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Biphasic haustorial differentiation of coffee rust (*Hemileia vastatrix* race II) associated with defence responses in resistant and susceptible coffee cultivars

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The objective of this study was to assess whether defence responses in coffee (*Coffea arabica*) were linked to a specific developmental stage of the rust fungus *Hemileia vastatrix*. Histological observations in compatible and incompatible high-yielding Brazilian coffee cultivars showed that the fungus produced 'pioneer' haustoria in adjacent and subsidiary stomatal cells soon after entering the stomata, followed by later developed 'secondary haustoria' which invade mesophyll cells. In the incompatible interaction between Race II and cv. Tupi, a strong and transient H_2O_2 generation at infection sites was detected at 39 h post inoculation (hpi) during secondary haustoria formation. In addition, clear-cut differences in defence gene expression between compatible and incompatible interactions were only observed during the secondary haustoria formation. Transcripts of the pathogenesis-related (PR) genes *CaPR1b* and *CaPR10* accumulated to maximal levels at 39 hpi (38- and 86-fold, respectively) in the incompatible interaction, but stayed at low levels in the compatible interaction. In contrast, the *CaWRKY1* gene and the *CaRLK* gene were only induced in the susceptible cultivar. These results indicated that the specific resistance of cv. Tupi was expressed after differentiation of the *H. vastatrix* secondary haustoria. Analysis showed no evidence of specific recognition of coffee rust at the pioneer haustoria stage, suggesting that haustoria components are not recognized by, or not secreted into, the subsidiary and adjacent cells of the stomata. Additionally, the present study provides new insights into the colonization process of the coffee rust fungus.

Keywords: *Coffea arabica,* pathogenesis-related genes, quantitative real-time PCR, reactive oxygen species

Introduction

Rust fungi are highly destructive parasites of cultivated plants. They are responsible for important yield losses in a variety of annual crops such as wheat, flax and barley, and also in perennial plantations such as pine, poplar and coffee. In general, the life cycle of rust fungi displays two parasitic stages: a dikaryotic one (aeciospore and urediniospore) and a monokaryotic one (basidiospore): generally the most important diseases are caused by the former (urediniospore) stage (Gold & Mendgen, 1991).

Rust fungi as obligate biotrophic pathogens depend on living host tissues for their growth and reproduction and form specialized structures called haustoria to allow intimate contact with living plant cells to obtain nutrients from host tissues. Rust fungi colonize tissues by spreading infection hyphae that form haustorial mother cells (HMCs), which are involved in cell wall penetration and the production of the haustorium. Through the invaginated plant cell plasma membrane, the haustorium coordinates the uptake of host water and nutrients, and also the signalling between host and parasite to establish and maintain compatibility (Voegele & Mendgen, 2003; O'Connell & Panstruga, 2006). Plant resistance against fungal colonization may occur before or after haustoria formation. Prehaustorial resistance that prevents formation of haustoria is very common in nonhost interactions (Heath, 1977). Posthaustorial resistance is often associated with resistance (*R*) gene-controlled specific recognition of the pathogen and is generally the rule in host plant-rust interactions (Heath, 1997). Posthaustorial resistance is typically expressed by a hypersensitive response (HR) after the formation of the first haustorium (Heath, 1997).

Recently, major insights have emerged from studies of haustoria-forming plant pathogens suggesting that

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the haustorium plays a critical role in delivering fungal effector proteins, including avirulence proteins, into the infected host cell (reviewed by O'Connell & Panstruga, 2006; Catanzariti *et al*., 2007). Among these was the avirulent protein AvrL567, from the flax rust fungus (*Melampsora lini*), which is recognized by the resistance proteins L5 and L6 of flax (*Linum usitatissimum*) triggering a necrotic resistance response (Dodds *et al*., 2004). Over the last few years, new Avr proteins were identified from haustorial extracts of flax rust (Catanzariti *et al*., 2006) and in other haustoria-forming pathogens (Armstrong *et al*., 2005; Ridout *et al*., 2006). Transgenic expression studies indicated that Avr proteins were recognized by disease resistance proteins inside host cells, suggesting that Avr proteins may be transported from the haustorium toward the host cell during infection (Catanzariti *et al*., 2006; Ridout *et al*., 2006). The time-course of haustoria formation may therefore be critical for specific host resistance responses to occur in particular plant-rust interactions.

The life cycle of *H. vastatrix* is essentially asexual, via cyclic urediniospore germination. In susceptible plants, the infection process has been well documented (Coutinho *et al*., 1993; Martins & Moraes, 1996; Silva *et al*., 1999). After appressoria differentiation over the stomata and penetration by means of an infection hypha, *H. vastatrix* develops a typical vesicle in an anchor shape with two, three or four lateral branches into the substomatal chamber. From each branch tip, a HMC is produced that gives rise to a haustorium which primarily infects the stomatal subsidiary and guard cells. Further on, the infection process involves colonization of mesophyll cells by intercellular hyphal growth, intense haustoria formation, and culminates in a large spore production in characteristic yellow-orange powdery lesions formed on the abaxial leaf surface.

The coffee rust interaction follows a 'gene-for-gene' model with nine plant resistance factors that are implicated in the recognition of the corresponding virulence genes in more than 45 rust races (Rodrigues *et al*., 1975). Histological observations suggested that *C. arabica* resistance may be expressed by a HR, with cell death of stomatal and mesophyll cells occurring at leaf infection sites associated with posthaustorial defence responses (Martins & Moraes, 1996; Silva *et al*., 2002). Construction of subtractive cDNA libraries, in studies aiming to investigate the molecular resistance responses associated with HR in *C. arabica*, allowed the identification of early expressed genes during coffee-rust interactions (Fernandez *et al*., 2004; Ganesh *et al*., 2006; Petitot *et al*., 2008). Among selected genes showing clear cut transcriptional changes was the *CaWRKY1* gene that displayed altered expression patterns in response to biotic and abiotic treatments (Ganesh *et al*., 2006; Petitot *et al*., 2008). *CaWRKY1* putatively encodes a WRKY protein, a family member of zinc finger-type transcription factors involved in the regulation of plant defence responses (Eulgem & Somssich, 2007). Other identified genes putatively involved in resistance signalling pathways were the *DSS6* gene encoding a receptor-like kinase (here renamed *CaRLK*), the *DSS22* gene encoding a salicylic acid-glucosyl transferase (here renamed *CaGT*) and the *CaR111* gene encoding a protein with yet unknown function (Fernandez *et al*., 2004).

One strategy for the development of cultivars resistant to coffee rust is the transfer of resistance genes from interspecific hybrids, via successive back-crosses into *C. arabica*. Accordingly, the most important hybrid between *C. canephora* and *C. arabica* is the Hibrido de Timor, a natural hybrid showing resistance to all rust races of *H. vastatrix* (Kushalappa & Eskes, 1989). Progenies from this hybrid originated the main *C. arabica* resistant varieties cultivated today (Rodrigues Jr *et al*., 2004). Among these varieties, the Brazilian high-yielding cultivar Tupi (IAC1669-33), commercialized since 2000, currently shows wide resistance against the races of coffee rust found in Brazil (Fazuoli *et al*., 2002).

The objective of this study was to assess whether defence responses in coffee resistance were linked to a specific developmental stage of *H. vastatrix*. This information is required to accurately determine the time-point of specific recognition of the rust pathogen by the coffee plant, which in turn should be useful for further isolating *H. vastatrix* avirulence components. Beside the resistant cv. Tupi (IAC1669-33), the *C. arabica* cv. Catuai (IAC 81), the susceptible parent of Tupi, was included in this work for comparison.

Materials and methods

Coffee plants, fungal isolate and inoculation

Brazilian high yielding *C. arabica* cultivars Tupi IAC1669-33 and Catuai IAC81 seeds were germinated in small plastic boxes (12 seeds per box) containing moistened vermiculite and kept in the dark in a growth chamber (25°C; 60% relative humidity (RH)). Three-week-old seedlings were transferred to soil pots and plants were kept in greenhouse conditions (24°C, 60% RH and 16 h light period). Urediniospores of *H. vastatrix* (race II) were received from the coffee rust collection maintained by the Centro de Investigação das Ferrugens do Cafeeiro (CIFC), Oeiras, Portugal. The race used in this study was previously purified by monospore isolation and characterized by its differential infection spectra on a set of 17 differential hosts as described in Rodrigues *et al*. (1975). For the rust assays, leaves of six-month-old plants were inoculated with freshly collected urediniospores (0·5 mg per leaf) of the *H. vastatrix* isolate CIFC 1427 (race II) by spreading with a camel hairbrush over the abaxial surface. Leaves were then sprayed with distilled water and the plants were kept overnight (*ca*. 18 h) in 50 L-plastic boxes in the dark, at 24 ± 1°C and 100% RH, to allow rust germination. After this period, plants were returned to standard conditions. At each collection time, germination of spores and appressorium formation were verified. Only assays with a germination rate above 80% were kept.

Evaluation of plant resistance

Coffee plant resistance to *H. vastatrix* was evaluated according to Eskes & Toma-Braghini (1981), who validated the method by comparing laboratory, nursery and field evaluations. Here, leaf discs (1·8 cm diameter) removed with a cork borer, were taken from healthy full-grown leaves and kept in plastic boxes on sterilized foam moistened with water. Each leaf disc was inoculated with one droplet of 0·025 mL *H. vastatrix* spore suspensions (1 mg spores per mL). Boxes were closed with a transparent glass cover and kept at 24°C without illumination. Glass lids were removed after 24 h to allow for evaporation of the infection droplets. Afterwards, discs were slightly wetted again and further incubated at approximately 1000 lx intensity of artificial light, with 12 h light period, 22 ± 2°C and 100% RH. Evaluation of the reaction type was made 50 days after inoculation (dai) using a 0 – 4 scale: 0 = immune; 1 = resistant; 2 = moderately resistant; 3 = moderately susceptible; 4 = susceptible. Each treatment was composed of twelve discs (one disc per plant) with four replications.

Microscopic observations

Germination of urediniospores and formation of appressorium were observed on small inoculated leaf pieces (2 cm^2), stained with fluorescent brightener (Sigma F-6259) for 5 min and examined under UV light with a Leitz Dialux 20 microscope equipped with a mercury bulb HB 100 W. Fungal structures exhibited a bright fluorescence at 435 nm.

Evaluation of fungal growth in the host tissues was performed using transverse cross sections of infected leaves made with a freezing microtome HM520 (Thermo Fisher Scientific) (Silva *et al.*, 1999). Fragments (18 μm thick) were stained in a 70°C warmed solution of cotton blue lactophenol (0·5%) for 2 min, washed twice in distilled water and mounted in equal volumes of glycerol/water (v/v). Observations were made with a microscope DMRXA (Leica).

Fungal stages inside leaf tissues were recorded from 25 to 40 infection sites per experiment, at 24, 30 and 36 h post inoculation (hpi), with three replications (plants). An infection site was defined as stomata with a fungal structure inside. The experimental procedure adopted for data analysis was a factorial survey approach (3 × 2) with the factors 'stages of development' and 'hours after inoculation' being analyzed by analysis of variance (ANOVA). Arcsine-transformed percentages and Tukey's test were used for the statistical analysis. All analyses were performed with the SISVAR 4·6 software (Ferreira, 2000).

Haustoria isolation

Haustoria were isolated from infected leaf tissues using the first steps of the procedure of Puthoff *et al.* (2008) except that the foliar mixture was directly filtered through a 100-μm sieve. After centrifugation, isolated haustoria in suspension were directly examined with a light microscope without further column purification. Non-inoculated leaves were used as controls. Auto-fluorescence of fungal tissues was used to assess the presence of haustoria.

Detection of H_2O_2

Detection of H_2O_2 was carried out using 3·3-diaminobenzidine (DAB) as substrate (Thordal-Christensen *et al.*, 1997) with minor modifications. At specific time-points after inoculation, leaves from resistant and susceptible cultivars were detached and placed in a solution containing 1 mg mL^{-1} DAB (pH 5·8) for 1 h after a brief vacuum infiltration. Leaves were cleared in 95% boiling ethanol for 30 min and stored in 50% ethanol. For microscopy, cleared leaf segments (2 cm^2) were mounted on glass slides in glycerol/water (v/v) solution and examined with a microscope DMRXA (Leica). Oxidation of DAB leads to its polymerization and deposition at sites of reactive oxygen species (ROS) generation as a reddish-brown staining. The number of stomata showing DAB staining was counted on two leaves per plant, with three plants for each cultivar for each time-point. The experiment was repeated twice at one-month intervals.

Kinetics, RNA extraction and RT-PCR

Inoculated leaves of cvs Catuai and Tupi were collected at 18, 21, 24, 36, 39, 42 and 45 hpi, immediately frozen by immersion in liquid nitrogen and stored at −80°C until RNA extraction. For each time-point, four leaves (one leaf per plant) were collected. Another four plants per time-point, kept in the same conditions as the inoculated plants, were mock-inoculated and used as control. Total RNA was extracted from leaf samples using the RNeasy Plant kit (Qiagen), with addition of an on-column DNase I digestion. First-strand cDNAs were synthesized from 1·5 μg of total RNA in 20 μL final volume, using Omniscript RT kit (Qiagen) and oligo-dT(18)-MN primer (Eurogentec) following the manufacturer's instructions.

Real-time quantitative PCR assays of gene expression

cDNA sequences of selected coffee genes were retrieved from an EST database (Fernandez *et al.*, 2004). Specific primers were designed from cDNA sequences using the Beacon Designer 5·0 software (Premier Biosoft International), with melting temperatures (Tm) of 58° ±5°C, primer lengths of 18 to 25 bp, and amplicon lengths of 75 to 200 bp (Table 1). Primers (reverse - R and forward - F) were synthesized by Eurogentec and used at 200 nM final concentration, with a tested efficiency ranging from 0·90 to 1·10 (data not shown). Real-time quantitative PCR was carried out on 1·25 ng cDNA in a 25 μL amplification mixture containing MESA GREEN Master Mix Plus

Table 1 Higher significant homologies (BlastX) and primer sequences of genes in coffee (*Coffea arabica*) evaluated by quantitative real-time PCR

Gene	Accession number	Best BlastX	Primer sequence (5′-3′)[a]
CaPR1b	DQ335594	PR1b (At2g14610) [*Arabidopsis thaliana*]	F- GATTACCTGGACGCCCATAA R- GCTGCCAGGTTTTCTCCATA
CaPR10	CF589103	CAC16166.1 PR10 [*Vitis vinifera*]	F- GCCACCATCCTTGAAGAGAA R- CAACTCTCTGCTTGGCAGTCT
CaR111	CF589193	AT5g12010/F14F18_180 hypothetical protein [*Arabidopsis thaliana*]	F- TCCAAATCGCTTCGACACC R- GTTGCGGTTTGTATATGGAGATTG
CaWRKY1	CO773974	AtWRKY6 (At1g62300) [*Arabidopsis thaliana*]	F- TGCAACAAGGACAGCACCAG R- CGTGATCGCGGCCGT
CaRLK	CF589181	gb|AAO42089.1| putative receptor protein kinase [*Arabidopsis thaliana*] (At5g25930)	F- ATGGGAGAAAAGAATGGCAGAAG R- GGCCAATTACAGTTTGAAAACACC
CaGT	CO773975	gb|AAF61647.1| UDP-glucose:salicylic acid glucosyltransferase [*Nicotiana tabacum*]	F- ACTCCAGCAACAACCACCATTA R- GAGACGTCTTGCAAGGTTTTGA
CaUbiquitin	AF297089	gb|AAF70460.1| polyubiquitin [*Populus tremula* x *Populus tremuloides*]	F- AACATTGAGGGTGGTTCTGTTC R- GCAGAAAACCAACTAAGACCTAACAA

[a]F: Forward; R: Reverse.

for SYBR Assay NO ROX (Eurogentec). The cycling conditions comprised 5 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s, 60°C for 20 s and 72°C for 40 s. Each assay was conducted in duplicate and included a non-template control. The *CaUbiquitin* gene (R. Rojas-Herrera, Centro de Investigacion Cientifica de Yucatan, Merida, Yucatan 97200, Mexico, personal communication) was chosen as the internal constitutively expressed control (normalization). The specificity of PCR products was checked by a melting curve analysis. Gene expression quantification was performed using the comparative Ct method as described in Ganesh *et al*. (2006). Relative quantification relates the PCR signal of the target transcript in the infected sample to that of the mock-inoculated sample at each time.

Results

Plant resistance evaluation

Response of cvs Tupi and Catuai to *H. vastatrix* race II was evaluated by recording rust urediniospore production on leaf discs (Fig. 1 and Table 2). The susceptible cv. Catuai showed the usual development of symptoms, with pale yellow lesions appearing on the discs about 15 days post infection (dpi), and production of urediniospores in lesions beginning at 19 dpi. About 94% of the inoculated discs showed lesions, and 100% of the lesions produced urediniospores 50 dpi (Table 2): cv. Tupi showed complete resistance to the tested isolate, exhibiting an immune reaction type (*i*) without any visible macroscopic lesion (Fig. 1).

Rust race II development in coffee leaves

By 18 hpi, 90% of urediniospores germinated, and an average of 50% formed appressorium over stomata (data not shown). By 24 hpi, the pathogen successfully entered into the stomata, reaching the substomatal chamber by a penetration hypha in about 22% of the infection sites and developing an anchor-shaped substomatal vesicle in *ca.* 15% of the cases in Tupi, and 10% in Catuai. At this time, no haustorium originating from an anchor HMC was detected (Table 3).

Careful examination revealed the presence of haustoria inside the subsidiary and adjacent cells of the stomata in a number of infection sites for the two coffee cultivars at 24 hpi (Fig. 2). Results showed

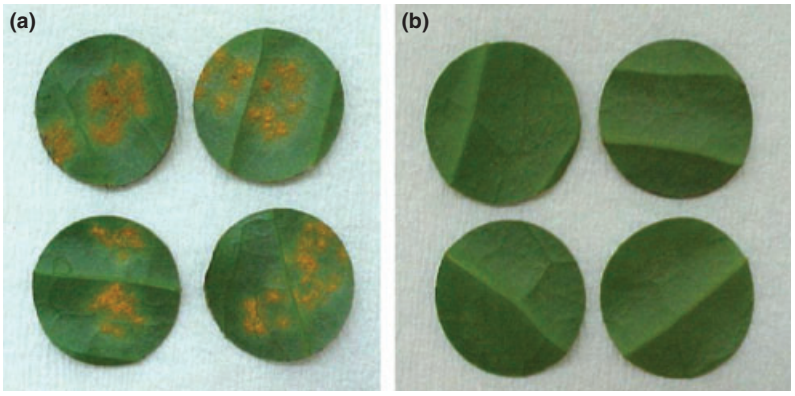

Figure 1 *Coffea arabica* leaf-discs 50 days post-inoculation, inoculated with *Hemileia vastatrix* race II showing (a) urediniospores on abaxial epidermis of the susceptible cv. Catuai (compatible interaction) and (b) resistant reaction type of cv. Tupi (incompatible reaction).

Table 2 Leaf-disc assay for evaluating resistance levels to coffee rust (*Hemileia vastatrix*) in *Coffea arabica* cvs Catuai (susceptible) and Tupi (resistant)

			Discs with lesion						Sporulation discs									
			Days after inoculation						Days after inoculation									
Cultivar	Replication	Number of discs	15	18	19	22	24	30	15	18	19	22	24	26	30	32	50	Reaction type[a]
Catuai	1	12	2	6	10	10	10	10	0	0	1	4	7	10	10	10	10	4
	2	12	3	9	9	11	11	11	0	0	0	3	6	10	10	10	11	4
	3	12	5	11	11	12	12	12	0	0	1	5	10	11	11	11	12	4
	4	12	5	10	11	11	11	12	0	0	0	4	8	10	11	11	12	4
Tupi	1	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Scale of 0–4, with 0 = immune and 4 = susceptible.

Table 3 Percentage of infection sites for each stage of *Hemileia vastatrix*, race II growth in leaves of *Coffea arabica*, cv. Tupi resistant (R) and cv. Catuai susceptible (S) at different time-points post inoculation

	Tupi (R)			Catuai (S)		
Fungal stages	24 hpi[a]	30 hpi	36 hpi	24 hpi	30 hpi	36 hpi
Pioneer hypha	36·5 (6·8)[b] a[c]	8·8 (3·9) b	0·0 (0·0) c	34·4 (5·4) a	8·7 (4·2) b	0·0 (0·0) c
Pioneer haustorium	26·2 (0·5) a	27·6 (8·5) a	11·3 (3·4) b	33·9 (3·0) a	34·7 (2·4) a	8·2 (3·7) b
Penetration hypha	22·7 (4·1) b	33·1 (3·3) a	8·3 (2·2) c	21·6 (3·9) ab	29·0 (4·2) a	14·1 (5·4) b
Anchor with HMC[d]	14·6 (2·8) b	20·4 (6·6) b	59·8 (10·1) a	10·1 (3·3) b	19·1 (9·2) b	52·8 (4·2) a
Secondary haustorium	0·0 (0·0) c	10·1 (4·2) b	20·5 (5·9) a	0·0 (0·0) c	8·7 (3·9) b	24·9 (2·8) a

[a]hpi: hours post inoculation.
[b]Standard errors of the means are in parentheses.
[c]Values for each fungal stage followed by different letter are significantly different according to Tukey's test ($P < 0·05$).
[d]HMC: Haustorial mother cell.

that haustorium production occurred as soon as the fungus entered into the stomata, and probably before reaching the substomatal cavity. After appressorium formation, the pathogen differentiated an infection hypha, named here 'pioneer' hypha, which formed haustorial mother cells (HMC) from which 'pioneer' haustoria were produced in the subsidiary or adjacent cells of the stomata (Fig. 2).

Subsequently, growth stages at each infection site were observed and identified as pioneer hypha, pioneer haustorium, penetration hypha, anchor with HMC and secondary haustorium (Fig. 3). At each infection site, only the most advanced stage was recorded.

By 24 hpi, the most frequent stages were pioneer hypha and pioneer haustorium (Table 3) which accounted for 63% of the observations in the resistant plants and 68% in the susceptible plants. The pioneer infection hypha was observed in a perpendicular orientation to the leaf surface (Figs 2d, 3).

The 30 hpi time period appeared to be a transition phase in the fungal development between penetration of stomata and mesophyll. Most of the infection sites (mean of 63%) displayed the pathogen in the stages of either pioneer haustoria or penetration hypha. The number of sites with *H. vastatrix* at the stage of pioneer hypha was fourfold lower than at 24 hpi. In one of five infection sites, the fungus had developed an anchor with HMC and haustoria were detected inside mesophyll cells in *ca.* 10% of sites. These haustoria were named secondary haustoria to distinguish them from pioneer haustoria.

By 36 hpi, the percentage of infection sites in the stage of anchor with HMC reached 53 and 60% in the compatible and incompatible interactions, respectively. The pioneer hypha stage was not observed at this time. The low number of infection sites with a pioneer haustorium and penetration hypha (9·5 and 11%, respectively) indicated that the fungal development occurred within the mesophyll. At 36 hpi, *H. vastatrix* secondary haustoria in the mesophyll of resistant and susceptible cultivars occurred in 21 and 25% of sites, respectively.

Statistical analysis was carried out on the percentage of infection sites for each stage of rust growth in leaves of cvs Tupi and Catuai at different time-points post inoculation (Table 3). No significant differences were observed in the fungal development between leaves of resistant and susceptible coffee cultivars until 36 hpi. Significant differences ($P < 0·05$) were verified between time-points (24,

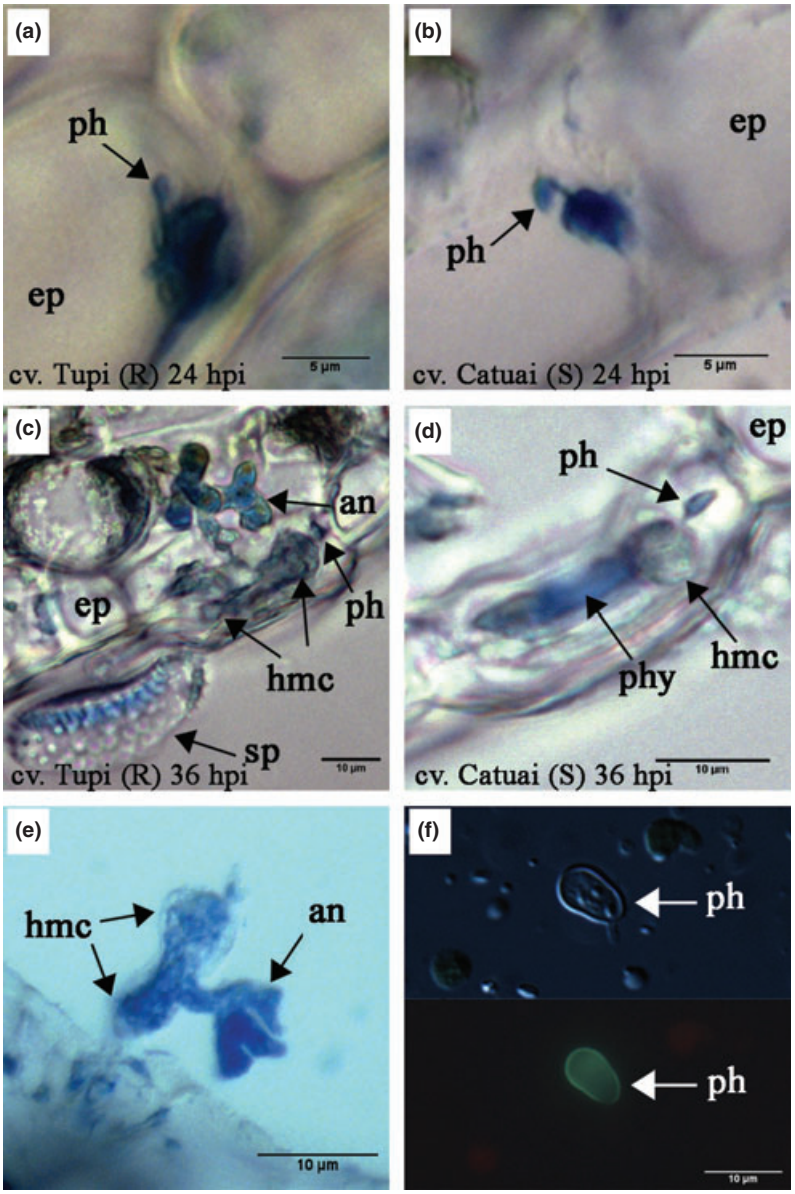

Figure 2 Infection structures of *Hemileia vastatrix* race II (a-e) and isolated pioneer haustorium (f) in *Coffea arabica* leaves. Haustorial mother cell and pioneer haustorium formed 24 hpi in an adjacent cell of a stomata in (a) the resistant cv. Tupi and (b) the susceptible cv. Catuai. (c) Haustorial mother cells and pioneer haustorium developed at the stomatal level; an anchor shape structure is localized in the substomatal cavity of the resistant cultivar at 36 hpi. (d) Pioneer hypha, haustorial mother cell and pioneer haustorium infecting a stomata subsidiary cell in the susceptible cv. Catuai. (e) Infection structures of *H. vastatrix* isolated from a coffee leaf at 36 hpi. (f) Pioneer haustoria isolated from leaves of the resistant cv. Tupi at 24 hpi, visualized with a light microscope (above) and exhibiting autofluorescence under UV-light (below). ph: pioneer haustorium; ep: epidermis; an: anchor; hmc: haustorial mother cell; sp: urediniospore; phy: pioneer hypha.

30 and 36 hpi) for each fungal stage in both cultivars, revealing the evolution of the infection process in the leaves (Table 3).

Isolation of haustoria

To validate histological observations on leaf cuttings, a quick procedure was used to isolate haustoria from 24 hpi infected leaves. Haustoria suspensions were obtained from Tupi and Catuai-inoculated leaves. The suspensions were highly enriched in chloroplasts with a few remaining fungal spores and hyphae. Isolated haustoria with bean-shaped or lobed bodies, with the haustorial neck still connected to the body, exhibited autofluorescence under UV-light and presented internal granular cytoplasmic content (Fig. 2f).

Histochemical detection of H_2O_2

The DAB staining method was performed on inoculated coffee leaves to determine if H_2O_2 accumulates in response to rust infection, since this is often associated with HR. Accumulation of H_2O_2 was detected in only a few subsidiary cells from 15 hpi and no differences between incompatible and compatible interactions were detected until 39 hpi (Fig. 4). At 39 hpi, intense DAB staining was observed in stomatal cells (both guard and subsidiary) and in adjacent epidermal cells of the resistant cv. Tupi (Figs 4, 5b). The number of coloured stomata increased to almost 200 per cm^2. In addition, DAB-stained reddish-brown chloroplasts were observed inside cells from the entire surface of inoculated leaves of cv. Tupi (Fig. 5a). Accumulation of H_2O_2 during the incom-

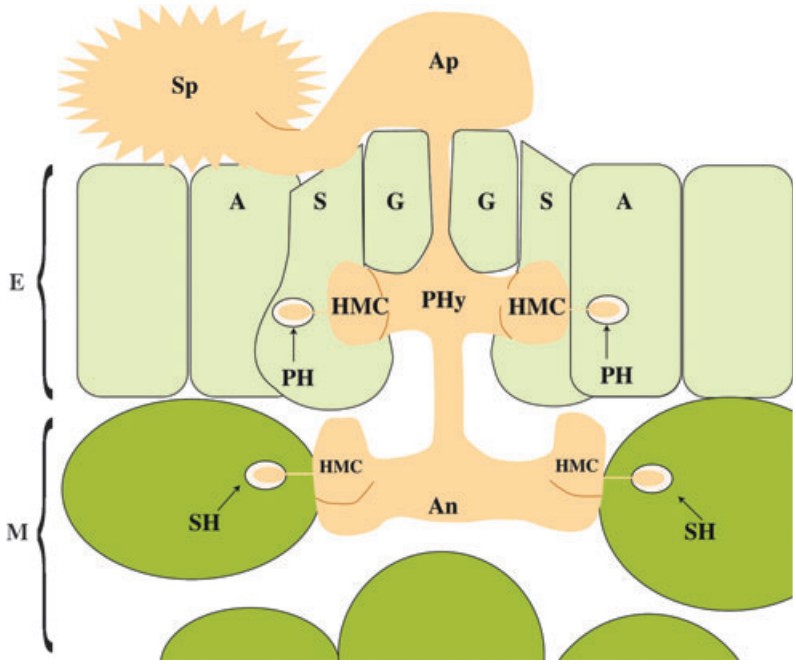

Figure 3 Diagram illustrating the early development of the dikaryotic stage of the coffee rust (*Hemileia vastatrix*). After urediniospore (Sp) germination and appressorium formation (Ap), the pathogen differentiates an intercellular pioneer infection hypha (PHy), which forms haustorial mother cells (HMC). From the latter, pioneer haustoria (PH) are formed inside the subsidiary (S) and adjacent (A) cells of the stomata. When infection arrives in the mesophyll (M), *H. vastatrix* develops a typical vesicle in an anchor shape (An) into the substomatal chamber. From each branch tip of the anchor, a HMC is formed from which a secondary haustorium (SH) invades the mesophyll cells. E: epidermis; G: guard cells.

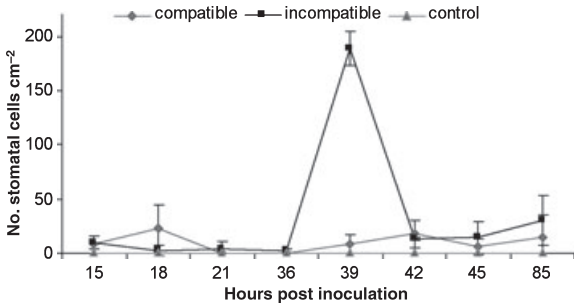

Figure 4 Number of stomata cells per cm^2 of *Coffea arabica* exhibiting H_2O_2 accumulation after inoculation with *Hemileia vastatrix* race II. At indicated time-points after inoculation, leaves from the resistant and susceptible varieties of *C. arabica* were detached and placed in a solution containing 1 mg mL^{-1} 3·3-diaminobenzidine (DAB) Oxidation of DAB leads to its polymerization and deposition as a red-brown stain at sites of reactive oxygen species generation. At each time-point, mock-inoculated leaves were used as control. Bars represent standard deviation (n = 3). Repeated experiments produced similar results.

patible interaction was transient, as the number of DAB-stained cells decreased (to around 20) by 42 hpi (Fig. 4). It was not possible to make observations directly into the mesophyll cells. In the susceptible cv. Catuai, no change in stomatal H_2O_2 accumulation was observed along the time-course studied.

Molecular analysis of host response

Molecular responses associated with haustoria differentiation were monitored by real-time RT-PCR of the mRNA levels of a set of defence-related genes at the time-points of pioneer (18, 21 and 24 hpi) and secondary (36, 39, 42 and 45 hpi) haustorial formation. The defence-related genes selected included *CaWRKY1*, *CaR111*, *CaGT* and *CaRLK* (Table 1). In addition, two genes putatively encoding pathogenesis-related proteins *CaPR1b* and *CaPR10* were chosen based on their specific expression in several plant-pathogen interactions (van Loon *et al.*, 2006).

Expression of genes was monitored in inoculated leaves and mock-inoculated leaves every 3 h at time-points corresponding to pioneer (Fig. 6a) and secondary (Fig. 6b) haustoria formation. The *CaUbiquitin* was used as internal control gene and was assayed in parallel with the defence-related genes.

During pioneer haustoria differentiation, a moderate to high activation of all genes occurred in rust-challenged leaves of both cultivars, indicating that the coffee plant fully responded to infection (Fig. 6a). Between 18 and 24 hpi, *CaPR1b* and *CaPR10* transcripts were strongly accumulated, peaking at 70- and 110-fold, respectively, in leaves of both resistant and susceptible cultivars, compared to control leaves. In the compatible interaction, the *CaPR1b* gene was induced earlier than in the incompatible interaction (18 and 21 hpi, respectively). The *CaPR10*

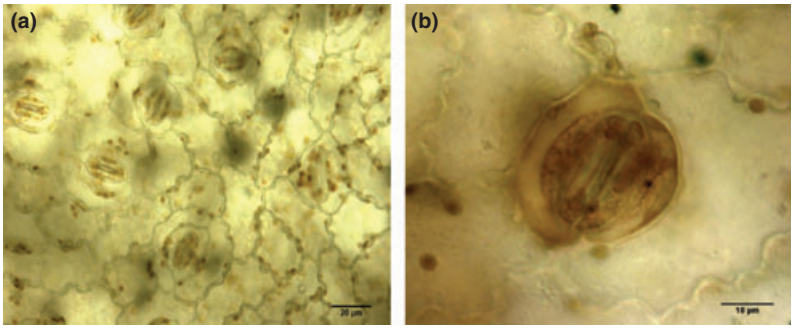

Figure 5 Subcellular localization of H_2O_2 in *Coffea arabica* leaves of the resistant cv. Tupi challenged with *Hemileia vastatrix* race II at 39 hpi. Detection of H_2O_2 was carried out using 3·3-diaminobenzidine (DAB) as the substrate. (a) Reddish-brown chloroplasts DAB-stained in epidermal cells. (b) Intense reddish-brown staining in the guard, subsidiary and adjacent cells of an infected stomata.

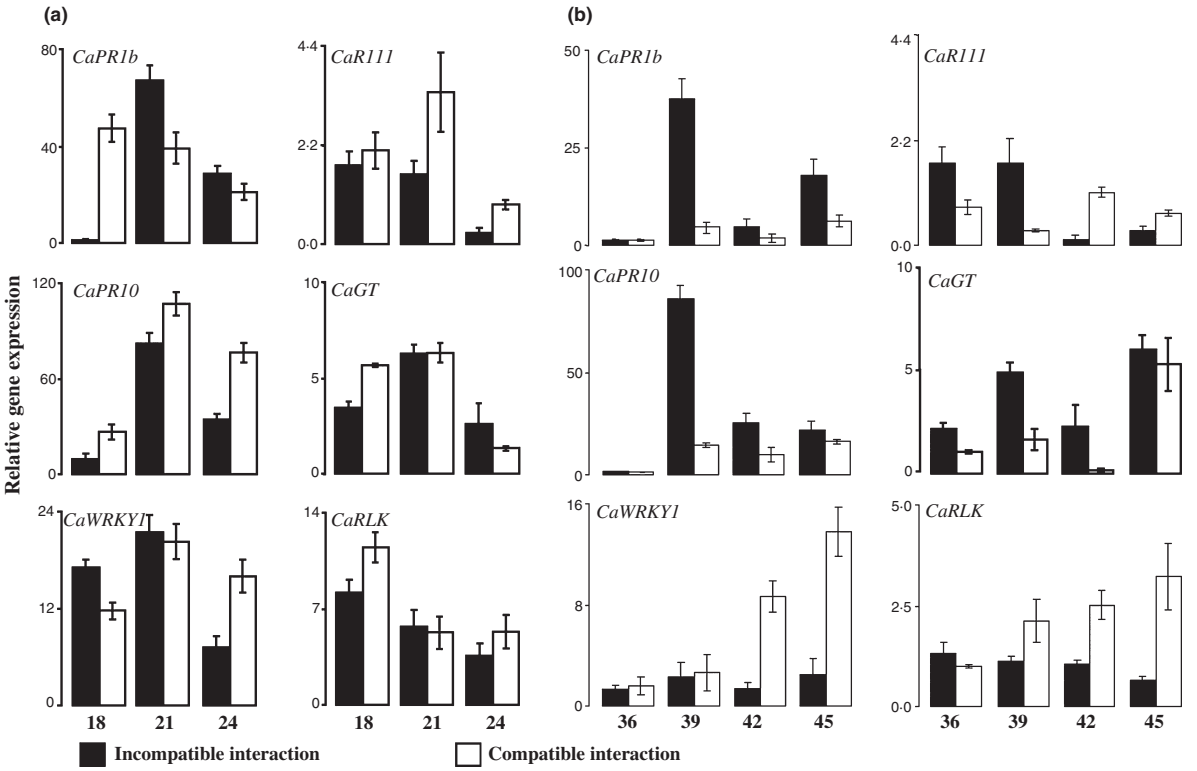

Figure 6 Quantitative real-time PCR analysis of the relative expression of *Coffea arabica* defence-related genes in leaves of cv. Tupi (resistant) and cv. Catuai (susceptible) challenged with *Hemileia vastatrix* isolate II, at the time-points (hours) of pioneer (a) and secondary (b) haustorium differentiation. Relative expression of each gene represents the ratio of the accumulated transcripts to *CaUbiquitin* transcripts used as internal control, by comparison to the mock-inoculated leaves (calibrator). Bars show the mean value ± SE.

gene showed a similar expression pattern in resistant and susceptible plants during pioneer haustorium formation. In the same way, the *CaWRKY1* gene was up-regulated in both interactions, displaying an average increase of mRNA levels of about 15-fold. The other genes peaked at 11- (*CaRLK*) and 6- (*CaGT*) whereas the *CaR111* gene displayed only a small and transient alteration of transcript accumulation.

At the time-points corresponding to secondary haustoria formation (36, 39, 42 and 45 hpi), resistant and susceptible cultivars presented remarkable differences in gene expression patterns (Fig. 6b). Transcripts of *CaPR1b* and *CaPR10* genes accumulated to maximal levels at 39 hpi (38- and 86-fold, respectively) in the incompatible interaction, but stayed at low levels in the compatible interaction. In contrast, the *CaWRKY1* gene and the *CaRLK* gene were only induced in the compatible interaction, the mRNA levels increasing gradually from 36 to 45 hpi (Fig. 6b). Changes in the *CaR111* mRNA levels were mostly undetectable, except for a marked suppression (8-fold) at 42 hpi in the incompatible sample. Finally, the *CaGT* gene remained moder-

ately activated (2- to 6-fold) throughout the incompatible interaction, but not in the compatible interaction from 36 to 42 hpi.

Discussion

In the early stages of infection, *H. vastatrix* race II was observed to have a different pattern of development than that observed for other rust fungi (Ferreira & Rijkenberg, 1989; Laurans & Pilate, 1999; Mendgen & Hahn, 2002; Moldenhauer *et al.*, 2006). Usually, the dikaryotic phase of rust fungi is characterized by a sequential growth that includes appressorium differentiation over stomata, indirect penetration via guard cells, formation of a substomatal vesicle (SSV), development of intercellular hyphae and differentiation of haustoria (Heath, 1997). Regularly, although with morphological differences depending on the rust species, SSV is formed into the substomatal chamber from the tip of the infection peg produced by the appressorium. Haustorial formation always occurs after differentiation of intercellular infection hyphae and HMC from the SSV (Hu & Rijkenberg, 1998; Ferreira & Rijkenberg, 1989; Laurans & Pilate, 1999; Rinaldi *et al.*, 2007; Moldenhauer *et al.*, 2008). The present study showed that *H. vastatrix* develops infection structures that seem to be unique among rust fungi. In host tissues, *H. vastatrix* race II differentiated haustoria in stomatal cells at a very early stage of plant infection, soon after entering into the stomata, and much earlier than previously described in the literature (Martins & Moraes, 1996; Silva *et al.*, 1999). After appressoria formation over the stomata, the pathogen differentiated a pioneer infection hypha with HMC that produced a pioneer haustorium in the subsidiary or adjacent cells of stomata, before penetration into the mesophyll.

This result suggests that *H. vastatrix* establishes a biotrophic interaction with its host within a few hours (< 24 h) after inoculation. Fungal growth was not arrested in the resistant variety after the pioneer haustoria were produced, instead *H. vastatrix* pursued similar development as in the susceptible cv. Catuai to form secondary haustoria inside mesophyll cells. These results are in accordance with previous observations in some other coffee-rust combinations (Rijo & Rodrigues, 1977; Martins & Moraes, 1996; Silva *et al.*, 2002) where the inhibition of fungal development was observed at higher frequency after haustoria were differentiated from the hyphal anchor inside the spongy mesophyll cells. Posthaustorial resistance is almost invariably observed in host plant-rust interactions (Heath, 1997; Hu & Rijkenberg, 1998; Rinaldi *et al.*, 2007; Moldenhauer *et al.*, 2008) and is characterized by the arrest of fungal growth after the formation of haustoria in mesophyll cells. Expression of resistance has not been detected in the first infection phases.

With the exception of *H. vastatrix*, all dikaryotic-stage rust fungi described so far preferentially form haustoria into the mesophyll cells, including the devastating *Puccinia recondita*, *P. striiformis, Melampsora lini*, *M. larici-populina* and *Phakopsora pachyrhizi*. (Koch & Hoppe, 1988; Kobayashi *et al.*, 1994; Hu & Rijkenberg, 1998; Rinaldi *et al.*, 2007; Moldenhauer *et al.*, 2008). Coffee leaf rust appears to be a remarkable model for leaf colonization, since stomata cells are being penetrated by haustoria very early before colonization of the mesophyll cells.

There is evidence that stomata are not passive openings for pathogen entry. In *Arabidopsis thaliana*, bacterially-induced stomatal closure is regulated by salicylic acid (SA), suggesting that stomatal defence is part of the SA-regulated immune system (Melotto *et al.,* 2006). Stomatal closure is also assumed to be part of the wheat innate immune system to delay the penetration of stripe rust fungus into leaves (Wang *et al.,* 2008). Thus, for pathogens passing by stomata, a successful infection might be first dependent on avoidance of guard cell defence machinery. Histological data obtained here suggests that *H. vastatrix* may overcome the stomata defence, not just passing through but also infecting subsidiary and adjacent cells of stomata.

In addition to these histological observations, intense DAB staining was verified at 39 hpi in the stomatal cells, surrounding epidermal cells and into chloroplasts of the inoculated epidermis of the resistant coffee cultivar. This suggests that major H_2O_2 production was triggered by the presence of the avirulent fungus in the mesophyll. Similar results were reported in other coffee rust post-haustorial incompatible interactions (Silva *et al.*, 2008). In the *C. arabica* cv. S4 Agaro × *H. vastatrix* race II interaction, accumulation of H_2O_2 and increase of peroxidase (POD) activity were detected 36–42 hpi (peaking around 40 hpi). The percentage of infection sites with POD activity decreased significantly at 48 hpi. Peroxidases use H_2O_2 for oxidizing various substrates and have been shown to be involved in plant resistance reactions to several pathogens. In other plant-rust interactions, only few data are available about the time-course accumulation of H_2O_2 (Tada *et al.*, 2004; Wang *et al.*, 2008). A rapid accumulation of H_2O_2 inside attacked and neighbouring mesophyll cells coincided with the development of haustoria of crown rust of oat and wheat stripe rust (Tada *et al.*, 2004; Wang *et al.*, 2008). In contrast with the coffee-rust interaction, H_2O_2 production in these cereal interactions was persistent, being detectable between 12 and 24 hpi for wheat stripe rust (Wang *et al.*, 2008) and between 36 and 60 hpi for crown rust of oat (Tada *et al.*, 2004). In the coffee-rust incompatible interaction, the strong and transient H_2O_2 release may be one of the first signals of successful pathogen recognition.

Interestingly, molecular responses of the host were detected concomitantly with *H. vastatrix* haustoria production suggesting that the plant was fully responsive to the fungus and that a molecular dialogue was being established between the host and pathogen. Remarkably, at the pioneer haustoria stage the defence-marker gene expression was not associated to specific detection of the avirulent fungus, since this was also activated in the compatible host response. In contrast, a distinct feature emerged at 39 hpi, during the secondary haustoria formation, where the

CaPR1b and *CaPR10* genes were only activated in response to the avirulent interaction, whereas the *CaWRKY1* and *CaRLK* genes only responded in the susceptible host. This differential gene expression response suggests that specific recognition by the host plant occurred during formation of secondary haustoria.

PR- proteins are part of the plant defence response machinery and *PR1b* is widely thought to be a molecular marker for the SA-dependent resistance signalling pathway in *A. thaliana* (Van Loon et al., 2006). The significant activation of the *CaPR1b* gene observed during rust infection in the resistant coffee cultivar may suggest that the SA-mediated resistance pathway occurs as also described for other biotrophic fungi-plant associations. WRKY proteins are a group of transcription factors (Eulgem & Somssich, 2007) acting as positive or negative regulators of basal defence and systemically acquired resistance (Dong, 2004). In rust-challenged coffee plants the *CaWRKY1* gene was rapidly activated during secondary haustoria formation in the compatible interaction, but did not remain activated in the incompatible interaction. It is thus likely that the putative WRKY transcription factor encoded by the *CaWRKY1* gene may operate as a negative regulator of disease resistance, preventing host immune responses in susceptible coffee plants.

The histological observations showed that there was no statistical difference in *H. vastatrix* development between the resistant and the susceptible cultivars until 36 hpi. Similarly, there was no difference in the expression of the defence-related genes and in the accumulation of H_2O_2 in stomatal cells between resistant and susceptible cultivars until this time. In leaf-disc assays, resistant cv. Tupi showed an immune reaction to *H. vastatrix* race II, with no visible macroscopic symptoms of fungal development. Therefore, as described for other rust fungi (Hu & Rijkenberg, 1998; Rinaldi *et al.*, 2007; Moldenhauer *et al.*, 2008), posthaustorial resistance in coffee is likely to occur after haustoria were produced inside the mesophyll cells. However, it should be noted that, contrary to what is commonly described in plant-rust interactions (Heath, 1997), the posthaustorial resistance of cv. Tupi to *H. vastatrix* is not expressed until after the production of the 'primary' haustorium (that occurred in the subsidiary and adjacent cells of stomata).

Recent studies indicate that in incompatible plant-biotrophic fungus interactions, *R* gene-controlled host resistance may be triggered by the specific recognition of haustorially produced Avr proteins (Catanzariti *et al.*, 2006; Ridout *et al.*, 2006). Induction of HR after formation of the first haustorium is well known in plant-rust interactions (Heath, 1997). In general, the HR form of cell death is a rapid and strong response of resistant plants to cellular invasion by biotrophic fungi. The histological analysis described here identified a biphasic haustorial differentiation of the *H. vastatrix* race II in both compatible and incompatible interactions. In the first phase, pioneer haustoria are formed in the subsidiary and adjacent cells of stomata from a first described infection structure named the pioneer hypha. In the second phase, secondary haustoria are produced in the mesophyll after differentiation of the anchor-shaped substomatal vesicle. Assuming that specific plant resistance is based on the recognition of a haustorially-produced Avr component, these observations would indicate that *H. vastatrix* race II Avr proteins are not specifically recognized by, or not secreted into, the epidermal cells of the stomatal region from pioneer haustoria. Tissue dependence of race-specific resistance responses was suggested in the barley-powdery mildew interaction (Schiffer *et al.*, 1997). Authors showed that some resistance genes that were normally functional in primary leaves were nonfunctional in coleoptiles. However, correlation of *Avr* gene expression with HR does not seem to be absolute in plant-pathogen interactions. Using green fluorescent protein (GFP) reporter expression, Ridout *et al.* (2006) showed that cell death did not always occur in barley cells expressing complementary *R* genes to powdery mildew *Avr* genes.

Future work aims to characterize the *H. vastatrix* secretome at the pioneer and secondary haustorial stage to identify proteins that may play a role in the coffee-rust interactions.

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