

# Differential expression of molecular rust resistance components have distinctive profiles in *Coffea arabica* - *Hemileia vastatrix* interactions

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**Abstract** Countering the economic hurdle caused by coffee leaf rust disease is most appealing at this time as it has posed a major threat to coffee production around the world. Establishing differential expression profiles at different times following pathogen invasion in both innate and acquired immunities unlocks the molecular components of resistance and susceptibility. Suppression subtractive hybridization (SSH) was used to identify genes differentially over-expressed and repressed during incompatible and compatible interactions

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between *Coffea arabica* and *Hemileia vastatrix*. From 433 clones of expressed sequence tags (ESTs) sequenced, 352 were annotated and categorized of which the proportion of genes expressed during compatible interaction were relatively smaller. The result showed upregulation and downregulation of various genes at 12 and 24 h after pathogen inoculation in both interactions. The use of four different databases in searching for gene homology resulted in different number of similar sequences. BLASTx against EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) database being with the maximum (100%) hits for all the annotated sequences. RT-qPCR analysis of seven resistance-signaling genes showed similar expression patterns for most of the genes in both interactions, indicating these genes are involved in basal (non-specific) defense during which immune reactions are similar. Using SSH, we identified different types of resistance related genes that could be used for further studies towards resistant cultivar development. The potential role of some of the resistance related proteins found were also discussed.

**Keywords** Coffee · Gene expression · Plant-pathogen interaction · RT-qPCR

## Introduction

Coffee leaf rust (*H. vastatrix*) is one of the main disease of coffee worldwide as its races are rapidly emerging and its aggressive isolates are presenting new

challenges. Therefore, the current level of epidemiology is being the severest than any time before in some regions (Avelino et al. 2015; Zambolim 2016). The triggering force behind the surge of new races is not well understood as urediniospores are asexual means of reproduction (Zambolim 2016). However, some reports have started to emerge attributing the hidden sexual spore with in the asexual urediniospore to be among the factors behind the evolution of different races (Carvalho et al. 2011). What is well known in any pathosystem is that the emergence of new races and resistance breakdown is related to co-evolution during plant-pathogen interaction. Plants develop the ability to recognize pathogens and develop elaborate defense mechanisms to avoid pathogen attack. Likewise, the pathogens evolve to avoid such recognitions leading to evolution race between the plant and the pathogen (Burdon and Thrall 2009).

In resistant cultivars, resistance proteins induce an immunity by which the network of defense systems recognize molecular signatures of the pathogen as a sign of invasion leading to cease of pathogen colonization (Saunders et al. 2012). The immune reaction could be manifested ranging from physical stress to death of the infected tissue (Nimchuk et al. 2003). The first line of defense is induced by pattern recognition receptors (PRRs), which recognizes microbe-associated molecular patterns (MAMPs) (Coll et al. 2011). MAMP-triggered immunity confers basal defense, the only defense in compatible interaction unlike in incompatible interaction in which cascades of pathogen specific immune reactions are eventually triggered (Jones and Dangl 2006; Muthamilarasan and Prasad 2013). The recognition of MAMPs followed by reaction of the defense system at the site of infection, induces rapid resistance signal transduction and death of local cells (hypersensitive response, HR) prompting strong protection against the invading pathogens in resistant cultivars (Rojas et al. 2014; Kushalappa et al. 2016). HR is often associated with the direct or indirect recognition of pathogen avirulence (*avr*) genes by the corresponding resistance (*R*) gene in incompatible interaction (Jones and Dangl 2006; Bozkurt et al. 2010). Ultimately, the activation of defense responses in the surrounding tissue and throughout the whole plant results in the development of systemic acquired resistance (SAR) (Hunt et al. 1996). In incompatible interaction, HR is manifested as an effective defense response in stopping biotrophic pathogen invasion and spread by programmed cell death

(Niks and Marcel 2009; Gill et al. 2015). Even though a hypersensitive-like cell death and accumulation of phenolic-like compounds are generally observed during compatible interaction, it does not result in effective defense response and lacks pathogen specificity (Gaudet et al. 2007; Freitas et al. 2014). Once pathogen components are recognized by the cognate receptors, cascades of signal transduction responses are followed which involve changes in calcium level, extracellular alkalization, production of reactive oxygen species (ROS), activation of kinases, transcriptional reprogramming and changes in hormone concentration (Seybold et al. 2014; Wu et al. 2014). During incompatible interaction, apoplastic secretome protein extraction in different organisms (Sheen 1998), differential expression screening studies in coffee-rust interaction (Diola et al. 2013; Guerra-Guimarães et al. 2015) and *A. thaliana* treated with fungus elicitors (Ndimba et al. 2003) indicated early expression of signaling genes.

Kinase associated protein phosphatase (KAPP) 2C family and the LRR receptor-like serine/threonine-protein kinase NIK1/protein NSP-interacting kinase 1 are involved in early signal perception of pathogen effectors (Sheen 1998; Afzal et al. 2008). The exact physiological role of kinase associated protein phosphatase 2C isoenzymes has not yet been fully understood though these kinase domains are featuring in defense systems of different organisms ranging from unicellular yeast (Maeda et al. 1993; Bögre 2003) to complex mammals (Flajolet et al. 2003). In *A. thaliana*, the kinase associated protein phosphatase (KAPP) is an enzyme that dephosphorylates the Ser/Thr receptor-like kinase (RLK) (Umbrasaitė et al. 2011). Pathogen infection is also marked by increased level of endogenous auxin/IAA triggering the expression of auxin binding and auxin-responsive genes to escalate the expression of resistance signaling genes through the cascading amplification of phosphorylation mediated by different kinases (Carna et al. 2014). On the other hand, auxin/IAA proteins favor pathogenesis by repressing the response of auxin resistance signaling as seen in *A. thaliana* (Padmanabhan et al. 2008). Similar studies show that the role of auxin-repressed protein in *A. thaliana* is linked to the concentration of auxin/IAA proteins (Ulmasov 1997; Korasick et al. 2014). In its entirety, the function of gibberellic acid (GA) signaling F-box protein or GA-insensitive dwarf2 (*gid2*), as known in rice (Sasaki et al. 2003), is less understood in establishing signal perception during compatible

interaction (Bari and Jones 2009). However, the role of GA in plant development and resistance signaling has started to emerge as it has a cross-talk with other hormones in inducing basal resistance and susceptibility. As reported in rice, GA has a negative role in basal resistance (Yang et al. 2008).

Histone proteins, on the other hand, are other essential proteins involved in regulating gene expression and transposon silencing in plants and animals (Law and Jacobsen 2010). They are molecular components of nucleosomes where methylation and acetylation plays regulatory role by determining the accessibility of chromatin to regulatory proteins (Ding and Wang 2015). In this context, therefore, the abundance of histone proteins (Ac-like transposase) is associated to the state of the plant immunity that signals the need for the expression of required genes against the pathogen effectors or limit the amplification of defense signals depending on the required response.

Coffee rust resistance is a condition when the defense system recognizes any of the 50 or so races of *H. vastatrix* nine virulence factors (v1–9) in the presence of the corresponding nine major R genes (S<sub>H</sub>1–9) (Rodrigues et al. 1975; Gichuru et al. 2012; Alwora and Gichuru 2014; Zambolim 2016). Crucial to the progresses achieved so far with regards to resistance coffee breeding is the discovery of Híbrido de Timor (HDT), a rust resistant natural interspecific hybrid between *C. arabica* and *C. canephora* (Bettencourt 1973). As HDT is resistant to coffee leaf rust and other major diseases (Pereira et al. 2005), it has been used as an important source of R genes in rust resistance coffee breeding programs (Bettencourt 1973; Rodrigues et al. 1975). Among the derivatives of HDT, CIFC 832/1 and CIFC 832/2 are of considerable importance in crossing with various rust susceptible coffee cultivars around the world as they are resistant to all races of *H. vastatrix* (Rodrigues et al. 1975; Diniz et al. 2012). Despite its susceptibility to coffee leaf rust (Guzzo et al. 2009; [www.ico.org/leafrust](http://www.ico.org/leafrust)), Catuaí IAC 44 is another important cultivar due to its high vigor, cup quality, wide adaptation capacity and high productivity (<http://www.consorciopesquisacafe.com.br/>). The transfer of rust and other disease R genes from HDT derived genotypes to susceptible but with high economic value coffee cultivars like Catuaí IAC 44 has an indispensable input in mitigating coffee rust damages around the world. Indeed, the ultimate objective of breeding programs in this regard is to develop resistant varieties without

compromising other agronomic qualities. To that end, expression profiling and molecular characterization of R genes could help open another level of understanding of phytopathosystem and in turn leads to durable rust resistance development against *H. vastatrix*.

Differential expression of resistance and resistance-signaling genes has been reported in compatible and incompatible interactions between coffee cultivars and *H. vastatrix* races (Nimchuk et al. 2003; Fernandez et al. 2004; Glazebrook 2005; Ganesh et al. 2006; Guzzo et al. 2009; Diniz et al. 2012; Diola et al. 2013). However, identification of resistance related genes differentially over expressed and repressed at a given time altogether during incompatible and compatible interaction is less exploited, hence of great importance to execute. Such efforts are essential inputs to have an insight into the understanding of how R genes function. It also paves a way to identify candidate R genes in developing resistant cultivars. Therefore, the objective of the present work was to identify coffee genes differentially upregulated and downregulated at 12 and 24 h after inoculation (h.a.i.) and quantify some resistance-signaling genes at 0 (control), 12, 24, 48 and 72 h.a.i. during compatible and incompatible interactions between *C. arabica* and *H. vastatrix*.

## Materials and methods

### Plant materials and pathogen inoculation

Coffee rust resistant genotype HDT (CIFC-832/2) and susceptible Catuaí IAC 44 were used in all the experiments. One-year-old greenhouse grown young Catuaí IAC 44 seedlings and clone derived HDT (CIFC-832/2) plants were used for pathogen inoculation. Race II of *H. vastatrix* fungus urediniospore was rubbed off against the intact abaxial leaf surface to induce immune challenge. Pathogen inoculated plants were immediately transferred to moist dark chamber at 22 °C (±2) and relative humidity near 100% (Cabral et al. 2016). For suppression subtractive hybridization assay, eight expression studies were carried out on two interactions in such a way that forward and reverse expression studies were set up at 12 and 24 h.a.i. In the same way, five expression studies (0, 12, 24, 48 and 72 h.a.i.) were setup for RT-qPCR validation of seven resistance-signaling genes (HT12F50, HT12F100, HT12R109, HT24F85, HT24F123, HT24F133, HT24R75) during

incompatible and compatible interactions. Uninoculated plants were used as control.

#### RNA extraction and cDNA synthesis

Collected leaves were immediately stored at  $-80\text{ }^{\circ}\text{C}$  until total RNA was extracted. Total RNA was extracted using Concert™ Plant RNA Reagent (Invitrogen) following the manufacturer's recommendations. Nanodrop (NanoDrop Technologies, Wilmington, DE, USA) scanning at 260/280 nm and 1.5% UltraPure™ agarose (Invitrogen) gel electrophoresis stained with ethidium bromide ( $0.5\text{ }\mu\text{g/ml}$ ) was routinely run to check the quantity and integrity of RNA before any downstream use (Online Resources 1 & 2). Subsequently, total mRNA was separated using Dynabeads® mRNA Purification Kit (DynaL Biotech-Life Technologies) and subjected to cDNA synthesis by SMART-PCR (polymerase chain reaction) cDNA Synthesis Kit (Clontech).

#### Suppression subtractive hybridization (SSH) and cloning of ESTs

cDNA subtraction and enrichment for selective amplification of differentially expressed genes during compatible and incompatible interactions was done using *Select cDNA Subtraction Kit* (Clontech). To separate and investigate upregulated and downregulated genes due to pathogen infection, cDNAs from the two contrasting expressions were labeled as tester and then as driver subsequently. First, cDNAs from incompatible interaction were labeled as tester and subtracted from mock inoculated (control) samples, which were labelled as driver, resulting in resistance related differentially expressed ESTs. This procedure was repeated for both interactions and at each point of time (12 and 24 h) after pathogen inoculation by shuffling the labelling of tester and driver otherwise (Diatchenko et al. 1996). Amplified ESTs were inserted into pGEM-T easy vector (Promega) and then incorporated into *E. coli* DH5 $\alpha$  by heat shock transformation. Plate LB (Luria-Bertani) medium with ampicillin (200 mg/ml), X-GAL (20 mg/ml) and 2% (w/v) IPTG (Isopropyl- $\beta$ -D-thiogalactoside) was used to select transformed white colonies. Selected white colonies were picked using toothpick, transferred to 3 ml liquid LB medium with ampicillin, and shaken at a speed of 180 rpm and temperature of  $37\text{ }^{\circ}\text{C}$  for 12–16 h. Plasmid DNA was extracted using centrifugation protocol of Wizard® SV Plus Minipreps DNA

Purification System (Promega). The quality and quantity of extracted plasmid DNA was measured by 1% gel electrophoresis and Nanodrop. Insertion of the DNA segment (clone) of the eight libraries was detected by PCR using SP6 and T7 primers of pGEM-T easy vector. PCR reaction was for 35 cycles in  $94\text{ }^{\circ}\text{C}$  for 30 s,  $45\text{ }^{\circ}\text{C}$  for 1 min and  $72\text{ }^{\circ}\text{C}$  for 2 min in a total reaction volume of  $25\text{ }\mu\text{l}$  using  $1\times$  PCR buffer,  $200\text{ }\mu\text{M}$  each dNTPs,  $0.4\text{ }\mu\text{M}$  of each primer,  $1.4\text{ mM}$   $\text{MgCl}_2$ , 1 unit Taq polymerase (Invitrogen), and  $200\text{ ng}$  plasmid DNA.

#### Sequencing of ESTs

433 clones were sequenced using 16-capillary 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA) fluorescence-based capillary electrophoresis system. Sp6 and T7 primers were used in sequencing PCR reactions. PCR reactions were based on BigDye® Terminator v3.1 cycle sequencing kit at a reaction condition of  $96\text{ }^{\circ}\text{C}$  for one min followed by 15 cycles of  $96\text{ }^{\circ}\text{C}$  and  $50\text{ }^{\circ}\text{C}$  for 15 s and extension reaction of  $60\text{ }^{\circ}\text{C}$  for 4 min in a reaction volume of  $20\text{ }\mu\text{l}$ . Sequence quality of  $>20$  QC was considered for downstream processing. Sequences were trimmed-off low quality, adapter, vector and primer sequences using Vecscreen server of NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/tools/vecscreen/>).

#### Annotation and homology search

Eight expression libraries were categorized into two groups as ESTs of incompatible and compatible interactions. The clones subtracted were subjected to BLASTx (Nucleotide Basic Local Alignment Search Tool) against ESTs available in NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), NCBI BLAST2GOx (BLAST for Gene Ontology), EMBL BLASTx, Brazilian Coffee Genome Project BLASTx (LGE, <http://bioinfo03.ibi.unicamp.br/coffea/>) and *C. canephora* EST data repositories. To avoid the potential contamination by fungal genes, BLASTx was run against ESTs of three related species of fungi; *Melampsora-laricis-populina* (54,445 ESTs), *Puccinia graminis* (269 ESTs) and *Hemileia vastatrix* (726 ESTs). All the three ESTs were accessed at <http://www.ncbi.nlm.nih.gov/nucest> (accessed on 11 August 2016) by searching for each species separately. Genes with homology to any of these three species were excluded from the libraries. Subsequently, redundant genes (7.81%) were

eliminated to avoid unnecessary duplications of identical genes within a library. Associated GOs (gene ontologies) were pooled together from all the accessed databases and grouped based on their function and cellular localization. A cutoff  $e$ -value of  $10^{-5}$  or less was considered significantly similar in annotating ESTs. Upregulated and downregulated genes were analyzed in both categories across all the databases. Putative and hypothetical functions of ESTs were searched in all the databases to map differentially expressed genes in all the libraries during the two contrasting interactions.

### Subcellular localization of ESTs

Protein subcellular localization of all 352 ESTs was done using TargetP 1.1 online localization prediction tool (<http://www.cbs.dtu.dk/services/TargetP/>) according to Emanuelsson et al. (2000). Significance cut-off for four different categories of subcellular localities were set to standard for plant network as 0.73 for cTP (chloroplast transit peptide), 0.86 for mTP (mitochondrial targeting peptide), 0.43 for SP (signal peptide, involved in secretory pathway) and 0.84 for others (other subcellular compartment).

### Quantitative analysis by RT-qPCR

Seven genes involved in resistance-signaling against *H. vastatrix* were selected from libraries constructed at 12 and 24 h.a.i. for quantitative validation (Table 1). Three of these genes were downregulated at either 12 or 24 h.a.i. while the others were upregulated at either of these time points during incompatible interaction as seen during identification of differentially expressed genes. Three reference genes (S24, UBQ10 and GAPDH), whose expressions were found to be stable (Cruz et al. 2009) were used. Primer design was done using NCBI primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) with preferential selection of primer pairs having minimum self-complementarity. The primers were *Sigma-Aldrich* made having 22–23 bp size (Table 1).

Two step RT-qPCR was carried out following the MIQE minimum standard guidelines for fluorescence based quantitative real-time PCR experiments (Bustin et al. 2009). cDNA was synthesized following Im-Prom-II™ Reverse Transcription System cDNA synthesis Kit (Promega) using 1 µg total RNA. Amplification of target fragments were optimized by testing various

annealing temperatures around  $T_m$  ranging from 54 °C to 66 °C using Applied Biosystems (Foster City, California 94,404, USA) thermocycler temperature gradient program. Amplification of expected targets were verified by 1% agarose gel electrophoresis. Primer concentration of 1 µM was selected for both reference and target genes in RT-qPCR quantification. Standard curve was developed using pooled cDNA from both cultivars serially diluted by a factor of 5 at five dilution points (1:1, 1:5, 1:25, 1:125 and 1:625) starting from 600 ng/µl. Real-time PCR reaction volume was 10 µl containing 2 µl H<sub>2</sub>O, 1 µl (1 µM) primer, 5 µl (50% v/v) SYBR green master mix and 1 µl (120 ng/µl) cDNA. Reaction parameters were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Finally melting curve stage was set to default conditions of Applied Biosystems 7500 Real Time PCR System (Foster City, California 94,404, USA). Primer specificity was confirmed by examining melting curve of reference genes in control samples (Online Resource 3a) and target genes (Online Resource 3b) at the four h.a.i. during efficiency test and final plate run, respectively. Amplification efficiency of  $\geq 81\%$  was used for both reference and target genes. Three biological and three technical replicates run for all genes quantified. For both interactions, three different plants were randomly selected and inoculated by freshly collected urediniospores for the four time points under investigation. No template controls were included for all target genes in all run plates. All the parameters of standard curve were used in relative quantification reactions. Technical errors within each biological replicate was tested using the quantification cycle ( $C_q$ ) values by using mean test while cDNA collected at 0 h from susceptible and resistant samples were used for comparison against the remaining four time points (Nicot et al. 2005). For all reference and target genes, technical replicate  $C_q$  values were averaged. Using the amplification efficiency (E) and threshold cycle of reference and target genes, relative expression was calculated in control and unknown sample in comparison to reference genes according to Pfaffl (2001) method.

### Statistical analysis

For the three biological replicates, three technical replicate  $C_q$  values were averaged and normalized to reference gene  $C_q$  values by qBase relative quantification tool (Hellemans et al. 2007). Before any

**Table 1** Primer sequences of genes validated by RT-qPCR<sup>a</sup>

Primer name (code)	Putative sequence identification	Annotation (LGE: Brazilian Coffee Genome Project, <a href="http://bioinfo03.ibi.unicamp.br/coffea/">http://bioinfo03.ibi.unicamp.br/coffea/</a> and EMBL-EBI: <a href="http://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html">http://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html</a> )	Sequence	Tm (°C)	Amplicon size (bp)
S24 <sup>b</sup>	GT021438	CarCTFrHh24FL2_17-C09-M13F similar to 40S ribosomal protein s24-1- <i>Arabidopsis thaliana</i>	F: 5'- GCCCAAATATCGGCTTATCA-3' R: 5'- TCITCTTGGCCCTGTCTTC-3'	63.5 63.3	92
UBQ10 <sup>b</sup>	KF56925	<i>Phaseolus vulgaris</i> clone BE1176 polyubiquitin mRNA, complete cDNA	F: 5'- CAGACCAAGCAAGGCTGATT-3' R: 5'- AGAACCAAGTGAAGGGTGGGA-3'	64.6 63.5	100
GAPDH <sup>b</sup>	FN431983	<i>Tectona grandis</i> partial g3pdhcy2 gene for glyceraldehyde-3-phosphate dehydrogenase	F: 5'- AGGCTGTTGGGAAAGTTCTTC-3' R: 5'- ACTGTTGGAACCTCGGAATGC-3'	63.4 64.0	70
HT12F50	EM_EST:GT003697	TransId-204,764 CACATN1 <i>Coffea arabica</i> cDNA clone CACATN1-1B26TVB similar to Auxin-binding protein ABP20 precursor - <i>Prunus persica</i> (Peach), mRNA sequence	F: 5'- ACTCGGGGCTCCACCCTTAATC-3' R: 5'- CACAGCGAAATCCAGACGGGCAA-3'	67.0 65.8	341
HT12F100	gb ABR18801	Kinase-associated protein phosphatase [ <i>Solanum peruvianum</i> ]	F: 5'- TGCTCCGCCATGTCATCACACA-3' R: 5'- GTCGCGGCCGAGGTGCAAAAGAAG-3'	66.7 68.6	116
HT12R109	KF588660	<i>Actinidia deliciosa</i> GA signaling F-Box (SLY1_12) gene, complete cds	F: 5'- CGGCGGCTTACTCGCTTACCT-3' R: 5'- TGCCGGAAGACTTGGCCGGAAC-3'	66.5 67.5	130
HT24F85	EM_EST:GR990444	TransId-100,452 CACAT45FR <i>Coffea arabica</i> cDNA clone CACAT45FR_32_B02_018.F similar to auxin-responsive protein / indoleacetic acid-induced protein 9 (IAA9), identical to SP:Q38827 Auxin-responsive protein IAA9 (Indoleacetic acid-induced protein 9)	F: 5'- GGAAAAGGAACCCGACAGCAGCA-3' R: 5'- TGCACAAGGAAAGGAAACAGGGG-3'	66.0 65.0	238
HT24F123	ref XM_004232861	PREDICTED: <i>Solanum lycopersicum</i> auxin-repressed 12.5 kDa protein-like, transcript variant 2 (LOC101258429), mRNA	F: 5'- CTCACCTGAAACGTGGCCTGGGA-3' R: 5'- GGTTGACAGGACTGGGTGGCATAAG-3'	68.3 65.4	136
HT24F133	ref XM_006338244	PREDICTED: LRR receptor-like serine/threonine-protein kinase NIK1 (protein NSP-INTERACTING KINASE 1-like (LOC102591832), mRNA)	F: 5'- CGGCAAGGTACACAAGAGAGCCA-3' R: 5'- AGCAGCGAACCCAGAAAAGGGGCAA-3'	66.4 67.2	154
HT24R75	gb AAD12209	Act-like transposase [ <i>Arabidopsis thaliana</i> ], similar to Histone H1 - <i>Plantago major</i> (Common plantain), mRNA sequence	F: 5'- ATGCGGAGAAAGAGGGGCTGGT-3' R: 5'- TTGGTCTTTGCCCTTCGCTGGA-3'	65.7 67.7	174

<sup>a</sup> The efficiency and R<sup>2</sup> of all primers were 0.81–0.99 and 0.915–0.996, respectively

<sup>b</sup> Reference genes (Cruz et al. 2009)

downstream analysis, primer dimer correction was performed. Cq values of target genes from control samples (0 h.a.i.) in both interactions were normalized to reference genes and subjected to one-way analysis of variance (ANOVA). Within interaction normalized mean comparison was made using Dunnett test while Tukey's multiple mean comparison was used to compare corresponding normalized means between interactions using GraphPad Prism version 7.00 for windows (GraphPad software, La Jolla California, [www.graphpad.com](http://www.graphpad.com)). Differential expression was shown as relative expression of a gene at a given time after pathogen inoculation in comparison to control sample as upregulated, downregulated or not changed significantly.

## Results

### Isolation of differentially expressed genes

Four expression libraries (HT12F, HT12R, HT24F and HT24R) during coffee rust resistant cultivar HDT C1FC832/2 and another four libraries (Ca12F, Ca12R, Ca24F and Ca24R) during susceptible cultivar Catuai Vermelo IAC 44 interaction with *H. vastatrix* were constructed. These acronyms were used to represent the cultivars used; how long (in hours) the plant was treated with the pathogen (*H. vastatrix* race II) before the samples were collected followed by F (upregulated genes) or R (downregulated genes). The cDNAs of differentially expressed genes from both genotypes were separated at 12 and 24 h.a.i. The number of upregulated genes in each library from incompatible interaction were comparable while the

number of downregulated genes at 24 h.a.i. were much lower in susceptible cultivar (Fig. 1). Nevertheless, for the other three libraries of compatible interaction, it followed the same trend as in incompatible interaction.

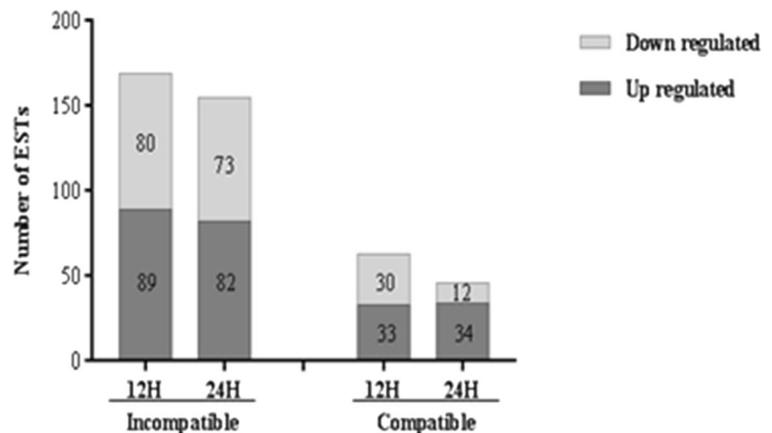
### Cloning and sequencing of ESTs

The number of isolated genes in each library was substantially greater than what they were after subsequent downstream processing. After repeated multiplication of white colonies on selective LB media, 433 ESTs (Fig. 1) were identified and sequenced. Redundant EST sequences were found in all subtraction libraries and excluded during annotation. After screening and eliminating redundant sequences, the number of non-redundant genes were 80% in upregulated libraries and 69% in downregulated libraries. The fragments sequenced were ranging from 77 bp to 1190 bp. However, only insert fragments with 154 bp or more were considered for homology search and further analyses. These EST sequences are available in Online Resource 4.

### Annotation and metabolic categorization

Non-redundant 352 ESTs differentially expressed due to pathogen inoculation in resistant and susceptible cultivars were annotated and categorized based on their metabolic roles (Online Resource 5). BLASTx search of these clones resulted in different number of matches in different databases (Online Resource 6). Matches were found for all the sequenced gene fragments in EMBL database though some hits (18.24%) were either not significant ( $e\text{-value} \geq 10^{-5}$ ) or matched with unrelated species and hence excluded. Contrarily, only 32.63% of the ESTs

**Fig. 1** Number of genes upregulated and downregulated during compatible and incompatible interaction at 12 and 24 h.a.i. before redundant ESTs were excluded



were with significant similarities using BLASTx against NCBI database. Yet, using BLAST2GO as homology search tool, the number of hits with significant similarities were different from simple BLASTx search (57.75% larger than the output by BLASTx). As these two databases were too robust, homology search was carried out in two more databases (Brazilian Coffee Genome Project, LGE EST and *C. canephora* EST) exclusively devoted to coffee and related genes. In this latter search, significant matches were found for 57.63% and 68% of the ESTs using BLASTx against LGE EST and *C. canephora* EST, respectively. Out of 352 ESTs with significant hits in any of the four databases searched (100% in EMBL, data not shown), 140 (39.55%) ESTs were shared between LGE, NCBI and *C. canephora* EST databases (Online Resource 6). We are interested to identify genes exclusively upregulated during each interaction after redundant ESTs were excluded. We found that almost all ESTs were interaction specific (Fig. 2).

Furthermore, to annotate and map GO terms associated with all ESTs (352) with significant similarities, a category of four broad biological function was considered for expression profiling. This classification was as follows; resistance and antimicrobial functions (A), resistance signal induction and transduction (B), cell maintenance and homeostasis (C) and no gene ontologies associated (D) (Fig. 3). In both interactions, no transcripts with resistance and antimicrobial functions were found in reverse libraries unlike gene transcripts involved in resistance-signaling, which were found in all but Ca24R library. Homology search in four databases resulted in no associated gene ontology (GO) terms for 33.21% of ESTs while the majority of the annotated ESTs (49.83%) were found to have cell maintenance and homeostasis role. In a separate data mining strategy; 619 LRR ESTs (<http://www.ncbi.nlm.nih.gov/nucest/?term=LRR>), 587

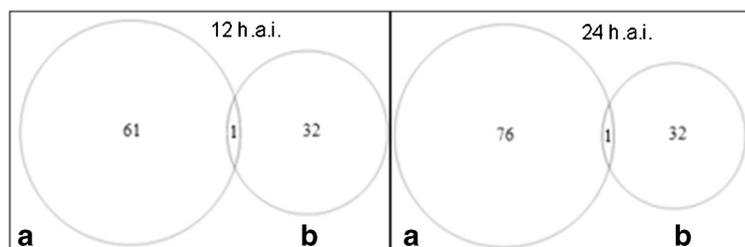
LRR GSSs (leucine rich repeat genome survey sequences, <http://www.ncbi.nlm.nih.gov/nucgss/?term=LRR>), 105,152 LRR proteins (<http://www.ncbi.nlm.nih.gov/protein/?term=LRR>), 231 NBS-LRR ESTs (<http://www.ncbi.nlm.nih.gov/protein/?term=NBS-LRR>), 222 NBS-LRR GSSs (<http://www.ncbi.nlm.nih.gov/nucgss/?term=NBS-LRR>) and 7011 NBS-LRR proteins of plants (all accessed on 10 August 2016) downloaded and BLASTed against the 352 sequenced ESTs. There was a significant (E-value  $10^{-5}$ ) BLAST hit for HT24F120 and HT24F133 against NBS-LRR EST and LRR protein, respectively. Similarly, cell component associated GO term search resulted in 42.6% of annotated ESTs with no functional site and localization in any of the databases mined (Fig. 4).

#### Upregulated and downregulated genes

The number of differentially expressed genes during incompatible interaction was greater than genes expressed during compatible interaction. Numerically, it was more than two times larger than what was expressed otherwise. Likewise, differentially downregulated genes were by far more abundant during incompatible interaction at both time points following pathogen inoculation (Fig. 1). Identification and annotation of all 352 ESTs showed most of these genes have cell maintenance and homeostasis role (Fig. 3). In both interactions, homology search resulted in higher number of significant matches for upregulated ESTs in all of the databases accessed (Online Resource 6).

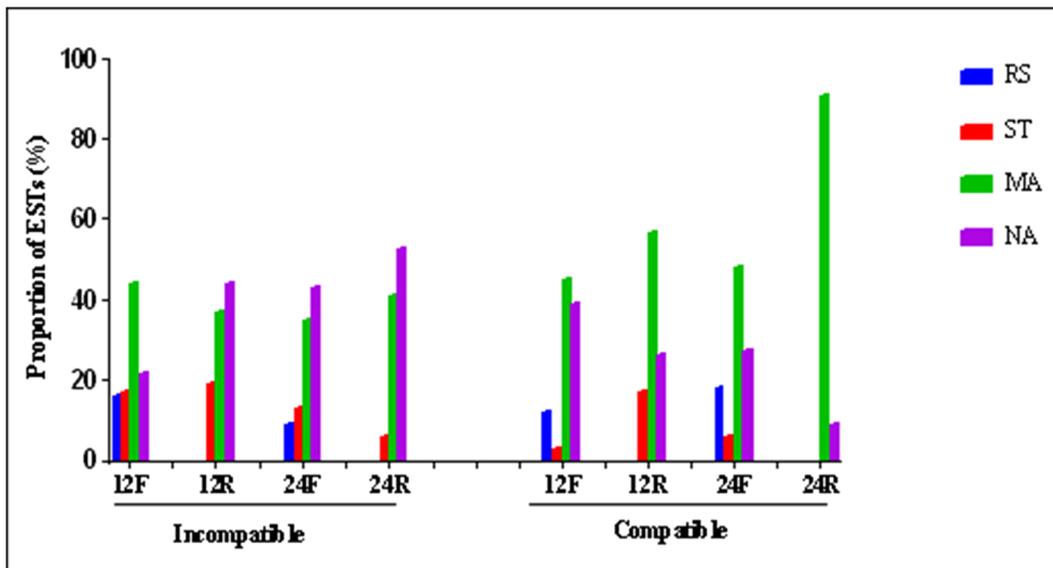
#### Subcellular localization prediction

Subcellular localization prediction of EST with significant hit ( $\leq 1 \times 10^{-5}$  e-value cutoff) resulted in 42.60%



**Fig. 2** Genes specifically and/or commonly expressed in both interactions (after redundant ESTs were removed). The number of differentially expressed genes at 12 h.a.i. during incompatible (a) and compatible interaction (b), and 24 h.a.i. during

incompatible (a) and compatible interaction (b). Overlapping regions show number of genes expressed in both libraries and h.a.i.: hours after pathogen inoculation



**Fig. 3** Proportion of different functional categories in each library as described in any of the four databases with  $\leq 10^{-5}$  E-value. RS = Resistance and antimicrobial function, ST = Signal induction

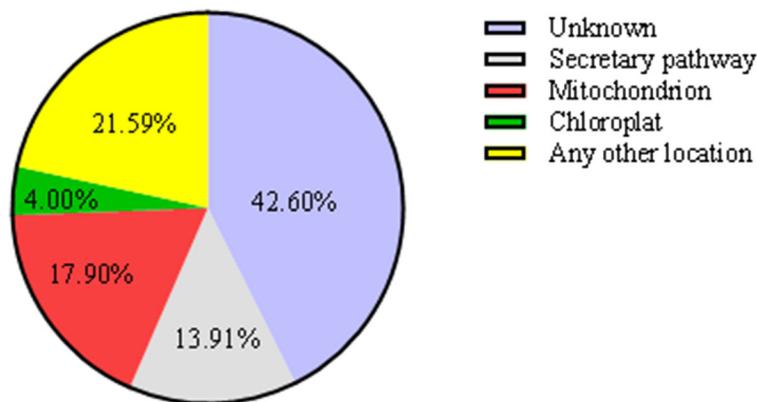
and transduction, MA = Maintenance and homeostasis, NA = No GO associated

unknown location using Targetp 1.1 plant network cut-offs (Online Resource 7). Proteins predicted as ‘any other location’ were the second largest proportion (21.59%) of proteins as shown by TargetP 1.1 subcellular prediction (Emanuelsson et al. 2000).

**RT-qPCR quantification**

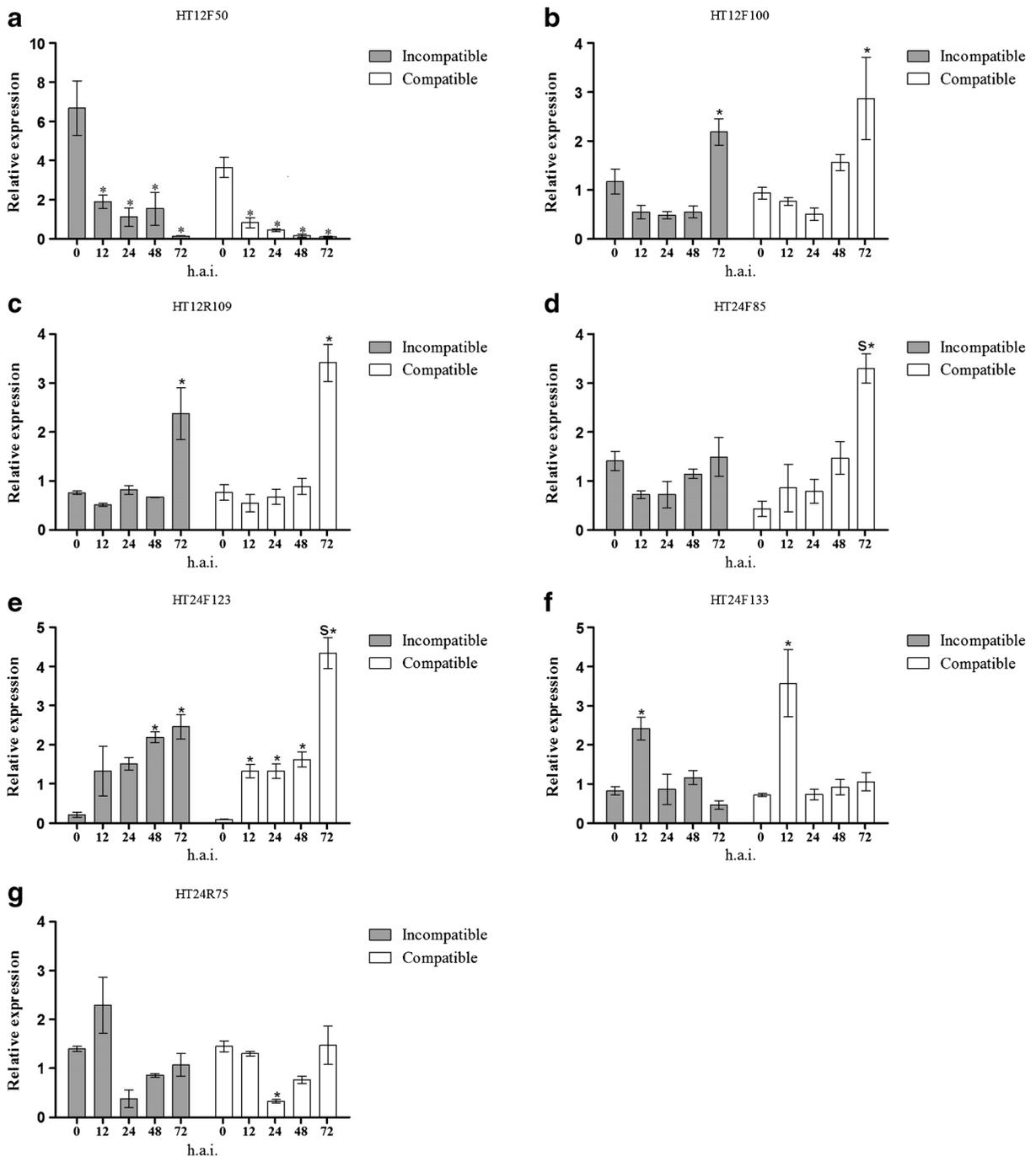
The seven target genes selected from subtractive hybridization libraries of incompatible interaction showed different expression levels along the pathogen treatment times, but the patterns, in general, were

similar during both interactions (Fig. 5a-g). HT12F50 showed consistent downregulation along the time course, 72 h.a.i. being its lowest point in both interactions. The levels of HT12F100 and HT12R109 transcript abundance was not significantly changed until 72 h.a.i., at which an elevated expression level was observed for both genes in the two interactions. Looking into inter-interaction comparison, the only significant difference in expression level was seen at 72 h.a.i. for HT24F85 and HT24F123 during compatible interaction. The level of HT24F123 expression was increasing with time after inoculation, while



**Fig. 4** Localization of differentially expressed genes as found in any of the databases mined. Subcellular localization was based on the presence of N-terminal sequences for all the categories.

Localization was done by using TargetP 1.1 server (<http://www.cbs.dtu.dk/services/TargetP/>).



**Fig. 5** RT-qPCR quantification of seven resistance-signaling genes (a-g) at 12, 24, 48 and 72 h.a.i. in resistant (HDT C1FC-832/1) and susceptible (Catuaí IAC 44) genotypes inoculated with *H. vastatrix* race II urediniospore. Quantities of transcripts were shown in relative expression compared with control (0-h) samples after Cq normalization against reference genes (S24, UBQ10 and GAPDH) by qBase relative quantification and Dunnett and

Tukey's mean comparisons of GraphPad Prism version 7.00. Results were normalized means  $\pm$  SEM of three replicates taken from three independent biological replicates \*Significantly upregulated or downregulated relative to uninoculated samples with in interaction ( $p < 0.05$ ). S Significant difference in expression level between the same h.a.i. across interactions

HT24F133 expression peaked at 12 h.a.i, when appressorium is supposed to form, but remaining constant in other times during both interactions.

## Discussion

Identification of differentially expressed genes due to pathogen inoculation showed different expression profiles in terms of the number and types of genes identified in both interactions similar to other work by Guerra-Guimarães et al. (2015). This specificity was observed at different hours for upregulated genes due to pathogen inoculation in samples from the same cultivar and yet the specificity was much more when comparing samples at different hours after pathogen inoculation in different cultivars with contrasting interactions. Similar results were reported using different cultivars for both interactions at different hours after pathogen inoculation (Fernandez et al. 2004; Guzzo et al. 2009).

Genes encoding enzymes that degrade fungal cell wall components were upregulated in both interactions. These pathogen related proteins (PR) include class III chitinase and acidic endochitinase, which are considered to be the front lines in defending against fungal pathogens, were also reported in other coffee cultivars and plant species (Guzzo et al. 2009; Legay et al. 2011; Martínez et al. 2012; Dolatabadi et al. 2014). Anti-fungal activity of chitinase enzymes against *H. vastatrix* and other species was reported in different plants (Jach et al. 1995; Martínez et al. 2012; Dolatabadi et al. 2014). Unlike in other rust resistant coffee cultivars treated with the same race of *H. vastatrix* (Fernandez et al. 2004; Guzzo et al. 2009) and resistance inducer ASM (Guzzo et al. 2009), the transcripts of  $\beta$ -1,3-glucanase gene was not found in both interactions. In tobacco, it has been reported that, chitinase and  $\beta$ -1,3-glucanase synergistically provide the maximum defense as chitinase is less effective in degrading the harder structure of chitin alone (Jach et al. 1995). The differential expression of other PRs like protease inhibitors and different types of antimicrobial genes in both interactions indicate that these genes are part of basal immunity which is characteristic of most plants (Jones and Dangl 2006; Guerra-Guimarães et al. 2015). Protease enzyme transcripts were found in upregulated libraries in both interactions at both points of time after inoculation, mainly to neutralize foreign proteins from the

pathogen as reported in grapevine (Legay et al. 2011). Chalcone synthase and polyubiquitin were the other genes upregulated during incompatible interaction with defense or cell maintenance functions (Fernandez et al. 2004). No ABC types of resistance protein transporter genes were induced in both interactions despite these proteins were reported in different coffee cultivars (Guzzo et al. 2009) and *Vitis quinquangularis* against *Erysiphe necator* (M. Gao et al. 2012).

As an anti-fungal agent owing to their oxidative action of phenolic compounds leading to cell wall lignification during HR, the activity of peroxidase enzyme has been reported to be elevated after 20 h of *H. vastatrix* inoculation in coffee during incompatible interaction (Silva et al. 2008). Peroxidase genes were activated at 12 and 24 h.a.i during incompatible interaction, yet the pick of their biological activity are to be refined. Catalase was another anti-fungal agent whose expression and activity was cofound in this work and others (Koç and Üstün 2012; Helepciuc et al. 2014). However, it was detected during compatible interaction only as one of the upregulated genes. On the other hand, NB-LRR are considered to be the main classes of major resistance proteins encoded by R genes in different coffee cultivars against bacteria (Kumar 2012) and *H. vastatrix* (Kobe and Deisenhofer 1994; Guzzo et al. 2009; Ribas et al. 2011) with direct and indirect resistance functions. Though no genes with the association of NB-LRR identity tags in their names were found in our work, like in other report (FERNANDEZ et al. 2004), these diversified R genes with NB-LRR feature are believed to characterize many of the genes in upregulated libraries. Evidently, differential expression of LRR receptor-like serine/threonine-protein kinase NIK1/protein NSP-interacting kinase 1-like (HT24F133), as validated by RT-qPCR quantification (discussed below) is an intuitive assertion for induction of signaling in response to NBS-LRR R gene(s) among the upregulated genes in this particular expression library (24 h. a.i., incompatible interaction). At least one EST (HT24F120) has shown to have similarity (96%) with NBS-LRR like EST motif (gi = 24,977,765), though this EST is not yet fully characterized and annotated to fully describe its exact role during such interaction (Vidal et al. 2010). Our result could also be explained by the fact that NBS-LRR genes are among the R genes whose expression levels are tissue specific (Carazzolle et al. 2011).

The expression of genes involved in signal perception and transduction as a vital component of

SAR was evident as they were expressed during both interactions. The proportions of these genes were comparable to genes with direct anti-fungal role in upregulated libraries of incompatible interaction (6.53% to 8.57%) while there were much less number of such genes in upregulated libraries of compatible interaction altogether (22 to 3 ESTs). The major functions of resistance-signaling genes is the linkage of effector recognition and defense responses through signal transduction involving secondary messengers (Nürnberger and Scheel 2001; Petre et al. 2014). Defense response mediated by ethylene and jasmonic acid are often considered to be the effective resistance response in developing basic or SAR immunity against necrotrophic pathogens like what salicylic acid does against biotrophs (Glazebrook 2005; Seifi et al. 2014). Ethylene and jasmonic associated signaling gene transcripts were found in both upregulated libraries during incompatible interaction ascertaining their involvement in defending biotrophic pathogens as well. The result also indicates that their defense signaling may not necessarily be independent of one another; but involved in different signaling pathways as synergistic and antagonistic regulatory interactions (Thaler et al. 2012; Mur et al. 2013; Zhu and Lee 2015). Auxin responsive genes were expressed at 12 and 24 h.a.i. during incompatible interaction in which case the repressive effect of auxin expression was limited to 24 h.a.i. which could route to activation of other signaling pathways as seen in *A. thaliana* (Kovtun et al. 2000). Different types of kinases and GTP-binding proteins known to characterize upregulated libraries in both interactions as seen in different cultivars and other plants (Guzzo et al. 2009; Medeiros et al. 2009; Gao et al. 2012). A large number of genes involved in signal transduction were downregulated at 12 and 24 h.a.i. during incompatible interaction and at 12 h.a.i. during compatible interaction. This selective repression of some signaling genes indicates that their expression is less important (or effective at low level) when compared to other signaling genes that are favored for up-regulation to counter the advancing pathogen in both basal and SAR defenses (Kovtun et al. 2000; Denancé et al. 2013).

Exclusive consideration of genes with annotations shows that the majority of the genes downregulated and upregulated were with cell maintenance and

homeostasis functions in both interactions (Fig. 3). These few but very important gene products may control the activity of several cell maintenance metabolisms during biotic and abiotic stresses (Chauhan et al. 2013). The number of both upregulated and downregulated genes involved in photosynthesis pathways are merely comparable (seven up- and eight downregulated) during incompatible interaction at the two time points studied. All of these genes are associated with starch biosynthesis metabolisms (Saithong et al. 2013). This result is similar to the work of Bilgin et al. (2010) in which pigment and light-reaction genes were downregulated while genes involved in redox reactions were upregulated following biotic stress. Moreover, most of induced genes were not R genes in both interactions. This could be explained from unique defense pathways pertaining to specific species/cultivar in responding to the pathogen invasion by few but effective R genes.

Most of the resistance related genes with putative function in all of the databases searched are localized in cytosol where chloroplast and its genes may take part in light-requiring signaling for HR development (Guttman et al. 2002; Jelenska et al. 2007). Moreover, catalase and peroxidase enzymes are localized in apoplast, cell wall and cytoplasm which make their anti-fungal role active at every site of attack (Silva et al. 2008). Different studies show that most signaling proteins such as kinases and resistance proteins with direct anti-microbial function have apoplastic, cytoplasmic and transmembrane localizations in most plants (Piedras et al. 2000; Zipfel 2009; Schneider and Collmer 2010). Class III chitinase was the most abundant anti-fungal gene product whose localization is in apoplast to directly block the establishment of fungal hyphae and induce fungal elicitors for additional expression of different types of chitinases (Neuhaus et al. 1996; Stotz et al. 2014; Jashni et al. 2015). Plant specific subcellular localization prediction indicated that most of the genes whose expression levels were influenced by pathogen inoculation were mitochondrial (17.90%). The result showed the expression of gene products linked to redox pathways are highly affected due to the metabolic cost of defense response (Nie et al. 2015). Gene products localized in mitochondrion and intimately linked chloroplast, including the different types of peroxidase species, are involved in HR resulting in the apoptosis due to ROS. Their temporal expression and regulation are globally linked to the nucleus by mitochondrial proteome for organelle communication (retrograde

signaling) (Schwarzlander et al. 2012). The fact that chloroplasts contain light-dependent reaction centers, the overall response of an infected plant is highly influenced by the chloroplast proteins in one or the other way. This is clearly evident as chloroplasts are the sources of stress induced hormones and different types of secondary metabolites induced in response to pathogen attack (Abramovitch et al. 2006; Delprato et al. 2015). Still for a reasonable number of functionally annotated ESTs, subcellular localization is not yet known (42.60%) indicating the importance further studies in this front.

Continuous generation of nucleotide and protein sequences has enriched databases and provided a great research potential for gene function prediction and annotation. There were different number of significant matches during homology search for most sequences in all libraries using EMBL and NCBI databases. On top of that, the discrepancy of GO terms was found in all databases; including the two coffee genome devoted databases, LGE and *C. canephora*, as well as EMBL and NCBI for a given EST. This result was in accordance to previous work on biological database integration (Gomez-Cabrero et al. 2014). A simple BLASTx search engine at LGE database resulted not only descriptions associated to each significant match, but also with associated GO terms unlike the same task at NCBI, EMBL and *C. canephora* which could only fetch short descriptions or simply an EST identification number. The development of standardized UniProt and structured GO annotation vocabulary incorporated with BLAST2GO, as per the objective of its inception (Camon 2004), provides an interface to deal with the biochemistry of annotated proteins. However, its restricted access to advanced level of annotation limits its fair availability to all users at different levels. The absence of a single run and unified access route to different databases forces the switch between different interactive interfaces, and to manually seek and combine results from different sources. Therefore, the development and availability of homology search tools like BLAST2GO and their integration to databases would help bypass the tedious and time consuming annotation works.

From the putative resistance related genes annotated, seven genes were selected and analyzed with RT-qPCR. For most of the RT-qPCR quantified genes, the overall expression trend showed increased level of transcript

abundance in later time points during both interactions. Kinase associated protein phosphatase (HT12F100) and LRR-receptor like protein kinase (HT24F133) are among the main signaling genes in response to different types of biotic stresses in different plant species (Sheen 1998; Durian et al. 2016). The expression of kinase associated protein phosphatase showed no significant change during the early hours of infection, with expression peak at 72 h.a.i., in both interactions. Studies in *A. thaliana* and other plants show that phosphatase proteins involved in defense signaling have negative regulation in plant innate immunity (Shi et al. 2013; Segonzac et al. 2014). Receptor-like kinases (RLK) are one of the major defense proteins, that are structurally diversified super families, evolved into LRR proteins with intracellular kinase domain (Goff and Ramonell 2007). The extracellular domain of these proteins are in continuous evolution to recognize the ever changing pathogen effectors (Kaku et al. 2006; Zhang et al. 2006). The concentration peak of HT24F133 at 12 h.a.i. during both interactions indicates that pathogen recognition and defense signaling occurred at the time of appressorial differentiation as also reported by Diniz et al. (2012). The coordinated activity of kinase associated phosphatases and RLK plays a decisive role in triggering resistance signaling (Alves et al. 2014). Delayed activation (after haustorial formation) of kinase associated phosphatase could be attributed to their negative modulator of stress-responsive signaling kinases at a time when elevated expression is no more required (Rodrigues et al. 2013). GA (gibberellic acid) signaling F-box gene (HT12R109), a hormone responsive gene was the other gene with expression pattern similar to kinase associated phosphatase. Post-haustorial activation of this gene may be associated to its involvement in host resistance development where HR is the major defense during the incompatible interaction (Ellis 2006). Based on our result, similar expression pattern during both interactions, GA signaling is one of the innate immunity component shared between the two cultivars. The involvement of F-box gene as regulators of defense responses has been reported in grapevine where it showed upregulated expression following *Botrytis cinerea* infection (Paquis et al. 2011). The role of GA in plant defense against pathogen attack either individually or in conjunction with other hormones has recently emerged (De Bruyne et al. 2014; Kazan and Lyons 2014). GA and its signaling f-box proteins also have a role in cross communication between signals to control development and disease defense (De Bruyne et al. 2014).

The expression of auxin-binding protein abp20 precursor (HT12F50) gene was consistently lowered at all time points, 72 h.a.i. being the lowest level during both interactions. In part, this result was similar to the work of Xue et al. (2015) on fusarium wilt in common bean in which auxin regulated protein was kept low until 72 h.a.i. during incompatible interaction. This protein precursor has been supposed to be transmembrane localized and controls the flow of auxin from cytosol to endoplasmic reticulum (Feng and Kim 2015). This gene is exceptional in that its deactivation was probably an important step for the other defense signaling genes to be upregulated. However, auxin-binding proteins were recently reported to have no role in either auxin signaling or in plant development stages in *A. thaliana* (Gao et al. 2015), hence its role in plant defense signaling is largely obscure (Feng and Kim 2015). Of all the expression patterns analyzed, a remarkably interesting defense signaling cross-talk was observed between auxin-responsive (HT24F85) and auxin-repressed protein-like (HT24F123) genes. Auxin responsive gene expression level was maintained at basal level throughout all the time points studied during incompatible and compatible (an exception is at 72 h.a.i.) interactions. The only change in expression level was at 72 h. a.i. during compatible interaction. However, the exact role of auxin responsive protein still remains less conclusive as it has complex hormonal cross-talk signaling role in plant defense against different pathogens (Carna et al. 2014; Verma et al. 2016). On the contrary, auxin-repressed protein-like gene expression was significantly increased during haustorial differentiation (48 h.a.i. and afterwards) while it was significantly elevated at all time points during compatible interaction. Auxin-repressed protein gene is a repressor of plant growth by inhibiting the expression of auxin responsive factor gene and R gene activator in tobacco (Zhao et al. 2014) and wheat (Song et al. 2015) against fungal pathogens. However, our result showed activation of this gene in resistant (at 48 and 72 h.a.i.) and susceptible (at all time points) plants. Therefore, as it was upregulated during both interactions, its role as one of the activator of R gene expression against *H. vastatrix* in coffee requires further studies. Steady upregulation of auxin-repressed protein during both interactions, along the time course in similar fashion, was an indication

that this signaling gene is indiscriminately involved in basal defense (Groszmann et al. 2015). When auxin expression is inhibited, the expression of auxin-responsive genes are expected to be low, leading to increased auxin-repressed gene expression (Tiwari 2004; Song et al. 2015). In host defense to biotrophic pathogen, downregulation of auxin responsive genes was reported to be part of salicylic acid (SA) defense signaling (Wang et al. 2007; Zhao et al. 2014). The exclusively coinciding upregulation of the two genes (auxin-responsive and auxin-repressed genes) at 72 h.a.i. during compatible interaction, which are supposed to be antagonistic otherwise, requires further studies.

A relatively different expression pattern was followed by Ac-like transposase (similar to histone H1) (HT24R75). The expression of this gene was not significantly affected at all time points studied during incompatible interaction unlike during compatible interaction in which it was significantly lowered at 24 h.a.i. The change in expression level of this gene may not be important to induce resistance during incompatible interaction while its downregulation at 24 h.a.i. during compatible interaction is also hard to neglect as it may contribute to some unsuccessful defense responses. According to Dereeper et al. (2013), substantial portion (11.9%) of *C. canephora* (diploid parent of *C. arabica*) genome is occupied by transposable elements. The role of AC-like transposase in plant defense has recently come to light as stress adaptive capacitor in *M. oryzae* (Chadha and Sharma 2014) and determinant of susceptibility under different phytopathosystems by enhancing gene expression or chromosome rearrangement (Hua-Van et al. 2011; Yu et al. 2012). In general, similar expression patterns (up or downregulation) of most genes (HT12F50, HT12F100, HT12R109, HT24F123 and HT24F133) validated by RT-qPCR showed that these genes are not essential to prevent the establishment of *H. vastatrix* and, therefore, they are involved in basal defense response. Such shared expression patterns of resistance related genes in host and non-host plants has been reported in barley against *P. graminis* (McGrann et al. 2009).

So far identification of differentially expressed resistance related genes has been reported by SSH during compatible and incompatible interactions between *C. arabica* and *H. vastatrix* (Fernandez et al. 2004; Guzzo et al. 2009). This work extended the scope by emphasizing on explicit identification of upregulated and

downregulated genes during compatible and incompatible interactions at 12 and 24 h.a.i. Moreover, the result paved a way forward in comprehensive understanding of some genes commonly over expressed and suppressed at different times in both interactions. Also, most of the genes upregulated and downregulated showed to be specific to a particular interaction. In general, the proportion of genes upregulated and downregulated in resistant cultivar showed that there was strong resistance metabolic dynamism in SAR for complete and long lasting resistance development during incompatible interaction. RT-qPCR analysis of seven resistance-signaling genes showed similar expression patterns for most of the genes in both interactions, indicating these genes are involved in basal (non-specific) defense during which immune reactions are similar.

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#### Compliance with ethical standards

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

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