Monitoring of the early molecular resistance responses of coffee (Coffea arabica L.) to the rust fungus (Hemileia vastatrix) using real-time quantitative RT-PCR

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Abstract

Molecular resistance responses of coffee (Coffea arabica L.) to the orange rust fungus Hemileia vastatrix were monitored by real-time quantitative RT-PCR analysis of gene expression. Significant activation of coffee genes by fungal infection could be observed around 12–16 h post inoculation (hpi) in the incompatible interaction. Microscopic observations indicated that, at this time, only a limited number of fungal germlings already differentiated a penetration hypha through the stomata. Activation of the CaWRKY1 gene, putatively encoding a WRKY transcription factor also occurred in the compatible interaction, but was delayed to 24 hpi. In contrast, activation of the CaR111 gene encoding a protein of unknown function only occurred in the incompatible interaction. The CaNDR1 gene, an homolog of the Arabidopsis non-race specific disease resistance (ndr1) gene was only poorly induced by fungal infection. Wounding and salicylic acid treatment markedly activated CaWRKY1, CaNDR1 and CaR111 gene expression. These results showed that specific transcriptional responses of coffee were detected before penetration of H. vastatrix into the leaf had occurred, and suggest a possible role of activated genes in the molecular resistance responses of coffee to the rust fungus.

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1. Introduction

Coffee leaf rust, caused by the fungus Hemileia vastatrix (Berkeley & Broome), is a major disease of coffee – Coffea arabica L. –, which constitutes about 70% of total coffee production. Naturally rust resistant coffee varieties display a specific plant resistance response called hypersensitive reaction (HR), a form of programmed cell death analogous to animal apoptosis [1,2]. In coffee, the HR is manifested by death of subsidiary and guard cells of stomata where the fungus penetrates [3,4]. In plant–pathogen interactions, activation of HR and plant defence responses is mainly triggered through the enhanced transcription of numerous genes coding for proteins displaying a wide array of biological activities [5–8]. These transcriptional changes can be usually monitored within the first hours following pathogen inoculation [7]. Few data are available on the defence gene induction triggered by pathogen infection in rust diseases [9–11]. Histological data obtained in the cowpea–rust (Uromyces vignae) pathosystem suggested that HR expression was linked to the host cell penetration by a fungal specialized structure (haustorium) [10,12]. Hence, in the coffee–rust pathosystem, the number of haustoria formed by H. vastatrix is usually reduced in resistant plants [3,4] and H. vastatrix infection is rapidly restricted by haustoria encasement with plant cell wall-derived materials [4]. However, careful examination of temporal patterns of fungal infection in resistant plants [4,13] indicated that, for a number (65%) of infection

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sites examined, fungal growth was stopped soon after entry into the substomatal chamber, before haustoria had differentiated. These data suggest that specific HR responses might be early activated to block fungal infection.

The aim of this study was to relate the time-course of coffee molecular resistance responses to *H. vastatrix* development into leaf tissues. We focused on three expressed sequence tags (ESTs) that were previously shown to be activated by rust infection [14]. Two of them putatively encoded signalling components of the plant resistance response pathway. EST DDS12 (here renamed *CaNDR1*) displayed high homology to the *Arabidopsis* non-race specific disease resistance (NDR1) protein, a key component of the signalling pathway governed by several resistance proteins [15]. EST DDS16 (here renamed *CaWRKY1*) putatively encoded a transcription factor of the WRKY plant specific family. Recent works evidenced that WRKY genes may be rapidly induced by pathogens and involved in the activation of plant defence responses [16–18]. Finally, we added to our analysis EST DDS23 (here renamed *CaR111*) that had homology to a protein of unknown function, and that may be a novel and interesting component of plant disease resistance. Reverse-transcription (RT) combined to real-time quantitative polymerase chain reaction (PCR) is the most sensitive technique for mRNA detection and quantitation currently available [19,20]. We present here the development of a real-time quantitative RT-PCR assay to analyze the relative resistance *S. H5* gene) were kept in the greenhouse. Sets of two leaves per plant and four plants per experiment were used. Wounding was performed by applying an average of seven transversal cuts per half-leaf using scissors. Wounded leaves were collected 1 h later. Non-wounded leaves were collected on another set of plants to be used as controls. SA-treatment was performed by infiltrating the entire leaf area with a 0.5 mM solution of SA using a needleless syringe. Water-infiltrated (mock-) as well as non-infiltrated leaves were used as controls. Leaves were collected 3 h after treatment.

2.3. Fungal colonization

Fungal growth in host tissues was observed by light microscope examination of leaf pieces as previously described in [4]. Counts of appressoria and fungal stages inside leaf tissues (stage 2: penetration hypha, stage 3: anchor and stage 4: haustorial mother cell (HMC) with haustoria) were recorded from 75 to 100 infection sites per experiment at various times after inoculation.

2.4. Genomic DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blotting

Fresh leaves of *C. arabica* were collected in the greenhouse and immediately frozen in liquid nitrogen. DNA extraction was performed using the DNEasy Plant minikit (Qiagen, France) following the manufacturer’s recommendations. Genomic DNA (10 μg) was digested with 50–60 units of the restriction enzymes EcoRI or Psrl (Promega, France) with the addition of 5 mM spermidine per reaction for 16 h at 37 °C. Restriction fragments were separated by electrophoresis in 0.8% agarose gels in 0.5 TAE buffer. DNA fragments were blotted onto NylonN+ membranes (Amersham, Les Ullis, France) by alkaline vacuum transfer (TE 80 TransVac, Hoefer Scientific Instruments, San Francisco, USA).

2.5. Labelling of probes and hybridization conditions

Hybridization probes used in this study were PCR-amplified cDNA fragments. Specific oligonucleotides were designed from the ESTs sequences and used to amplify plasmid cDNA inserts by PCR. After purification using QIAquick PCR purification kit (Qiagen, France) DNA probes (50 ng) were labelled with 32P-dCTP by random-priming, hybridized to membrane-bound DNA fragments and detected by autoradiography according to the manufacturer’s specifications (Megaprime kit and hybridization buffer, Amersham, France).

2.6. RNA extraction and RT-PCR

Total RNAs were extracted from coffee leaves using the RNeasy Plant kit (Qiagen, France) completed by a DNase treatment. Quality of RNA were checked on denaturing agarose gel. RNA samples were quantified using the RiboGreen RNA Quantitation kit (Molecular Probes, Interchim, France) and...
Hitachi F2500 Fluorescence Spectrofluorimeter (Tokyo, Japan). A control PCR was run on extracted RNA samples to check the absence of genomic DNA. First-strand cDNAs were synthesized from 1 μg of total RNA in 20 μl final volume, using Omniscript RT kit (Qiagen) and oligo-dT(18)-MN primer (Eurogentec, Angers, France) following the manufacturer’s instructions.

2.7. Real-time quantitative PCR assays

Specific gene primers were designed from the cDNA sequence of each gene to be analyzed using the Primer Express 1.5 software (PE Applied Biosystems) following the manufacturer’s guidelines. The CaUbiquitin gene (Rojas-Herrera et al., unpublished) was used as internal constitutively expressed control (reference gene). Primers (reverse-R and forward-F) and [5′] 6-FAM, [3′] TAMRA-labelled probes (-P) listed in Table 1 were synthesized by Eurogentec and used at 200 nM final concentration. PCR reactions were performed in 25 μl final volume according to the manufacturer’s instructions (qPCR Mastermix Plus, Eurogentec) and three replicates of the PCR assay were used for each sample. Real-time quantitative PCR was conducted on an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA, USA). PCR cycles were as follows: 1 cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles each of 15 s at 95°C, and 1 min at 60°C. Reactions performed without template did not result in any product.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
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<td>CaR111</td>
<td>CF589193</td>
<td>R111-F</td>
<td>TCCAAATCGCITT CGACACC</td>
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<td></td>
<td>R111-R</td>
<td>GTTCCGGTTGTAT ATGGAGATTG</td>
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<tr>
<td></td>
<td></td>
<td>R111-P</td>
<td>CCCGGAGAGGCT CCCCCGT</td>
</tr>
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<td></td>
<td></td>
<td>NDR1-R</td>
<td>CAGCATCGGCAA CACCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NDR1-P</td>
<td>CAGACCCAGCACG CAGTGCAGG</td>
</tr>
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<td>CO773974</td>
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<td></td>
<td></td>
<td>WRKY1-P</td>
<td>TTGGTTGTTGCC ACCGGAGGAAA</td>
</tr>
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<td>Ubiquitin</td>
<td>AF297089</td>
<td>Ubi-F</td>
<td>AACATTGAGGGTG TGCATTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubi-R</td>
<td>GCAGAAAAACCACT AAGACCTAACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubi-P</td>
<td>TCATCTTTGTCTCG CTCAGAGG</td>
</tr>
</tbody>
</table>

2.8. Quantitative PCR data analysis

Quantitative PCR data analysis was achieved using the SDS software Version 1.7 (Perkin-Elmer-Applied Biosystems). The threshold cycle (Ct) values of the triplicate PCRs were averaged and relative quantification of the transcript levels was performed using the comparative Ct method [22]. Relative quantification relates the PCR signal of the target transcript in the infected sample to that of the control uninfected sample at each time. The fold change in cDNA (target gene) relative to the reference gene (Ubiquitin) was determined by the following formula: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{Ubi})_{sample} - (Ct_{Target} - Ct_{Ubi})_{control}$. For each gene tested, the PCR efficiency of the amplification reaction was first checked to ensure that the ΔCt between the endogenous reference gene and the gene of interest would not change when different amounts of total sample is added. PCR efficiencies were determined as described in [22]. For all genes tested, efficiencies varied from 93 to 100% (data not shown).

2.9. Statistical analysis

Data concerning fungal growth were presented as the combined values of two experiments because no significant differences were found between them. Arcsine-transformed percentages and the Student’s test for statistical analysis were used.

Data concerning quantitative gene expression were averaged from three independent time-course experiments. Variance analysis (ANOVA) and Tukey tests were performed for detecting changes in the relative gene expression patterns for each treatment (time and plant–fungus interaction phenotype).

3. Results

3.1. Estimation of gene copy number in the C. arabica genome

To verify that the candidate ESTs corresponded to single genes, and that RT-PCR experiments would only result in the amplification of the desired gene, we conducted Southern blots experiments on C. arabica genomic DNA. Coffee DNA was digested either with the restriction enzyme EcoRI (no site in CaWRKY1 and CaNDR1 sequences, one site in CaR111 sequence), or with the PstI enzyme (one site in the CaWRKY1 and CaNDR1 sequences, no site in CaR111 sequence). For all genes tested, only 1–2 major bands hybridized to the radiolabeled EST probe (Fig. 1) indicating that the corresponding genes do not belong to multigene families and may be present in one copy per haploid genome of the tetraploid species C. arabica.

3.2. Coffee gene expression during H. vastatrix infection

To characterize the response of C. arabica to H. vastatrix infection, we monitored the mRNAs levels of the three candidate genes during the early stages of the plant–fungus interaction.
interaction by real-time quantitative RT-PCR assays. Time-course experiments (9, 12, 16, 24 and 48 hpi) were conducted using the Caturra variety challenged with avirulent or virulent H. vastatrix isolates either eliciting an incompatible reaction (resistance) or a compatible reaction (susceptibility), respectively. The CaUbiquitin gene chosen as internal reference of gene expression was assayed in parallel with the candidate genes. The relative changes in gene expression are presented in Fig. 2. No difference in gene expression could be detected between control and inoculated plants before 12 hpi (data not shown). Between 12 and 16 hpi, depending on time-course experiments, a marked (>5-fold) induction of the CaR111 and CaWRKY1 genes by fungal infection was evidenced. For convenience, data obtained at 12 and 16 hpi were pooled. Statistically significant differences (P < 0.05) in the relative expression of the two genes were found between the compatible and incompatible interactions. CaR111 was induced by the avirulent H. vastatrix strain around 12–16 hpi but weakly activated by the virulent strain. Activation of the CaWRKY1 gene occurred during both interactions, but was higher in the incompatible samples around 12–16 hpi and higher in the compatible samples at 24 hpi. Concerning the CaNDR1 gene, only a poor induction (<3-fold) was observed at 12–16 hpi and occurred in response to both virulent and avirulent pathogen (no statistically significant difference). The three analyzed genes returned to their basal expression level at 24 hpi for CaR111 and CaNDR1 (Fig. 2), and 48 hpi for CaWRKY1 (data not shown).

3.3. Coffee gene expression during wounding and SA-treatment

The three genes were substantially induced in coffee leaves by wounding and by SA-treatment (Figs. 3 and 4). For CaR111 and CaWRKY1, the relative expression levels generated by heavily wounding leaves (Fig. 3) were much higher than those obtained by fungal inoculation (Fig. 2) or by SA-treatment (Fig. 4). In the SA-treatment assays, water-infiltrated control plants displayed enhanced expression of the target genes compared to untreated control plants. However, the relative expression levels of the three genes in SA-treated plants were in average three times higher than that in water-infiltrated control plants (Fig. 4).

3.4. Fungal colonization

Microscopic examination of fungal growth in host tissues were monitored from 12 hpi when urediniospores of both races had germinated and developed an appressorium over a stomata (Table 2). At this time, only a limited number (15–20%) of fungal germlings had already differentiated a penetration hypha.
through the stomata (stage 2). Fungal infection of leaf tissues significantly differed between incompatible and compatible interactions from 16 hpi (P ≤ 0.05) and 24 hpi (P ≤ 0.01). In the incompatible interaction, fungal development was slowed from 16 hpi and ceased after 30 hpi with most of the infection sites blocked at stage 2. No haustoria at all were formed by the avirulent *H. vastatrix* strain. In contrast, fungal development of the virulent *H. vastatrix* strain reached stage 3 (anchor development) at 24 hpi and stage 4 (haustoria) at 36 hpi. Two days after inoculation, the virulent rust fungus had differentiated at least one haustorium in a third of the infection sites examined (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Time after inoculation (h)</th>
<th>Coffee–rust interactions</th>
<th>Frequency (%) of infection sites with the fungal growth stages</th>
<th>x_w</th>
<th>r-Value</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
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<td>12</td>
<td>I</td>
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<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>80</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>I</td>
<td>73</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
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<tr>
<td></td>
<td>C</td>
<td>22</td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

I = incompatible interaction; C = compatible interaction; stage 1: appressorium; stage 2: appressorium with penetration hypha; stage 3: appressorium and penetration hypha with anchor; stage 4: haustorial mother cell with haustorium; x_w = weighted average (x_w = (85 × 1) + (15 × 2) + (0 × 3) + (0 × 4)/100 = 1.15).

* Student’s test (ns: non-significant; *P ≤ 0.05; ***P ≤ 0.001).

## 4. Discussion

In this study, we used real-time quantitative RT-PCR for assessing the relative level of transcripts of three defence-related genes in coffee after challenge with *H. vastatrix* and after abiotic treatments. Considering the possibility that these genes belong to a large gene family, and that PCR would result in the amplification of several alleles, we assessed the single-copy status of the genes in the *C. arabica* genome by a preliminary Southern blot experiment.

Analysis of the three genes expression in time-course experiments showed that they were transiently induced during the coffee–rust interaction. Enhancement of their mRNA levels was observed during the early stages (12–24 hpi) of pathogen infection. In addition, monitoring of the mRNAs levels of the *CaR111* and *CaWRKY1* genes showed they were significantly increased in the incompatible samples, as compared to the compatible samples (Fig. 2). Cytological observations of coffee leaves revealed that in the incompatible interaction the fungus had stopped its growth before reaching the haustorial stage (Table 2). These data, together with the early specific transcriptional changes that we observed suggest that recognition of the pathogen may occur before penetration of the fungus into the substomatal chamber. In other plant–rust interactions, host specific resistance responses are typically observed concurrently with the formation of the first fungal haustoria [10,12]. Previous cytological data conducted on the resistant *C. arabica* var. S4 Agaro challenged by *H. vastatrix* race II indicated that the fungus was able to develop HMCs in only 36% of the infection sites [4]. In the interaction between the coffee variety Caturra and *H. vastatrix* race VI (this study), the fungus even not reached this stage. The resistance phenotype observed in Caturra leaves has been described as immune in previous works [23,24] and may be related to a non-host resistance reaction. Hence, *H. vastatrix* race VI is only virulent on some *Coffea* species other than *C. arabica*, and the variety Caturra is known to be susceptible to all other *H. vastatrix* races. Nevertheless, specific HR-cell death and other accompanying resistance mechanisms have been observed in Caturra leaves challenged by race VI [13]. These data suggest that distinct mechanisms of resistance signalling may operate in coffee to stop pathogen infection.

Hypotheses on the putative function of the three genes that we studied may be derived from literature. The coffee genes were activated by rust infection as well as by wounding and SA-treatment. Many defence-related genes also react to abiotic stimuli [21]. Microarrays analyses in model plants have identified components of resistance signalling pathways that are activated after specific recognition of the pathogen and that may be used to engineer plants with enhanced disease resistance [5,6,25,26]. For instance, the NDR1 gene conferred enhanced resistance to virulent *P. syringae* isolates when artificially overexpressed in *A. thaliana* [27]. Northern blot analysis showed that NDR1 expression in *A. thaliana* is quickly induced (two times) in response to pathogen inoculation [15]. The real-time quantitative RT-PCR data of *CaNDR1* expression in the *C. arabica–H. vastatrix* interaction indicated similar
induction level in the pathogen-treated samples. No data is available on the expression patterns of NDR1 in response to wounding or SA-treatment. The exact role in disease resistance of the A. thaliana NDR1 protein is still unknown but its location in the plasma membrane suggests either NDR1 could directly interact with the pathogen or act as a transducer of pathogen signals [27]. Based on sequence homology and expression pattern similarities with A. thaliana NDR1 it is tempting to hypothesize for a putative role of CaNDR1 in coffee disease resistance signalling.

Transcriptional activity of genes is modulated by the specific binding of transcription factors to DNA promoter sites. WRKY transcription factors are a major group of plant proteins implicated in the regulation of several biological processes, including pathogen defence [16–18]. In A. thaliana, 49 out of 72 WRKY genes were transiently activated upon pathogen infection or SA-treatment [16]. In parsley, recent work evidenced that WRKY proteins are present as a complex pool of low-abundant preformed proteins in the cell nucleus and that de novo synthesis is quickly induced in response to a pathogen-derived elicitor [17]. The promoter element (W-box) that binds WRKY proteins has been found in several plant defence-related genes [6,17]. Here we showed that the CaWRKY1 gene is activated soon after H. vastatrix inoculation, wounding or SA-treatment. In addition, delayed expression of the gene in the compatible interaction as compared to the incompatible interaction was evidenced (Fig. 2). It has been shown recently that differences in timing and quantitative level of defence gene induction may account to a large degree for the phenotype of host responses towards an avirulent or avirulent pathogen [8]. Cloning and characterization of the entire coffee CaWRKY1 gene will offer new insights into the possible function of the CaWRKY1 protein in the activation of coffee resistance responses to pathogens.

Finally, concerning CaR111, no inference on its putative function in resistance signalling may be made in the absence of homology with any protein of known function. However, the specific activation of CaR111 in response to the avirulent rust strain suggests that this gene might be an interesting factor in the signalling pathway of host resistance in C. arabica.

In conclusion, the present study has shown that specific transcriptomic responses may be identified in coffee challenged with the rust fungus at an early stage of infection. Real-time quantitative RT-PCR data have indicated that the genes may be differentially expressed in response to the virulent or avirulent rust pathogen. Future work will aim at understanding the role of the CaWRKY1, CaNDR1 and CaR111 gene in the mechanism of coffee resistance to pathogens. Transgenically enhanced expression of genes involved in the induction of basal plant defence responses may lead to improved durability of host resistance to leaf rust and possibly also other pathogens in coffee.

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