



Proteomics changes during the incompatible interaction between cowpea and *Colletotrichum gloeosporioides* (Penz.) Penz and Sacc

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ABSTRACT

Anthraxnose represents an important disease of cowpea [*Vigna unguiculata* L. (Walp.)] caused by the hemibiotrophic fungus *Colletotrichum gloeosporioides* that drastically reduces cowpea field production. In this study we investigated some biochemical aspects underlying the incompatible interaction between a resistant cowpea genotype and *C. gloeosporioides* using a proteomic approach. Analyses of two-dimensional gel electrophoresis patterns and protein identification indicate *C. gloeosporioides* infection-dependent cowpea leaf proteome changes associated with metabolism, photosynthesis, response to stress, oxidative burst and scavenging, defense signaling, and pathogenesis-related proteins. Moreover the *C. gloeosporioides* responsive proteins interaction network in cowpea revealed the interconnected modulation of key cellular processes involving particularly antioxidants proteins, photosynthetic apparatus forming proteins and proteins of the energetic metabolism that interact with each other suggesting that their expression changes are also important for resistance of cowpea to *C. gloeosporioides*.

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1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important legumes for the human kind and has long been used as food particularly because its seeds provide vitamins, minerals, and good quality dietary proteins ranging from 23% to 32% of the seed dry weight, besides to be rich in lysine and tryptophan [1]. In addition, the leaves of cowpea are a good source of micronutrients like folates and minerals such as calcium (Ca), sodium (Na), potassium (K), phosphorus (P), iron (Fe) and zinc (Zn) [2]. Moreover, cowpeas can tolerate adverse conditions such as drought, high temperatures, poor soils [3], and salinity conditions [4] to a certain extent. These traits are in agreement with cowpea adaptation to the semi-arid regions of the tropics and subtropics. Unfortunately, cowpea field production is limited by a number of constraints including diseases caused by viruses, bacteria, fungi, nematodes, insects, and parasitic flowering plants [5,6].

Anthraxnose is the disease caused by fungi of the genera *Colletotrichum* and the species *Colletotrichum gloeosporioides* is one of the most important fungal pathogen of cowpea. The affected plants present reddish-brown spots on the leaf veins that spread to all organs in the late stage of the disease [7]. High humidity and warm weather are among the factors that contribute to the success of infection. This explains the high incidence of anthracnose in the tropics especially during the rainy season. In leaves, the infection process begins by spore germination and apressoria development, forming the infection structure at the infection site. Typical lesions on susceptible cowpea plant enlarge rapidly and coalesce to girdle stems, peduncles, petioles and shoot [7]. These symptoms are not visible in cowpea genotypes resistant to Anthracnose. Therefore, understanding the defense mechanisms underlying resistance is crucial in plant breeding programs toward the development of resistant plants, currently the most environment friendly and cost-effective way to manage plant diseases [8,9]. It is worth mentioning that most of the mechanisms of plant resistance to pathogens are associated to the differential expression of proteins that act as key molecules in defense responses to infection [10–12].

We have previously reported that the cowpea resistance to the hemibiotrophic fungus *C. gloeosporioides* is correlated, at least

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in part, to the hypersensitive response reaction (HR), H_2O_2 accumulation, enhanced phenylalanine ammonia lyase (PAL) activity with the consequent increase in phenolic compound deposition, enhanced cell wall lignification, and callose (papilla) formation during the early stages of infection [7]. Here, additional biochemical and molecular aspects underlying cowpea resistance were investigated by studying the leaf proteomic changes during the incompatible interaction of cowpea with *C. gloeosporioides*, at different time points post infection, using two-dimensional electrophoresis in association with ESI-TOF/TOF MS/MS, and database-searching for protein identification.

2. Materials and methods

2.1. Plant material

Seeds of a cowpea genotype (BR-3) resistant to *C. gloeosporioides* (LPVD-1 isolate) were obtained from The Brazilian Enterprise for Agricultural Research (EMBRAPA), Piauí, Brazil. Seeds were surface disinfected with 1% (v/v) hypochlorite (0.05% active chloride) for 3 min, rinsed exhaustively with distilled water and soaked in distilled water for 10 min. Seeds were germinated in Germtest® paper humidified under sterile condition. Three days later, germinated seeds were sown in 0.8 L pots (three per pot) containing water washed autoclaved (120 °C, 1.5 KGF, 30 min) river sand. The plants were kept in a growth chamber with a 12:12-h light–dark photoperiod, photosynthetic active radiation of (PAR) 200 $\mu\text{mol}/\text{m}^2/\text{s}$, at $32 \pm 2^\circ\text{C}$ day/ $27 \pm 2^\circ\text{C}$ night cycle and $70\% \pm 5$ relative humidity (RH). Plantlets were watered with sterile distilled water for up to 3 day after sowing and then watered with five times diluted Hoagland and Arnon [13] nutritive solution modified as described by Silveira et al. [4].

2.2. *C. gloeosporioides* inoculation

Inoculum was obtained from the local fungus collection at Federal University of Ceara, Brazil. To preserve its pathogenicity *C. gloeosporioides* was maintained in the leaves of a susceptible cowpea genotype (TE97) cultivated in a growth chamber under the same photoperiod, PAR, temperature range and RH described above. Once the disease symptoms were visible, the fungus was isolated from the infected leaves and cultivated on potato/dextrose/agar (PDA) medium in Petri dishes maintained in acclimatized room at $25 \pm 4^\circ\text{C}$. After 12-day-old cultures, in which the fungus had proliferated throughout the diameter of the Petri dishes, spores were taken with the aid of a Drigalski spatula and suspended in sterile water. The suspension was filtered through nylon mesh for hyphae retention, the spore concentration determined in a Neubauer chamber and adjusted to 2×10^6 spores mL^{-1} . This suspension was used to inoculate 12-day-old randomly selected cowpea plants. 50 μL spore suspension drops (2.5×10^6 spores mL^{-1}) were deposited on each side of the midrib of the adaxial surface of fully expanded primary leaves and gently spread over the leaf surface using a fine brush. In control plants, sterile water was used under the same conditions. After inoculation, RH was adjusted to $90 \pm 5\%$. 24, 48 and 72 h post inoculation (HPI), the primary leaves of control and inoculated plants were collected and stored at -80°C until used for protein extraction and proteome analysis. The experiment was performed to obtain three biological replicates for both the inoculated and the mock-inoculated group.

2.3. Protein extraction

This was performed as previously described by Yao et al. [14], with modifications. 2 g of fungus inoculated or control leaf

material were ground to powder in liquid nitrogen and 15 mL of 10% (m/v) TCA and 2% (v/v) β -mercaptoethanol in acetone was added for homogenization. After centrifugation at $15,000 \times g$, 15 min, 4°C , the supernatant was discarded, and the precipitate thrice washed with cold 2% (v/v) β -mercaptoethanol in acetone and centrifuged as above. The precipitate obtained was solubilized with 6 mL of 100 mM Tris–HCl pH 8 buffer, containing 30% (m/v) sucrose, 2% (m/v) SDS, 1 mM PMSF and 1 mM PVPP at 4°C for 10 min and centrifuged at $10,000 \times g$, 4°C , 10 min. To the resulting supernatant an equal volume of Tris–phenol was added and the mixture centrifuged as above. The upper phenol phase was withdrawn and 6 volumes of 0.1 M ammonium acetate/methanol solution were added and gently mixed. After incubation for 2 h at -20°C , the mixture was centrifuged ($15,000 \times g$, 15 min, 4°C) and the precipitate thrice washed with cold 80% (v/v) acetone and air-dried. This precipitate was dissolved in the hydration solution [7 M urea, 2 M thiourea, 2% (m/v) CHAPS, 65 mM DTT and 0.5% (v/v) IPG buffer, pH 3–10], the protein concentration measured [15] using a standard curve prepared with known concentration of bovine serum albumin (BSA) and absorbances read in a Genesys 10S UV-vis spectrophotometer (Thermo Scientific, USA). The protein samples (leaf extracts) recovered were stored at -20°C until used for proteome analysis.

2.4. Two-dimensional gel electrophoresis

The leaf extract containing 400 μg protein in 250 μL of 7 M urea, 2 M thiourea, 65 mM DTT, 2% (m/v) CHAPS, 0.5% (v/v) IPG buffer (pH 3–10) and 0.002% (m/v) bromophenol blue were applied on a pH 3–10, immobilized pH gradient (IPG) 13-cm strip (GE Healthcare) and incubated for 17-h at 25°C . The isoelectric focusing was performed on Ettan™ IPGphor 3™ apparatus (GE Lifesciences Piscataway, NJ, USA) using the following protocol (38 kVh total): 30 min at 200 V, 500 V (500 Vh), gradient to 1000 V (800 Vh), gradient to 8000 V (11,300 Vh), and 8000 V (22,900 Vh). For the second dimension, each strip was immersed in 5 mL of 50 mM Tris–HCl pH 8.8, containing 6 M urea, 20% (v/v) glycerol, 2% (m/v) SDS, 1% (m/v) DTT and 2.5% (m/v) iodoacetamide for 15 min. The strips were placed on the top of homogeneous SDS-PAGE gels (12.5%), sealed with agarose (0.5%, m/v) prepared in the SDS-PAGE running buffer and run at 250 V (20 mA/gel) in a Hoefer SE 600 system (GE Lifesciences, USA) coupled to a circulating bath set at 10°C , for 7 h. After electrophoresis, protein spots were stained with colloidal coomassie brilliant blue (CBB), as described by Candiano et al. [16] with minor modifications. 2D-PAGE gels were washed three times (20 min each) in a solution containing phosphoric acid (2%, v/v) and ethanol (30%, v/v), followed by three further washes in 2% (v/v) phosphoric acid and incubation in a solution composed of phosphoric acid (2%, v/v), ethanol (18%, v/v) and ammonium sulfate (15%, m/v), containing CBB G-250 (2%, v/v, final concentration). Reagents used for electrophoresis and gel staining were purchased from Bio-Rad Laboratories (USA), GE Lifesciences (USA) and/or Sigma–Aldrich (USA). The 2D analysis was repeated three times for each sample.

2.5. Image acquisition and differential analysis

2D gels were scanned at 300 dpi (ImageScanner II; GE Lifesciences, USA), saved as tagged image file format (.tiff) and analyzed with PDQuest software, version 7.3.0 (Bio-Rad, Rockville, MD, USA). Three replicates of cowpea leaf maps for each time point (24, 48 and 72 HPI) to both inoculated and control conditions were evaluated in match sets totalizing 18 gels from each sample type. The gel image with the greatest number of spots and the clearest pattern was chosen as a reference map and spots were then matched across all gels. Protein quantities were given as parts per million of the total integrated optical density of spots in the gels, according

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