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ORIGINAL PAPER

# A new set of differentially expressed signaling genes is early expressed in coffee leaf rust race II incompatible interaction

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**Abstract** New races of coffee rust are overcoming resistance genes available in germplasm and cultivated cultivars and bringing recently some coffee-producing countries in severe economic challenge. The objective of this study was to identify the genes that are linked to host resistance to the major coffee rust race II. In our study, we have identified and studied a segregating population that has a single monogenic resistant gene to coffee rust. Coffee leaves of parents, resistant, and susceptible genotypes of the $F_2$ generation plants were inoculated with pathogen spores. A differential analysis was performed by combined cDNA-AFLP and bulk segregant analysis (BSA) in pooled samples collected 48 and 72 h postinoculation, increasing the selectiveness for differential gene expression. Of 108 differential expressed genes, between 33,000 gene fragments analyzed, 108 differential expressed genes were identified in resistant plants. About 20 and 22 % of these resistant-correlated genes are related to signaling and defense genes, respectively. Between signaling genes, the major subclass corresponds to receptor and resistant homolog genes, like nucleotide-binding site leucine-rich repeat (*NBS-LRR*), *Pto-like*, *RLKs*, *Bger*, and *RGH1A*, all not previously described in coffee rust responses. The second major subclass included kinases, where two mitogen-activated kinases (MAPK) are identified. Further gene expression analysis was performed for 21 selected genes by real-time PCR gene expression analysis at 0, 12, 24, 48, and 72 h postinoculation. The expression of genes involved in signaling and defense was higher at 24 and 72 h after inoculation, respectively. The *NBS-LRR* was the more differentially expressed gene between the signaling genes (four times more expressed in the resistant genotype), and thraumatin (PR5) was the more expressed between all genes (six times more expressed). Multivariate analysis reinforces the significance of the temporal separation of identified signaling and defense genes: early expression of signaling genes support the hypothesis that higher expression of the signaling components up regulates the defense genes identified. Additionally the increased gene expression of these two gene sets is associated with a single monogenic resistance trait to to leaf coffee rust in the interaction characterized here.

**Keywords** *Hemileia vastatrix* · Host-specific resistance · R-genes · Transcript-derived fragments · Defense response · Signaling in response to pathogens

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## Introduction

Coffee leaf rust, which is caused by the biotrophic fungus *Hemileia vastatrix*, is the major disease of coffee with losses in production that may range from 35 to 50 %, if not properly controlled (Zambolim et al. 2002). *H. vastatrix* is considered one of the most ancient rust lineages (Grasso et al. 2006), and

resistance of coffee involve some strategies as old as the occurrence of rust, making this system one important research field to be explored about coevolution of pathogen–host genomes. As an example, in coffee rust incompatible interactions, hypersensitive reaction can occur as early as at the appressorial stage, making this a unique feature among rusts (Silva et al. 2008). The hypersensitive response (HR) is expressed by death of guard and subsidiary cells in stomata, so early as the fungus developed an appressorium (Siva et al. 2006). The resistance to this disease is is expressed by a rapid hypersensitive cell death at leaf infection sites (Rodrigues et al. 1975; Silva et al. 2006). In resistant coffee plants, fungus growth usually terminates after formation of the first haustoria in mesophyll cells (Silva et al. 2006). The use of resistant cultivars has been the most efficacious strategy to control this disease. However, the coffee pathosystem includes 45 pathogenic races of *H. vastatrix* in the world (Várzea and Marques 2005). In Brazil, a previous study differentiated 14 race I, but race II is the most widely distributed (Cabral et al. 2009). In our Hibrid de Timor germplasm, derived from seed brought from Timor Island, this resistance was defined as monogenic (Brito et al. 2010) or polygenic (Capucho et al. 2009) depending of plant genotype. In coffee leaf, rust race-specific resistance appears to be determined by at least nine genetic factors (called $S_H1$ to $S_H9$), alone or in combination, and virulence is determined by factors v1 to v9 (Bettencourt and Rodrigues 1988). The resistance factors $S_H1$, $S_H2$, $S_H4$, and $S_H5$ were identified in the germplasm of *C. arabica* (Rodrigues et al. 1975). The other factors were introgressed from other species, including $S_H6$, $S_H7$, $S_H8$, and $S_H9$ from *C. canephora* (Rodrigues et al. 1975) and $S_H3$ from *C. liberica* (Prakash et al. 2004). Besides these, at least two resistance genes not yet characterized were detected. The Híbrido de Timor (HT) germplasm is the main source of resistance to *H. vastatrix*. These plants originated from the spontaneous cross between *C. arabica* and *C. canephora*. The HT germplasm has at least five dominant genes ($S_H5$, $S_H6$, $S_H7$, $S_H8$, and $S_H9$) that can differ between HT derivatives. The HT CIFC 832/1 has the five genes and other HT derivatives, as the Catimor group have $S_H$ (5, 6, 7, and 9), $S_H5,8$, $S_H$ (5, 6, and 9), $S_H6$, and $S_H5$ (Bettencourt et al. 1992). The Brazilian germplasm has a collection of HT, which contains important sources of gene for disease resistance. The HT UFV 427-15, which belongs to this germplasm, was previously study by Brito et al. (2010) and Diola et al. (2011). They found that the resistance of this HT to race II of *H. vastatrix* was determined by one dominant gene that is still unknown. The information about the inheritance of the vertical resistance of coffee is significant; however, the characterization of the genetic factor and the mode of signaling and defense response are needed to understand the mechanisms of evolution breaks of resistance and overcoming virulence. Studies aiming to understand the complex interaction of coffee and *H. vastatrix* are important to increase the knowledge of the resistance and to get efficient control of this disease. In general, when the pathogen infects the plants, they are able to protect themselves against pathogens by the induction of highly sophisticated and usually complex defense mechanisms (Li et al. 2010).

Guzzo et al. (2009) have produced subtracted cDNA libraries and sequenced genes distributed in induced genes by acibenzolar-*S*-methyl (ASM) in a susceptible cultivar and in an incompatible interaction (HT 831/*H. vastatrix* race II). In this last class, the authors have gotten definitive evidences by real-time PCR of eight resistance-associated genes, including a glucanase, ABC transporter, lipoxygenase, and an *NBS-LRR* resistance gene. Ramiro et al. (2010) had characterized several members of WRKY transcription factors. Diniz et al. (2012) has compared host (race II) and nonhost rust (*Uromyces vignea*) using HT 832-2 and has characterized the gene expression of 11 genes by real-time PCR, previously selected as potentially involved in rust response. The reported cellular and molecular resistance responses of HT 832/2 to these rust fungi were interpreted by the authors as if common immunity components are shared between host and nonhost resistance. Ramiro et al. (2010) have used a different incompatible coffee leaf interaction (*C. arabica* var. Caturra/race VI) and identified 10 WRKY transcription factors that are induced in the Caturra genotype, which were not reported in the compatible interaction (*C. arabica* var. Tupy/race II).

In a compatible interaction, this process occurs 12 h after inoculation (Vieira et al. 2012). In resistant varieties, a sequence of molecular signals at the plasmatic membrane is required to control fungal development (You-Xiong et al. 2008). Effective pathogen recognition, which is triggered by conserved pathogen elicitors (Duplessis et al. 2011), is the first step towards resistance; this broad-range immunity is mediated by high-affinity plant cell surface receptors (Nishimura and Dangl 2010). During the process of fungus recognition, genes belonging to the nucleotide-binding site leucine-rich repeat (*NBS-LRR*) class (R-genes, Duplessis et al. 2011), which coffee gene family was characterized by Noir et al. (2001) play important roles in the activation of resistance via a signaling cascade (Ganesh et al. 2006). R-genes contain highly conserved structural domains (NBSs) and hypervariable domains (LRRs) (Leng et al. 2010); the functions of these domains include recognition of the pathogenic elicitors and activation of defense response proteins, the pathogenesis-related proteins (PRs) (Li et al. 2010), and a series of other proteins (Guzzo et al. 2009; Vieira et al. 2012).

The cultivation of large areas with coffee offers a special environment for rapid coffee rust evolution, and recently, new races with large numbers of susceptible phenotypes in Brazil have been detected (Cabral et al 2009). In fact, at the end of 2012, three countries have declared state of emergence due to the uncommon losses caused by coffee leaf rust

(Cressy 2013) and the recently reported breakdown of HDT832/1and HDT832/2 resistance in an Indian germplasm bank (Prakash et al. 2010) illustrates this quick evolution and great genetic variability of H. vastatrix populations. Taking in account that an average of 10 years are needed to breed a new coffee variety, detailed molecular characterization of plant responses to incompatible rust races represents an urgent need for the understating of coffee resistance to leaf rust. This effort could be very useful to help breeders in the challenge of the development of resistant cultivars to the new races arising nowadays. Therefore, the purpose of this study was to identify genes involved in host-specific resistance that are activated during the incompatible interaction between coffee plants and *H. vastatrix* race II pathotype. Here, we identified a new set of 108 genes related to resistance of HT using cDNA-AFLP coupled to BSA strategy and detailed temporal analysis of 21 of these genes by real-time PCR to demonstrate that signaling and defense temporal profile expressions are distinct.

## Materials and methods

### Plant material and fungus inoculation

The resistant parent Híbrido de Timor UFV 427-15, the susceptible parent Catuaí Amarelo UFV 2143-236 (IAC 30), and a population of 224 $F_2$ plants were characterized for resistance to *H. vastatrix*. The pathotype used was a race II, characterized in a series of differentiating coffee tree clones as described by Cardoso (1986) and has been maintained in the mycology collection of UFV. Leaf discs with diameters of 2.5 cm, obtained from completely developed young leaves from each plant, were placed in germination boxes (plastic box 11×11×3 cm) containing moistened foam and nylon. The leaf discs were then inoculated by applying ten 5.0-μL drops of uredinospores in distilled water (2.0 mg $mL^{-1}$) over the abaxial surface of each leaf disc. Phenotyping was performed using two replicates of eight leaves from one plant (Fig. 1a). Uredinospore germination was induced in the dark for 24 h, followed by a photoperiod of 12 h at 22 °C (±2). The disease evaluation was performed at 18, 24, and 36 days, taking into account the presence and absence of uredinospores (Fig. 1). To comprise the different groups, five $F_2$-resistant and five susceptible genotypes were selected. A new inoculation was performed in these genotypes, and samples were collected at 0, 12, 24, 48, and 72 h postinoculation for real-time PCR analysis and at 48 and 72 h for complement DNA-amplified fragment-length polymorphism (cDNA-AFLP). The germination and grower of the *H. vastatrix* were monitored with a stereomicroscope (10× magnification).

### RNA extraction, and cDNA and double-stranded DNA syntheses

Total RNA was extracted using Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Total RNA was quantified in a spectrophotometer adjusting the concentrations to 300 ng $\mu L^{-1}$. The physical integrity of the RNA was evaluated by 1 % agarose gel electrophoresis.

To remove contaminating DNA, total RNA was treated with DNase I (RQ-DNase I, Promega, Madison, WI, USA) at a concentration of 1 U of DNase I per microgram of total RNA. The samples for cDNA-AFLP included 4 μg of total RNA for each genotype and 1 μg for qRT-PCR. cDNA was synthesized using the SuperScript II kit (Invitrogen) with the indicated concentration and volume adjustments (1 reaction=1 μg of total RNA). Two control samples (without reverse transcriptase) were included. Synthesis was validated by amplification with a primer for the coffee plant ubiquitin gene, and the product was visualized by 1 % agarose gel electrophoresis.

The double-stranded DNA (dsDNA) was synthesized according to a modified protocol proposed by D'Alessio and Gerard (1988). For every 80 μL of cDNA reaction for each genotype, 4.0 U of RNase H (Promega, Madison, WI, USA), Second-Strand enzyme buffer (Invitrogen, Carlsbad, CA, USA), 0.25 mM of each dNTP, 0.5 mM of DTT, 20 U of T4 DNA ligase (Epicentre Biotechnologies, Madison, WI, USA), and 20 U of *E. coli* DNA polymerase I (Promega) were added, and the final reaction volume was adjusted to 240 μL with water. The reaction was incubated for 1 h at 12 °C, followed by 1 h at 22 °C. Then, total RNA was removed by adding 20 ng of RNase A (Promega) incubating at 37 °C for 30 min. The samples were purified using the phenol–chloroform method and diluted in 6 μL of 1× TE buffer.

### TDF identification

Then, 200 ng of dsDNA from each sample was digested with only the restriction enzyme *Eco*RI (Promega) for 8 h at 37 °C. The ligation reaction was performed using 50 ng of the adaptor primer ds*Eco*RI 5′ CTAGTAGACTGCGTACC 3′, 3 U of T4 DNA ligase (Promega), and 1× DNA ligase buffer in a final volume of 20 μL. The ligation reaction was performed overnight at 8 °C. The reactions were then diluted in 1× TE buffer at a ratio of 1/10 and stored at −20 °C until use. The cDNA-AFLP technique was conducted according to the method described by Bachem et al. (1996). Preselective amplification was performed with 50 ng of the *Eco*RI 5′ GACTGCGTACCAATTCN 3′ primer. The following reagents were added to obtain a volume of 25 μL:2.5 μL of diluted digestion reaction, PCR buffer 1×, 1.25 mM of $MgCl_2$, 0.25 mM of each dNTP (Promega), and 1.5 U of Taq DNA polymerase (Phoneutria, BH, Brazil). The

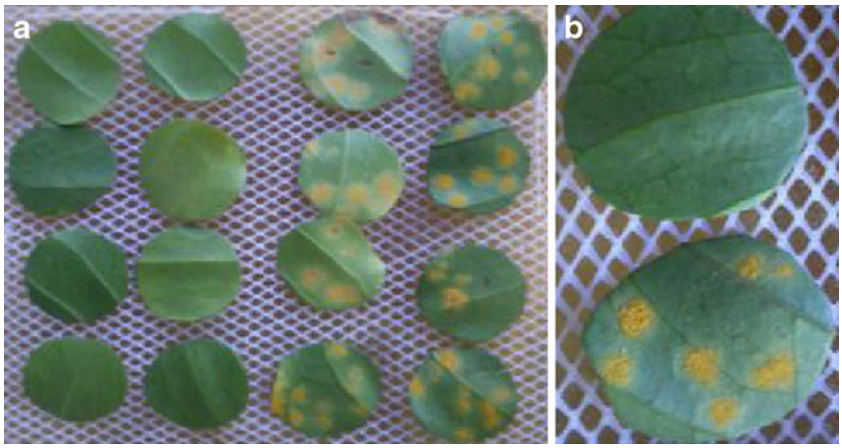


**Fig. 1** Phenotypic characterization of coffee plant resistance to the pathotype of race II of *H. vastatrix*. **a** Phenotyping replica set containing eight leaf discs from each genotype 24 days after inoculation; resistant (*left*) and susceptible (*right*). The presence of spores indicates a complete reproductive cycle and compatible plant–pathogen interaction. **b** Magnified image of contrasting genotypes

PCR cycling protocol consisted of 24 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s. The amplification reactions were diluted 40×. Half of each reaction for the $F_2$ generation genotypes was used to generate the resistant and susceptible genotypes in the systems using bulked analysis segregant (BSA). The other half was used in individual selection reactions and transcript-derived fragment (TDF) confirmation. Selective amplification was performed by touchdown PCR consisting of 13 cycles of 94 °C for 30 s, 65 °C (−0.7 °C after each cycle) for 30 s, and 72 °C for 60 s, followed by 26 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s in a volume of 20 μL containing 4.0 μL of the 40× dilution of preselective reaction and the following reagents: Taq DNA polymerase buffer 1×, 1.25 mM of $MgCl_2$, 0.25 mM of each dNTP (Promega), 50 ng of each selective primer, and 1.5 U of Taq DNA polymerase (Phoneutria). The reaction products were separated by 6 % denaturing polyacrylamide gel electrophoresis (19:1) at 1,800 V for 2.5 h, and silver nitrate staining was performed as described by Brito et al. (2010). After drying, an image of the gel was digitally captured and used to analyze DNA polymorphisms. The primer combinations that were polymorphic between parents and pools with contrasting resistance (Fig. 2a) were analyzed individually for each genotype (Fig. 2b) to validate the connection between the TDF and the defense response.

## TDF cloning and sequencing

To recover the polymorphic bands from the polyacrylamide gel, each TDF was marked in resistant parents and $F_2$ plants, rehydrated, removed from the plate, and macerated in 40 μL of 1× TE buffer, according D'Alessio and Gerard (1988). A new PCR was performed with 5 μL of this template solution using the same concentrations of reagents as for the selective

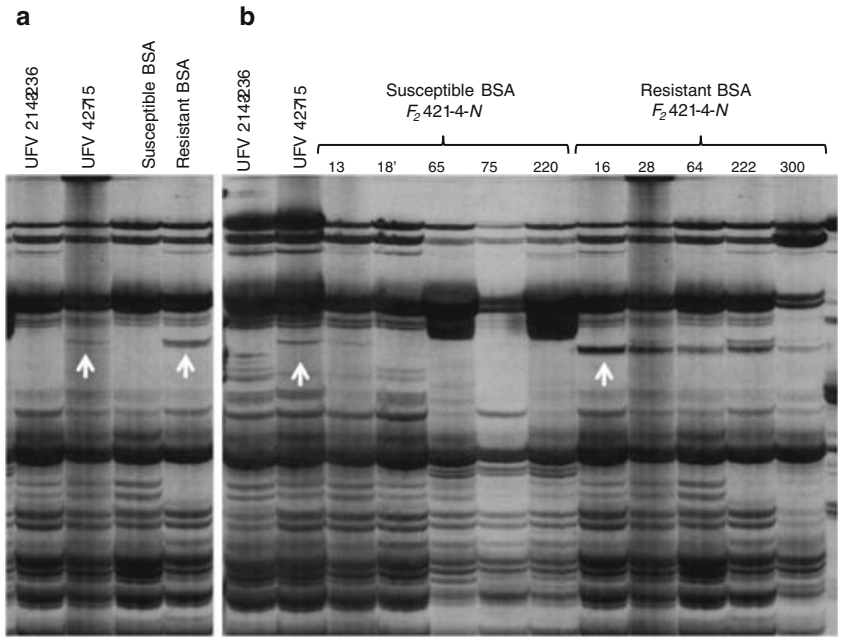


**Fig. 2** Polymorphisms between resistant and susceptible plants, parent, and $F_2$ generation, indicated by the *arrows* for the ECTA/EACG primer combination. **a** Pooling and polymorphism selection. **b** Confirmation of polymorphism by the decomposition of pools in individual PCRs

amplification of cDNA-AFLP in a final volume of 50 μL and using a program of 32 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. The PCR product was separated by 1 % agarose gel electrophoresis at 70 V for 25 min. The band was excised from the gel and purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, NY, USA). After this process, 16 ng of the purified DNA fragment was used for ligation into the pGEM-T® plasmid following the manufacturer's instructions (Promega). *E. coli* DH5α-competent cells were transformed by thermal shock, and plasmid DNA was extracted using the alkaline lysis method (Clemson and Kelly 2003). Sequencing was performed on a MegaBACE ABI PRISM 1000 sequencer (GE Healthcare).

### TDF evaluation of similarity

A similarity comparison was performed using the National Center for Biotechnology Information (NCBI) (www.ncbi.org) and the Brazilian Coffee Genome Project (Projeto Brasileiro do Genoma Café—https://alanine.cenargen.embrapa.br/coffEST) databases and the BLASTn and BLASTx programs (Altschul et al. 1990). Comparisons were made between the degrees of similarity of the TDF nucleotide sequences and the genes and ESTs. Sequences that had an *E* value $>1.10^{-5}$ were considered to have no significant similarity. Functions, species of origin, and NCBI accession numbers were assigned to each TDF.

### Differential analysis of TDFs by real-time PCR

Only sequences that were similar to genes linked to the signaling and activation pathways of the defense response were selected. Specific primers for 21 TDFs were obtained using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi), with sizes of 20 bases and melting temperatures of approximately 56 °C. These primers produced amplicons with sizes between 80 and 140-base pairs. All primers (Table 1) were used in primer-specific reactions at a final concentration of 5 μM for each primer. PCRs with final volumes of 20 μL each consisted of 1× Taq polymerase buffer (High-Fidelity PCR Buffer), 3.0 mM of $MgCl_2$, 25 μM of dNTPs, 0.04 μL of ROX, 0.08 μL of BSA, 2.0 μL of 1:10,000 diluted SYBR Green, and 0.25 U of Platinum Taq DNA polymerase (all reagents are from Invitrogen). Four technical replicates and four biological replicates (two for parents and blocks) were analyzed for each treatment (resistant and susceptible). Quantitative real-time PCR was performed in a Step One Plus thermocycler (Applied Biosystems, Foster City, CA, USA). Primer validation was obtained using a relative standard curve with three technical replicates of resistant genotype samples at 48 h after inoculation in 1:5 serial dilutions at four concentrations starting from the cDNA reaction (diluted one, five, 25, and 125 times). Each reaction used 0.28 μL of the cDNA synthesis solution. The PCR program was 90 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min for the amplification stage, and a cycle of 60 °C+0.3 °C every 20 s until 95 °C was reached for the dissociation curve. Primer sets with amplification efficiencies above 0.95 were accepted. The following three constitutive genes were used as reference genes: Ca Ubi (ubiquitin), Ca Tub (α-tubulin exon 2), and Ca actin (actin), as reference was estimated as the average between them, according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al. 2010). Relative quantification cycle (Cq) was calculated by the comparative cycle threshold ($\Delta\Delta C_T$) method on the Step One Plus thermocycler using the values of $\Delta\Delta C_T$ program and the same cycle program used for the relative standard curve. The experimental design consisted of analyzing the resistant and susceptible genotypes in two biological replicates (parents and blocks) at 0, 12, 24, 48, and 72 h after inoculation, with four homogeneous technical replicates. Step One Plus software version 2.0 (Applied Biosystems) was used to analyze the data according to Ali-Benali et al. (2005). The errors relating to the technique concerning the homogeneity of the four technical replicates within each biological sample were tested by Bartlett's test ($p<0.05$) using the $C_T$ values. The level of expression ($R$) was calculated by the following formula: $R=2^{-\Delta\Delta Ct}$, where $^{-\Delta\Delta Ct}=(\Delta C_T \text{ of target})-(\Delta C_T \text{ of endogenous gene})$. Samples from resistant genotypes at time 0 h were used for comparison (Nicot et al. 2005).

For the quantitative comparison ($R_q$) between genes and genotypes, the relative expression based in data $C_q$ were evaluated by a one-way analysis of variance (ANOVA), and the means were compared using Duncan's test ($p<0.05$).

To isolate differentially expressed genes or groups according to the coexpression of all genes in contrasting genotypes, a Spearman's correlation analysis was performed to compare the intensities of temporal gene expressions with each other, within the same genotype, and between genotypes. The statistical analyses were performed using the STATISTICA 8.0 program (StatSoft® 2009).

## Results

### Characterization of resistance to coffee leaf rust

Twenty-four days postinoculation, *H. vastatrix* uredinospores were detected on the leaves of susceptible genotypes but not on resistant genotypes (Fig. 1). The ratio of resistant to susceptible genotypes was 3:1 (Mendelian character), indicating that one gene is involved in the resistance of the HT to pathotype of race II of *H. vastatrix*.

**Table 1** Primers used for the amplification of selected TDF sequences in the quantitative study of gene expression associated with resistance to coffee leaf rust

| Primer (Ca TDF) | GenBank accession | Amplicon (bp) | Sense sequence (5′→3′) | Antisense sequence (5′→3′) |
|---|---|---|---|---|
| 1 POX | GT029982.1 | 80 | ACGATACCCAAGCGGAACTC | CCTGGTATCCCTGTACCTGTC |
| 2 RGH1A | GT029983.1 | 107 | CGTCAAGCTCGCTTTGCTCA | TTTGTGCAGCTCGCCGAAAG |
| 14 GR | GT029995.1 | 108 | CGCATGGTATGGTAGCGTCG | ATCCAGGGTCGAGAGCTTGC |
| 19 MEK2 | GT030000.1 | 115 | CCGTGACGTCGACAACCCTA | TGGATCCCTTGAGCAGAGCC |
| 24 CyP450 | GT030005.1 | 107 | ACATCGTGGCAAGAAGGCAA | CGACTGGAAGCTGCCCAATG |
| 41 UbiE2 | GT030022.1 | 95 | GCGGCCTAACAAAATGCTGC | ATTACAGGCCAACACCAGGC |
| 44 PR5 | GT030025.1 | 94 | GCACCCTGCCCCATTTATGC | GTGCCGATGGATTTCAGCCC |
| 48 RLK | GT030029.1 | 121 | CCATCGGTATTTTGCGCGAC | TGCTGGACCCTCGTAGCTTG |
| 53 Begr1 | GT030034.1 | 98 | ACGACAAGGTATCAGCCGGG | GTCGCGCAAAATACCGATGG |
| 54 Pto-like | GT030035.1 | 129 | ATGCCGCGGTAAACTTCGTG | CTGCTTAGCCTGCCCAAAGC |
| 56 SAMsI | GT030037.1 | 95 | CTCTGGTCAAGCCCCCTCAG | AAAGTGGTTCAGCGCACACC |
| 58 MAPK3 | GT030039.1 | 127 | TACGTCAGGCACACTCCCAC | GATCAATGGCCACGGGATGG |
| 61 SAG12 | GT030042.1 | 117 | TCGCTGTTTGCCGGTTTCTG | GCATCGTAGGAAACACGCCC |
| 74 ERB2 | GT030055.1 | 110 | AGCGGAGGGACTCGATATGC | GCTTCCGGTCCTACCCTTCC |
| 77 NBS-LRR | GT030058.1 | 88 | CCAGTGGCTAATGCTCTGCC | TGGACCAGCGTGCATCATCT |
| 81 MAPK2 | GT030062.1 | 112 | TGGGAGGGTCCAGCCAATTC | GCGACTTACGGTGTTGGAGG |
| 87 CDPK | GT030068.1 | 129 | AGGAGTGGGTATGTTGGCACA | TCCCTTGAACAGCAACGCAAC |
| 89 CBP | GT030070.1 | 95 | TACGGCGTATTCACCGGTCC | GCAAGGGAATGAGGGTTGCG |
| 90 Aco1 | GT030071.1 | 83 | AGCAAAGGGACATTCAGCGA | TTCCTAACCGTGTTGCTGGC |
| 98GPX4 | GT030078.1 | 100 | TCCCTGGTGGCATTCCCTTC | TTCGGCCTTAAAGCGTGTGC |
| 99 bZIP56 | GT030080.1 | 86 | TTCGCTTTGTGGCGGACCTA | TCCTGCCGAAGACTGAGAGC |
| Ca-Actin | FJ426886 | 95 | CTTGAAATACCCCATTGAGC | ACAGGATGTTCTTCTGGAGC |
| Ca-α-tubulin | AF363630.1 | 128 | TTTGGGCTTCAGATTAGCAC | GCATTCATAACCAACACCCT |
| Ca-ubiquitin | AF297089.1 | 85 | CAAAGGCTCATCTATGCTGG | GATGAAGAACAGAACCACCC |

*POX* peroxidase, *RGH1A* resistance gene analogs 1A, *GR* glutathione reductase, *MEK2* mitogen-activated protein kinase 2, *CyP450* cytochrome P-450, *UbiE2* ubiquitin-conjugating enzyme E2, *PR5* pathogenesis-related thaumatin-like protein, *Bger1* beta-glucan elicitor receptor, *Pto-like* Pto R protein kinase-like, *SAMsL S*-adenosyl-L-methionine synthetase-like, *MAPK3* mitogen-activated protein kinase 3, *SAG12* senescence-associated gene 12–cysteine type peptidase, *ERB2* ethylene-responsive element binding protein 2, *NBS-LRR* nucleotide binding site–leucine-rich repeats, *MAPK2* mitogen-activated protein kinase 2, *CDPK* calcium-dependent protein kinase 5, *CBP* calmodulin-binding protein, *Aco1* ACC oxidase ACO1, *GPX4* glutathione peroxidase, *bZIP56* transcription factor bZIP56

## Major gene ontology classes associated with resistance

Using the cDNA-AFLP technique combined with BSA, 512 selective primer combinations, 256 of each *Eco*RI GNN/*Eco*RI TNN and *Eco*RI CNN/*Eco*RI ANN were analyzed; these combinations generated 33,685 amplified transcript-derived fragments (TDFs) in several sequencing gels (data not shown). On average, 66 amplicons were generated by each primer combination, and the sizes of the amplicons ranged between 100 and 2,300 bp. A total of 1,380 polymorphic amplicons were identified. However, only 489 amplicons were present in only the parent and resistant group (BSA resistant), and 362 of these amplicons were selected for validation based in at least a band intensity five times stronger in all member (Fig. 2), where the expression of these genes were tested in each individual of the two groups (BSA resistant, and BSA susceptible). Among these amplicons, only 108 were confirmed as differential TDFs associated to resistance. From these TDFs, 62 were obtained from the *Eco*RI GNN/*Eco*RI TNN primer combination, and 46 were obtained from the *Eco*RI CNN/*Eco*RI ANN primer combination. The average size of these selected TDFs was approximately 500 bp.

By performing sequence similarity comparisons of TDFs using BLASTn and BLASTx (www.ncbi.gov), 93 sequences from the NCBI database were identified as similar and 15 were not similar to genes deposited in GenBank. The sequences were recorded and entered into the GenBank EST database with consecutive GenBank accession numbers (see http://www.ncbi.nlm.nih.gov/dbEST/: GT029982 to GT030089). The gene ontology performed using similarity analysis from the functional characterization (Fig. 3) showed that defense genes represent ~22 % of the sequenced TDFs (24 genes), while signaling pathway genes behave ~20 % (21 genes) of the sequenced TDFs.

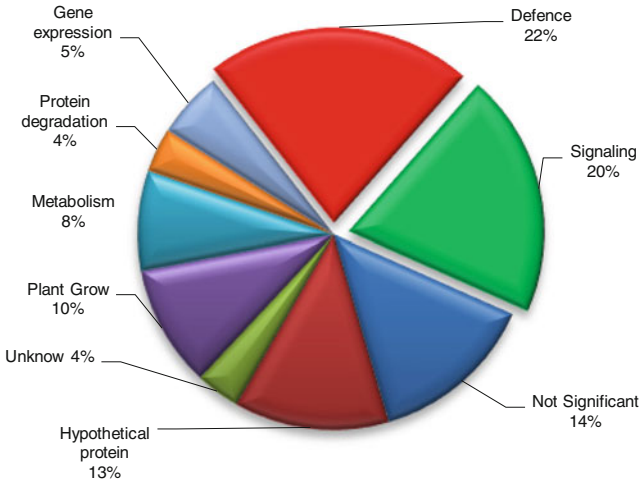


**Fig. 3** Putative comparative classification according to the biological function of genes expressed by TDFs with sequences entered into databases, based on the similarity of known sequences detected by queries using BLASTn. (White and Kerlavage 1996)

### Validation of cDNA-AFLP/BSA results with gene expression analysis using real-time PCR

We have selected 21 of the 93 genes with high homology to know genes (Table 1). The housekeeping primers have good stability in the presence of these biotic stress and experimental procedure used (*M* value of 0.1243, estimated by geNorm—http://medgen.ugent.be/~jvdesomp/genorm/). The highest level of expression of genes related to transcription factors and genes responsible for signaling and pathogen recognition was at 24 h after inoculation (Fig. 4). In this class, genes that were more expressed were *NBS-LRR*, *RGH1A*, and *MEK*, with approximately 12.0, 9.3, and 8.6 times. Among the genes that signal pathogen infection, the *NBS-LRR* gene was expressed by the resistant genotype (Fig. 4) 4.82 times more. Not less interesting was the response of the kinases MEK, MAPK2, and CDPK with higher differential induction (2.49-, 4.27-, and 5.44–fold, respectively, in resistant genotypes). Between transcription factors, the TDF that encoded β-Zip showed a significant difference among the contrasting genotypes (4.5-fold more expressed). As a group, the genes that had differential expression related to defense response were more expressed at 72 h after inoculation. The defense response genes showed no significant changes in expression between resistant and susceptible genotypes, except the protein pathogenesis-related gene encoding (Traumatin-like, class III) at 17.4 fold compared to control and 5.31 times compared to susceptible and resistant genotypes (Fig. 4).

### Temporal profile of gene induction after infection

Correlation analysis (Supplement 1) of the expression transcriptional pattern of the 21 genes in the relation to gene coexpression in time and intensity allowed us to classify the genes in three different classes (*p* value between 0.1 and 0.01). Class I includes the transcriptional factors EREB1 and bZIP which induction modulation was similar, having a transient increase of expression between 12 and 24 h. The second class include several resistance genes (*NBS-LRR*, *RGH1A*, *RLK*, *Berg1*, and *Pto-like*), kinases homologs (MEK, MAPK3, MAPK2, and CDPK), and signaling protein calmodulin-binding protein (CBP), all included in the signaling gene group. *NBS-LRR* has the highest induction rate in this group and a clear peak of gene expression at 24 h, which is also similar to the other receptor genes of this group. The third group is composed mainly of defense response proteins and pathogenesis-related proteins including the PR5, a higher induced gene in this work. This last class has a peak of gene expression at 72 h or more. The genes of this group coexpressed between themselves but not with other groups. Positive correlations were observed between defense response genes and signaling genes, but these correlations were not strong (Supplement 1).

## Discussion

In coffee leaf rust incompatible interaction, our genetic analysis has proven that just one gene is involved, making this interaction ideal to identify and to study a specific group of defense associated with coffee resistance to leaf rust race II pathotype. This pathogen is one of the most ancient rust lineages (Grasso et al. 2006). As an example, in coffee rust-incompatible interactions, hypersensitive reaction can occur as early as at the appressorial stage, making this a unique feature among rusts (Silva et al. 2008). As a consequence, resistance of coffee involve some plant strategies as old as the rust does, making this system one important research field to be explored to better understand coevolution of rust–host genomes.

### CDNA-AFLP coupled to BSA analysis reduces the presence of false positives

In this study, the combined techniques were applied to characterize the expression of 33,685 gene fragments in two parental genotypes and in two sets of five individuals each of susceptible and resistant plants (resistant and susceptible BSAs). With this wide analysis, 1,380 different expressed fragments were identified but only 108 differently expressed genes were confirmed as linked to resistance (close to 1.4 % of the polymorphic fragments). This notable reduction of detection of false positives confirms the advantage of combining cDNA-AFLB with BSA analysis, where not only detection of differentially expressed genes are performed but also more importantly, consistently differentially expressed in different individuals of a segregating population. This strategy has been rarely used, but also has been proved to be efficient in other

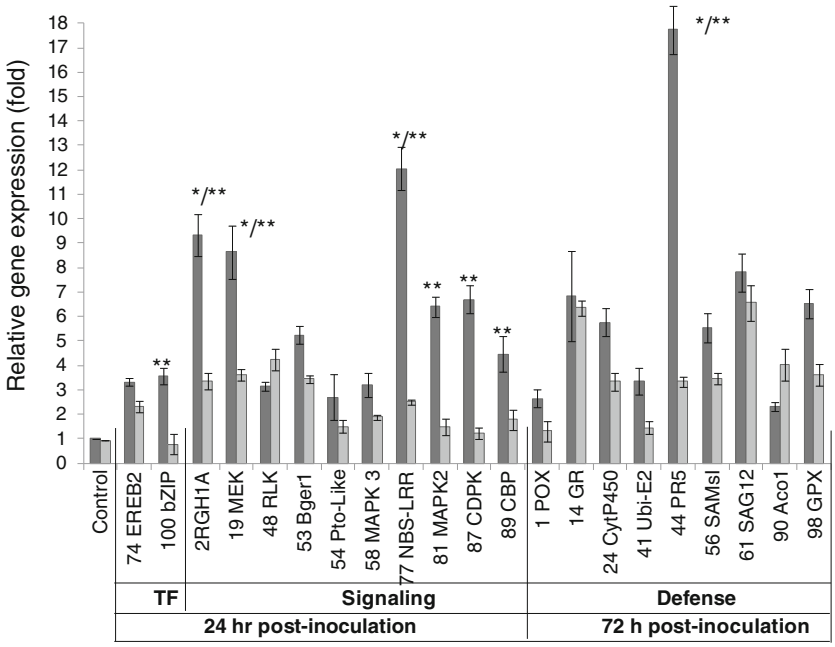


**Fig. 4** Qualitative analysis of activation and gene expression in coffee plant resistance to pathotype of race II of *H. vastatrix*. Maximum intensities of expression in resistant genotypes, showing genes from the resistance response signaling pathway and transcription factors at 24 h after inoculation and defense genes at 72 h after inoculation (susceptible genotype without significant differences). Significant differences in the relative expression levels of genes were measured by one-way ANOVA with Duncan's mean separation test at 0.05 probability. Between genes (*one asterisk*) and gene expression between genotypes (*two asterisks*)

studies of other plant–pathogen interactions (Guo et al. 2006). The low cost and simplicity of this strategy make more advantageous the use of cDNA-AFLP to study transcriptional changes in gene expression when characterized and segregating population of contrasted parents for the desired trait are available.

## A new set of coffee leaf rust resistance signaling genes are identified

We complement the study of Guzzo et al. (2009), which has sequenced genes differentially expressed (SSH method) in the incompatible interaction using HT and race II spores in libraries done with RNA extracted 72 h after infection. These results did not overlap our data, since we have used another HT genotype, originating from segregating plants from seeds harvested in Timor Island brought directly to the UFV coffee germplasm bank. The genotype used here is that which has a single dominant resistant gene to race II, whereas the HT-CIFC 832/1 has five different resistance genes. Another important difference is that we have analyzed differential expression not restricted to 72 h, but using pooled samples collected 48 and 72 h postinoculation. Between genes analyzed in the cited work, 22 % were grouped as resistance mechanism (56 genes) and 30 % were grouped as cell maintenance and development (12 genes), but only four genes were confirmed by real-time PCR. The use of BSA represents a second confirmation verifying that 108 resistance-specific associated genes (Fig. 2) have at an least expression five times higher in all five different resistant plants (as exemplified in Fig. 2b). Furthermore, 21 genes were confirmed by real time. The genes reported here are different from those described by Guzzo et al. (2009) and Diniz et al. (2012). Our work data added to the results of the two other citations raising the interesting result of the involvement of two different resistance genes (85 % nucleotide identity) and several transcription factors and kinases. Whether the functions of these genes are dependent of each other will be very important to explore. *Arabidopsis* could be used to analyze the function of heterologous disease-resistant genes (Azinheira et al 2010; Cheng et al 2013), and interfamily transfer of NB-LRR genes confers resistance to other pathogens (Narusaka et al 2013a, b), since coffee transformation has low efficiency. This recent discovery suggests that whereas the R-genes are highly genotype specific, downstream components of R-genes are highly conserved between different plant species (Narusaka et al. 2013a, b). Otherwise, rapid transient overexpression or antisense of these genes in coffee (Ribas et al 2011) is also an interesting strategy to elucidate more detailed data about the signaling network involved in disease resistance. Ramiro et al. (2010) have identified several WRKY transcription factors. Subtraction methods (our work, Guzzo et al. 2009) were both unable to find any WRKY transcription factors using these different subtraction methods, which suggest that this transcription factors could be more important in basal responses, like nonhost resistance. Temporal profiling and correlational analysis clearly distinguish different patterns for signaling and defense genes.

The rust fungi that develop their hyphae normally activate the defense responses 12 h after infection in coffee trees (Azinheira et al. 2010; Vieira et al 2012). Ganesh et al. (2006) and Vieira et al. (2012) have studied morphophysiological cellular changes and found that pathogen recognition occurs moments after fungal penetration. At this initial period after infection, nonhost resistance genes are

mainly expressed. Host resistance genes are expressed later and recognize race-specific pathogen effectors. This response only occurs after the pathogen have survived several successively induced basal plant defenses (Uma et al 2011). Our selection of differential expressed genes has focused on genes expressed at 48 and 72 h after infection. However, all signaling genes characterized (nine genes, except the *Pto-like* R-gene) have a peak of gene expression at 24 h after infection. Curiously, the higher expressed gene was a *NBS-LRR* gene, which was 14 times more expressed in the resistant genotype than in the susceptible one, and was the second highest induced gene in HdT genotype. The *NBS-LRR* gene described by Guzzo et al. (2009) was more expressed at 48 h in other incompatible interactions with coffee leaf rust, and it is possible that this latter induced R-gene acts downstream of the gene characterized here. Alternatively, since other incompatible interaction is described, other specific R-gene expressed at 24 h at HT-CIFC 832/1 will act upstream of the R-gene described by Guzzo et al. (2009). Considering that other three resistant genes (*2RGHA1*, *Berg1*, and *Pto-like*) are also induced, but at a lower level (no more than 50 % of the induction observed for the NBS-LRR), these differences and temporal profile suggest that this gene could be a good candidate for a single resistant gene that our genetic analysis has identified (Brito et al. 2010; Diola et al. 2011). The differential expression of just one gene from this class is responsible for triggering the activation of resistance responses in barley (Rostoks et al. 2004), sugarcane (You-Xiong et al. 2008), and poplar (Zhang et al. 2008).

Azinheira et al. (2010), also studying resistance against *H. vaxtratris* race II, a prehaustorial rust resistance, and using *Arabidopsis thaliana* as a model system of heterologous gene for the molecular dissection of coffee responses to leaf rust indicate triggering of the induction of a set of defense-related enzymes peaking at 18 and 42 h postinoculation. Altogether, we can suggest that the host race-specific-induced recognition event occurs before 48 h, probably between 18 and 24 h. Before this period, defense responses probably are nonhost resistance. A correlation analysis of all temporal profiles reinforces the observation that almost all signaling genes studied have a higher expression at 24 h and are probably coregulating. Between then, there are four R-genes and five kinases (e.g., MEK, RLK, 2MAPKs, and CDPK), which have between two- to sevenfold higher expression than in an compatible interaction, induction similar to the values reported by Asai and Yoshioka (2008) and Diniz et al. (2012), and which are important resistance signaling elements of plant resistance (Jalali et al. 2006; Zhao and Qi 2008).

A corelational analysis separates defense genes as a separate group of signaling genes. During these processes, groups of genes undergo simultaneous and interdependent activation and are coexpressed in time and intensity similarly in resistant plants (Jia et al. 2000; Trinchieri 2003). This pattern of gene expression in resistant genotypes revealed coexpression during the period of infection, suggesting that the genes may be involved in signaling and defense response pathways. Almost all of these defense genes (seven of nine) have a latter peak of gene expression, which occurs at 48 and/or 72 h. The signaling genes identified here could modulate this late expression pattern, suggesting an increase in the expression of these genes. Between these seven genes, we have mainly antioxidative enzymes, and the protein related to pathogenesis PR5 thraumatin-like of the chitinase group showed the highest relative expression among all analyzed genes. PR-5 proteins are enzymes (chitinases) that hydrolyze chitin, a β-1,4-linked polymer of *N*-acetylglucosamine (Vasconcelos et al. 2011). The PR proteins, PR-5, are potent antifungal proteins, and higher expression of PR could be a mechanism that contributes to resistance (Vasavirama and Kirti 2012). Because chitin constitutes a large fraction of the cell wall of certain classes of phytopathogenic fungi, these enzymes have been suggested to potentially play a role in defense against these plant pathogens (Li et al. 2010) by causing cell collapse and fungus death (Guzzo et al. 2009; Vasconcelos et al. 2011).

The robustness of the use of cDNA-AFLP coupled to BSA analysis of an incompatible interaction involving a single gene have showed to be sensible enough to isolate good candidates for the single resistant gene and associated signaling pathway elements. The temporal compartmentalization of identified signaling and defense genes and the early expression of signaling genes support the hypothesis that higher expression of the signaling components upregulates the defense genes and that this increased gene expression is associated with a single monogenic resistance to leaf coffee rust disease in the incompatible interaction characterized here. Our high density map of this single resistance gene (Diola et al 2011) have allowed us the identification of three overlapped bacterial artificial chromosomes in this HT genotype, one of them where the NBS-LRR-77 is localized, and, together with data presented here, make this gene a good candidate for a resistance gene. The complete new set of 108 resistance-associated genes to race II of *H. vastatrix* could be an important tool to analyze gene expression in other coffee rust-incompatible interactions in order to dissect the mechanism of resistance to coffee leaf rust, an emergent new thread to global coffee production nowadays.

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