Integrated Cytologic and Proteomic Analysis of Coffea Arabica – Hemileia Vastatrix Interactions

L. GUERRA-GUIMARÃES¹, A. VIEIRA¹, I. CHAVES², V. QUEIROZ¹,³, C. PINHEIRO², J. RENAUT⁴, L. SILVA¹, L. ZAMBOLIM⁵, C. RICARDO², M. D. C. SILVA¹

¹CIFC/BioTrop/IICT, Oeiras, Portugal
²ITQB/UNL, Oeiras, Portugal
³UFES, Alegre, Brazil
⁴CRP - Gabriel Lippmann, Luxembourg
⁵UFV, Viçosa; Brazil

SUMMARY

Coffee leaf rust, caused by the fungus Hemileia vastatrix Berk & Br., is the most widespread disease of Coffea arabica L. cultivars. Coffee – rust interactions are governed by the gene-for-gene relationship, being the resistance conditioned at least by nine major dominant genes (S₁H₁-S₁H₉) singly or associated. A cytologic and proteomic approach was used to study C. arabica – H. vastatrix compatible and incompatible interactions. In the incompatible interactions the first leaf cytological changes corresponded to hypersensitive host cell death (HR), observed in more than 50% of infection sites, 48h after inoculation. Protein multiplex two-dimensional fluorescence differential gel electrophoresis (Refraction-2D™), was performed with 30 µg of leaf protein in 13cm gel strips with immobilized pH 4-7 gradient. Differentially expressed polypeptides were found in the ranges of 4.8 to 5.6 pI values and 45 to 60 kDa MW. The identification of the proteins by matrix assisted laser desorption/ionization time of flight-mass spectrometry (MALDI – TOF/TOF MS) followed by homology search in several NCBI databases provided insights into the molecular characteristics of proteins specific for each of the interactions. With this methodology we found proteins that are potential candidates for resistance markers and will be further validated.

INTRODUCTION

Coffee leaf rust, caused by the fungus Hemileia vastatrix Berk & Br., is the most widespread disease of Coffea arabica L. cultivars, causing serious damage. Although fungicides can provide adequate control, breeding for resistance is environmentally and economically the most appropriate and sustainable strategy to fight this disease.

The resistance of coffee to H. vastatrix is predominantly post-haustorial (the fungus ceases its growth at different stages of the infection, but more frequently after the formation of the first haustorium), and is associated with rapid host cell death (hypersensitive reaction – HR). The early increase of activity of oxidative enzymes such as lipoxygenases (LOX), peroxidases (POD) and superoxide dismutases (SOD) as well as other enzymes (e.g. phenylalanine ammonia-lyase and chitinases) have also been detected during the early expression of resistance in some coffee genotypes [1,2,3,4]. In this study an integrative cytological and proteomic approach was used to identify apoplastic proteins that are potential candidate markers for resistance. To this purpose we used C. arabica genotype S4 Agaro inoculated with two different rust races to establish a compatible (susceptible) and incompatible
(resistant) interactions. At least 9 protein spots (associated with signal pathways, stress and defense and proteolysis) showed increased expression in the resistant sample, comparatively to control or susceptible samples.

MATERIALS AND METHODS

Biological material

Coffee plants (C. arabica L.) of the genotype S4H4S1S5 (S4 Agaro) grown in greenhouse conditions were inoculated with fresh urediospores of H. vastatrix, races II (v5) and XV (v4,5) establishing an incompatible (resistant) and a compatible (susceptible) interaction, respectively. The infected leaves were collected at different time points after inoculation.

Light microscope observation of fresh tissues

Pre-penetration fungal growth stages (germinated urediospores and appressoria formation over stomata) were visualized on leaf pieces, as previously described. For time course studies of fungal growth and plant cell responses, cross sections of infected leaf fragments, cut with a freezing microtome, were submitted to blue lactophenol staining and an epifluorescence test. Observations were made with a microscope Leica DM-2500 microscope equipped with a mercury bulb HB 100W, u.v. light and blue light.

Protein extraction

Protein from intercellular fluid (IF) of C. arabica leaves (healthy and inoculated) were obtained. The IF fraction was desalted and concentrated on centrifugal filter Vivaspin2 (Sartorius) followed by purification in PD SpinTrap G-25 column (GE Healthcare). Protein content was measured using a modified Bradford assay.

Proteomics analysis

Multiplex two-dimensional fluorescence differential gel electrophoresis analysis was performed using Refraction-2D™ labelling kit (DyeAGNOSTICS). Fluorescent protein labelling was performed using G-Dyes (G-100, G-200, G-300) according to the standard workflow of DyeAGNOSTICS. Three biological replicates were prepared for each sample. Each sample was dye-swap labelled, to avoid artefacts due to preferential labelling and the internal standard was created by pooling an aliquot of all biological samples. Protein samples (30 µg per sample) were run in 13 cm long IPG strips, pH 4–7 (GE Healthcare). IEF was performed using the Ettan IPGphor (GE Healthcare) for a total of 33000 Vh at 20 ºC and a maximum current setting of 50 µA per strip. After IEF, the SDS-PAGE was run using the Hoefer SE 600 Ruby apparatus (GE Healthcare) at 10 mA for 15 min and then at 20 mA at 20 ºC until the bromophenol blue dye front had run off the gel. After electrophoresis, Ref2D gels were visualized and scanned at 700V using the FLA-5100 Fluorescent Image Analyzer (Fuji Film). The image gel analysis was carried out using the Progenesis Samespot 2D software. The differentially expressed spots (relative to the control) were isolated from the gel and the proteins identified by matrix assisted laser desorption/ionization time of flight-mass spectrometry (MALDÍ – TOF/TOF MS), followed by homology search in several NCBI databases.
RESULTS AND DISCUSSION

The microscopic analyses, 24h after inoculation, revealed that the percentages of germinated urediospores (70-75%) and of appressoria formed on stomata (54-56%) were similar in both resistant and susceptible samples. The majority (70-80%) of appressoria gave rise to penetration hyphae and in the susceptible the fungus pursued its growth without apparent inhibition, contrarily to what occurred in the resistant sample. The first sign of resistance (detected 24h after the inoculation), was cytologically expressed by the hypersensitive plant cell death (HR), as monitored by cell autofluorescence and/or browning. This response began to be observed in only the guard cells or in both guard and subsidiary cells at the infection sites where the fungus reached the stage of appressorium or penetration hypha (Figure 1). Death of subsidiary and mesophyll cells invaded by a haustorium was observed from 48hpost-inoculation. At this time, more that 50% of infection sites exhibited dead cells. The HR, the common expression of resistance of gene-for-gene interactions, has been associated with many events, including activation of defence genes, production of reactive oxygen species (ROS), increase in activity of oxidizing enzymes and the synthesis of pathogenesis-related proteins (PR proteins) [2,3,4,10,11]. The multiplex technology (Ref2D) allows the relative assessment of differences in protein levels between two or more samples.

Figure 1. Resistant plant, light microscope observation, epifluorescence test (blue light). Infection site showing autofluorescence of guard cells (arrows) associated with a penetration hypha (h), 24h after inoculation; Ap= appressorium (bar = 10 µm).

However, this technology remains a challenging approach in non-model plants, since problems can arise at several points; particularly, artifacts related to inappropriate saturation labeling of protein spots. An additional problem we faced was the incomplete labeling of proteins of lower molecular weight (MW). Due to Ref2D technological specificities and the characteristics of the sub-cellular fraction (intercellular fluid), only a minimal fraction of the total cell proteome could be visualized. At 48h after infection it was possible to detect differentially expressed spots (pI from 4.8 to 5.6; MW from 45 to 60 kDa; Figure 2).

Multivariate analysis (principal components analysis, PCA) revealed a clear separation of control (healthy leaves), susceptible and resistant samples along the 1st axis, which explains 87.1% of the total variance (data not shown). The susceptible sample is closer to the control than to the resistant one. Considering all samples, a total of nine spots with a fold change threshold of 2.1 were selected as differentially expressed. The spots 1-5 were identified as different isoforms of aspartic proteinase nepenthesin-2 and the spots 6-9 as germin-like proteins, showing a higher expression in the resistant than in the control or susceptible samples (Figure 2).
The aspartic proteases superfamily is widely distributed in all living organisms and is one of the most important superfamilies of proteolytic enzymes. They are expressed in several plant organs and in the digestive fluids of carnivorous plants, being implicated in protein processing and/or degradation, in plant senescence, stress response, programmed cell death, and reproduction. Aspartic proteinase nepenthesin-2, has been associated to protein catabolism, folding, sorting and degradation and recently with rice resistance to sheath blight disease. The germin and germin-like proteins (GLP) family is a large and considerably heterogeneous group of proteins, expressed in several plant organs and developmental stages, and in response to a number of abiotic and biotic stresses. Three different enzymatic activities, oxalate oxidase (OxO), superoxide dismutase (SOD) and ADP-glucose pyrophosphatase or phosphodiesterase (AGPPase), have been associated with these proteins. Grapevine VvGLP3 is specifically induced in epidermal cells in response to powdery mildew infection; a proportion of the VvGLP3 protein is targeted to the extracellular space and shown to catalyze superoxide dismutation. These data suggest a potential role for VvGLP3 in the penetration-based defense response against powdery mildew infection. In Brassica napus, GLP are likely to participate in the Sclerotiorum sclerotiorum induced apoplastic formation of H$_2$O$_2$ and may act in concert with NADPH oxidases and peroxidases, enzymes known to execute the apoplastic oxidative burst in response to pathogen stress. The apoplastic localization of these proteins in combination with the H$_2$O$_2$ generating SOD activity offers a role in cell-wall fortification through the cross-linking of proteins and carbohydrates. Taken together, the cytological and the proteomic data reinforce the involvement of the apoplastic SOD activity and the enhanced cross-linking of cell wall components in the establishment of coffee resistance response. Further, this is the first time that proteinases, the aspartic proteinase nepenthesin-2, were referred as being associated with HR of coffee to leaf rust. Both enzymes are potential candidates for resistance markers and will be further validated.

Figure 2. Representative proteomics Refraction-2D analysis of the apoplast fraction from C. arabica leaves. Circled gel spots evidence intense protein expression, due to H. vastatrix infection. Spots 1-5 represent the aspartic proteinase nepenthesin-2 proteins and spots 6-9 the germin-like proteins. Insert boxes show detailed changes in protein accumulation for the different samples: R-resistant, S-susceptible and C-control.
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