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ORIGINAL PAPER

# Identification of coffee *WRKY* transcription factor genes and expression profiling in resistance responses to pathogens

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**Abstract** In plants, WRKY proteins are a group of transcription factors existing as a gene superfamily that play important roles in regulation of defense response pathways. To assess the diversity of this protein family in coffee (*Coffea* spp.), data mining methods were used on a set of around 200,000 coffee expressed sequence tags. A total of 53 different putative *WRKY* genes were obtained, but only 22 unigenes encoding a protein with a WRKY domain were identified, eight of which are supported by full-length cDNA sequences. Alignment of WRKY domain sequences of the coffee unigenes and 72 *Arabidopsis thaliana WRKY* genes showed that the 22 coffee WRKY members were distributed among the main *A. thaliana* WRKY subgroups and shared conserved peptide domains. To assess the involvement of *WRKY* genes in coffee defense response pathways, their expression was analyzed under biotic (nematode and rust fungus infection), hormonal (salicylic acid, methyl-jasmonate), and wounding treatments, leaf senescence, and fruit development. Five members of WRKY groups IId and III were regulated only by pathogens and hormone treatments. Although a significant correlation of *WRKY* genes expression after MeJA and rust treatments was observed, expression of coffee genes involved in JA biosynthesis and lipoxygenase (EC 1.13.11.12) activity assays did not support the involvement of JA in the early coffee resistance responses to the rust pathogen. The five WRKY transcription factor members identified might play important roles as regulators of pathogen resistance responses and could be useful for improving coffee tolerance to various biotic stresses.

**Keywords** Rust disease · *Coffea arabica* · Regulator genes

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## Introduction

The coffee plant *Coffea arabica* L. is one of the key export and cash crops in tropical and subtropical developing countries, and much efforts are devoted to improve this perennial crop for important agronomical traits (Etienne et al. 2002). In particular, *C. arabica* endures high production losses due to pests and diseases. Coffee leaf rust caused by the fungus *Hemileia vastatrix* (Berkeley & Broome) is one of the most destructive diseases of *C. arabica* and is widely distributed in all coffee production regions of the world (Bettencourt and Rodrigues 1988). Resistance of *C. arabica* varieties to leaf rust is conditioned by gene-for-gene interactions (Rodrigues et al. 1975; Bettencourt and

Rodrigues 1988) and is expressed by a rapid hypersensitive cell death at leaf infection sites (Rodrigues et al. 1975; Silva et al. 2002). The HR is expressed by death of guard and subsidiary cells in stomata where the fungus developed an appressorium and a penetration hypha (Silva et al. 2002). In resistant coffee plants, fungus growth usually terminates after formation of the first haustoria in mesophyll cells (Martins and Moraes 1996; Silva et al. 2002; Ramiro et al. 2009). Activation of several defense marker/related genes in coffee plants was detected during rust resistance response, and this activation coincided with pathogen entry in the stomata and mesophyll layer (Fernandez et al. 2004; Ramiro et al. 2009). Among rust-responsive genes identified was *CaWRKY1*, encoding a putative WRKY transcription factor that displayed altered expression patterns in response to biotic and abiotic treatments (Ganesh et al. 2006; Petitot et al. 2008).

In plants, the WRKY transcription factors belong to a major group of DNA-binding proteins and function as transcriptional activators as well as repressors in a number of developmental and physiological processes (Ülker and Somssich 2004; Eulgem 2006). In particular, WRKY transcription factors can regulate plant responses to biotic stresses (reviewed in Pandey and Somssich 2009). Plant immunity is associated with the accumulation of hormones acting as defense signal molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; reviewed in Bari and Jones 2009). SA-mediated signaling pathway has been associated with resistance to biotrophic pathogens which feed on living host tissue during all or part of their infection cycle. ET- and JA-mediated signaling pathways, on the other hand, often mediate plant defense against necrotrophic pathogens that promote host cell death at early stages of infection (Glazebrook 2005). Pathogen infection or treatment with SA has been shown to induce rapid expression of a great number of *WRKY* genes from *Arabidopsis thaliana* and rice (*Oryza sativa*) (Dong et al. 2000; Ryu et al. 2006). Only little information is available regarding JA activation of *WRKY* genes (Ryu et al. 2006; Mao et al. 2007; Skibbe et al. 2008). All *WRKY* genes that have been functionally assigned to the regulation of plant disease resistance were shown to be transcriptionally activated by pathogens, including *AtWRKY*3 and *AtWRKY*4 (Lai et al. 2008), *AtWRKY*7 (Kim et al. 2006), *AtWRKY*11 and *AtWRKY*17 (Journot-Catalino et al. 2006), *AtWRKY*18, *AtWRKY*40, and *AtWRKY*60 (Xu et al. 2006), *AtWRKY*22 and *AtWRKY*29 (Asai et al. 2002), *AtWRKY*33 (Zheng et al. 2006), *AtWRKY38* and *AtWRKY62* (Kim et al. 2008), *AtWRKY*41 (Higashi et al. 2008), *AtWRKY*53 (Murray et al. 2007), and *AtWRKY*70 (Li et al. 2004). For example, AtWRKY70 was reported to function as a convergence node for JA- and SA-mediated signal transduction during plant defense (Li et al. 2004), acting as an activator of SA-induced genes and also as a repressor of JA-responsive genes. This gene also enhanced resistance to the fungal biotroph *Erysiphe cichoracerum* but reduced resistance to fungal necrotroph *Alternaria brassicicola* (Li et al. 2004, 2006). By contrast, AtWRKY33 functions as a negative regulator of SA responses and as a positive regulator of JA/ET-mediated pathways and plays an important role in disease resistance to necrotrophic fungal pathogens (Zheng et al. 2006). Disruption of *AtWRKY33* results in enhanced susceptibility to necrotrophic fungal pathogens and impaired expression of JA/ET-regulated defense genes (Zheng et al. 2006).

So far, genomic and functional annotations revealed 74 *WRKY* genes in *A. thaliana* (Ülker and Somssich 2004) and 102 in rice (Ross et al. 2007). WRKY proteins are characterized by the presence of one or two DNA-binding domains which comprise the conserved WRKYG[Q/K]K core motif (Rushton et al. 1996; Eulgem et al. 2000). Based on both the number of WRKY domains and features of a Zinc-finger-like motif, these proteins can be classified: WRKY proteins with two WRKY domains belong to group I, whereas most WRKY proteins with one WRKY domain and a $C_2H_2$ zinc finger motif belong to group II, and WRKY proteins with one WRKY domain and a $C_2HC$ zinc finger motif belong to group III (Eulgem et al. 2000).

Identification of *WRKY* genes that play important roles in plant defense responses in several plants suggest that the regulation specificities of some WRKY proteins might be conserved (Liu et al. 2004, 2005). The aim of this study was to identify *WRKY* genes in the coffee genome by data mining large sets of coffee expressed sequence tags (ESTs) (Lin et al. 2005; Vieira et al. 2006) and to identify those involved in pathogen resistance processes based on their expression patterns. Our data show that coffee genome encodes at least 22 members of this family, and they belong to types I, II, or III *WRKY* genes. We analyzed their expression under biotic (nematode and fungus infection), abiotic (wounding), phytohormones (SA, MeJA) treatments, and during leaf senescence and fruit development. These data allowed identifying a set of *WRKY* genes involved in coffee resistance/defense responses to the leaf-rust pathogen and to highlight the potential involvement of SA and JA in rust resistance signalization.

## Experimental procedures

### Database searches and gene annotation

Coffee genes were retrieved from ESTs databases by keyword searches of annotated unigenes. For coffee *WRKY* genes, multiple BLAST searches using the WRKY domain sequence were also performed. The databases searched

included (1) the Brazilian Coffee Genome Project ESTs database (http://www.lge.ibi.unicamp.br) which comprises more than 30,000 unigenes (210,000 ESTs) isolated from 37 cDNA libraries made from *C. arabica* tissues under several physiological conditions (Vieira et al. 2006), (2) the *Coffea canephora* ESTs database developed from five cDNA libraries made from coffee leaves, cherry, and seeds (http://www.sgn.cornell.edu), and comprising more than 13,000 unigenes (47,000 ESTs) (Lin et al. 2005), and (3) the IRD *C. arabica* EST database made of 1,900 unigenes from defense-specific subtractive cDNA libraries (Fernandez et al. 2004; Lecouls et al. 2006). Homology to *A. thaliana* WRKY sequences present in GenBank were searched using basic local alignment search tools (BLASTN and BLASTX; Altschul et al. 1990).

## Sequence alignment and phylogenetic constructions

The protein sequence corresponding to each coffee *WRKY* unigene was predicted using the EditSeq program of the Lasergene software package (DNAStar, Inc., Madison, WI, USA). Search for the specific DNA-binding protein domain (WRKYGQK sequence followed by a C2H2- or C2HC-type of zinc finger motif) (Eulgem et al. 2000) was manually performed on the protein sequences. The C-terminal WRKY domain sequences (68 amino acid residues) of 72 *A. thaliana* and 22 *C. arabica WRKY* genes were aligned, and a phylogenetic tree was constructed using the MegAlign program with ClustalW and default settings (Gap opening 10, gap extension 2, Blosum weight matrix).

## Plant material—biotic and abiotic treatments

Coffee plants (*C. arabica*) were grown in potting soil in a greenhouse. The following varieties were used in this study: Caturra (resistant to *H. vastatrix* race VI), IAPAR59 (resistant to *Meloidogyne exigua*), Tupi IAC1669-33 (resistant to *H. vastatrix* race II).

Biotic treatments were performed on 6-month-old plants. For rust assays, *C. arabica* leaves were challenged with *H. vastatrix* isolates eliciting an incompatible interaction (race VI with *C. arabica* var. Caturra or race II with *C. arabica* var. Tupi) as described in Fernandez et al. (2004). Plants sprayed only with water were used as control. An experiment with three replicated assays was conducted using 24 plants for each assay. Leaf samples were collected 18 h post-inoculation (hpi) for the Caturra × race VI interaction, and 39 hpi for the Tupi × race II interaction. Root-knot nematode infection was performed by inoculating *M. exigua* juveniles (J2) on *C. arabica* IAPAR59 (ten replicated plants) as described in Lecouls et al. (2006). Plants only inoculated with water were used as control. Roots tips (5-mm long) were collected 3 and 5 days after inoculation (dpi).

Abiotic and hormonal treatments were performed on coffee (*C. arabica* var. Caturra) leaves of the same physiological state than leaves used for rust inoculation. Two leaves per plant and four plants per experiment were used, and experiments were repeated three times. Wounding was performed by applying an average of seven transversal cuttings per half-leaf using scissors. Wounded leaves were collected 30 min later. Non-wounded leaves collected on different plants were used as controls. SA treatments were performed either by infiltrating leaves with a 0.5-mM solution of SA using a needless syringe or by spraying leaves with a 5-mM solution of SA. In preliminary experiments, we tested several SA concentrations (from 25 µM to 2 mM), and we chose the highest dose that did not induce necrosis. Water-infiltrated or water-sprayed (mock control) as well as non-infiltrated leaves were used as controls. Leaves were collected 1 and 3 h after treatment. Methyl jasmonate (MeJA) treatments were performed by enclosing plants for 24 h into an airtight 50-L plastic chamber containing 10 µl of pure MeJA on a piece of a filter paper as described in Avdiushko et al. (1995). For the control, plants were kept in similar chambers without MeJA.

Senescent leaves showing visible signs of yellowing were harvested from the lower part of Caturra plants. Young leaves from the same plants were collected and used as controls. Biological samples originated from three independent experiments.

Fruit development: coffee berries were collected at five different physiological states of ripeness from one adult plant of *C. arabica* var. Obatã, maintained under field conditions at the Experimental Center of Instituto Agronômico, Campinas (SP), Brazil. The stages of berry development were defined by days after anthesis (d.a.a.), and they were as follows: pin heads (15 d.a.a.), endosperm expansion (55 d.a.a.), green cherry-immature (115 d.a.a.), light green immature (165 d.a.a.), and red cherry (190 d.a.a.). Fruits were collected from flowering (end of September 2005) up to complete maturation (April 2006). Samples used for *WRKY* genes expression assays were pinheads and mature cherries.

Plant materials collected after treatment were immediately frozen by immersion in liquid nitrogen, pooled and stored at −80°C until RNA extraction.

## RNA extraction and RT-PCR

Total RNA was extracted from coffee fruit, leaf, or root samples using the RNeasy Plant kit (Qiagen, France), with addition of an on-column DNase I digestion. For quantification, the absorbance from 1:100 RNA samples were detected using the Spectronic Genesys 8 UV/Visible Spectrophotometer (Spectronic Instruments, Cheshire, England), and 1% agarose gel was run to visualize the quality of the RNA. Absence of DNA in RNA samples was checked by running a

test PCR with *CaUbiquitin* primers. 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.

### Real-time quantitative PCR assays of gene expression

Specific primers were designed from the coffee cDNA sequences using the Beacon Designer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), with melting temperatures ($T_m$) of 58°±5°C, primers lengths of 18 to 25 bp, and amplicon lengths of 75 to 200 bp. When possible, primers were designed from the 3′ region of the gene and outside of the conserved DNA-binding domain to ensure gene specificity. Primers listed in Table 1 suppl. (supplemental material) were synthesized by Eurogentec and used at 200 nM final concentration. Quantitative RT-PCR was performed using the Stratagene MX3005P with MxPro v 3.00 software for Comparative Quantitation (Stratagene, La Jolla, CA). Quantitative PCR was carried out on 1.25 ng cDNA in a 20-μL amplification mixture containing MESA GREEN Master Mix Plus for SYBR Assay NO ROX (Eurogentec). The cycling conditions comprised 2 min at 50°C followed by 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s. Each assay was conducted in duplicates and included a no-template control. The *CaUbiquitin* gene (Rojas-Herrera et al., unpublished) was chosen as the internal constitutively expressed control (normalization). Dilutions of cDNA (1:10 to 1:1,280) from a reference sample was used to construct a relative standard curve. For all primer pairs tested, efficiencies varied from 0.90 to 1.10 (data not shown). The specificity of PCR products was checked by melting curve analyses and only primers sets producing a single sequence-specific peak in the dissociation curve were conserved. Data were analyzed using the MxPro software package to obtain the relative expression levels of coffee genes. Based on the comparative Ct method, data are either expressed as fold changes to calibrator average or as log (base 2) fold changes to calibrator average. Data were analyzed by ANOVA and Tukey's test ($a$=0.05). Statistical analyses were performed by using the SISVAR 4.6 software (Ferreira 2000).

For expression cluster analysis, all hierarchical clusters were calculated and drawn using the TIGR MeV (Saeed et al. 2003) software provided by TIGR (http://www.tigr.org/software).

### Evaluation of specific lipoxygenase (LOX, EC 1.13.11.12) activity in coffee leaf extracts

Coffee extracts were prepared according to the procedure described by Jalloul et al. (2002) on 1 g frozen leaf tissue. The extract (0.5 ml) was incubated for 20 min at 25°C with 1 ml 250 mM sodium phosphate buffer (pH 7) and 5 μl of an ethanolic solution of linoleic acid (100 mM). The LOX reaction was stopped by addition of 70 μl $HClO_4$ (12 N), and hydroperoxy fatty acids were extracted in 2 ml hexane. After vigorous shaking, the absorbance at 234 nm of the upper organic phase was measured (spectrophotometer Uvikon 922; Kontron, Marseille, France) against a blank containing hexane. LOX activity was expressed in picokatal $mg^{-1}$ protein using a molar extinction coefficient for conjugated dienes of 25,000 $M^{-1}$ $cm^{-1}$. The soluble protein content of the extracts was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

## Results

### Identification of putative *WRKY* genes in coffee

Among more than 200,000 coffee ESTs, 313 ESTs were identified with BLAST homologies to WRKY proteins. After clustering and annotation, 53 different putative *WRKY* genes were obtained (data not shown). All these putative *WRKY* genes are classified based on sequence similarity searches, refined by multiple alignments, and conserved motifs were identified when possible. Only 22 sequences out of those 53 possess the structures typical of the WRKY family, including the diagnostic WRKYGQK signature and the zinc-finger motif. The sequence of the other unigenes either did not cover the WRKY domain (22 genes) or ended within the domain (nine genes), thus impairing further analyses. The 22 unigenes encoding a protein with one or two WRKY domains that were undoubtedly identified are listed in Table 1. Two unigenes pairs displayed high sequence homology. The *CaWRKY13* and *CaWRKY14* unigenes shared 96% nucleotide identity and differed by one amino acid replacement in the WRKY domain. The same occurred for the *CaWRKY19* and *CcWRKY20* unigenes. It is thus possible that they are allelic copies in the *C. arabica* and *C. canephora* genomes or homeologous copies of a same gene in the tetraploid *C. arabica* genome.

### Classification of *WRKY* genes on the basis of the WRKY domains

Classification of genes is important for the functional analysis of a gene family. Out of the 22 coffee *WRKY* sequences, two (*CaWRKY15*, *CcWRKY17*) encoded two WRKY domains. The *CaWRKY18* sequence spanned the N-terminal domain of group I WRKY proteins and the *CaWRKY16* sequence covered only the C-terminal domain. The *CaWRKY18* sequence was thus excluded from the

**Table 1** List of 22 coffee unigenes encoding putative WRKY transcription factors

| Coffee clone | Origin | *AtWRKY* best BlastX | *AtWRKY* ortholog | *WRKY* group | Accession number |
|---|---|---|---|---|---|
| *CaWRKY1* | *C. arabica* | 6 | 6 | IIb | DQ335599 |
| *CaWRKY2* | *C. arabica* | 57 | 57 | IIc | GT732236;GT732237;GT687640; GW428999 |
| *CaWRKY3* | *C. arabica* | 75 | 75 | IIc | GT715378;GT715379;GT669686; GT684003;GT675420;GT675421; GT675422;GT675423 |
| *CaWRKY4* | *C. arabica* | 21 | 21, 39, 74 | IId | GT704950;GT699101;GT708285; GT714188;GT723892;GT726133; GT721669;GT696023;GT696024; GT683342;GW430652;GW429682; GW452273 |
| *CcWRKY5* | *C. canephora* | 21 | 21, 39, 74 | IId | DV710913 |
| *CaWRKY6* | *C. arabica* | 7 | 15 | IId | GW460548;GW439927;GT671542; GT700942;GT688469;GT722306 |
| *CaWRKY7* | *C. arabica* | 11 | 11,17 | IId | |
| *CaWRKY8* | *C. arabica* | 27 | 22,27,29 | IIe | GT685360;GW436389 |
| *CaWRKY9* | *C. arabica* | 14 | 14,35 | IIe | |
| *CcWRKY10* | *C. canephora* | 69 | 69 | IIe | DV693086 |
| *CaWRKY11* | *C. arabica* | 53 | 30 | III | GW489409;GW472658;GW489686; GW467855 |
| *CaWRKY12* | *C. arabica* | 53 | 41 | III | GW487300;GT715908 |
| *CaWRKY13* | *C. arabica* | 70 | 54, 70 | III | GW428837;GW469454 |
| *CaWRKY14* | *C. arabica* | 54 | 54, 70 | III | GT730216;GT688262;GT688263; GT733537 |
| *CaWRKY15* | *C. arabica* | 33 | 33 | I | GT725660;GT721476;GT721477; GT704531;GT707389;GT702040; GT702041;GW490838;GT695761; GT674787;GT704614 |
| *CaWRKY16* | *C. arabica* | 33 | 33 | I | |
| *CcWRKY17* | *C. canephora* | 33 | 33 | I | DV678740 |
| *CaWRKY18* | *C. arabica* | 33 | 4,3 | I | GW452866;GW464006;GW435767 |
| *CaWRKY19* | *C. arabica* | 40 | 40 | IIa | GW490173;GW439047;GW438774; GW472647;GW481654 |
| *CcWRKY20* | *C. canephora* | 40 | 40 | IIa | DV706804 |
| *CcWRKY21* | *C. canephora* | 40 | 18, 40, 60 | IIa | DV709595.1 |
| *CaWRKY22* | *C. arabica* | 40 | 18, 40, 60 | IIa | GT685546;GT684702 |

phylogenetic analysis based on C-terminal WRKY domain sequences. Alignment of the C-terminal WRKY domain from the 21 coffee amino acids sequences together with those from the 72 *A. thaliana WRKY* proteins allowed to classify coffee genes within the three main WRKY groups already defined in *A. thaliana* (Eulgem et al. 2000) (Fig. 1 and Table 1). A high conservation of both the WRKY motif and the zinc-finger motif was observed between the two plant species (Fig. 1suppl.). All coffee amino acids sequences shared the conserved WRKYGQK peptide in the WRKY domain. *WRKY* coffee genes of group III had a $C_2HC$-type zinc-finger motif (C-$(X)_7$-C-$(X)_{23}$-H-X-C). All other coffee *WRKY* genes had a $C_2H_2$-type (C-$(X)_n$-C-$(X)_{23}$-H-X-H), with *n* (4 or 5). Four unigenes were assigned to group I of *A. thaliana* WRKY proteins, 14 to group II, and four to group III. Representatives of each *Arabidopsis* WRKY proteins subgroups (IIa, b, c, d, and e) were found in coffee.

Based on the best BLASTX homology result obtained with the *A. thaliana* referenced *WRKY* genes and on the phylogenetic tree, we assigned orthologs for the coffee *WRKY* genes. For several genes that matched closely related *A. thaliana* WRKY proteins, orthologs could not be defined unambiguously. For instance, *CaWRKY13* and *CaWRKY14* matched the *AtWRKY54* and *AtWRKY70* genes. On the other hand, several unigenes matched the same Arabidopsis WRKY protein, including AtWRKY33 and AtWRKY40 but encoded coffee proteins with several

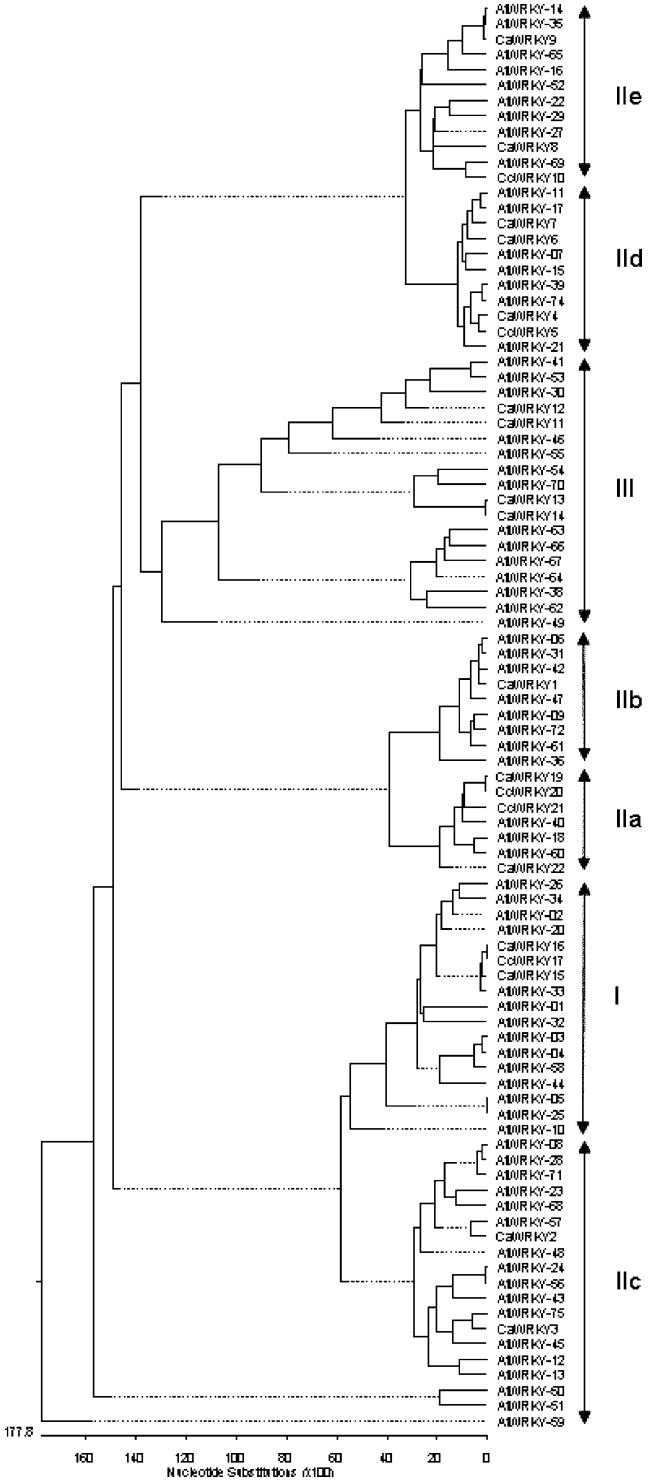


**Fig. 1** Phylogenetic tree of 21 members of the coffee WRKY family and 72 members of the AtWRKY family

amino acid divergences. For instance, the *CaWRKY19*/*CcWRKY20* unigenes pair and the *CcWRKY21* unigene best matched AtWRKY40 but exhibit eight amino acids differences in their WRKY domain (Fig. 1suppl.).

## Conserved motifs between Arabidopsis and coffee WRKY proteins

Contigs spanning full-length cDNA sequences were obtained from the Brazilian unigene database for at least one member of each WRKY group and subgroup identified, except for group IIe. A total of eight full-length clones were analyzed, including the previously cloned *CaWRKY1* (Petitot et al. 2008). For group IIe proteins, the almost complete CaWRKY8 sequence (lacking around 40 aa at the C-terminal end) was added to the analysis of conserved motifs (Table 2suppl.). The full-length open-reading frames varied from 208 to 572 amino acids (Table 2suppl.). Automatic and manual searches for conserved patterns allowed the detection of structural motifs related to those identified in Arabidopsis WRKY proteins (Table 2suppl., Fig. 2) (Eulgem et al. 2000). With few exceptions, amino acid sequences were not strictly conserved between Arabidopsis and coffee, and even between coffee sequences from the same group. For instance, the group IId-specific HARF motif was found in the CAWRKY6-deduced protein sequence that strictly corresponded to the Arabidopsis consensus sequence RTGHARFRR[A/G]P (Eulgem et al. 2000). In CAWRKY4, another group IId protein, the motif found in the same protein region could be related to the HARF motif only by three amino acids (TLGHARVRKV). Likewise, the putative nuclear localization sequence (NLS) KAKKxxQK found in Arabidopsis group IIc proteins

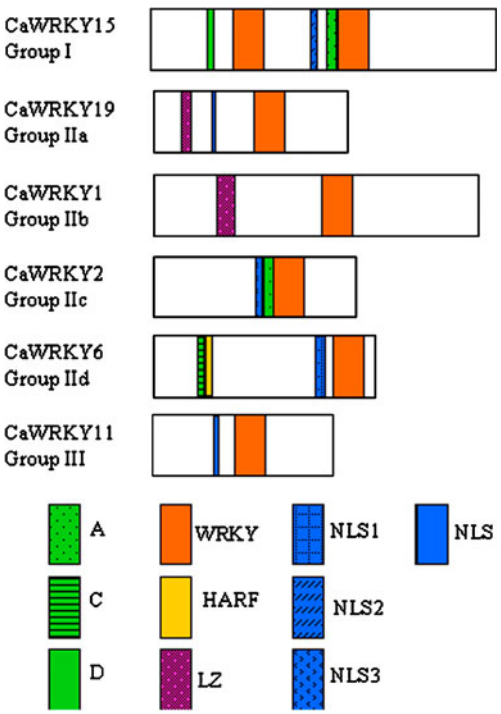


**Fig. 2** Schematic diagrams of conserved motifs found in coffee WRKY peptide sequences

(Eulgem et al. 2000) was strictly conserved in the CaWRKY2 deduced protein sequence. However, only the KSGKKKADKK motif could be found in the CaWRKY3 putative sequence at the same location. Basic stretches associated to NLS were found in all full-length cDNA sequences examined and were related to motifs 1, 2, and 3 described in Arabidopsis. Distribution of NLS1, 2, and 3 motifs in *A. thaliana* WRKY groups and subgroups was retained in CaWRKY proteins (Fig. 2). In contrast, the calmodulin binding domain (CaMBD) VSSFK (K/R) VISLL (Park et al. 2005) found in groups IId, IIe, and III AtWRKY proteins was only observed in the group IId CaWRKY proteins.

## Patterns of coffee *WRKY* genes expression in *C. arabica*

Most of the coffee *WRKY* genes identified (15 out of 22) were retrieved from the Brazilian Coffee Genome Project, which includes 27 cDNA libraries made from *C. arabica* tissues under several physiological conditions (Vieira et al. 2006). In a preliminary analysis, ESTs-derived expression profiles (digital Northern) of all putative *WRKY* genes were assessed to verify a possible preferential expression in specific conditions. Using the method of Audic and Claverie (1997) which allows the calculation of the probability of differential expression of a given gene between two libraries, no gene having significant ($P<0.05$) differential expression was observed (data not shown). Since only a few cDNA libraries contained hormonal- or pathogen-treated tissues, the patterns of expression of the coffee *WRKY* genes were investigated in *C. arabica* plants in pathogen resistance and after several hormonal treatments. *WRKY* genes expression patterns in *C. arabica* were assessed by quantitative real-time RT-PCR assays using gene-specific primers. Reliable and specific amplification was obtained for only 17 out of the 22 *WRKY* genes. Specific primers could not be designed for the related sequences *CaWRKY13*/*CaWRKY14* and *CaWRKY19*/*CcWRKY20*. In addition, the *CaWRKY19*/*CcWRKY20* unigenes pair and the *CcWRKY21* unigene shared 92% sequence identity and we did not succeed in designing gene specific primers. Gene expression results are, therefore, referred hereafter using this double (*CaWRKY13*/*14*) or triple (*CaWRKY19*/*20*/*21*) denomination for designing these two groups of genes. For the other genes (*CaWRKY9*; *CaWRKY16*), as no transcript could be detected in control plants, further relative quantification in treated plants, whenever amplification occurred, was not possible. In total, 17 primer pairs were used to evaluate the expression of 17 genes or groups of genes.

## Constitutive expression

Basal levels of *WRKY* genes expression were first assessed in leaves and roots of *C. arabica* plants using data obtained in control plants. *WRKY* genes expression levels were normalized to *CaUbiquitin* transcript accumulation. Basal expression levels in roots and leaves varied between genes (Fig. 2suppl.). For instance, in roots, there was a 180-fold change difference ($P<0.05$) between *CaWRKY15* and *CaWRKY22* expression levels. Significant differences were also observed in the expression patterns between roots and leaves for 13 out of the 17 genes tested. However, as different coffee cultivation conditions were used for the SA treatment and the nematode infection assays, conclusions related to organ-differential expression may not be possible.

## Relative expression

Data of log2-transformed relative expression ($-\Delta\Delta Ct$) of coffee genes are shown in Table 2. Genes were considered

**Table 2** Relative expression of coffee *WRKY* members in response to several treatments. Data are log2-transformed (or $-\Delta\Delta Ct$) value of gene expression in treated plants relative to control plants

| Clone | *H. vastatrix* race II | *H. vastatrix* race VI | MeJA | AS(3h) infilt. | AS(3h) pulv. | Nematode 3dpi | Nematode 5dpi | wounding | fruit maturation | leaf senescence |
|---|---|---|---|---|---|---|---|---|---|---|
| CaWRKY1 | 1.20 | 3.90 | 5.79 | 1.77 | 2.77 | 0.18 | -0.19 | 5.39 | 2.87 | 6.49 |
| CaWRKY2 | -0.31 | -0.15 | 1.72 | 0.08 | 0.72 | 2.09 | 0.61 | 1.20 | 0.74 | 1.98 |
| CaWRKY3 | 5.21 | 6.83 | 6.20 | 3.34 | 4.37 | 0.20 | -0.29 | 0.56 | 4.85 | 8.84 |
| CaWRKY4 | 0.12 | 0.26 | 0.72 | 1.07 | 0.76 | 0.51 | 0.26 | 0.49 | -0.19 | 0.61 |
| CcWRKY5 | -0.03 | 0.49 | -0.90 | 0.55 | 0.76 | 0.46 | -0.39 | -0.61 | 0.93 | -1.36 |
| CaWRKY6 | 1.15 | 1.68 | 3.24 | -0.05 | 3.34 | 1.58 | 2.02 | 1.18 | 1.77 | -2.18 |
| CaWRKY7 | 0.11 | 2.04 | 2.02 | 1.48 | 2.38 | 0.78 | 1.11 | 2.04 | 0.82 | -0.25 |
| CaWRKY8 | 0.93 | 2.98 | 0.23 | 1.08 | -0.11 | 0.57 | -0.33 | 1.06 | 5.71 | 4.69 |
| CcWRKY10 | 1.59 | 1.20 | 3.12 | 0.90 | 1.99 | 1.94 | 1.09 | 0.62 | 2.19 | 1.52 |
| CaWRKY11 | 1.16 | 2.20 | 3.61 | 0.57 | 0.84 | 1.64 | 0.98 | 1.94 | 1.20 | 1.99 |
| CaWRKY12 | 1.19 | 2.94 | 3.08 | 2.37 | 2.65 | 2.18 | 1.90 | 1.10 | -1.08 | -0.60 |
| CaWRKY13/14 | 3.02 | 2.77 | 5.35 | 8.57 | 5.48 | 2.19 | 1.63 | 1.91 | -0.77 | -0.84 |
| CaWRKY15 | 1.08 | 2.68 | 3.68 | 1.74 | 4.08 | 1.15 | 0.00 | 3.14 | 3.91 | 2.20 |
| CcWRKY17 | 2.51 | 3.89 | 4.80 | 1.78 | 4.15 | 1.04 | 1.42 | 3.38 | 3.86 | 1.10 |
| CaWRKY18 | -0.29 | 0.00 | -0.15 | 0.32 | -0.46 | 0.40 | 0.00 | -0.84 | 0.76 | 0.01 |
| CaWRKY19/20/21 | 2.14 | 4.58 | 6.08 | 3.99 | 5.83 | -0.99 | -1.10 | 5.16 | 3.99 | 6.51 |
| CaWRKY22 | 3.51 | 4.77 | 1.78 | 11.54 | 9.92 | 0.40 | 0.29 | 2.07 | 1.18 | 7.43 |

color code log2(ratio)
<-2
<-1
not diff
>1.0
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>3.0

as activated or repressed when values were superior to 1 (2-fold activation) or inferior to −1 (2-fold repression), respectively.

*Fruit maturation, leaf senescence*

Some *WRKY* genes involved in pathogen defense were also shown to be involved in death-related processes such as senescence (Robatzek and Somssich 2001; Miao et al. 2004; Murray et al. 2007). Expression level of the 17 coffee *WRKY* genes was investigated in senescent leaves (SL) and during berry development (fruit maturation (FM)). Collected data showed that more than half of genes tested were activated during fruit maturation and leaf senescence (11 FM- and 11 SL-activated; Table 2). Also, only a few of those were repressed (two FM- and two SL-repressed). All except one FM-activated gene were also SL-activated; nevertheless, repressed genes were distinct between FM and SL.

*Pathogen resistance*

Rust-resistant *C. arabica* plants were challenged with *H. vastatrix* isolates eliciting an HR around 18 hpi (*H. vastatrix* race VI with *C. arabica* var. Caturra) (Ganesh et al. 2006; Silva et al. 2008) or 39 hpi (*H. vastatrix* race II with *C. arabica* var. Tupi) (Ramiro et al. 2009). The nematode-resistant *C. arabica* cv IAPAR59 was inoculated with *M. exigua* juveniles and samples were collected at 3 and 5 dpi that corresponded to the onset of HR (Anthony et al. 2005).

Seventy-eight percent and 55% of the *WRKY* genes were activated in the resistance responses to rust and nematodes, respectively (Table 2). In general, induction levels were much higher in rust-challenged plants (from 2- to 100-fold) than those triggered by nematodes which reached only an average of 3-fold change (around 1.5 ΔΔCt).

*Wounding and hormonal treatments*

*WRKY* genes expression patterns were also assessed after a 24-h treatment with MeJA, 30 min after wounding (W), and SA 3-h treatments (Table 3). Seventy-eight percent and 67% of the *WRKY* genes responded to MeJA and SA treatments, respectively. Except for two genes (*CaWRKY3* and *CcWRKY10*), *WRKY* genes that responded to MeJA were also activated after wounding. Interestingly, considerable overlap was found between genes responding to MeJA and to SA: all genes activated by SA were also activated by MeJA.

In order to classify coffee *WRKY* genes according to the alteration range of their expression patterns, the average relative expression was calculated over all treatments (Fig. 3). A statistically significant correlation (Pearson $R=-0.74$, $t=-4.98$; $p<0.001$) was found between the average relative expression and the basal level of expression for each gene examined. Genes showing the lowest level of basal expression (*CaWRKY3*, *CaWRKY22*, *CaWRKY13*/*14*) were found to be the most upregulated upon several treatments. In contrast, those genes that showed elevated basal expression levels in roots or leaves (*CaWRKY4*, *CaWRKY18*, *CcWRKY5*) did not display alteration in any treatment.

Hierarchical cluster analysis

Expression data of the 17 *WRKY* genes were clustered with the TIGR multi-experiment viewer with the HCL algorithm and using Euclidean distance and complete linkage. Statistical significance of clustering was verified by bootstrap analysis using the ST algorithm and K-mean/K-median support analysis. Expression data were compiled for all treatments, including rust and nematodes, SA and MeJA, wounding, senescence, and fruit maturation (Fig. 4). From this analysis, we can distinguish groups of genes

**Table 3** Summary of regulation patterns of rust-induced *WRKY* members in coffee plants

| Clusters | Clone | Rust | MeJA | Wounding | Fruit maturation | Leaf senescence | SA | Nematode |
|---|---|---|---|---|---|---|---|---|
| 1 | *CaWRKY6* | + | + | | | − | + | + |
| 1 | *CaWRKY12* | + | + | | − | | + | + |
| 1 | *CaWRKY11* | + | + | | | | | + |
| Unassigned | *CaWRKY13/14* | + | ++ | | | | ++ | + |
| 4 | *CaWRKY15* | + | + | + | + | + | + | + |
| 3 | *CaWRKY19/20/21* | ++ | ++ | ++ | + | ++ | ++ | − |
| 3 | *CaWRKY1* | ++ | ++ | ++ | + | ++ | + | |
| Unassigned | *CaWRKY22* | ++ | | + | | ++ | +++ | |
| Unassigned | *CcWRKY17* | ++ | ++ | + | + | | ++ | + |
| Unassigned | *CaWRKY3* | ++ | ++ | − | ++ | +++ | ++ | |

+ moderately activated; ++ activated; +++ strongly activated; − repressed

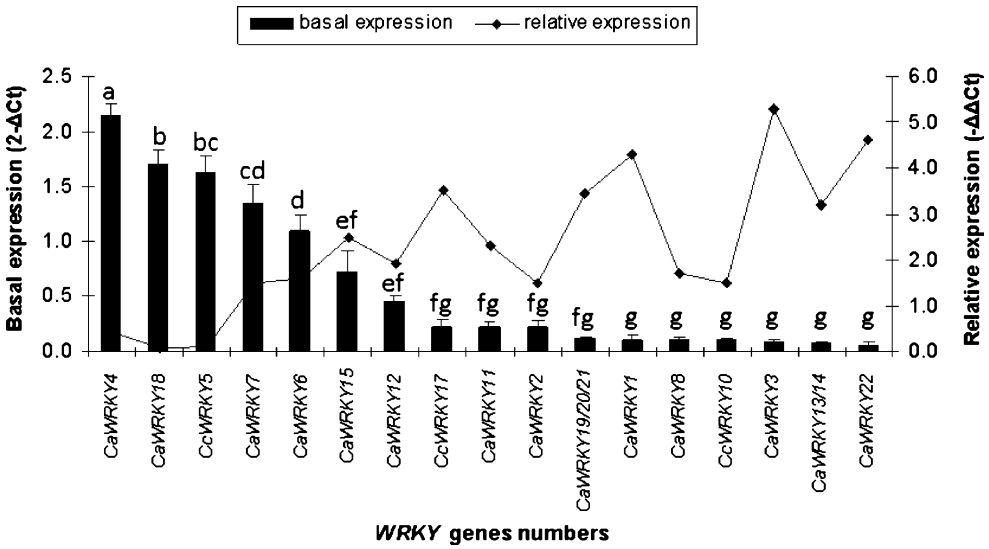


**Fig. 3** Basal expression levels in coffee leaves and relative expression average of coffee WRKY members calculated over all treatments. Note that basal expression data refer to the relative expression of *WRKY* genes to that of the *CaUbiquitin* gene in leaves ($2^{-\Delta Ct}$). Relative expression data of *WRKY* genes are log2-transformed fold change values of relative gene expression ($-\Delta\Delta Ct$). On each histogram bar, values with the same letter are not significantly different according to Tukey's test ($P<0.05$)

displaying the same alteration of expression patterns over all treatments. Only four clusters were consistently formed over 60% in 100 K-mean/K-median runs (Table 2, Fig. 4).

A first cluster was made of five genes, including *CaWRKY6*, *CaWRKY7*, *CaWRKY12*, *CaWRKY11*, and *CcWRKY10*. These genes were moderately (1–3 ΔΔCt) activated by pathogens, SA, MeJA, and some of them by wounding. *CaWRKY11* was activated by MeJA but not by SA.

A second cluster of four genes was made of the *CaWRKY4*, *CcWRKY5*, and *CaWRKY18* genes that did not display alteration in any treatment, and *CaWRKY2* which displayed little activation by MeJA, wounding, and in leaf senescence.

A third cluster grouped *CaWRKY1* and *CaWRKY19/20/21* genes. These genes were highly activated (average of 3–5 ΔΔCt) in all treatments except by nematodes where they were slightly repressed (*CaWRKY19/20/21*) or poorly altered.

The fourth cluster was made of *CaWRKY15* and *CaWRKY8* that were activated neither by pathogens nor SA. *CaWRKY8* was strongly activated (4–5 ΔΔCt) in leaf senescence and in mature fruits only.

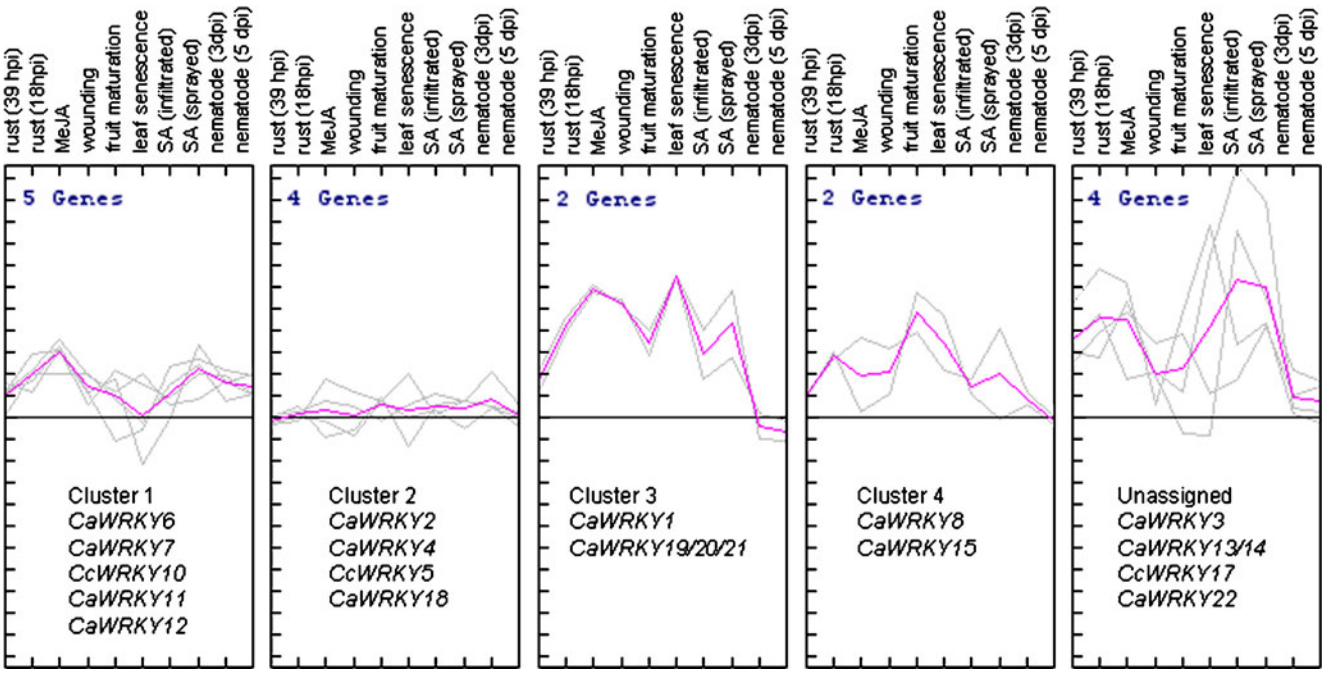


**Fig. 4** K-mean/K-median cluster analysis of 17 coffee *WRKY* genes differentially expressed in resistant coffee varieties. Treatments were on the x-axis (from left to right): rust pathogen (*H. vastatrix*) inoculation (race II, race VI), MeJA, wounding, fruit maturation, leaf senescence, SA (infiltrated, pulverized), nematode (*M. exigua*) infection (3, 5 dpi). Relative expression ($-\Delta\Delta Ct$) is on the y-axis. Genes were clustered with The Institute for Genomic Research (TIGR) multi-experiment viewer (MEV 4.01) using a 60% confidence value

Finally, four other coffee *WRKY* genes could not be assigned to a specific cluster. They were *CaWRKY3*, *CaWRKY13/14*, *CcWRKY17*, and *CaWRKY22*. This set comprises genes activated in response to pathogens (rust and/or nematodes) and to hormonal treatments. *CaWRKY13/14* was strongly activated by SA and MeJA.

## A role for JA in coffee resistance to rust disease?

Statistical analysis of gene expression showed a significant correlation (*t* test, $P<0.01$) between senescence, fruit maturation, MeJA, and rust treatments (all combinations) for all *WRKY* genes tested.

To investigate the involvement of JA in rust resistance responses, the expression of genes related to JA biosynthesis, including allene oxide synthase (*CaAOS*) and 13-Lipoxygenase (*Ca13-LOX*) (Laudert and Weiler 1998; Liavonchanka and Feussner 2006), was monitored during the time-course of HR development (18, 21, and 24 hpi) in Caturra plants challenged with *H. vastatrix* race VI. In addition, the total lipoxygenase activities were quantified in coffee leaves at 24 hpi.

Genes putatively encoding allene oxide synthase and lipoxygenase enzymes were retrieved from the HarvEst coffee database. Concerning *AOS*, clone CGN-51620 (accession number DV704753) that best matched allene oxide synthase proteins was used to designate primers for qPCR assays. Concerning lipoxygenases, 10 unigenes with BLASTX homology to LOX proteins were found. Nucleotide and deduced amino acid alignments showed that the 10 unigenes grouped into six clusters (data not shown). In plants, LOXs (linoleate/oxygen oxidoreductase, EC 1.13.11.12) constitute a large gene family of fatty acid dioxygenases that are classified with respect to their positional specificity of linoleic acid oxygenation (Liavonchanka and Feussner 2006). Two unigenes could be assigned to 13-LOX proteins (accession number DV704189) and 9-LOX proteins (accession number DV711018), when those sequences were analyzed using the Sloane's determinant (Sloane et al. 1991). To verify their involvement in JA biosynthesis, the *AOS* and *LOX* transcripts were quantified in coffee leaves after wounding, SA, and MeJA treatments. As expected, the *CaAOS* and *Ca-13-LOX* genes were activated after a 24-h MeJA treatment and by wounding, but not in response to SA (Fig. 3suppl.). In contrast, the *9-LOX* transcript levels were not altered by wounding or MeJA treatment but decreased with the SA treatment.

In coffee leaves challenged with rust isolate VI, only little induction of the *9-LOX* gene could be found at 24 hpi (Fig. 5a). The *CaAOS* and *13-LOX* did not show induction of their transcript levels during the time-course of HR development, and the 13-LOX gene even appeared slightly repressed at 21 hpi (Fig. 5a). At the biochemical level, quantification of total LOX activity showed no variation between control and rust-challenged coffee plants at 24 hpi indicating that no activation of lipoxygenases occurred at this time (Fig. 5b).

## Rust-induced WRKY genes

Expression of *WRKY* genes was also evaluated during the time-course of HR development in Caturra plants chal-

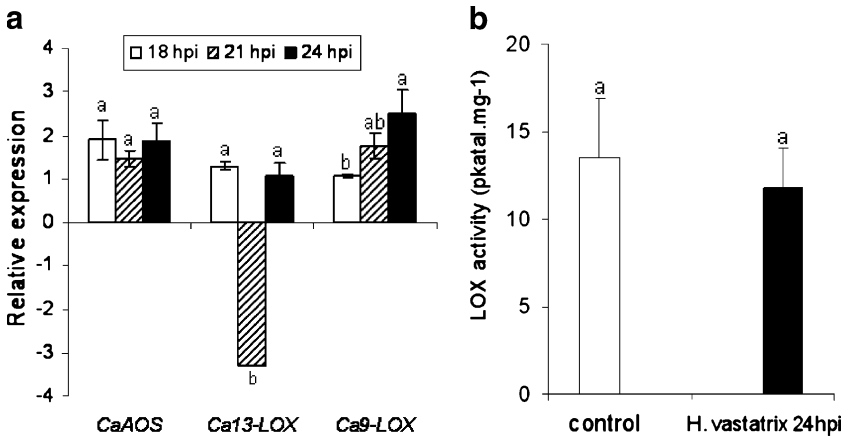


**Fig. 5** **a** Quantitative RT-PCR Analysis of allene oxide synthase (*CaAOS*), 13-Lipoxygenase (*Ca13-LOX*), 9-Lipoxygenase (*Ca9-LOX*) genes expression in *C. arabica* cv. Caturra leaves challenged with *H. vastatrix* race VI (incompatible interaction). Leaves were collected 18, 21, and 24 hpi. Data are fold change values of relative expression to mock-inoculated plants. Each *point* represents the mean and SD values from three replicated experiments; **b** total lipoxygenase (EC 1.13.11.12) activity in *C. arabica* cv. Caturra leaves, after *H. vastatrix* race VI (incompatible interaction) inoculation (24 hpi) or control untreated plants, was measured using C18:2 as substrate according to the UV method reported in "Experimental procedures" section. Protein concentration of samples was measured by the Bradford method, and each point represents the mean and SD of the specific activity from three replicates. On each histogram bar, *values with the same letter* are not significantly different according to Tukey's test ($P<0.05$)

lenged with *H. vastatrix* race VI. Twelve *WRKY* genes were chosen, including the 10 *WRKY* genes that displayed activation in previous rust assays and two genes that did not display alteration in any treatment (*CaWRKY4* and *CaWRKY18*). As expected, the latter were not induced by rust inoculation (Fig. 6). Two major patterns of activation during HR were obtained ($p<0.05$). A first group of genes was moderately (2–5-fold) activated all along the time-course analysis, including *CaWRKY6*, *CaWRKY11*, *CaWRKY12*, *CaWRKY13/14*, and *CaWRKY15*. The other five genes (*CaWRKY1*, *CaWRKY3*, *CcWRKY17*, *CaWRKY19/20/21*, and *CaWRKY22*) showed stronger (10–20-fold) activation patterns, and, except *CaWRKY3* and *CaWRKY22*, were transiently activated between 18 and 24 hpi.

Table 3 shows compilation of these results together with the expression data in other treatments. Interestingly, those genes that are strongly activated during rust resistance responses are also strongly activated in almost all treatments. In contrast, genes that are moderately activated after rust inoculation display specific patterns of expression in response to different treatments, except gene *CaWRKY15*. Genes *CaWRKY11*, *CaWRKY12*, *CaWRKY6*, and *CaWRKY13/14* are activated by pathogens (rust and nematodes) and hormonal treatments (SA and/or MeJA) but not by wounding, senescence or fruit maturation. They were clusterized together in group 1, or unassigned (*CaWRKY13/14*) by TMEV analysis. These genes may be specific regulators of coffee responses to pathogens. Interestingly, these genes belong to WRKY groups IId and III only.

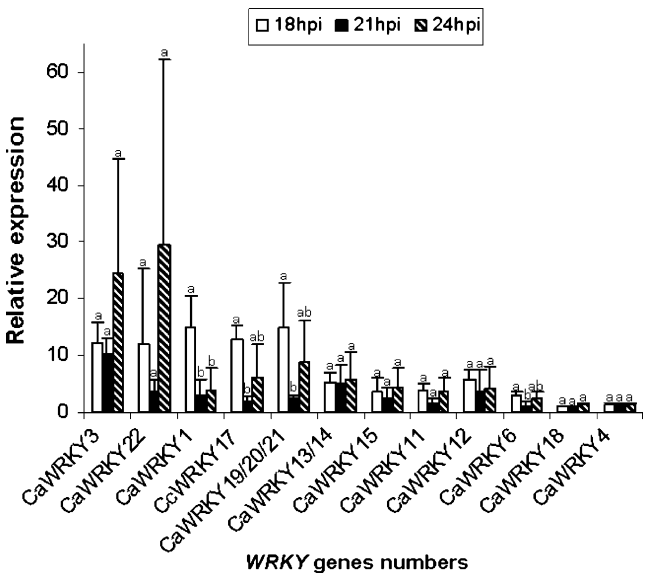


**Fig. 6** Quantitative RT-PCR analysis of *WRKY* genes expression in *C. arabica* cv. Caturra leaves challenged with *H. vastatrix* race VI (incompatible interaction). Leaves were collected 18, 21, and 24 hpi. Data are fold change values of relative expression to mock-inoculated plants. Each point represents the mean and SD values from three replicated experiments. For each gene, *values with the same letter* on *histogram bars* are not significantly different according to Tukey's test ($P<0.05$)

## Discussion

Determination of genes involved in plant immune responses, and their functions, is one of the major goals in plant biology. In particular, WRKY transcription factors can regulate plant responses to pathogens (reviewed in Pandey and Somssich 2009). So far, mutational approaches have been used to analyze WRKY functions in Arabidopsis. However, probably due to gene redundancy, many of these mutational insertions did not produce any detectable phenotype. Therefore, expression profiling and definition of genes specifically or preferentially expressed in response to pathogens may complement the genetic and molecular approaches. In coffee, the generation of EST collections and the quantitative real-time PCR can produce reliable, high-quality data (Fernandez et al. 2004; Lin et al. 2005; Petitot et al. 2008; Simkin et al. 2008). We analyzed a large number of ESTs to identify WRKY transcription factor genes which are specifically expressed in response to pathogens. For this, expression of identified coffee *WRKY* genes was monitored during resistance response to the rust fungus *H. vastatrix* and to the *M. exigua* nematode. In parallel, *WRKY* genes expression after hormonal treatments and during developmental processes was also assessed in order to identify genes displaying specific patterns of expression.

Fifty-three different putative *WRKY* genes were retrieved from the coffee ESTs database set, and 22 sequences could be unambiguously assigned to the WRKY family of plant genes (Table 1). It is likely that a large part of the remaining ESTs which sequence did not cover the whole WRKY domain are also true *WRKY* genes and that the number of coffee *WRKY* genes could be estimated to around 50. Nevertheless, this number is much lower than the 74 genes in Arabidopsis and 102 in rice (Ülker and Somssich 2004; Ross et al. 2007). Firstly, the diploid genome of coffee (*C. canephora*) is estimated to be of approximately 690 Mb in size (Noirot et al. 2003) and has not been sequenced yet. Genome size of *C. arabica* is around 1,200 Mb, Arabidopsis is around 150 Mb and rice around 450 Mb (Bennett and Leitch 2005). The *WRKY* gene analysis was performed on a ESTs dataset which may lead to greatly underestimate the number of coding genes. In Arabidopsis and rice, only 62% and 53%, respectively, of the genes were covered by ESTs data (Zhang and Wang 2005; Ross et al. 2007). Secondly, some *WRKY* genes are unique to some species, which had been confirmed in Arabidopsis and rice. And even within *O. sativa* species, a divergence in the number of *WRKY* genes was found, with 98 genes identified in the

subspecies japonica and 102 in the indica rice (Ross et al. 2007).

In addition, it may be difficult to conclude about the exact *WRKY* genes number in coffee by exploring the *C. arabica* transcriptome. *C. arabica* is an allotetraploid resulting from a recent hybridization between two wild diploids *Coffea* species (Carvalho 1952; Hamon et al. 2009). The *CaWRKY13* and *CaWRKY14* unigenes and the *CaWRKY19* and *CcWRKY20* unigenes, respectively differed by only one amino acid replacement in the WRKY domain. It may be possible that they are gene homeologs in the *C. arabica* genome or allelic sequences from *C. arabica* and *C. canephora*. However, *CaWRKY1* homeologous and allelic sequences in several *Coffea* species displayed strictly identical WRKY domain sequences (Petitot et al. 2008). In Arabidopsis, some closely related WRKY proteins like AtWRKY6 and AtWRKY31 differ by only one amino acid change in the WRKY domain (Eulgem et al. 2000). Some rice *WRKY* genes encoded identical WRKY domains although the nucleotide sequences were not identical, and they were located in different chromosomes (Zhang and Wang 2005). And finally, the rice OsWRKY45 alleles differ by one amino acid change in the WRKY domain (Tao et al. 2009). Thus, the total number of coffee *WRKY* genes will remain unknown until the sequencing of the coffee genome is completed.

A high conservation of peptidic motifs was observed between coffee and Arabidopsis WRKY proteins (Fig. 1suppl.). We did not find any sequence variant as previously described in rice (Zhang and Wang 2005). As observed in Arabidopsis (Eulgem et al. 2000), there are several groups of WRKY proteins in *C. arabica* (Table 1). The classification of the WRKY family in Arabidopsis is not completely based on phylogenetic analysis. Group 2 *Arabidopsis* WRKY domains are subdivided into five subgroups that form three distinct clades (Zhang and Wang 2005). Representatives of each IIa, b, c, d, and e subgroups were found in coffee (Fig. 1 and Table 1).

Several protein motifs are conserved among WRKY proteins from angiosperm (both mono and dicotyledonous) plants suggesting a conservation of protein function between plant genera. For each group and subgroup, the specific peptidic motifs found in Arabidopsis proteins, outside of the WRKY domain (Eulgem et al. 2000), were also found in coffee (Fig. 2). For instance, basic stretches related to NLS were identified in all full-length cDNA sequences examined, and the distribution of NLS1, 2, and 3 motifs in *A. thaliana* WRKY groups and subgroups was also retained in CaWRKY proteins (Fig. 2). The HARF motif, which function is still unknown, is found in group IId WRKY proteins of all plants species examined, including Arabidopsis (Eulgem et al. 2000), rice (Xie et al. 2005), barley (Mangelsen et al. 2008), and coffee (this study). In contrast, the CaMBD VSSFK (K/R) VISLL (Park et al. 2005) found in groups IId, IIe, and III AtWRKY proteins was only found in the group IId of coffee WRKY proteins.

Analysis of expression patterns may help to elucidate the function of regulated genes. Expression of a large number of evaluated coffee *WRKY* genes was affected by pathogen and hormonal treatments, which is in accordance with a regulatory role of WRKY proteins in biotic stress responses (Eulgem et al. 2000). In particular, putative pathogen response regulators could be identified from a set of coffee *WRKY* genes that responded to rust, nematodes, MeJA, and/or SA treatments but not to wounding, senescence, or fruit maturation (Table 3). Plant hormones play important roles in regulating signaling networks involved in plant responses to biotic stresses. Significant progress has been made in identifying key components and understanding the role of SA, JA, and ET in plant defense signaling pathways (Glazebrook 2005; Bari and Jones 2009). In this study, pathogens and hormonal treatments allowed identification of a set of *WRKY* genes (*CaWRKY6*, *CaWRKY11*, *CaWRKY12*, and *CaWRKY13/14*) which may be specific regulators of coffee responses to pathogens.

Interestingly, coffee gene *CaWRKY6* belongs to WRKY group IId, and *CaWRKY11*, *CaWRKY12*, and *CaWRKY13/14* belong to WRKY III. In *A. thaliana*, members of WRKY groups IId and III are involved in regulating resistance pathways to bacterial and fungal pathogens. For instance, expression of four members of the IId subfamily (*AtWRKY7*, *11*, *15*, and *17*) was induced upon challenge with virulent and avirulent strains of *Pseudomonas syringae* pv *tomato* (*Pst*) (Journot-Catalino et al. 2006). Mutant analysis showed that *AtWRKY11* and *17* are negative regulators of *A. thaliana* basal resistance to *Pst* and that complex cross-regulation occurred within the AtWRKY group IId to fine-tune jasmonic acid-dependent plant defense responses (Journot-Catalino et al. 2006). Coffee gene *CaWRKY6* displayed best amino acid sequence homology with *AtWRKY7*. Comparison of gene expression patterns in Arabidopsis and coffee may help assigning potential orthologs to coffee WRKY members. After inoculation with virulent or avirulent *Pst*, *AtWRKY11*, *AtWRKY7*, and *AtWRKY17* showed similar expression profiles, with a rapid and transient induction that peaks 2 h after inoculation. In contrast, *AtWRKY15* induction was sustained between 2 and 12 h after inoculation in the incompatible interaction with *Pst*. In addition, none of the AtWRKY group IId members responded to wounding or stress treatment (Journot-Catalino et al. 2006), and only *AtWRKY15* is induced by MeJA (transcriptome database at Genevestigator [Hruz et al. 2008]). All these data thus suggest that *CAWRKY6* is ortholog with *AtWRKY15* and not *AtWRKY7*. WRKY group IId transcription factors interact with calmodulin (Park et al. 2005), a well-characterized primary Ca(2+) sensor in eukaryotes. Pathogen infection

generates alterations of calcium ions flux across the cell membrane and Ca(2+) is known to act as a second messenger of plant defenses (Ma and Berkowitz 2007). The way CaM affects WRKY group IId function(s) is not known, but alteration of the DNA-binding ability of members of the bHLH transcription factor family that bind to CaM has been demonstrated, suggesting direct downstream target gene transcription modifications (Corneliussen et al. 1999). Coffee CAWRKY6 amino acid sequence exhibit conserved CaM-binding sequences described in AtWRKY group IId members, suggesting that it may also be regulated by CaM when Ca(2+) modifications occurs in plant cells.

Concerning AtWRKY group III members, the majority are responsive both to pathogen infection and to salicylic acid (Kalde et al. 2003). Coffee genes *CAWRKY11*, *CAWRKY12*, *CaWRKY13*, and *CaWRKY14* displayed best amino acid sequence homology with *AtWRKY30*, *41*, and *53* (*CaWRKY11* and *CaWRKY12*), and *AtWRKY54* and *70* (*CaWRKY13* and *CaWRKY14*; Fig. 1suppl.). AtWRKY53, AtWRKY54, and AtWRKY70 are direct targets of Non-expresser of pathogenesis-related genes 1 (Wang et al. 2006), a transcriptional cofactor required for several different types of plant immune responses (Pieterse and Van Loon 2004). AtWRKY70 was identified as a node of convergence for integrating SA- and JA-mediated signaling events during plant response to pathogens (Li et al. 2004, 2006). In addition, mutant analysis showed that AtWRKY70 and AtWRKY54 have overlapping roles in counteracting accumulation of SA (Wang et al. 2006). Because *AtWRKY70* expression is MeJA-repressed (Li et al. 2004), it is likely that *CaWRKY13* and *CaWRKY14* are not the coffee orthologs of *AtWRKY70* but rather of *AtWRKY54*. A role in flagellin-mediated signaling pathway was demonstrated for *AtWRKY41* (Higashi et al. 2008) and in basal resistance against *P. syringae* for *AtWRKY53* (Murray et al. 2007). In addition, functional analysis of *AtWRKY53* showed its involvement in leaf senescence (Miao et al. 2004; Miao and Zentgraf 2007). *AtWRKY41* and *53* genes are induced by SA, but this is not the case for *AtWRKY30* (Kalde et al. 2003). Similarly, coffee gene *CaWRKY11* was not activated by SA treatments and may be, therefore, an ortholog of *AtWRKY30*. Also, a 4-h MeJA treatment reduced expression of *AtWRKY53* (Miao and Zentgraf 2007), and a 3-h MeJA treatment did not affect expression of *AtWRKY41* (Higashi et al. 2008). In contrast, expression of the coffee gene *CaWRKY12* was activated by both SA and 24-h MeJA treatment. However, a 3-h MeJA treatment did not induce the *CaWRKY12* gene (data not shown), suggesting that *AtWRKY41* may be ortholog with *CaWRKY12*.

Activation of defense responses regulated by the SA-dependent signaling pathway is generally associated with resistance to biotrophic pathogens that feed and reproduce on live host cells, whereas JA activates defense against necrotrophic pathogens that kill host cells for nutrition and reproduction (Glazebrook 2005). Rust pathogens and root-knot nematodes are biotrophs that require keeping their plant host alive to perform their life cycle. In this study, although a significant correlation of *WRKY* genes expression after MeJA and rust treatments was observed, expression of coffee genes involved in JA biosynthesis and lipoxygenase (EC 1.13.11.12) activity assays did not support the involvement of JA in the early coffee resistance responses to the rust pathogen (Fig. 5a and b). In contrast, activation of several genes involved in the SA-dependent resistance signaling pathway, including *PR1b*, suggested that SA may play a role in the coffee resistance responses to *H. vastatrix* (Ramiro et al. 2009).

Another interesting point derived from gene expression studies was that coffee *WRKY* genes with elevated basal expression levels (*CaWRKY4*, *CcWRKY5*, *CaWRKY18*) did not react to any treatment at the transcriptional level (Fig. 3). Searches in the GeneInvestigator database indeed showed that expression of their close orthologs *AtWRKY 3, 4, 21*, *39*, and *74* was not altered by several biotic and abiotic treatments, except for *AtWRKY 74* in drought-stress assays. These data suggest that these genes may be involved in general transcriptional processes. However, a recent study indicated that *AtWRKY3* and *AtWRKY4* can have a positive role in plant resistance to necrotrophic pathogens and *AtWRKY4* has a negative effect on plant resistance to biotrophic pathogens (Lai et al. 2008). To our knowledge, no data are available about the function of *AtWRKY 21*, *39*, or *74*.

In conclusion, the *WRKY* gene family analysis in coffee plant presented here identified at least 22 members in the genome. Expression analysis allowed selection of five members that may play central roles in the transcriptional network activated in immune responses of *C. arabica*. This is the first report demonstrating involvement of WRKY transcription factors in biotic stress in coffee plants. These data may be useful for improving coffee varieties for achieving enhanced and durable resistance to pathogens.

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