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# High throughput transcriptome analysis of coffee reveals prehaustorial resistance in response to *Hemileia vastatrix* infection

**Juan Carlos Florez[1] · Luciana Souto Mofatto[2] · Rejane do Livramento Freitas-Lopes[1] · Sávio Siqueira Ferreira[1] · Eunize Maciel Zambolim[1] · Marcelo Falsarella Carazzolle[2] · Laércio Zambolim[3] · Eveline Teixeira Caixeta[1,4]**

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**Abstract**

***Key message*** **We provide a transcriptional profile of coffee rust interaction and identified putative up regulated resistant genes**

*Abstract* Coffee rust disease, caused by the fungus *Hemileia vastatrix*, is one of the major diseases in coffee throughout the world. The use of resistant cultivars is considered to be the most effective control strategy for this disease. To identify candidate genes related to different mechanism defense in coffee, we present a time-course comparative gene expression profile of Caturra (susceptible) and Híbrido de Timor (HdT, resistant) in response to *H. vastatrix* race XXXIII infection. The main objectives were to obtain a global overview of transcriptome in both interaction, compatible and incompatible, and, specially, analyze up-regulated HdT specific genes with inducible resistant and defense signaling pathways. Using both *Coffea canephora* as a reference genome and de novo assembly, we obtained 43,159 transcripts. At early infection events (12 and 24 h after infection), HdT responded to the attack of *H. vastatrix* with a larger number of up-regulated genes than Caturra, which was related to prehaustorial resistance. The genes found in HdT at early hours were involved in receptor-like kinases, response ion fluxes, production of reactive oxygen species, protein phosphorylation, ethylene biosynthesis and callose deposition. We selected 13 up-regulated HdT-exclusive genes to validate by real-time qPCR, which most of them confirmed their higher expression in HdT than in Caturra at early stage of infection. These genes have the potential to assist the development of new coffee rust control strategies. Collectively, our results provide understanding of expression profiles in coffee—*H. vastatrix* interaction over a time course in susceptible and resistant coffee plants.

**Keywords** Coffee rust · Transcriptome · Biotrophic interaction · Disease resistance

Juan Carlos Florez and Luciana Souto Mofatto have contributed equally to this work.

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✉ Eveline Teixeira Caixeta
eveline.caixeta@embrapa.br

[1] Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), BioCafé, Universidade Federal de Viçosa, Campus Universitário, Avenida P.H. Rolfs, s/n, Viçosa, MG, Brazil

[2] Laboratório de Genômica e Expressão, Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, Distrito de Barão Geraldo, Campinas, SP 13083-970, Brazil

[3] Departamento de Fitopatologia, Universidade Federal de Viçosa, Campus Universitário, Avenida P.H. Rolfs, s/n, Viçosa, MG, Brazil

[4] Embrapa Café, Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa, Campus Universitário, Avenida P.H. Rolfs, s/n, Viçosa, MG, Brazil

## Introduction

Coffee is a worldwide-appreciated beverage and an important commodity for several countries. *Coffea arabica* and *Coffea canephora* are the two most economically important species (Davis et al. 2011). *C. arabica*, a natural alotetraploid (2n = 4x = 44) hybrid between the two diploids species *C. canephora* and *C. eugenioides* (Lashermes et al. 1999),

accounts for 70% of the world production (Davis et al. 2011). One of the major problems in coffee production is the coffee rust disease, which can cause yield losses up to 50% (Zambolim 2016). The causal agent of this disease, *Hemileia vastatrix*, is a biotrophic fungus, which it is entirely dependent on the cells of living plants for its growth and reproduction. The fungus produces several specialized structures to colonize the plant. After spore germination, the germ tube differentiates to apressoria, which, in turn, became penetration hyphae at the stomata. This structure will reach the substomatic chamber where it will differentiate to haustoria that will invade adjacent cells altering plant metabolism to meet its nutritional needs and, thus, completing the fungus lifecycle. This mode of interaction involves a prolonged and effective suppression of the host immune system and, at the same time, the induction of host-specific genes for its establishment as biotrophic fungus (Schulze-Lefert and Panstruga 2003; Voegele and Mendgen 2003; Guerra-Guimarães et al. 2015). Therefore, in coffee—*H. vastatrix* pathosystem, identification of genes involved in the plant defense mechanism triggered by the presence of the pathogen is indispensable to find new defense genes to develop resistant cultivars.

Plant defense against pathogens starts with the recognition of pathogen-associated molecular patterns (PAMPs) by transmembrane proteins named pattern recognition receptors, PRRs (Jones and Dangl 2006). This step activates the PAMP-triggered imunity (PTI). Pathogen can overcome this initial defense by secreting small extracellular proteins called effectors (De Wit et al. 2009). However, plants can recognize these effectors direct or indirectly by a resistance (*R*) protein, and induce a second defense phase, named Effector-triggered immunity (ETI), which is more efficient than PTI (De Wit et al. 2009). Each *R* gene recognize a specific effector (called *Avr* gene, for Avirulent) (Flor 1942), therefore, plant immunity is related to the presence of specific alleles of *R* genes. Some pathogens have evolved their effectors proteins either by losing an *Avr* gene, or diversifying its genes into different effector protein and thus, it can suppress ETI and successfully colonize the host (Jones and Dangl 2006).

Cytological and biochemical studies have shown coffee activates some defense mechanisms when attacked by *H. vastatrix*. One such mechanism is the hypersensitive response (HR), a type of ETI, which is associated with the deposition of callose, phenolic compounds (flavonoid and chlorogenic acid), and cell wall lignification (Silva et al. 2002). Other types of responses involve enzymes such as lipoxygenase and peroxidase that stimulate the pathway of phenylpropanoids. PR proteins (pathogen related proteins), as β-1,3-glucanase and chitinase, are also found in association with resistance of some coffee cultivars to *H. vastatrix* race II (Silva et al. 2002; Fernandez et al. 2012). Molecular methodologies as suppression subtractive hybridization (SSH), 454 pyrosequencing and RT-qPCR have been used to identify several genes putatively involved in host resistance. Some of the expressed sequence tags (ESTs) found in these studies encode for proteins involved in resistance, stresses, defense and signal transduction pathways (i.e. chitinases, beta-1, 3 glucanases, PR10, lipoxygenase type-AP2 and WRKY transcription factors). Also, activity of oxidative enzymes (lipoxygenase, peroxidase and superoxide dismutase), phenylalanine ammonia lyase, chitinase and glucanase were detected in the resistance reaction to *H. vastatrix* race II (Fernandez et al. 2004, 2012; Ganesh et al. 2006; Diniz et al. 2012). On the other hand, coffee resistance against *H. vastatrix* is governed by at least nine major dominant genes ($S_H1$–$S_H9$) that have the corresponding virulence genes ($v1$–$v9$) in the pathogen (Rodrigues et al. 1975; Bettencourt and Rodrigues 1988; Várzea and Marques 2005). However, resistance breakdown by virulence factor *v5*, which is present in the race II of *H. vastatrix* and at least thirty other races, has been reported in most commercial coffee cultivars with $S_H5$ gene (Rodrigues et al. 1993).

Until 2005, more than 50 races of *H. vastatrix* had been broadly described worldwide (Várzea and Marques 2005; Zambolim 2016), in Brazil 15 races were identified: I, II, III, VII, X, XIII, XV, XVI, XVII, XXI, XXII, XXIII, XXIV, XXV or XXXI, and XXXVII (Cabral et al. 2009; Capucho et al. 2012). The *H. vastatrix* races recently discovered have been able to infect derivatives of Híbrido de Timor (HdT), a natural hybrid originated from *C. arabica* and *C. canephora* crossing (Bettencourt 1973; Rodrigues et al. 2004). HdT is the main source of resistance used in breeding programs throughout the world (Gichuru et al. 2012; Zambolim 2016). The presence of such rust virulent races in the field represents a serious risk to coffee production worldwide. An example is the cultivar Oeiras MG 6851, originated from a crossing between *C. arabica* cv. Caturra (CIFC 19/1) and HdT (CIFC 832/1) and released as an important rust resistant cultivar (Pereira et al. 2000). Twelve years after its release, the resistance was broken by race XXXIII of *H. vastatrix* (Capucho et al. 2012). Thus, to study the dynamics of the interaction between the pathogen and the plant through transcriptome profiling will help to understand the supplanting of plant resistance by new physiological races of the fungus.

Despite all the efforts so far, there is limited information available on the transcriptome analysis to reveal which defense genes are involved in response to *H. vastatrix* infection. Transcriptome profiling studies are essential to fully understand the biological pathways that are activated in various physiological conditions or stages of an organism development (Wang et al. 2009; Ozsolak and Milos 2010). Expression profiling of host and pathogen can provide a new understanding of this interaction, and allow the identification of virulence genes in the pathogen or defense pathways in the host cells (Westermann et al. 2012; Boyd et al. 2013).

Here, we report a time-course high throughput transcriptome analysis of a susceptible and resistant genotypes challenged by *H. vastatrix* infection. In this approach, we could identify genes related to both compatible (susceptible) and incompatible (resistant) interactions that are important for disease development and resistance. This knowledge will help in better understanding the molecular basis of resistance to specific *H. vastatrix* races and develop new coffee rust control strategies.

## Materials and methods

### Plant inoculation, library preparation and RNA sequencing

Two coffee genotypes were used in this study: *C. arabica* cv. caturra vermelho CIFC 19/1 (susceptible) and Híbrido de Timor CIFC 832/1 (resistant). They are the parents of cultivar Oeiras, the one that have the resistance supplant by the race XXXIII of *H. vastatrix*. Plants (juvenile leaves from second pair) were inoculated with spores of *H. vastatrix* race XXXIII and sampled at 0, 12, 24, 96 h after inoculation (hai) and 17 days after inoculation (dai), totaling 10 different samples (5 time points for each of the 2 genotypes). Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen) and cDNA synthesis was performed using the Mint-2 cDNA Synthesis kit (Evrogen), according to the manufacturer's instructions. After this step, the samples were normalized with TRIMMER – cDNA Normalization kit (Evrogen), to reduce the abundant transcripts, especially rRNAs. RNA sequencing was performed using MiSeq platform (Illumina) at University of North Carolina Chape Hill, NC, USA, with TruSeq DNA Sample Preparation protocol (Illumina). This sequencing produced 2x250pb paired-end reads for each genotype and time sampled, and these ten libraries were sequenced in 10 MiSeq runs, each run comprising all libraries in a multiplex reaction, using a different barcode for each library.

### Quality assessment and overlapping paired-end reads

Read quality was assessed with FastQC software version 0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The strategy used to improve read quality and length was overlapping the forward (R1) and reverse (R2) reads using the PEAR software (Zhang et al. 2014), with T-30 parameter. Reads which did not overlap (unassembled) were filtered using Clean Solexa software, to remove low-quality regions ($Q < 20$). Finally, $Q > 20$ assembled and unassembled reads were concatenated and submitted to transcriptome assembly as single-end reads (Fig. 1).

### Transcriptome assembly

The ten libraries were mapped against a reference genome. Despite *C. arabica* genome is not available to date, *C. canephora* had its genome recently made available (Denoeud et al. 2014). Since *C. canephora* is an ancestral of *C. arabica*, we used *C. canephora* as a reference genome. An index file of *C. canephora* was created with Bowtie2 version 2.2.5 software, and mapped against the set of reads with each library using Tophat2 version 2.0.13 (Trapnell et al. 2009). The assembly of each library was created with Cufflinks (Trapnell et al. 2010), pooled together with Cuffmerge software and annotated with Cuffcompare. For this latter process, we used the *C. canephora* GFF3 and CDS (*Coding DNA Sequence*) files, which contains structural and functional genes. The set of annotated transcripts was analyzed using RSEM (*RNA-Seq by Expectation Maximization*) version 1.2.20 software (Li and Dewey 2011) to estimate the abundance of genes. The Fasta, GFF3 and CDS files of *C. canephora* genome were downloaded at http://www.coffee-genome.org/coffeacanephora.

The next step was to get reads that mapped against *H. vastatrix* and subtracted from unmapped reads against *C. canephora*, using a partial genome of *H. vastatrix* (http://www.bioinformatics.cenicafe.org/index.php/wiki/CoffeeRustHybridDraftAssembly_Contigs). Additionally, the unmapped reads against *H. vastatrix* were subtracted from the mapped *C. canephora* (Fig. 1). The purpose of this strategy was to get reads unique to *C. arabica*. Based on the result, we produced a de novo assembly using Trinity software (Grabherr et al. 2011; Haas et al. 2013). Subsequently, the obtained transcripts were analyzed with the Transdecoder (cut ORFs > 250 pb) and compared with the non-redundant databases (-NR-NCBI) (*e-value* 1e−5) by BLASTX. In addition, transcripts were submitted to BLASTX against the annotated genomes of *Arabidopsis thaliana, Vitis vinifera* and *Solanum lycopersicum* (http://www.phytozome.net). Similarly, the set of annotated transcripts obtained by de novo methodology was analyzed using RSEM (*RNA-Seq by Expectation Maximization*) version 1.2.20 software (Li and Dewey 2011) to estimate the abundance of genes.

### Identification and annotation of differentially expressed genes

Read counting and normalization (FPKM, *fragments per kilobase of exon per million mapped reads*) obtained in previous steps were used to analyze differential expression of genes using RSEM software two statistical packages (DESeq, Anders and Huber 2012 and EDGER; Robinson et al. 2010) were used to identify differentially expressed genes at time points 12 hai, 24 hai, 96 hai and 17 dai

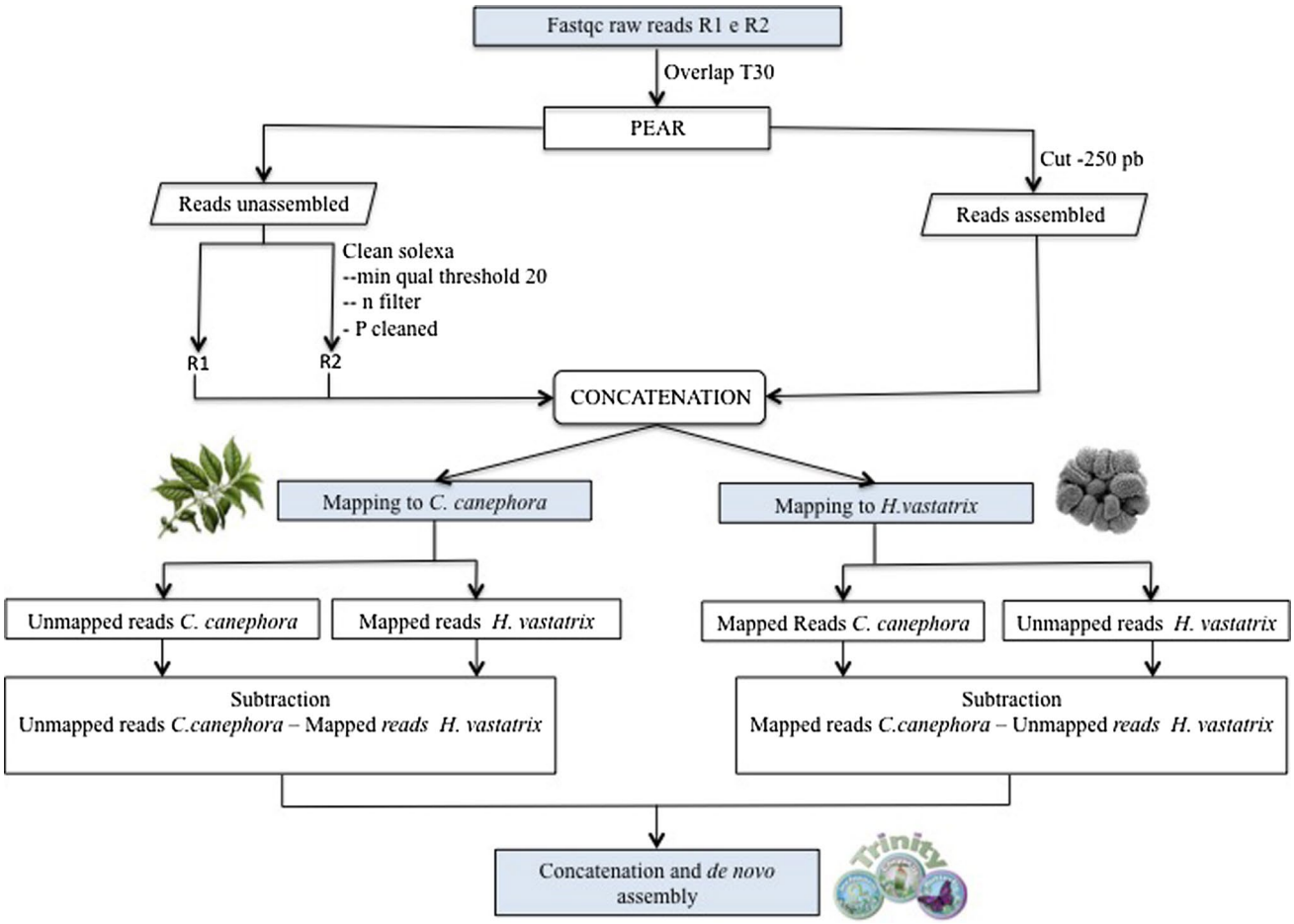



**Fig. 1** Pipeline of filtering raw reads by quality using the PEAR and Clean Solexa software. *R1* Read forward, *R2 read reverse* and strategy used for de novo assembly

compared to 0 hai (control) for each genotype separately. The list of differentially expressed genes was filtered by $Log_2$ *fold change* $\geq 0.5$ and $Log_2$ *fold change* $\leq -0.5$, with $q$ value of 0.1. To assess the variability between samples, PCA plot was performed using the statistical R package, with reads count table from RSEM. Cluster analysis was performed using Cluster 3.0 software (De Hoon et al. 2004), starting from normalized read counts. Functional annotation related to biological process, molecular function and extracellular component was performed using BLAST2GO (Conesa et al. 2005).

## Candidate gene selection and validation by real time qPCR

Only differentially expressed genes identified by both statistical packages (DESeq and EDGER) were used for quantitative real time PCR validation. Initially, the up- and down-regulated genes within each genotype and at all contrasting times were identified (Fig. 2). Within the intersection of each group, a new intersection between the up-regulated and down-regulated genes was done in susceptible and resistant cultivars for all the time points monitored. Then with the unique genes of the HdT (resistant) in each time, a last intersection was separated in order to select important genes related to plant defense (Fig. 2).

The qPCR was conducted to compare the expression pattern of some selected genes during compatible (Caturra—*H. vastatrix*) and incompatible (Híbrido de Timor—*H. vastatrix*) interactions. Primers flanking the sequence of each candidate gene were designed (Table 1) with *Primer-BLAST* software of NCBI (available at: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the following parameters: Amplicon length between 90 and 150 bp, primer size: $20 \pm 2$ bp, annealing temperature (Ta) between 55 and 60 °C, GC content of $\pm 50\%$. Reference genes used were *GADPH* (glyceraldehyde-3-phosphate dehydrogenase), *UBQ10* (polyubiquitin 10) and *S24* (ribosomal protein) (Cruz et al. 2009), see Table 1. Primer efficiency was tested by developing a standard curve of five dilution points of cDNA (1:5),

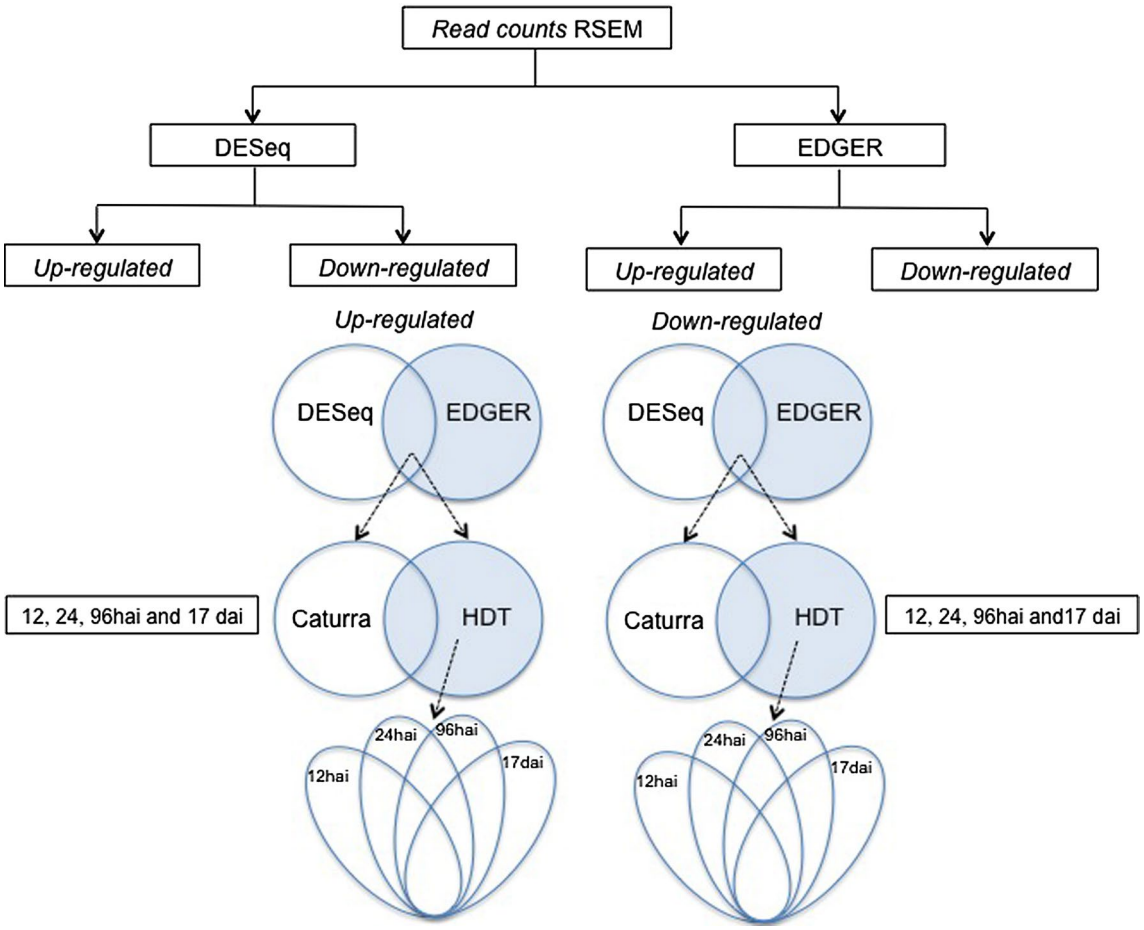



**Fig. 2** Pipeline strategy to select candidate genes. *HDT* Híbrido de Timor, *hai* hours after inoculation, *dai* days after inoculation

and primer efficiency (E) was calculated from the slope (a) of standard curve [E = 10(−1/a) − 1] with the Cts obtained for each dilution.

Using a different set of plants of those used for transcriptome study, total RNA was extracted from leaves of Caturra and Híbrido de Timor genotypes infected with *H. vastatrix* at 0, 12, 24 and 96 hai. Three biological and three technical replicates were performed for each cDNA sample. Reverse transcription was conducted using the ImProm-II™ Reverse Transcriptase system (*Promega, Madison, USA*), with 1 µg of total RNA. The qPCR reactions were carried out in 7500 Real Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA), in final volume of 10 µl with 50 ng/µl of cDNA and 100 nM of forward and reverse primers in 1x *GoTaq® qPCR Master Mix* (*Promega, Madison, USA*) as final concentrations. Reaction parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and melting curve stage was set to default conditions. The expression levels of the candidate genes were calculated using qBase software (Hellemans et al. 2007). All statistical analyses were carried out using the GraphPad Prism (version 5; GraphPad Software Inc.; La Jolla, CA, USA). To determine significant differences between control and treated samples, the one-way ANOVA with Dunnet test ($p < 0.05$) was performed. Means were compared using the Tukey test ($p < 0.05$).

## Results and discussion

### Read quality filtering, mapping and assembly

Transcriptome of coffee during compatible (Caturra) and incompatible (HdT) interactions with *H. vastatrix* race XXXIII was analyzed, using various bioinformatics tools. Firstly, a total of 103,031,664 2X250 pair-end reads were generated from ten libraries sequenced by the Illumina Miseq platform (see Table 2). The overlap between paired reads was performed producing 70,450,936 overlapped reads. This approach generated assembled (overlapped) and unassembled (R1 and R2) 300-bp reads, which were concatenated generating a total of 110,135,873 single-end reads

**Table 1** Sequences of primers used for qPCR experiments

| Gene | Name sequence primer | Annealing temper (°C) | Product size (bp) | Publication |
|---|---|---|---|---|
| Receptor-like serinethreonine-protein kinase SD1-8 | RLSKsd1 F: 5′-ACTCGGGGCAAGGAATAG AAGA-3′ | 60.8 | 92 | This study |
| | RLSKsd1 R: 5′-GCAACAACCTAAAAGGCG AACTAAA-3′ | 60.7 | | |
| Putative late blight resistance protein homolog R1A-6 | R1A-6 F: 5′-TCCGAAGGTTGTTATGGC TTTGG-3′ | 61.3 | 150 | This study |
| | R1A-6 R: 5′-TGGCACCACTCGTAGTTC TTTGT-3′ | 62.3 | | |
| Putative E3 ubiquitin-protein ligase PUB24 | E3ubiq F: 5′-AAATTCTCGGGTACAGTT GGGG-3′ | 60.2 | 112 | This study |
| | E3ubiq R: 5′-TTGCCTTATCCTTGAGGT GCGA-3′ | 62.0 | | |
| Putative probable receptor-like protein kinase At5g39020 | RLKat5 F: 5′-GATACATGGCTCCTGAGTTGT TCT-3′ | 60.3 | 119 | This study |
| | RLKat5 R: 5′-TGCATTTACATTTCTCCTCCT TCCT-3′ | 60.2 | | |
| Premnaspirodiene oxygenase | Premna F: 5′-ACGGGAAAGAGGACCATT GAAGA-3′ | 61.5 | 105 | This study |
| | Premna R: 5′-AACTGATAAAGGGGCAGG AGGA-3′ | 60.8 | | |
| NB-ARC domain-containing disease resistance protein | NB-ARC F: 5′-ACGGGGAATTGTCGAAGG TGTT-3′ | 62.4 | 111 | This study |
| | NB-ARC R: 5′-ATGCAGGGATTTCATGGT CCTC-3′ | 60.4 | | |
| Ethylene-responsive transcription factor 1B | Eth F: 5′-CCCTTCGTGATATGAAATGCGGT-3′ | 61.3 | 135 | This study |
| | Eth R: 5′-CCGCCTCTCTTGCAATTTTGTG-3′ | 60.9 | | |
| Cationic peroxidase 2 | Perox F: 5′-TTGGGGGACATACGATTGGAAC-3′ | 60.3 | 111 | This study |
| | Perox R:5′-GAGAAAGAAAAGAGGGACTGA TGGA-3′ | 60.2 | | |
| Putative disease resistance protein RGA1 | RGA1 F: 5′-TCCTTGTTCTTGATGATGTGT GGA-3′ | 60.2 | 124 | This study |
| | RGA1 R:5′-ACTGGTTGTAGACGAGTAGTG AGA-3′ | 60.5 | | |
| Putative ankyrin repeat-containing protein At3g12360 | Anky F: 5′-GGAAGGAACCCTCTTCATGTT GCT-3′ | 62.8 | 112 | This study |
| | Anky R: 5′-TGGTCTCCCCACGTCTAGTCT TTT-3′ | 63.0 | | |
| Putative basic helix-loop-helix (bHLH) DNA-binding superfamily protein | bHLH F: 5′-CATATTCAGGCGCTACAGGTG AGA-3′ | 62.2 | 102 | This study |
| | bHLH R: 5′-GTTCTTCCAGCCCATTTA GCAGA-3′ | 60.8 | | |
| Putative disease resistance-responsive (dirigent-like protein) family protein | DRR F: 5′-CGGTCGAAGCCAAGGTCTTTA TGT-3′ | 63.1 | 108 | This study |
| | DRR R: 5′-TCCAATGTGCTACCGTTGTAT TGT-3′ | 60.8 | | |
| Pathogen-related protein | PRP F: 5′-TCTAGGCATCAACGGGAGGAAA-3′ | 61.1 | 120 | This study |
| | PRP R: 5′-TTCGCAGGATTGTAAACCCGAA-3′ | 60.5 | | |

**Table 1** (continued)

| Gene | Name sequence primer | Annealing temper (°C) | Product size (bp) | Publication |
|---|---|---|---|---|
| *Coffea arabica* cDNA clone CA00-XX-LV5-041-E04-QH, mRNA sequence | GAPDH F: 5′-AGGCTGTTGGGAAAGTTC TTC-3′ | 63.4 | 70 | Cruz et al. (2009) |
| | GAPDH R: 5′-ACTGTTGGAACTCGGAAT GC-3′ | 64.0 | | |
| S24 ribosomal protein | S24 F: 5′-GCCCAAATATCGGCTTATCA-3′ | 63.5 | 92 | Cruz et al. (2009) |
| | S24 R: 5′-TCTTCTTGGCCCTGTTCTTC-3′ | 63.3 | | |
| UBQ10 polyubiquitin 10 | UBQ10 F5: 5′-CAGACCAGCAGAGGCTGA TT-3′ | 64.6 | 100 | Cruz et al. (2009) |
| | UBQ10 R5: 5′-AGAACCAAGTGAAGGGTG GA-3′ | 63.5 | | |

**Table 2** Total raw and concatenated reads from coffee—*H. vastatrix* interaction libraries

| Source | Library | Total paired end reads | Total overlapping reads | Total concatenated sinlge-end reads |
|---|---|---|---|---|
| Susceptible | 0 hai | 6,129,221 | 2,845,924 | 7,525,660 |
| | 12 hai | 12,468,894 | 10,226,867 | 11,323,418 |
| | 24 hai | 9,574,685 | 6,896,300 | 9,621,706 |
| | 96 hai | 6,708,346 | 4,750,954 | 6,695,765 |
| | 17 dai | 12,285,892 | 9,483,293 | 11,679,975 |
| Resistant | 0 hai | 13,647,518 | 8,071,594 | 16,351,660 |
| | 12 hai | 11,164,309 | 8,925,786 | 11,478,694 |
| | 24 hai | 15,530,942 | 8,253,540 | 19,494,216 |
| | 96 hai | 6,179,620 | 3,030,221 | 7,471,180 |
| | 17 dai | 9,342,237 | 7,966,457 | 8,493,599 |
| | Total | 103,031,664 | 70,450,936 | 110,135,873 |

*hai* hours after inoculation, *dai* days after inoculation

(Fig. 1; Table 2). As such, the quality and average length of number of reads was increased and improving the chance to obtain good transcriptome assembly.

Since *C. arabica* reference genome has not made available to date, we used a genome from a closely related species, i.e. *C. canephora*, as a reference for read mapping. In the susceptible genotype, read mapping against *C. canephora* genome showed a decrease of alignment along the infection time course, starting from 48% (0 hai) and reducing to 33% (17 dai) (Fig. 3a). In contrast, resistant library alignment was almost constant at all time points (Fig. 3b). On the contrary, when mapping was made against *H. vastatrix* partial genome (Cristancho et al. 2014), the mapping of reads in the susceptible genotype during the early hours of infection (12 and 24) was 3%, then started to increase at 96 hai, up to 8% in the 17 dai library (Fig. 3a). This final library showed a high percentage of reads mapped against *H. vastatrix* genome due to increased fungus biomass at this time point. Approximately 20 days after the start of the infection process, hyphae forming a large mycelial density, appearing on the outside through the stomata, a uredosporic serum in a "bouquet" occurs in the area of penetration (Silva et al. 2006). In resistant genotype the effect was the opposite; the amount of the fungus reads aligned began to decrease from 12 hai and onwards (Fig. 3b).

The transcriptome assembly using *C. canephora* reference genome produced 28,119 contigs with an N50 value of 1626 (Table 3). Approximately 50% of the total reads of each HdT' libraries mapped to *C. canephora* genome, which is higher than observed for the susceptible libraries (Fig. 3). This higher mapping percentage in the resistant libraries is probably due to HdT origin, since it is a natural hybrid between *Coffea arabica* and *C. canephora* (Bettencourt 1973; Rodrigues et al. 2004), therefore a larger part of its genome derives from *C. canephora*. As a complement to the strategy using *C. canephora* as a reference, and in order to increase the number of contigs to have a greater coverage of the transcriptome, de novo assembly of unaligment reads was adopted in this study. We obtained a total of 15,040 contigs with N50 of 1317 (Table 3). A total of 43,159 contigs

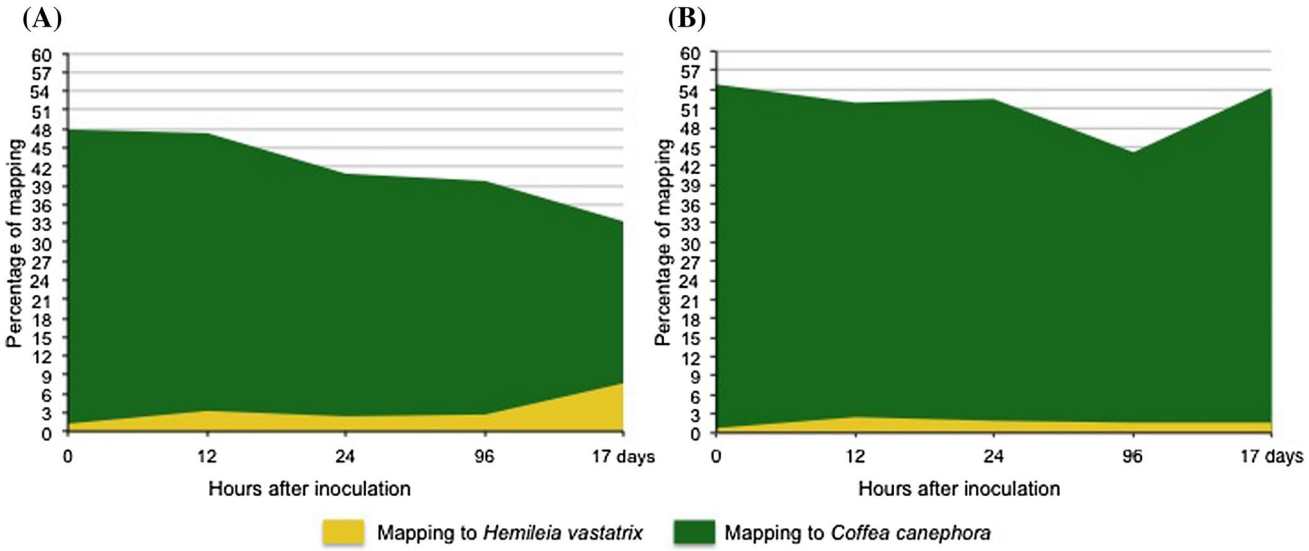



**Fig. 3** Percentage of reads mapping against *Coffea canephora* (green) and *Hemileia vastatrix* (yellow) reference genomes during infection course. **a** Susceptible libraries, **b** resistant libraries

**Table 3** Statistical assembly parameters in *C. canephora* and the de novo assembly

| Assembly | Total contigs | Mean size (bp) | N50 | # Total base pair |
|---|---|---|---|---|
| *C. canephora* | 28,119 | 1296 | 1626 (7148 contigs) | 36,468,733 |
| De novo | 15,040 | 1274 | 1317 (5370 contigs) | 1,961,987 |

were produced using *C. canephora* genome reference and de novo assembly. This number is much greater than reported by Fernandez et al. (2012) in a transcriptome analysis of *C. arabica* CIFC H147/1 (resistant) during the interaction with *H. vastatrix* using the 454-pyrosequencing platform, the only published study reporting transcriptome profiling of this pathosystem. The authors produced 352,146 reads assembled into 13,951 contigs with an average size of 631 bp. The functions of the majority of the genes were related to caffeine or chlorogenic acid biosynthesis, and genes associated to plant defense response such as *WRKY* transcription factor, pathogenesis-related (PR) proteins (1,3-b-glucanases, PR1b, PR-5 of the thaumatin-like protein family and chitinases). Similar genes were founded in our experiment; however, other HdT exclusive genes such as NAC, MYB, Beta-glucosidase, F-box protein and peroxidase were found involved in defense mechanisms at early hours (12 and 24) of *H. vastatrix* infection.

In addition to obtaining a greater number of contigs, we analyzed both compatible and incompatible coffee—*H. vastatrix* interaction with the aim of providing a better understanding of gene expression profile. Moreover, here, we used Illumina sequencing which has less low error rate, compared to 454-pyrosequencing platform (Loman et al. 2012). Recently, other transcriptome studies of coffee, however not involving plant-pathogen interactions, showed similar number of contigs assembled as in this present study. Leaf and fruit transcriptome analysis of *Coffea eugenioides* produced 36,935 contigs using Illumina HiSeq platform (Yuyama et al. 2016). Also Mofatto et al. (2016) obtained a total of 41,512 contigs from *C. arabica* transcriptome, comparing the molecular responses to drought in two commercial cultivars using 454—pyrosequencing and Sanger platforms. The expression profiles (read counts) in all libraries were submitted for PCA analysis in order to evaluate the intrinsic variation between libraries. A distinction between the group of resistant and susceptible genotype libraries was observed when *C. canephora* was used as a reference for transcriptome assembly (Fig. 4). The results from de novo assembly (data not shown) are similar to the result elucidated by *C. canephora* assembly. The PCA clearly revealed that the greatest changes in host transcriptome were caused by the fungal infection following the interaction process. In particular, in susceptible genotype 17 dai library was well separated from all other libraries.

## Profiling of differentially expressed genes (DEGs)

Plants are in a constant struggle with pathogens, and use different defense mechanisms to preserve their integrity, such as PTI and ETI. In coffee—*H. vastatrix* interaction, the pathogen establishes a biotrophic relation with the host

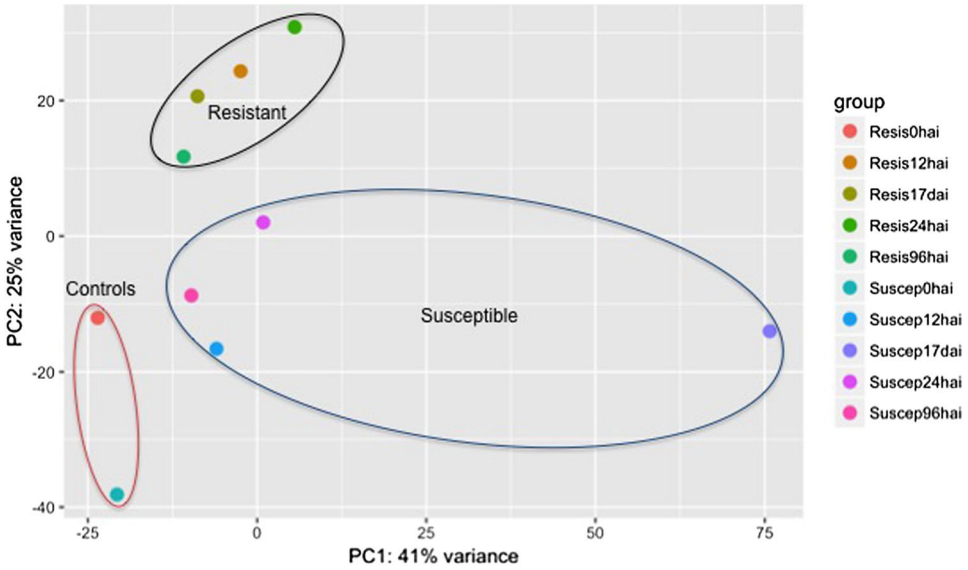



**Fig. 4** Principal component analysis (PCA) of susceptible and resistant genotype libraries during the interaction with *H. vastatrix*

within the first hours after infection (< 24 hai). Two main groups of expression levels can be observed from the heatmap (Fig. 5a). The first group correspond to the early infection events (12 and 24 hai) and HdT responded to the attack of *H. vastatrix* with larger number of up-regulated genes than Caturra, which is indicated as a higher red intensity in heatmap (Fig. 5a) and total number of up-regulated genes (Fig. 5b), which shows around 500 more up-regulated genes than in Caturra. In the second group, both genotypes had similar pattern expression during 96 hai. However, at 17 dai the expression of some genes was increased in resistant genotypes unlike in susceptible genotypes. The markedly up-regulation of genes in HdT may reflect the exploitation of cellular resources and / or the activation of defense responses (Grenville-Briggs and West 2005). In fact, it has been reported that coffee plants with complete resistance to *H. vastatrix*, fungus growth ceases in the early stages of infection where there is a disruption of the cytoplasmic contents of the fungal infection structures (Diniz et al. 2012). Therefore, this overall up-regulation of transcription might be a direct consequence of fungus attack and it is responsible for a coordinate and effective defense.

Generally, sets of genes in plants are induced against a biotic or abiotic stress condition. This response may be early or late, but the first response is key to induce signaling cascades for expression of genes involved in defense mechanisms (Kumar and Kirti 2011). For the majority of pathosystems, all this process starts with the recognition or perception, signal transduction, followed by the activation of defense genes (Oliveira et al. 2016). By inducing a hierarchical network of genes that regulate the expression of resistance genes, the advancing pathogen within the plant is suppressed (Kushalappa et al. 2016). Here, we found several DEGs encoding receptors in the transcriptome of HdT such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs) in the initial stage of infection (data not shown). These receptors could be related to pathogen perception, such as PAMPs recognition by PRRs, which leads to a signaling cascade that alerts the plant to the presence of a pathogen and induces PTI (Ishiga et al. 2013). This PTI response frequently involves ion fluxes, the production of reactive oxygen species, protein phosphorylation, ethylene biosynthesis, and callose deposition (Boller and Felix 2009). Linked to such functions during the early hours of HdT-*H. vastatrix* interaction, we found genes such as calcium-transporting, peroxidase, ethylene-responsive transcription and callose synthase.

Additionally, we confirmed that the early response of HdT to the *H. vastatrix* attack was related to pre-haustorial resistance as suggested (Heath 1977; Mellersh and Heath 2003; Niks and Rubiales 2002; Freitas et al. 2014). Usually the haustoria formation of *H. vastatrix* begins after 24 hai in the host. Although, in coffee is also possible to found post haustorial resistance, the type of response depend of the coffee genotype (Silva et al. 2006). The pre-haustorial response was also observed in the barley-*Puccinia graminis* f. sp. *tritici* pathosystem, during this interaction the resistance gene *RPG1* was expressed a few minutes after inoculation with stem rust races (Nirmala et al. 2010). More recently, in the same pathosystem it was observed early responses to avirulent stem rust races in wheat carrying resistance genes *Sr5* and *Sr36* involved callose deposition in stomatal guard cells (Wang et al. 2015). It is possible that the genes identified here as induced in early hours in HdT may trigger a rapid and efficient response for the plant defense, and these mechanisms may involve pre-haustorial resistance in

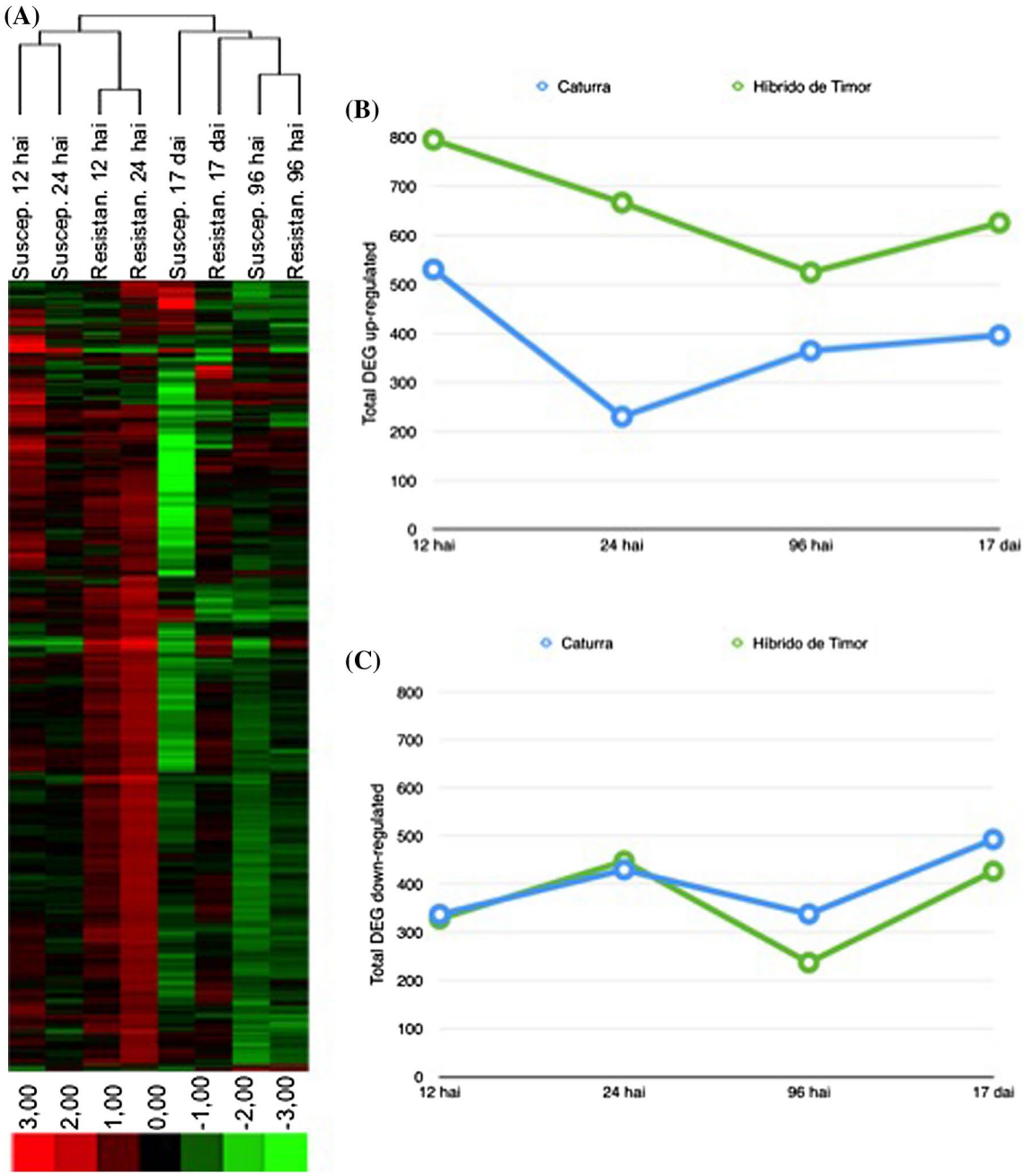



**Fig. 5** Expression profile in Híbrido de Timor (resistant) and Caturra (susceptible) inoculated with *H. vastatrix*. **a** Hierarchical clustering according to the changes in expression level along the time course. Cluster analysis was performed using Cluster 3.0 software. Each column represents the $Log_2$ fold change in transcript levels in coffee at the indicated times. Up-regulated and down-regulated genes are shown in red and green, respectively. The intensity color scale indicates the level of expression. **b, c** Total up and down-regulated genes, respectively

coffee-*H. vastatrix* interaction, in the same way found for barley and wheat pre-haustorial defense to *P. graminis*. Niks and Rubiales (2002) suggest that combining different resistance mechanisms acting at different stages of the infection process could provide multiple barriers that are not easily overcome by simple race-type changes of the pathogen. In addition, different members of a host plant species contain different *R* gene complements. Rust infections can therefore lead to either a resistant or a susceptible outcome, depending upon the plant and pathogen genotypes involved (Bettgenhaeuser et al. 2014).

In the case of a group of genes in susceptible plant, more up-regulated genes at 12 hai (Fig. 5a) may be associated with basal resistance of the plant (Jones and Dangl 2006). In the following time points, however, the level of gene expression was suppressed (Fig. 5a). Note that in all time points, HdT showed a higher gene expression, which can be noticed by the color intensity in heatmap (Fig. 5a). This shows that transcription programming in the resistant genotype is completely different from susceptible genotype. On the other hand, the number of down-regulated genes is comparable in both genotypes (Fig. 5b). Plant–pathogen interactions are complex processes that trigger a series of molecular responses at several expression levels. While resistant plants initiate responses in incompatible interactions, susceptible plants can also launch a series of basal defense responses in compatible interactions. Although they present similar expression profiles, defense gene induction in compatible interactions occurs later than that in incompatible interactions (Balaji et al. 2008; Lara-Ávila et al. 2012).

BLAST2GO analysis was performed for genes up-regulated at 12 and 24 hai in both resistant and susceptible genotypes (Fig. S1). These time points are when actual biotrophic interactions are assumed to occur. The result presented for the biological process category, the two genotypes showed the existence of up-regulated genes associated with programmed cell death but at different time points (12 hai in susceptible and 24 hai in resistant genotype). It could be inferred that programmed cell death is a delayed response to efficiently deter the ingression and establishment of the invading pathogen. A microscopic study analyzing the same pathosystem that we used (HdT CIFC 832/1 and Caturra CIFC 19/1 inoculated with *H. vastatrix* race XXXIII) revealed 30% of HR sites in both genotypes at 12 hai. However, this same study showed an increase HR to 70% in HdT at 24 hai and maintained 30% in Caturra (Freitas et al. 2014). On the other hand, for the molecular function category for the majority of the genes (70%) were represented by genes involved in catalytic activity in resistant genotype. Of remarkable difference that can be noticed is the higher proportion of up-regulated genes predicted as cell wall component under the cellular component category in resistant genotype as part of structural resistance.

On the other hand, analysis of differentially expressed genes using *C. canephora* genome reference, represented using venn diagrams in all time courses between Caturra and HdT, allowed to identify one set of up-regulated genes unique to HdT for each time of infection (Fig. 6a–d), the results from de novo assembly (data not shown) are similar to the result elucidated. However, within each time it was possible to observe that both genotypes have some genes in common (Fig. 6a–d), some of which could be associated with basal resistance in both genotypes, considering that Caturra does not show vertical resistance against *H. vastatrix* (Silva et al. 2006). Basal resistance is a kind of defense response mechanism common in plants when they are attacked by biotrophic fungi (Niks et al. 2015), and that is initiated during the early phases of pathogen infection (Gill et al. 2015). Finally, one more intersection of this pool of genes allowed us to choose 13 genes exclusively up-regulated in HdT, which showed importance within the plant defense mechanisms. This selection was made from 219, 129 and 118 genes corresponding to 12, 24 and 96 h after infection in HdT, respectively (Fig. 6e).

The qPCR analysis of 13 different types of genes related to plant defense mechanisms were conducted at 0, 12, 24 and 96 hai in susceptible and resistant coffee genotypes. Expression pattern in qPCR was similar to transcriptome analysis for most of the studied genes (Fig. S2). A similar correlation between qPCR and transcriptome profiling was reported by Rubio et al. (2015). Both analyzes have their own strengths and limitations, the different expression levels between Transcriptome and qPCR could be caused by the bioinformatics process performed in the Transcriptome analysis, which includes several factors that can affect the reproducibility of quantitative expression profiles, including alignment choices, estimation of transcript expression, etc. (Labaj et al. 2011). The first group of analyzed genes was transcription factors (*Putative basic helix-loop-helix bHLH DNA-binding superfamily protein*, and *Ethylene-responsive transcription factor 1B*), which showed early expression in HdT in contrast to Caturra (Fig. 7a, b). The *bHLH* (Fig. 7a) presented a high peak expression at 12 hai in the incompatible interaction. The results suggest that this gene may be involved in regulating the expression of some resistance genes or defense mechanisms only in resistant genotype. Kundu et al. (2015) evidenced that the expression profiling of transcription factor of this same family (bHLH) may play a complementary and/or overlapping role in enhancing expression of downstream components of the defense pathway.

The second transcription factor, *Ethylene-responsive transcription factor 1B* (ERF) presented two expression peaks (at 12 and 24 hai) in resistant genotype and also significantly higher when compared to susceptible genotype (Fig. 7b). Usually, the transcription factors are activated by external stimuli and subsequently regulate the expression of genes involved in plant defense. A number of ERF genes confer tolerance to various biotic stresses when expressed in different plants under different conditions (Licausi et al. 2013). There is evidence that ERFs induce transcription resistance genes, pathogenesis-related (PR) genes, osmotin, chitinase and b-1,3-glucanase (Licausi et al. 2013). This kind of gene has been selected through evolution to regulate a series of stress-response pathways and could be used in genetic engineering for the breeding of plants with specific traits related to stress tolerance (Licausi et al. 2013). Ganesh et al. (2006)

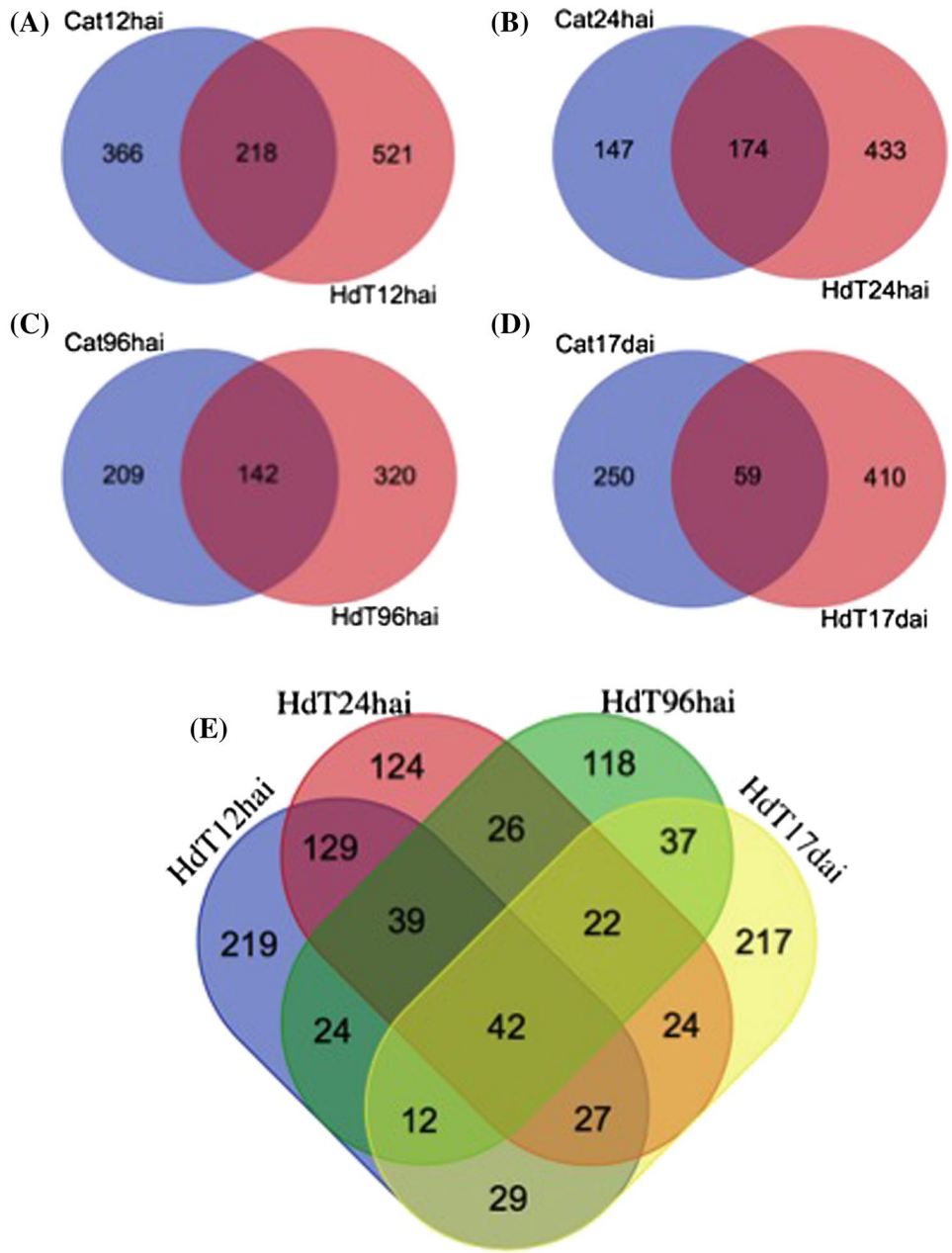



**Fig. 6** Number of up-regulated genes (DEGs) of the *C. canephora* assembly in Caturra and HdT in response to *H. vastatrix* infection. **a** Venn diagram displaying the number of up-regulated DEGs between Caturra (Cat) and HdT at 12 hai. **b** Up-regulated DEGs between Cat and HdT at 24 hai. **c** Up-regulated DEGs between Cat and HdT at 96 hai. **d** Up-regulated DEGs between Cat and HdT at 17 dai. **e** Up-regulated DEGs exclusive to HdT at all times investigated

evaluated the expression of three defense related genes during coffee—*H. vastatrix* interaction, including a transcription factor. These genes showed that they were transiently induced during early stages (12–24 hai) of pathogen infection. They reported that two of the three genes (*CaR111*, gene encoding a protein of unknown function and *CaWRKY1*, a transcription factor) were up-regulated in incompatible samples, which suggest their involvement in defense mechanism.

The group of genes related to recognition of pathogen elicitor proteins [*Serine-threonine protein-receptor-like kinase SD1-8* and *Probable putative protein receptor-like kinase* (At5g39020 homolog)], exhibited a constant expression in HdT during infection process while the expression in Caturra was delayed (24 hai) (Fig. 7c, d). Such behavior are associated with signal perception through their extracellular domain and propagate the signal through the intracellular kinase domain to activate *R* genes (Afzal et al. 2008). A similar expression profiling was found with the resistance gene group: *Putative late blight resistance protein homolog R1A-6, NB-ARC domain-containing disease resistance protein* and *Putative disease resistance protein RGA1* (Fig. 7e–g). These genes group are most likely involved in the activation of plant defense mechanisms and their expression during

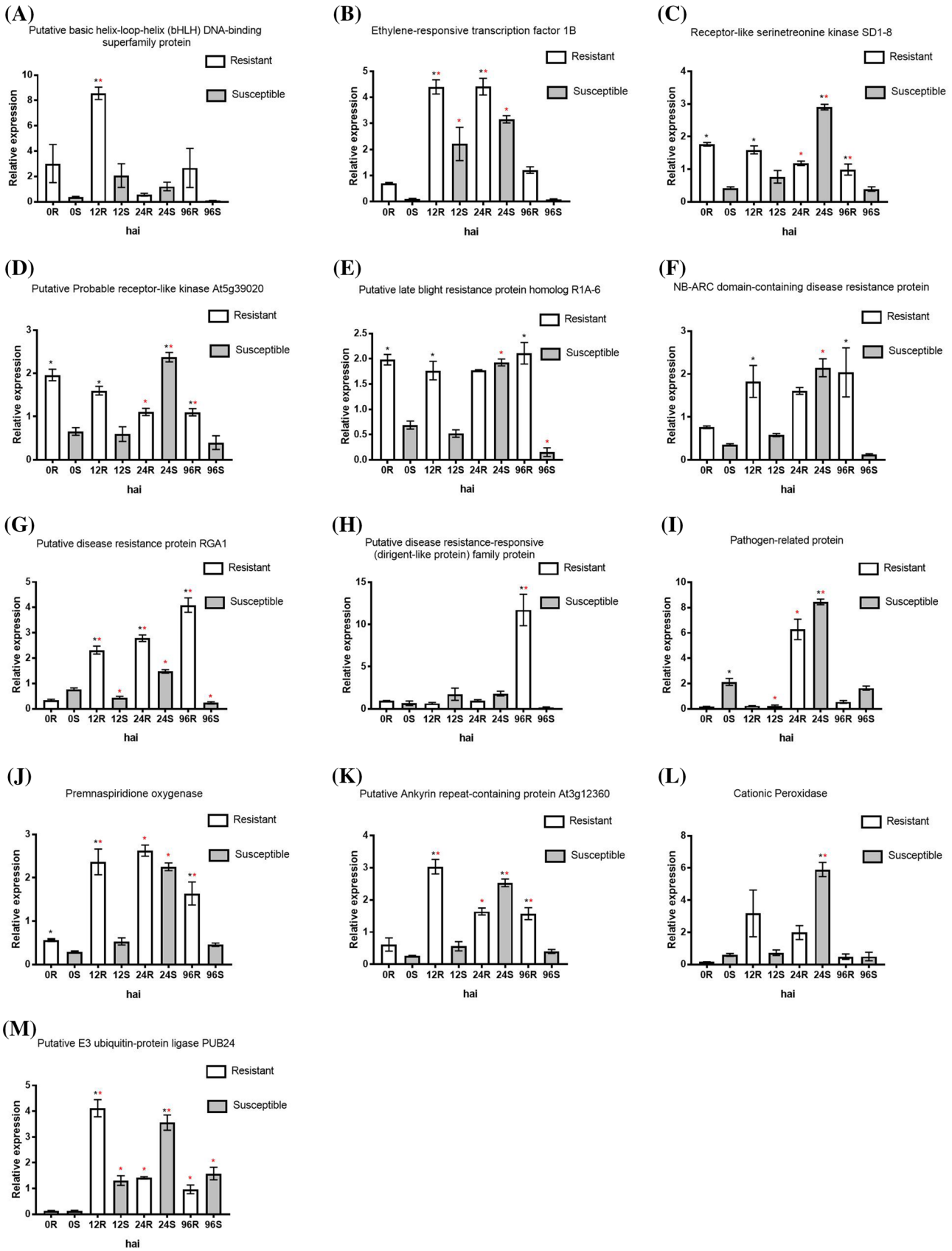
all time course, is possibly associated with controlling the differentiation of several fungal structures. In coffee, Diniz et al. (2012) evaluated the expression of *CaRLK*, which is involved in recognition, signaling and defense, showed that this gene was activated in the early events of the HdT 832/2—H. vastatrix interaction. *CaRLK* showed expression peak at 6 to 12 hai, a period when appressoria and penetration hypha differentiate. According to this report, the second highest expression peak was when the anchors and haustoria mother cells differentiate (21–24 hai). In the present work, *RLK* was continuously expressed along all the time courses studied in resistant genotype unlike in the susceptible genotype in which the expression was delayed. The result suggests that this gene is one of the key defense components which is in accordance to the work of (Diniz et al. 2012).

In the case of *Putative disease resistance-response* (dirigent-like protein) family protein, it presented a unique and high relative expression at 96 hai (Fig. 7h). This might be a case of an *R*-genes that is highly genotype specific (Narusaka et al. 2013) and downstream activated by signaling cascades (Hammond-Kosack and Jones 1997). The PR protein showed high expression in both genotypes at 24 hai, however the susceptible was higher than resistant genotype (Fig. 7i), which suggests that in general these types of genes are important in the defense of plants against pathogens, mainly in susceptible plants. Several types of PR proteins in resistant and susceptible wheat genotypes were also reported as up-regulated (Xin et al. 2012). The PR protein expression is often triggered by pathogen infection, limiting the pathogen progression (Silva et al. 2006).

Another group of genes analyzed by qPCR were *Premnaspiridione oxygenase, Putative Ankyrin repeat-containing protein* At3g12360, *Cationic peroxidase* and *Putative E3 ubiquitin-protein ligase PUB24*. The first gene encodes solavetivone, a potent antifungal phytoalexin (Takahashi et al. 2007). Generally, phytoalexins are organic compounds, which possess antimicrobial or repellent activities (Jeandet et al. 2013). Their production starts a few hours after the pathogen or pest attack (Pedras et al. 2011). The results found here are in agreement with the literature, since in the resistant genotype *Premnaspiridione oxygenase* up-regulation starts at 12 hai while in the susceptible genotype it only starts at 24 hai (Fig. 7j). The putative ankyrin repeat-containing protein followed a similar expression pattern (Fig. 7k). Functional characterization of *Ankyrin repeats proteins* across species have indicated a conserved role for them in protection against pathogen and disease resistance, as promoter of Systemic Acquired Resistance (SAR) (Sharma and Pandey 2016). This protein is important for transducing the Salicylic acid (SA) signal. In some *R–avr*-mediated interactions, SA is required for the *R* gene-dependent host programmed cell death (called the hypersensitive response, HR) and/or for disease resistance (Lu 2003). Perhaps, the induction of *Ankyrin repeat-containing protein* in the resistant plant, within the first hours after infection, promotes the production of SA, important compound to trigger HR.

The *Cationic peroxidase gene* is related with the accumulation of lignin-like compounds and reduction in pathogen multiplication in leaves and onset of the HR. We found this gene up-regulated at 12 hai in HdT and at 24 hai in Caturra (Fig. 7l). Its rice homologue is also induced at 24 hai during incompatible interaction with *Xanthomonas oryzae* pv oryzae pathogen (Young et al. 1995) and during compatible interaction at 48 hai. The early expression of cationic peroxidase in HdT may be involved in most important responses in coffee HR (Silva et al. 2002). *Putative E3 ubiquitin-protein ligase PUB24* is related to different positive and negative functions in different steps of plant defense such as regulation of RLKs (Duplan and Rivas 2014), involved in recognition of pathogen elicitor proteins (Kenn 1999; Ebel 1997; Shibuya and Minami 2001; Peck 2003). Wang et al. (2006) demonstrated that the interaction of *E3 Ubligase* gene with a *RLKs* type protein triggered cascades of defense gene activation in rice plant against *Xanthomonas oryzae* pv oryzae. Here, we found a high gene expression of *Putative E3 ubiquitin-protein ligase PUB24* at 12 hai (Fig. 7m) in the resistant plant at the same time of an up-regulation of an *Serine-threonine protein-receptor-like kinase SD1-8* and *Probable putative protein receptor-like kinase* (At5g39020 homolog) genes (Fig. 7c, d). Therefore, due to their expression pattern, it is reasonable to hypothesize that these three genes might be involved in a defense mechanism similar to that found in rice against *X. oryzae* pv oryzae, where coffee E3 ubiquitin-ligase acts together with these two receptor kinases to activate the signaling cascade leading to defense response.

Taken together, the qPCR expression analysis between resistant and susceptible genotypes confirmed that genes such as *putative basic helix-loop-helix bHLH DNA-binding superfamily protein, ethylene-responsive transcription factor 1B, putative disease resistance protein RGA1, putative disease resistance-response (dirigent-like protein) family protein*, and *premnaspiridione oxygenase* showed higher expression at early stage upon challenge with the biotrophic pathogen *H. vastatrix* in resistant plant. In contrast, no such up-regulation in early hours was detected in susceptible genotype. These genes could represent potential candidates for different biotechnological and effective management tools for operational application in new strategies for controlling coffee rust. In general, the regulation of gene expression is a dynamic process. We confirmed that early expression patterns of up-regulated genes in HdT are directly related to prehaustorial resistance. This transcriptome study paves


(A)
Putative basic helix-loop-helix (bHLH) DNA-binding superfamily protein
(B)
Ethylene-responsive transcription factor 1B
(C)
Receptor-like serinetreonine kinase SD1-8
(D)
Putative Probable receptor-like kinase At5g39020
(E)
Putative late blight resistance protein homolog R1A-6
(F)
NB-ARC domain-containing disease resistance protein
(G)
Putative disease resistance protein RGA1
(H)
Putative disease resistance-responsive (dirigent-like protein) family protein
(I)
Pathogen-related protein
(J)
Premnaspiridione oxygenase
(K)
Putative Ankyrin repeat-containing protein At3g12360
(L)
Cationic Peroxidase
(M)
Putative E3 ubiquitin-protein ligase PUB24
Resistant
Susceptible
Relative expression
0R 0S 12R 12S 24R 24S 96R 96S
hai

◂**Fig. 7** Validation of candidate genes by qPCR. Y-axis represent the relative expression at various times after *H. vastatrix* inoculation in susceptible and resistant coffee genotypes. Error bars = SEM, n = 3 independent biological replicates. Red asterisk (*) shows significantly up-or down-regulated relative to uninoculated samples (0 hai) within interaction as defined by one-way ANOVA followed by Dunnett test ($p < 0.05$); black asterisk (*) shows significant difference in expression level between the same hai between interactions defined by Tukey test ($p < 0.05$)

a way for more detailed work on coffee resistance to *H. vastatrix*, which will be important for future development of plants with certain adaptive characteristics to coffee rust.

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**Author contributions** JCF, LCM and MFC analyzed the sequencing data for transcriptome assembly; RDLFL and EMZ developed the biological experiments for the sequencing; JCF and SSF developed the qPCR experiments; Wrote the manuscript draft: JCF, SSF and ETC edited and revised the manuscript; ETC and LZ formulated the idea.

**Compliance with ethical standards**

**Conflict of interest** The authors have no conflict of interest to declare.

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