spots 24, 45, 48, 85, 87, 153, and 170) are molecular chaperones that are found in all organisms and are natural defenses against various types of stress, e.g., oxidative stress caused by drugs or environmental changes (Wandinger et al., 2008).

Oxidoreductases were observed as well (spots 18, 82, 108, 109, 111, 112, 120, 121, 132, and 148; Figure 1; Table 1). Oxidoreductases are involved in antioxidant defense, the maintenance of intracellular redox balance and the repair of damage caused by oxidative stress (Xu, 2005). Mur et al. (2004) observed that PR-10 gene expression was influenced by the presence of reactive oxygen species (ROS) and observed an overexpression of AoPR-10-GUS in transgenic *Arabidopsis thaliana* when the plants were exposed to salicylic acid, an important inducer of ROS, or in the presence of *Pseudomonas syringae* virulence factor. These

oxidoreductases may be expressed by the fungus to withstand the oxidative damage caused by TcPR-10, reducing the expression of TcPR-10 at 120 min. These results indicate that ROS have a potential negative influence on TcPR-10 expression. ROS, which are highly potent oxidants, are produced during normal metabolic processes or by an unfavorable environment. Within the cell, ROS react with macromolecules, including lipids, carbohydrates, DNA and proteins, to trigger molecular damage, such as DNA mutations, lipid peroxidation, and protein oxidation, eventually leading to cell death and the progressive aging of the organism (Heller and Tudzynski, 2011). Eukaryotic cells contain mechanisms to respond to and protect against stress conditions, including neutralization of the stress, pausing of the cell cycle, alterations in translation, and the repair of damage to apoptotic or necrotic pathways (Thompson et al., 2008). The stress induced in fungal cells by drugs such as TcPR-10 triggers some defense mechanisms against oxidative stress, of which antioxidants are relevant endpoints.

Also at 30 min, we observed the suppression of the expression of phosphoketolase, a probable stress response protein (reduced >100-fold). The two substrates of this enzyme are D-xylulose 5-phosphate and phosphate, and its three products are acetyl phosphate, D-glyceraldehyde-3-phosphate and H₂O (Ronne, 1995).

60-min stress

The entire synthesis process must proceed within 30 min with an intense production of ATP. The following proteins were induced (>100-fold): glyceraldehyde-3-phosphate dehydrogenase (oxidoreductase), ATP-dependent protease ATPase subunit HslU (heat shock stress response), ATP synthase subunit alpha (mitochondrial, ATP catabolic process), phosphoglycerate kinase phosphoprotein, S-adenosylmethionine synthase 2 (one-carbon metabolism), pyruvate kinase (carbohydrate metabolism), chaperone protein DnaK 5, (stress response), and acetyl-CoA acetyltransferase.

Acetyl-CoA acetyltransferase plays a major role in ketone body metabolism as a mitochondrial enzyme involved in both amino acid degradation and fatty acid oxidation. It catalyzes the last step in breaking down isoleucine by converting 2-methyl-acetoacetyl-CoA into propionyl-CoA and acetyl-CoA. During ketolysis, this enzyme converts an acetoacetyl-CoA into two molecules of acetyl-CoA. Note that there is a cytosolic form of the enzyme (ACAT2) that can link two molecules of acetyl-CoA to acetoacetyl-CoA as an early step in ketogenesis and fatty acid biogenesis. We did not observe any suppression of the expression of this protein (reduced >100-fold) at 60 min.

120-min stress

At 120 min under the stress caused by the presence of TcPr-10, the fungus began the process of DNA replication/repair and the synthesis of new mRNA. We observed the activation of the pentose phosphate pathway to produce ribose and the repression of the fatty acid degradation pathway. At this time, the most highly induced (>100-fold) proteins were ubiquitin-conjugating enzyme (post-replication repair) and transaldolase of the pentose phosphate shunt (stress response).

Ubiquitin-conjugating enzymes, also known as E2 enzymes and more rarely as *ubiquitin-carrier enzymes*, perform the second step in the ubiquitination reaction that targets a pro-

tein for degradation via proteasome. The ubiquitination process covalently attaches ubiquitin, a small protein of 76 amino acids, to a lysine residue on the target protein. Once a protein has been tagged with a single ubiquitin molecule, subsequent rounds of ubiquitination form a polyubiquitin chain that is recognized by the 19S regulatory subunit of the proteasome, triggering the ATP-dependent unfolding of the target protein that allows its passage into the 20S core subunit of the proteasome, where the active sites of the protease degrade the target into short peptide fragments for recycling by the cell (Moudry et al., 2012). Transaldolase of the pentose phosphate shunt (stress response) is an enzyme (EC 2.2.1.2) of the non-oxidative phase of the pentose phosphate pathway (Trujillo et al., 2013).

Other proteins and functional analysis

At 30 and 60 min after treatment with TcPR-10, we identified two proteins related to the process of autophagy, GTP-binding nuclear protein and autophagy-related protein. Autophagy is the process by which intracellular compounds are transported to the lysosome and vacuoles for cellular degradation, and the subunits generated after degradation are then reused by the cell, thereby establishing a feedback mechanism that allows the maintenance of homeostasis (Yorimitsu et al., 2007). ROS are stress-inducing signaling molecules that are produced during autophagy (Scherz-Shouval et al., 2007); therefore, the action of the TcPR-10 protein on *M. perniciosa* may be related to the expression of the GTP-binding nuclear protein and autophagy-related protein in all treatments in an attempt to detoxify the cell. According to Pozuelo-Rubio (2010), autophagy is modulated by 14-3-3 proteins, which exhibited the highest expression in the control treatment (0 h) and decreased expression over time. Conversely, the highest expression levels of autophagy markers were observed at 60 min.

Among the proteins expressed by the fungus in response to TcPR-10-induced stress were proteins involved in the maintenance of fungal wall integrity against the oxidative damage caused by the antifungal protein. The alpha-1 subunit of the 26S proteasome is an ATP-dependent protease that prevents the accumulation of degraded proteins (Vierstra, 2009) and therefore provides greater resistance to oxidative stress. However, it is possible that the observed decrease in the expression of this subunit over time correlated with an increase in the amount of oxidized proteins.

The proteins in the largest class that responded to TcPR-10 exposure were involved in several mechanisms (Figure 3). Our functional analysis showed that the majority of differentially expressed proteins identified by MS were involved in stress response (29%) and cell metabolism (22%), indicating a fungal response to maintain homeostasis and suggesting possible mechanisms of action of TcPR-10 upon and after contact.

Several PR-10 proteins with antifungal activities have been described in the literature, for example, maize PR-10 proteins (Xie et al., 2010) and JcPR-10a (Agarwal et al., 2013). Pungartnik et al. (2009) investigated mutants of *S. cerevisiae* DNA repair, membrane transport, metal transport, and antioxidant defense genes to elucidate the ribonucleic and antifungal activities of TcPR-10 against *M. perniciosa* and *S. cerevisiae*. This study showed that TcPR-10 activities involving synthesis, modification or degradation may confer multiple biological functions to individual genes. The involved proteins were related to biosynthesis, carbohydrate metabolism, assembly, folding, translational regulation, and amino acid biosynthesis and transport. These modifications and their impacts on virulence, intracellular signal cascades and other biological processes represent potential areas for further investigation.

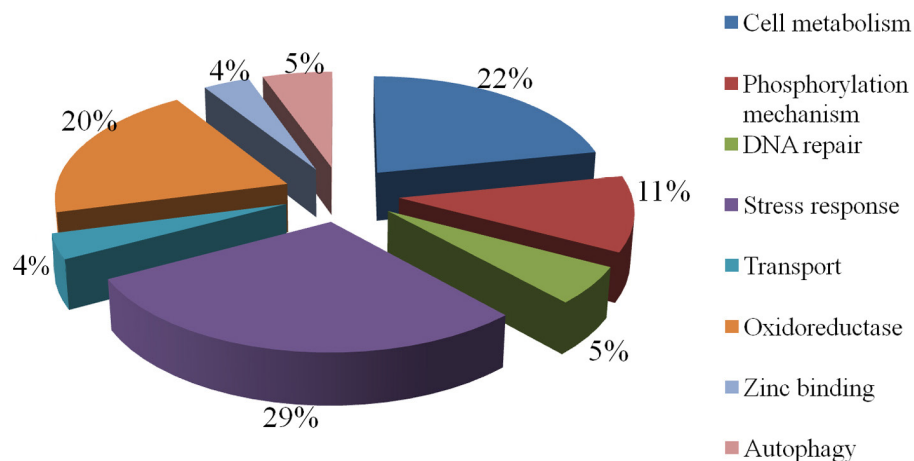


Figure 3. Representation of the distribution of differentially expressed proteins identified in *Moniliophthora perniciosa* according to biological process. Categorizations were based on information provided by the online resource UniProt classification system.

We observed that TcPR-10 induced interesting metabolic responses in the fungus at all times analyzed. There was a large increase in the abundance (% vol) of several proteins, with values above 100X compared with the control treatment. TcPR-10 has a strong ribonuclease action that induces a reaction in the fungus *M. perniciosa* in the initial attempt to eliminate the toxic effects of this antifungal compound. Thus, at the 30- and 60-min time points, corresponding to the initial period of stress, the major induced proteins are related to the mobilization of ATP and the activation of proteins with antioxidant action. At 120 min, the cells begin the process of repairing the damage caused by TcPR-10, which is degraded over time, activating the pentose phosphate pathway and repressing the fatty acid degradation process. Globally, the proteins identified in this analysis are activated to detoxify the cell and to generate energy to ensure its survival. Thus, we observed that TcPR-10 can overcome the resistance of the fungus, acting as an effective antifungal agent for the periods tested.

In summary, this study describes the first proteomic analysis of the phytopathogenic fungus *M. perniciosa* in response to the TcPR-10 protein of cocoa. The identification of proteins that are differentially expressed after treatment with TcPR-10 provides new information that may be used to unravel the molecular basis of the pathogenesis of these fungi in the presence of antifungal molecules. The findings in this study, namely of the expression of proteins involved in DNA repair, zinc binding and the ROS response and, in particular, stress response proteins, together demonstrate the great potential for TcPR10 to be used as an antifungal agent against *M. perniciosa*. Further research is required to analyze the behavior of the differentially expressed proteins at other time points and to identify additional proteins involved in the TcPR-10 response.

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