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Histological and proteomics analysis of apple defense responses to the development of *Colletotrichum gloeosporioides* on leaves

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ABSTRACT

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is an important fungal pathogen known to cause glomerella leaf spot (GLS). The objective of this study was to analyze the infection of *C. gloeosporioides* on leaves of apple cv. Fuji (resistant) and cv. Gala (susceptible) and apply proteomics techniques to study the apple defense responses 48 h after inoculation (h.a.i.). On both of cultivars, *C. gloeosporioides* started to germinate at 3 h.a.i. on adaxial surface and produced appressoria adhering to epidermal cell juxtapositions. Histological analysis showed more stratified parenchyma in leaves of cv. Fuji than cv. Gala associated with differences in the chemical composition of cell walls. Total and unique proteins expressed by cvs. Fuji and Gala at 3, 12, 24 and 48 h.a.i. were detected by comparative proteomes analysis. A total of 42 unique proteins expressed at 24 and 48 h.a.i. were identified by MALDI/TOF mass spectrometry, and most of these proteins were identified as directly involved in defense responses.

Introduction

Glomerella leaf spot (GLS), caused by the fungal pathogen *Colletotrichum gloeosporioides*, is currently one of the most important pathogens affecting apple industry in Brazil [1]. This disease causes defoliation and thereby reduces production. The use of chemical control has resulted in increased production costs and negative environmental externalities [2]. Thus, one of the current priorities of apple breeding in southern Brazil is related to the development of new apple varieties resistant to GLS, which would reduce the use of chemical pesticides and, consequently, reduce the impact on human health and the environment [3].

The incidence of GLS is favored by relatively high temperature (above 20 °C) and long periods of leaf wetness. Under such conditions, the symptoms may become visible 45 h post-infection [4]. However, when at temperatures as low as 12 °C, the symptoms may occur since the periods of leaf wetness are sufficiently long. In the process of infection, appressoria adhere to the host surface by emitting a germ tube and then colonizing the host tissue [5]. Depending on environmental or host conditions, *C. gloeosporioides* may not penetrate the host surface immediately after the formation of appressoria and the pathogen remains in a quiescent state until condition become favorable [6].

A known and efficient defense mechanism activated in plants after the recognition of a pathogen, is the synthesis of pathogenesis-related proteins (PR proteins). Several PR proteins have been identified, such as peroxidases, β-1,3-glucanases, chitinases class I and II, RNAses, among others [7]. Peroxidases are enzymes that play a role in lignification, suberization, cross-linking of cell wall structural proteins, and self-defense against pathogens [8]. Glucanases and chitinases are enzymes that hydrolyze β-1,3-glucans and chitine, respectively, the major components of cell walls of fungi and bacteria [9]. In apple plants, ulvan-induced resistance to GLS is associated with enhanced peroxidase activity [10], and an increased glucanases activity has been associated with resistance against fire blight [11].

Proteomics studies during plant–pathogen interactions are of great importance because most pathways used either by plants or

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pathogens are based on changes in the regulation of gene and protein expression [12]. It is possible to reveal post-translational modifications in proteins related to the plant immune system after triggering plant defense mechanisms during pathogenic challenge. Proteomics approaches based on 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technology combined with other analytical instruments, such as tandem mass spectrometry (MS, MS/MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), allow the establishment of proteome maps that explain specific aspects of host–pathogen interactions [13].

The present work aimed at to study the infection process of *C. gloeosporioides* on the apple leaves of cvs. Fuji and cv. Gala, as well as to perform a comparative histological and proteomics analysis of apple leaf tissue in response to the pathogen development.

Material and methods

Inoculation with Colletotrichum gloeosporioides isolate SJ197

Twenty plants of each cultivar grafted on M.7 rootstock were used for inoculation with the SJ197 apple pathogenic isolate of *Colletotrichum gloeosporioides*. This isolate was previously isolated from leaves with high incidence of GLS at EPAGRI Estação Experimental de São Joaquim (Santa Catarina State, Brazil – 28°16′33″S 49°56′4″W). Inoculum was prepared at 5.0×10^5 conidia/ml, and leaves were sprayed on their adaxial and abaxial surfaces with DeVilbiss n°5. Subsequently, the plants were transferred to an inoculation chamber at 25 °C (±1 °C) and 90% relative air humidity. Finally, five plants per treatment were selected randomly to collect material for analysis, in a total of five treatments (0 and 3, 12, 24 and 48 h.a.i.). As controls, five non inoculated plants of each cultivar were used.

Sample collection and preparation for microscopic analysis

Four leaf discs of 9 mm^2 were cut from the three upper leaves selected out of three plants of each treatment. The samples were fixed in 2.5% paraformaldehyde, 0.1 M phosphate buffer (pH 7.2), for 24 h at 4 °C. Subsequently, they were washed twice in 0.1 M phosphate buffer for 15 min each and then dehydrated through a series of washes with aqueous solutions of increasing ethanol concentrations (30%, 50%, 70%, 90% and 100%). The pre-infiltration was performed by immersing the samples in a pre-infiltration solution (100% ethanol, Leica® Historesin Embedding Kit, 1:1 v/v) for 15 h at 25 °C. Subsequently, the samples were immersed in pure Leica® infiltration solution for 24 h and finally included in the infiltration solution containing Leica® hardener (15:1 v/v) and oriented in gelatin capsules. Cross-sections 8 μm thick were cut with a Slee Technik® rotatory microtome. After distending on glass slides, the sections were stained for 60 s in aqueous solution containing 0.5% toluidine blue. Finally, the slides were covered with cover-slips and observed under light optical microscopy (OLYMPUS BX40).

Sample preparation for scanning electronic microscope (SEM)

Leaf discs were obtained following the same procedure described above. The leaf discs were fixed in 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2), for 24 h at 4 °C. Subsequently, the samples were washed twice in 0.1 M phosphate buffer (pH 7.2) for 15 min and dried through a series of washes with aqueous solutions of increasing acetone concentrations (30, 50, 70, 90 and 100% acetone). Samples were washed twice for 15 min. The total dehydration of samples was performed in a critical point Balzers CPD-030. Subsequently, the samples were mounted on stubs and coated with two layers of gold in an "Ion Sputter" Balzers-Union SCD-040. Finally, the observations were performed in a JEOL JSM-6390LV Scanning Electron Microscope (SEM).

Extraction and quantification of total protein from apple leaf tissue inoculated with C. gloeosporioides

The extraction of total proteins from leaf tissue was performed following the method of Carpentier et al. (2005) with modifications [14]. In brief, 0.1 g of leaf tissue collected from each of the selected seedlings per treatment was ground to powder with the aid of liquid nitrogen. The macerated material was homogenized with 1.0 ml of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 1.0 ml of buffer-saturated phenol (pH 7.8) by vortexing for 15 min. The homogenates were centrifuged for 15 min at 15,000 g, 4 °C. The phenolic phase was recovered and homogenized with 1.0 ml of extraction buffer by vortexing for 15 min. The homogenate was centrifuged for 15 min at 15,000 g, 4 °C. The phenol phase was collected. After precipitation with 100 mM of ammonium acetate in methanol (1:5 v/v), proteins were maintained 12–14 h at −20 °C. The tube was centrifuged for 10 min at 15,500 g at 4 °C. The pellet was washed three times with 0.5 ml of pure methanol and three times with 0.5 ml of acetone. Finally, the proteins were solubilized in 0.3 ml of solubilization buffer (7M urea, 2 M thiourea, 2 mM PMSF, 0.5% Pharmalyte® pH 3–10, and 2% Triton X-100) by mild vortexing and stored at −20 °C. Protein quantification was carried out in a UV-HALO DB20 (DYNAMICA) spectrophotometer, following the methodology proposed by Bradford et al. (1976), as modified by Ramagli & Rodriguez (1985) [15,16].

Two-dimensional gel electrophoresis (2DE)

Isoelectric focusing was carried out in strips of 13 cm, pH 3–10 at linear scale (GE Healthcare, 17-6001-14). The strips were rehydrated in 250 μl of rehydration buffer (7 M Urea, 2 M Thiourea, 2% Triton X-100, 2% CHAPS, 0.5% IPG buffer pH 3–10, 0.002% Bromophenol blue, and 1% DTT) containing 700 μg of protein for 12–14 h at room temperature. The strips were focused on an Ettan-IPGphor 3 isoelectric focusing unit (GE Healthcare). After IEF, strips were equilibrated for 20 min in equilibrium solution (75 mM Tris-HCl pH 8.8, 6M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS, and 0.002% (w/v) Bromophenol Blue) containing 1% (w/v) DTT, followed by 20 min in equilibration buffer containing 2.5% (w/v) Iodoacetamide. Electrophoresis was performed in polyacrylamide gels (12%) under denaturing conditions (375 mM Tris-HCl pH 8.8, 12% acrylamide, 0.32% bis-acrylamide, 0.1% SDS, 0.1% ammonium persulfate, and 0.04% TEMED). Gels were stained with Coomassie blue (1% Coomassie Blue G-250, 0.1 N H_3PO_4, 0.6 M $(NH_4)2SO_4$, and 20% methanol) and stored in 20% ammonium sulfate at 4 °C. The gels were scanned in ImageScanner® (GE Healthcare) and analyzed using ImageMaster 2D Platinum® software (v. 6.0). The bands were identified and selected based on the comparative analysis of the gels (Smooth = 6; Min. Area = 18; Saliency = 90.000). Bands unique to treatments and cultivars concomitantly were selected from the comparisons between gels of the treatments of each cultivar and between treatments among cultivars, simultaneously. For the analysis it was selected the highest-quality of three 2D gels generated by protein sample, giving a total of five comparative 2D gels per treatment.

Protein digestion and MALDI-TOF-MS analysis

Selected bands were manually cut out of the gels and destained with 1.5 ml of Decoloration Solution (50% Acetonitrile, 25 mM