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Sub-genomic origin and regulation patterns of a duplicated *WRKY* gene in the allotetraploid species *Coffea arabica*

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Abstract

The extensively cultivated coffee species *Coffea arabica* is an allotetraploid resulting from a recent hybridization between two wild diploid *Coffea* species. We describe here the first identification and functional assessment of homoeologous gene copies in *C. arabica*. When cloning the *CaWRKY1* gene encoding a transcription factor of the WRKY superfamily associated with plant defense responses to pathogens (Ganesh *et al.*, 2006), two distinct gene copies (*CaWRKY1a* and *CaWRKY1b*) were obtained from *C. arabica*. Southern blots experiments and phylogenetic analysis of the *WRKY1* gene in related diploid *Coffea* species showed that *CaWRKY1a* and *CaWRKY1b* are homoeologous sequences in the allopolyploid coffee genome, and are probably close descents of the extant *C. canephora* and *C. eugenioides WRKY1* genes. To verify if *CaWRKY1a* and *CaWRKY1b* were both functional, gene expressions were monitored in *C. arabica* plants challenged by the rust fungus *Hemileia vastatrix*, the root-knot nematode *Meloidogyne exigua* and after several abiotic treatments. Real-time quantitative PCR assays showed that *CaWRKY1* homoeologs were concomitantly expressed and displayed the same altered patterns of expression in leaves and roots during biotic and abiotic treatments. These results suggest that *CaWRKY1a* and *CaWRKY1b* were functionally retained in the coffee genome after allopolyploidization and that no functional divergence occurred between the duplicated genes in the *C. arabica* genome. This study provides the first molecular data on subgenome-specific expression in allopolyploid coffee. The origin of the *C. arabica* subgenomes is discussed with regards on the probable progenitors of this important crop species.

Key Words : allopolyploidy ; *Coffea* ; disease resistance ; gene regulation ; coffee phylogenetics ; WRKY transcription factor ;

Introduction

Coffee belongs to the large botanical family *Rubiaceae*, which includes tropical trees and shrubs growing in the lower storey of forests. *Coffea* is by far the most important member of the family economically, and *C. arabica* (Arabica coffee) accounts for over 70% of world coffee production. *C. arabica* is a tetraploid ($2n = 4x = 44$) and may have resulted from a natural hybridization between two wild diploids *Coffea* species (Carvalho, 1952). Polyploids are common in certain plant and animal taxa, and the genetic and evolutionary consequences of genome duplication have been recently reviewed (Comai, 2005). In particular for allotetrapolyploids, it is expected that most genes are present in two homoeologous forms, highly similar but non-identical. The gene redundancy may lead to gene silencing or to the functional divergence of duplicated genes (Adams and Wendel, 2005 ; Chen and Ni, 2006). Recent studies using microarray and quantitative gene expression analyses identified progenitor-dependent (or genome-specific) gene regulation in allotetraploid cotton (Udall *et al.* 2006 ; Yang at al., 2006), and in synthetic *Arabidopsis* allotetraploids (Wang *et al.*, 2006). In allotetraploid cotton, up to 43% of homoeologous genes appear to be differentially transcribed in leaves (Adams *et al.*, 2003 ; Udall *et al.* 2006). Significant genome-specific regulation was evidenced in the cotton allotetraploids containing AADD genomes, where ESTs from the AA-subgenome were predominantly accumulated in leaves (Udall *et al.* 2006) and in ovules during fiber cell development (Yang *et al.*, 2006). In the hexaploid wheat, SSCP analysis of expressed genes in leaves or roots revealed that a significant proportion of homoeologs (27 % and 26 %, respectively) were not expressed (Bottley *et al.* 2006). Therefore, genome-specific gene regulation may be a general consequence of polyploidization in many allopolyploid plants.

In *C. arabica*, no data at all is available about gene redundancy and about the relative contribution of duplicated gene pairs in the transcriptome. As of July 2007, the coffee Expressed Sequence Tags (ESTs) collection in the public database (http://www.ncbi.nlm.nih.gov) contained only 1000 *C. arabica* ESTs, mostly obtained from cDNA libraries of rust-challenged *C. arabica* leaves (Fernandez *et al.*, 2004). Coffee leaf rust is caused by the fungus *Hemileia vastatrix* (Berkeley & Broome) and is one of the most destructive diseases of *C. arabica* (Bettencourt and Rodrigues, 1988). Natural 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 the leaf infection sites (Rodrigues *et al.*, 1975 ; Silva *et al.*, 2002). Among the ESTs isolated, several genes showed up-regulation of transcript accumulation in coffee leaves after *H. vastatrix* challenge (Fernandez *et al.*, 2004; Ganesh *et al.*, 2006). One of them, DSS16 (renamed *CaWRKY1*), displayed homology to WRKY transcription factors genes. The WRKY transcription factors belong to a major group of DNA - binding proteins in plants, and function as transcriptional activators as well as repressors in a number of developmental and physiological processes (Eulgem *et al.*, 2000 ; Robatzek and Somssich, 2002 ; Zhang *et al.*, 2005). In particular, WRKY transcription factors have been associated with plant defence responses to biotic and abiotic stresses (reviewed *in* Ülker and Somssich, 2004; Eulgem, 2006). Recent studies have shown involvement of specific WRKY proteins in plant defence responses and SAR. For instance, constitutive expression of Arabidopsis *WRKY18* or *WRKY70* in transgenic plants leads to enhanced disease resistance to virulent pathogens (Chen and Chen, 2002 ; Li *et al.*, 2004 ; Wang *et al.*, 2006) and knock-out mutants of *AtWRKY70* or *AtWRKY33* showed increased susceptibility to

necrotrophic fungal pathogens (AbuQamar *et al.*, 2006 ; Zheng *et al.*, 2006). Identification of WRKY genes that play important roles in plant defense responses in several plants suggests that the regulation specificities of some WRKY proteins might be conserved (Liu *et al.*, 2004; Liu *et al.*, 2005).

In *C. arabica*, the *CaWRKY1* gene was differentially activated in rust-challenged leaves of resistant *versus* susceptible coffee plants, with a higher and earlier activation in resistant plants (Ganesh *et al.*, 2006). Activation patterns of *CaWRKY1* are coincident with fungal entry into the plant stomata and precede hypersensitive cell death. CaWRKY1 is closely related to members of group IIb of *Arabidopsis* WRKY proteins, including AtWRKY6. Since AtWRKY6 was identified as an important downstream component of signalling pathways involved in resistance to pathogens and in senescence (Robatzek and Somssich, 2001, 2002), we wanted to investigate whether *CaWRKY1* also plays important roles in coffee defense responses.

As a first step towards the functional analysis of the CaWRKY1 protein, the objective of this study was to explore the genomic status of the *CaWRKY1* gene in *C. arabica*. First, we predicted that two homoeologous *CaWRKY1* loci should exist in the tetraploid coffee genome. To test this hypothesis, full-length cDNA and DNA sequences of the *CaWRKY1* gene were cloned in *C. arabica* and compared with orthologous *WRKY1* DNA sequences we obtained from a set of related diploid *Coffea* species. Second, to assess the genome-specific regulation of the homoeologous *CaWRKY1* genes in *C. arabica*, we tested their expression patterns and quantified mRNAs accumulation using real-time quantitative reverse-transcription Polymerase Chain Reaction (qRT-PCR)

Materials and methods

Plant material - biotic and abiotic treatments :

Coffee plants were grown in potting soil in a greenhouse (24°C day, 22°C night, 65% RH). The following species were used in this study : *C. arabica* var. Caturra, *C. arabica* var. IAPAR59, *C. canephora* T3561, *C. eugenioides* DA60, *C. congensis* CC54, *C. liberica* EB58, *C. racemosa* IA57 and *C. humilis* G59.

Biotic treatments were performed on 6-months old plants. For rust assays, *C. arabica* var. Caturra leaves were challenged with *H. vastatrix* isolates eliciting an incompatible interaction (race VI) or a compatible interaction (race II) as described in Fernandez *et al.* (2004). Plants only sprayed with water were used as control. Biological samples originated from at least three independant experiments conducted in the greenhouse at different periods of the year. Root-knot nematode infection was performed by inoculating *Meloidogyne exigua* juveniles (J2) on the resistant *C. arabica* var. IAPAR59 and the susceptible var. Caturra as described in Lecouls *et al.* (2006). Roots tips (5-mm long) were collected 2, 3 and 5 days after inoculation.

Abiotic treatments were performed on coffee leaves of the same physiological state than leaves used for rust inoculation. Wounding was performed by applying an average of 7 transversal cuttings per half-leaf using cissors. Wounded leaves were collected 15, 30 and 60 min later. Non-wounded leaves collected on different plants were used as controls. SA - treatments were performed by infiltrating leaves with a 0.5 mM solution of SA using a needless syringe. 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 (mock-control) as well as non-infiltrated leaves were used as

controls. Leaves were collected 1h, 3h and 7h after treatment. Senescent leaves showing visible signs of yellowing were harvested from the lower part of Caturra plants. Two leaves per plant and 4 plants per experiment were used and experiments were repeated twice.

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

Genomic DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blotting :

Fresh coffee leaves 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. For each plant sample, 10 µg of genomic DNA was digested with 50-60 units of the restriction enzymes *Eco*RI or *Pst*I (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 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, U.S.A.).

Labelling of probes and hybridization conditions :

Specific oligonucleotides were designed from the DSS16 sequence and used to amplify plasmid cDNA insert by PCR. After purification using QIAquick PCR purification kit (Qiagen, France), the DNA probe (50 ng) was labelled with ^{32}P-dCTP by random-priming, hybridized to membrane-bound DNA fragments (at 65°C overnight) and detected by autoradiography according to the manufacturer's specifications (Megaprime kit and hybridization buffer, Amersham, France).

Full length cDNA cloning by RACE experiments :

Specific oligonucleotides (DSS16-5R and DSS16-3R) were designed from the DSS16 sequence (Genbank accession CF589188) for RACE experiments (Table 1 and Figure 1). 3'RACE and 5'RACE experiments were conducted by combining Omniscript RT kit (Qiagen) and SMART PCR cDNA synthesis kit (Clontech). The resulting PCR product was cloned into pGEM-T easy vector (Promega, France) and sequenced (Genome Express, France). Full length cDNA (FL cDNA) clones were obtained by PCR amplification using two oligonucleotides (CATW5 and CATW3) designed from 5'- and 3'RACE sequences. The resulting 2-kb PCR product was cloned and sequenced.

Cloning of genomic CaWRKY1 sequences :

CaWRKY1 sequences were obtained by PCR experiments on *C. arabica* var. Caturra DNA using CATW5 and CATW3 primers. The PCR products (around 2.5 kb) were cloned and sequenced.

Quantitative gene expression analysis :

RNA extraction and qRT-PCR were performed as described in Ganesh *et al.* (2006). Specific oligonucleotides were designed in the 3' - part of the *CaWRKY1* cDNA sequences that discriminated *CaWRKY1a* and *CaWRKY1b* transcripts (Table 2). The same TaqMan probe (WRKY1-S) was used for detecting both amplicons. Primers were carefully checked for absence of cross-amplification on *CaWRKY1a* and *CaWRKY1b* plasmid clones at various concentrations. 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 (Livak and Schmittgen, 2001). Relative quantification related the PCR signal of the target transcript

in each sample to that of the control sample at each time. For absolute quantification of cDNA molecules, the threshold cycle (Ct) values of the triplicate PCRs were averaged and the copy number of each cDNA was estimated from calibration curves data obtained on calibrated amounts (10^2, 10^3, 10^4 and 10^5 copies) of purified plasmids bearing the cloned gene tested. The *CaWRKY1a* and *CaWRKY1b* gene copy numbers were normalized to the *CaUbiquitin* gene chosen as internal reference of gene expression. Linear regression analysis was used to calculate the correlation coefficient between the *CaWRKY1a* and *1b* gene copy number obtained in each experiment, and over all experiments.

Cloning of WRKY1 sequences in related Coffea spp. :

Fresh leaves of *C. arabica* var. Caturra, *C. canephora*, *C. eugenioides*, *C. congensis*, *C. liberica*, *C. racemosa* and *C. humilis* were used for genomic DNA extraction (DNEasy Plant minikit, Qiagen, France). Partial genomic *WRKY1* sequences were obtained by PCR experiments on DNA of each *Coffea* spp. using CATW5 and DSS16-5R primers (Fig. 1). The resulting 2-kb PCR products were cloned and sequenced.

Bioinformatic analysis of CaWRKY1 sequences :

Homology to sequences present in international databases were searched using Basic local alignment search tools (BLASTN and BLASTX) (Altschul *et al.*, 1997). Sequences were aligned using the MegAlign tool contained in the Lasergene software (DNASTAR, Inc., Madison, WI, USA) using ClustalW algorithm (slow-accurate option). Search for specific protein domains were performed on Pfam database website (http://www.sanger.ac.uk/Software/Pfam).

Phylogenetic analysis of CaWRKY1 sequences :

Parsimony analyses with unweighted, unordered characters were conducted with PAUP 4.0b10 (Swofford, 2002) in Macintosh environment. Bootstrap analysis was performed using 1000 replicates and unrooted consensus trees were constructed.

Results

Full –length cDNA isolation of two CaWRKY1 genes :

CaWRKY1 was originally isolated as a differentially expressed sequence fragment (DSS16) during infection by the rust fungus *H. vastatrix* (Fernandez *et al.*, 2004). Specific oligonucleotides designed from the DSS16 sequence were used for 3' and 5' RACE cloning experiments. Full length – cDNA clones were further obtained and two distinct FL - cDNA sequences (named *CaWRKY1a* and *CaWRKY1b*) were identified (GenBank accessions DQ335599 and DQ335598, respectively). Clone *CaWRKY1a* (1974 bp) and clone *CaWRKY1b* (1960 bp) contained an open reading frame (ORF) encoding a predicted polypeptide of 573 aminoacids and 572 aa, respectively (Fig. 2). The two sequences shared 97.5 % nt identities and 98.3 % aa identities. *CaWRKY1a* and *CaWRKY1b* nucleotide sequences varied by 12 base pairs insertions / deletions (Indels) and 7 single nucleotide polymorphisms (SNPs) in their 5' - untranslated region (5'UTR) (128 bp and 116 bp, respectively). ORFs differed by 1 Indel and 18 SNPs leading to a total of 10 aa changes in the predicted polypeptide sequences (Fig. 2). The indel that we observed in the ORF sequence of *CaWRKY1a* and *CaWRKY1b* resulted from a microsatellite (3 bp repeated - GCA) repeat polymorphism (4 and 3 repeats, respectively). Primers designed from this *CaWRKY1* microsatellite (or simple sequence repeats (SSRs)) amplified homologous loci in six *Coffea* species and was found polymorphic among *C. canephora* accessions (Poncet, V., pers. com.).

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Homology searches with the amino acid sequences of the FL – cDNA clones revealed a highly significant similarity (BlastP E value = 3e-113) to proteins belonging to the *A. thaliana* WRKY transcription factors subgroup IIb (Eulgem *et al.*, 2000). Both CaWRKY1a and CaWRKY1b deduced proteins matched the same *A. thaliana* WRKY factor, AtWRKY6 (At1g62300). The CaWRKY1a and CaWRKY1b aa sequences were characterised by the presence of a single WRKY domain (from position aa 315 to position aa 367) containing the core motif WRKYGQK together with a C2H2 – type zinc finger motif in the C – terminal region (Fig. 2). In addition, a potential leucine – zipper –motif (LZ) (from position aa 139 to position aa 181) and a nuclear localization signal (NLS) were identified (Eulgem *et al.*, 2000). Search for other peptide motifs, such as the coactivator or repressor motifs described in other WRKY proteins (Eulgem *et al.*, 2000 ; Xie *et al.*, 2005) did not yield significant results.

Cloning of CaWRKY1a and 1b genes :

Two distinct *CaWRKY1* genomic sequences sharing 96.7 % of nucleotide identity were obtained from *C. arabica* DNA. The two genes exhibited a similar structure and consisted of six exons and five introns (Fig. 1). Nucleotide sequences differed in numerous SNPs as well as in several Indels mainly in the first and last intron. As for all *A. thaliana* WRKY genes (Eulgem *et al.*, 2000), the WRKY domain - encoding region in *CaWRKY1* was interrupted by an intron. Interestingly, the five intron positions were conserved in *CaWRKY1* genes and some *A. thaliana* WRKY genes of subgroup IIb (namely At1g62300, At4g04450 and At4g22070).

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Phylogenetic analysis of the coffee WRKY1 gene in the Coffea genus :

To assess whether *CaWRKY1a* and *CaWRKY1b* were homoeologous sequences or paralogous sequences, we conducted a genetic diversity analysis of the coffee *WRKY1*

gene in the *Coffea* genus. Six *Coffea* species (*C. canephora*, *C. eugenioides*, *C. congensis*, *C. liberica*, *C. racemosa* and *C. humilis*) closely related to *C. arabica* were chosen based on previous phylogenetic analyses conducted with several DNA markers (Lashermes *et al.*, 1997, 1999 ; Cros *et al.*, 1998). PCR experiments using CATW5 and DSS16-5R primers enabled amplification of a 2-kb fragment overlapping the 5'- non transcribed region, most of the coding sequences and the 5 intronic sequences (Fig. 1). Depending on the coffee species, one or 2 homologous sequences of the *CaWRKY1* gene were obtained from *C. canephora* (2), *C. eugenioides* (2), *C. congensis* (1), *C. liberica* (1), *C. racemosa* (2) and *C. humilis* (1) genomic DNAs (GenBank accessions DQ335600 to DQ335619). Alignment of the sequences (1540-bp total length) showed that *WRKY1* sequences essentially differed by numerous SNPs as well as distinct Indel events in the 5'UTR and the first two intronic sequences (Fig. 1, supplementary material). *WRKY1* nucleotide sequences similarity ranged from 94.4 to 99.3 % between *Coffea* species (data not shown). The highest similarities (99.9 %) were observed between *CaWRKY1a* and *canephora1 WRKY1* sequences and between *CaWRKY1b* and *eugenioides1-1 WRKY1* sequences. Alignment of the deduced aminoacid sequences (324 aa in total) showed a strict conservation of CaWRKY1a and CaWRKY1b with *congensis*, and *eugenioides* WRKY1, respectively. CaWRKY1a displayed only one aminoacid difference with *canephora* WRKY1 (data not shown).

Because Indels (Table 3) may severely bias phylogenetic inferences derived from molecular sequences (Felsenstein, 1981), the DNA sequence (including introns) of each clone was cleared of Indels for phylogenetic analyses. Parsimony analysis conducted on the resulting DNA sequences (1475 characters, 45 parsimony - uninformative, 48 parsimony - informative) produced four parsimonious trees requiring 106 evolutionary

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steps with a consistency index of 0.95, indicating a low level of homoplasy. The phylogenetic tree obtained clearly separated the two *C. arabica WRKY* sequences into distinct phylogenetic clades (Fig. 3). *CaWRKY1a* grouped with the *canephora* and *congensis* sequences while *CaWRKY1b* was closely related to the *eugenioides* sequences. The highest bootstrap value (100 %) was assigned to the major branches. In addition, all but one Indels (Indel no. 14) manually assigned on the cladogram clearly supported the tree topology (Fig. 3). The close relationships observed between the *C. arabica*, *C. canephora* and *C. eugenioides WRKY1* sequences strongly suggest that *CaWRKY1a* and *CaWRKY1b* are homoeologous sequences resulting from the allopolyploidization event that gave rise to the *C. arabica* species.

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Genomic organization of the CaWRKY1 genes in the Coffea sp. genome :

To verify that the *CaWRKY1* gene corresponded to a single copy gene, we conducted Southern-blot experiments on coffee genomic DNAs. Coffee DNA samples were digested with the restriction enzyme *Eco*RI, for which no site was detected in the *CaWRKY1* sequence, and with the *Pst*I enzyme (1 site in the DSS16 sequence ; 3 sites in the *CaWRKY1* sequence, Fig. 1). Depending on the coffee species tested, one or two *Eco*RI–digested DNA fragments strongly hybridized to the *CaWRKY1* probe (Fig. 4). *CaWRKY1* probe detected two fragments in *Eco*RI-digested DNAs of *C. liberica* and *C. racemosa* and one sharp band (ca. 4-kb) in other *Coffea* species. An additional weakly-hybridizing 1300-bp band was detected in all coffee DNAs tested. The DSS16 probe contains a small part of the 3'end of the conserved WRKY DNA binding domain (11-bp), and might therefore weakly hybridize to another coffee *WRKY* gene. In *Pst*I-digested DNAs, the radiolabeled EST probe hybridized to two fragments of the expected sizes (1900- and 300-bp, respectively) for all species (Fig. 3suppl.). Although

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the possibility of duplicated genes cannot be ruled out, our data suggest that the *WRKY1* gene may be present as one copy (per haploid genome) in the genome of coffee species. In addition, *Eco*RI-restriction fragment length polymorphisms (RFLPs) were detected between the 7 coffee species tested (Fig. 4). *C. liberica* and *C. racemosa* displayed higher-sized hybridizing fragments than *C. arabica*, *C. canephora*, *C. eugenioides*, *C. congensis* and *C. humilis*, suggesting allelic variation around the *WRKY1* locus.

CaWRKY1a and CaWRKY1b expression patterns :

Quantitative real-time PCR was used to investigate the respective contribution of *CaWRKY1a* and *1b* genes to the *C. arabica* transcriptome, and more specifically during plant defence responses. Transcript accumulation of each gene was monitored during plant infection with the coffee rust pathogen, the root-knot nematode *M. exigua*, and upon several abiotic treatments. Mock-inoculated plants or non-treated plants were used as controls to assess the relative expression level of each gene under each treatment. The *Ubiquitin* gene chosen as internal reference of gene expression was assayed in parallel with the candidate genes. Absolute quantification of the *CaWRKY1a* and *CaWRKY1b* mRNA levels in coffee leaves or roots allowed to assess the transcript copy number of each gene in each sample.

Results showed that both *CaWRKY1a* and *CaWRKY1b* genes were induced by rust infection, root-knot nematode infection, in senescent leaves, by wounding or SA – treatments (Fig. 5). For all treatments, similar amounts of *CaWRKY1a* and *CaWRKY1b* transcripts were detected in each sample (Table 4). A statistically significant correlation (Pearson $R=0.92$, $p<0.001$) between the expression of the two genes was obtained across 44 data points measured (including rust, SA and wounding responses),

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suggesting that *CaWRKY1a* and *CaWRKY1b* genes are coregulated (Fig. 2, supplementary material).

In rust assays, time-course experiments were conducted using the Caturra variety challenged with *H. vastatrix* isolates either eliciting an incompatible interaction (resistance) or a compatible interaction (susceptibility). The relative changes in gene expression showed that *CaWRKY1a* and *CaWRKY1b* genes were induced between 12 and 16 hours post-inoculation, depending on time-course experiments. Statistically significant differences ($P < 0,05$) in the relative expression of the *CaWRKY1* genes were found between the compatible and incompatible interactions.

CaWRKY1a and *CaWRKY1b* genes were also differentially activated by *M. exigua* infection in the resistant and susceptible coffee varieties. At 3 days after inoculation, *CaWRKY1* genes were activated (mean 1.7-fold) in the resistant coffee plants and down-regulated (mean 0.8- fold) in the susceptible plants.

For abiotic treatments, the highest activation of *CaWRKY1* genes was found in senescing leaves and in leaves wounded for 30 min (Fig. 5). After 1h-wounding, *CaWRKY1* activation peak reached up to 100-fold (data not shown).

To verify whether regulation of *CaWRKY1* was conserved in the closest *C. arabica* diploid relatives, wounding experiments were performed on *C. canephora* and *C. eugenioides*. *C. canephora* and *C. eugenioides WRKY1* transcripts were detected using *CaWRKY1a* and *CaWRKY1b* primers, respectively. Results presented in Fig. 5 showed that a 30-min wounding treatment activated *C. canephora* and *C. eugenioides WRKY1* gene expression.

Discussion

The aim of this study was to identify homoeologous copies of the *CaWRKY1* gene in the *C. arabica* genome, and to assess their genetic relatedness and functionality. Using primers designed from the DSS16 sequence previously isolated (Fernandez *et al.*, 2004), we conducted a systematic cloning of full-length cDNA or DNA sequences of the *WRKY1* gene in *C. arabica* and six other coffee species. Among the DNA sequences obtained from allotetraploid *C. arabica*, we identified two distinct copies of the *CaWRKY1* gene named *CaWRKY1a* and *CaWRKY1b*. In the diploid coffees, we isolated one or two *WRKY1* allelic sequences, depending on the species. Southern-blot analysis (Fig. 4) and *CaWRKY1*-derived SSR analysis in *Coffea* spp. (Poncet V., pers. com.) showed that the *WRKY1* gene was located at a single locus in the *Coffea* spp. genome. The close phylogenetic relationships observed between *CaWRKY1* and the orthologous *WRKY1* from other diploid *Coffea* species (Fig. 3) strongly suggested that *CaWRKY1a* and *CaWRKY1b* are homoeologous sequences originating from each of the two parental sub-genomes of the tetraploid Arabica coffee species.

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Molecular data obtained so far from the coffee genome suggested that the Arabica coffee species formed recently and that low divergence occurred between the two constitutive genomes of *C. arabica* and those of its progenitor species (Lashermes *et al.*, 1999). Among the extant diploid coffee species ($2n = 2x = 22$), *C. congensis*, *C. eugenioides* and *C. canephora* are thought to be most closely related to the tetraploid species (Raina *et al.*, 1998 ; Lashermes *et al.*, 1999). A higher level of polymorphism was thus expected between the *C. arabica* homoeologous sequences than among them and their ancestor *Coffea* sequences. In this study, we indeed isolated in the *C. arabica* genome two homoeologous copies of the *CaWRKY1* gene that were distantly-related, sharing only 96 % nt identity. The phylogenetic analysis of the *WRKY1* gene in the

Coffea genus showed that the two arabica *WRKY1* homoeologs were genetically closely-related to some diploid coffee *WRKY1* genes (Fig. 4).

The origin of the *C. arabica* genome has been explored using cytological analyses coupled to Genomic In Situ Hybridization (Raina *et al.*, 1998 ; Lashermes *et al.*, 1999). Data showed that *C. arabica* is an amphidiploid formed by hybridisation between two wild diploid species, including *C. eugenioides*, and a member of the canephoroid species, either *C. congensis* (Raina *et al.*, 1998) or *C. canephora* (Lashermes *et al.*, 1999). In our study, we found that *CaWRKY1b* was genetically very close to the *C. eugenioides WRKY1* gene, and a strict conservation of WRKY1 protein sequences was retained. The *CaWRKY1a* homoeolog grouped together with the two closely-related *C. canephora* and *C. congensis WRKY1* sequences. A higher similarity was observed between *CaWRKY1a* and *canephora WRKY1* sequences (99.9 %). In contrast, alignment of the deduced aminoacids sequences showed a strict conservation of CaWRKY1a with the *congensis* WRKY1 sequence and one aminoacid difference with the *C. canephora* WRKY1 sequence (99.7 % identity). Whether *C. canephora* or *C. congensis* is the closest relative of *C. arabica* may still be questionable. As discussed in Lashermes *et al.* (1999), the taxonomic boundaries among the diploid species belonging to the canephoroid group are unclear since highly fertile hybrids can be easily obtained by crossing species (Berthaud and Charrier, 1988). The *Coffea* accessions we used in this study were carefully chosen among the IRD coffee genetic resources available to best fit the current species description. At the present time, wild *C. arabica* populations are only found in some highlands of Ethiopia, Kenya and Sudan, where lies the primary center of genetic diversity of the species. In contrast, diploid species are either restricted to central African areas (*C. congensis*), highlands of Uganda and neighbouring countries

(*C. eugenioides*) or widely distributed in the tropical lowlands forests of West and central Africa (*C. canephora*), but never coexist with wild *C. arabica* (Berthaud and Charrier, 1988). Palaeoecology records indicate that climate changes affected the distribution of plant species in African tropical forests during the late quaternary period (Prentice *et al.*, 2000). As suggested by Lashermes *et al.* (1999), the present distribution of *C. arabica* may thus rather reflect its preservation in a refuge area, than the geographic origin of the speciation. Interspecific hybridization may have taken place in the past in other areas such as Uganda, where sympatric populations of *C. canephora* and *C. eugenioides* are still found (Thomas, 1944 ; D. Byesse pers. com.). The diploid accessions we used in this study originated from Central Africa areas (*C. canephora* and *C. congensis*) and Kenya (*C. eugenioides*). When available, it would be interesting to analyse some wild *canephora* accessions from Uganda in order to compare their genetic relatedness with *C. arabica*.

In *C. arabica*, resistance to leaf rust and nematodes (*M. exigua*) is expressed by an hypersensitive reaction (HR) at the infection sites (Silva *et al.*, 2002 ; Anthony *et al.*, 2005). Plant cell death may occur as soon as 24 h post inoculation (p. i.) and parasite growth usually ceased in the early stages of the infection process (Silva *et al.*, 2002; Anthony *et al.*, 2005). Microarray analysis in model plants such as *A. thaliana* showed that plant disease resistance involves the reprogramming of cellular functions to fight off pathogen attacks. A large number of genes that mediate resistance signalling and defence reactions are subject to transcriptional regulation (Maleck *et al.*, 2000 ; Tao *et al.*, 2003). The activities of the genes may be up- or down-regulated and follow defined temporal programmes. In *C. arabica*, we showed that *CaWRKY1* mRNA levels are selectively increased during resistance to *H. vastatrix* or *M. exigua*, and wounding or

SA treatments. *CaWRKY1a* and *CaWRKY1b* were concomitantly expressed and displayed the same altered patterns of expression in leaves and roots during coffee responses to rust, nematodes, and other abiotic treatments. Both *CaWRKY1* homoeologous genes therefore contributed to the transcriptomic expression of coffee defense responses to pathogens, evidencing that the duplicated genes are functional in the *C. arabica* genome.

In allopolyploid species, gene redundancy may lead to loss of function of excess copies, through wholesale deletion or mutation to pseudogenes, or more subtly, by epigenetic silencing of homoeologs (Adams and Wendel, 2005). Alternatively, gene duplication may be resolved by subfunctionalization within the ancestral gene pair. Altered gene expression, including silencing and up- or downregulation of one of the duplicated genes, may be common in natural allopolyploids, and the pattern of this bias has been shown to be organ-specific in cotton and wheat (Adams *et al.* 2003; Bottley *et al.*, 2006 ; Udall *et al.* 2006). In *C. arabica*, similar amounts of *CaWRKY1a* and *CaWRKY1b* transcript levels were found in *C. arabica* leaves and roots under all treatments, suggesting that the homoeologous genes undergo the same transcriptional control. Considering that *C. arabica* resulted from a recent hybridization (Lashermes et al., 1999), the apparently similar expression pattern of the two genes is perhaps not so surprising. It may be possible that the two inherited *WRKY1* gene copies were functionally conserved after the polyploidization event that gave rise to the *C. arabica* species. However, further experimental validation on their function would be required to verify that both genes are truly functional homologs.

Recent studies provided evidence that WRKY proteins have regulatory functions in plant responses to pathogen infection (Eulgem, 2006). An ortholog of *CaWRKY1* is

AtWRKY6, which is involved both in the activation of plant defense responses and in senescence-related processes in *A. thaliana* (Robatzek and Somssich, 2001). *AtWRKY6* was highly expressed in senescent leaves and after *P. syringae* inoculation or flagellin treatments (Robatzek and Somssich, 2001). In addition, the WRKY6 protein acted as a positive regulator of *PR1* expression (Robatzek and Somssich, 2002). *AtWRKY6* over-expression lines displayed enhanced *NPR1* and *PR1* mRNAs accumulation, and several W boxes were found within the *PR1* promoter (Robatzek and Somssich, 2002). The *PR1* upstream regulator *NPR1* is positively regulated by several WRKY in *A. thaliana* (Yu *et al.*, 2001) and AtWRKY6 may directly, or indirectly, participate to its regulation. In addition to rust- and nematode-activated expression, *CaWRKY1* homoeologs displayed the same patterns of expression than *AtWRKY6*, under SA - treatment, wounding or in senescent leaves (Robatzek and Somssich, 2001), suggesting that the function of this WRKY transcription factor in several physiological traits may be conserved across plant genera.

Future work will aim at understanding the role of the *CaWRKY* gene in the mechanisms of *C. arabica* resistance to pathogens. Additionally, several other *WRKY* genes have been identified in the coffee plant, with potential implication both in pathogen resistance and developmental processes (Fernandez *et al.*, 2006). Coffee is an important cash crop, and thus genetic insights into genes and their regulation specificities have the potential to be directly exploited to assist genetic improvement programs and to better manage coffee genetic resources.

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Legends :

Figure 1. 1a. Primer positions for *CaWRKY1* gene cloning from the DSS16 sequence. 1b. *CaWRKY1a* and *CaWRKY1b* genomic structures. Exons are indicated by boxes and introns are shown as broken lines. Arrows indicate the translational initiation sites and the star indicates the aa insertion/deletion position. Note that the WRKY domain is interrupted by an intron.

Figure 2. Alignment of *CaWRKY1a* and *CaWRKY1b* deduced amino-acid sequences. The open boxes indicate the domains corresponding to WRKY factors from group IIb (NLS: nuclear localisation signal. LZ: leucine Zipper, WRKY domain) Arrowheads represent intron positions. The stars represent the zinc finger motif. Non-conserved aa are shown as grey boxes. Microsatellite position is indicated by black dots.

Figure 3. Unrooted phylogenetic tree (maximum parsimony) of *WRKY1* genes in *Coffea* species. Numbers above branches are bootstrap values (100 replicates). Dark bars (Indels mutations) were manually assigned to each branch but were not taken into account for the phylogenetic analysis.

Figure 4. Genomic DNA of *C. arabica* var. Caturra, *C. liberica*, *C. congensis*, *C. canephora*, *C. eugenioides*, *C. racemosa and C. humilis* digested with *Eco*RI and probed with ^{32}P-labeled DNA fragment of *CaWRKY1*. Arrows on the left indicate the estimated DNA band sizes (in bp).

Figure 5. Relative expression level of *CaWRKY1a* and *CaWRKY1b* coffee genes measured in *C. arabica*, *C. canephora* and *C. eugenioides* after wounding (30 min leaf treatment), and in *C. arabica* after rust inoculation (avirulent *H. vastatrix*, 17 hours post-inoculation), nematode inoculation (avirulent *M. exigua*, 3 days post-inoculation), in senescing leaves and after 3h of salicylic acid (SA) treatment. Amounts of cDNAs were calibrated using the *CaUbiquitin* gene as reference. Gene expression in the treated plants was relative to that of the control plants (settled to 1).

Table 1. List of primers used for *CaWRKY1* gene cloning.

Table 2. List of primers used in real-time quantitative PCR assays.

Table 3. Sequence and location of Indels in the *WRKY1* gene sequences of *Coffea* sp.

Table 4. Example of *CaWRKY1a* and *CaWRKY1b* transcript quantification (copy number.μg^{-1} RNA) in *C. arabica* leaves after rust inoculation, salicylic acid (SA) treatment, and wounding.

Supplementary materials :

Figure 1 : Alignment of the *WRKY1* gene sequences (5' part) in several *Coffea* species.

Supprimé : s

Figure 2 : Pearson correlation between *CaWRKY1a* and *CaWRKY1b* gene expression across 44 data points measured (including rust, SA and wounding responses).

Figure 3. Genomic DNA of *C. arabica* var. Caturra, *C. liberica*, *C. congensis*, *C. canephora*, *C. eugenioides*, *C. racemosa and C. humilis* digested with *Pst*I and probed with ^{32}P-labeled DNA fragment of *CaWRKY1*. Arrows on the right indicate the estimated DNA band sizes (in bp).

Table 1

Primer name	Primer sequence (5'-3')
DSS16-3R	TACTCAAACTCCAAATCCATTACAA
DSS16-5R	ACCTGATGCATTTGTGGGTTAGCCA
CATW5	CCTCTTTAGAATACTGCAGCCTGA
CATW3	TGAACATGTTAACTATGTTCAGCC

Table 2

Gene	primer	sequence	start location	Amplicon size (bp)
CaWRKY1a	WRKY1a-F	TGCAACAAGGACAGCACCAG	1654	40
	WRKY1a-R	CGTGATCGCGGCCGT	1718	
	WRKY1-S	CATCATTCGCTGACACGCTTAGCGC [5']6-FAM [3']TAMRA		
CaWRKY1b	WRKY1b-F	TGCAACAAGGACAGCACCAC	1639	45
	WRKY1b-R	TCAGCTGTGATCGCGGC	1708	

Table 3

INDEL no.	type	sequence	location	*CaWRKY1b*	*eugenioides* 1	*eugenioides* 2	*liberica*	*humilis*	*racemosa* 1	*racemosa* 2	*congensis*	*canephora* 1	*canephora* 2	*CaWRKY1a*
1	insertion	GG	5'UTR	-	-	-	-	-	-	-	+	+	+	+
2	insertion	AAGATC	5'UTR	-	-	-	-	+	-	-	-	-	-	-
3	deletion	TAG	5'UTR	-	-	-	-	-	+	+	-	-	-	-
3'	deletion	T	5'UTR	+	+	+	+	+	-	-	-	-	-	-
4	insertion	GCT	5'UTR	-	-	-	+	-	-	-	-	-	-	-
4'	insertion	ACT	5'UTR	-	-	-	-	+	-	-	-	-	-	-
5	insertion	AACTTGATA	5'UTR	-	-	-	-	-	-	-	-	-	+	+
6	insertion	GTCT	1st intron	-	-	-	-	-	-	-	+	+	+	+
6'	insertion	GTC	1st intron	-	-	-	-	-	+	-	-	-	-	-
7	insertion	A	1st intron	-	-	-	-	-	-	-	+	+	+	+
8	insertion	T	1st intron	-	-	-	-	-	-	-	+	+	+	+
9	deletion	CA	1st intron	-	-	-	-	-	-	-	+	+	+	+
10	deletion	T	1st intron	-	-	-	-	-	-	-	+	+	+	+
11	deletion	GCTTACC	1st intron	-	-	-	-	-	-	-	+	+	+	+
12	deletion	AT	2nd intron	-	-	-	+	-	-	-	-	-	-	-
13	deletion	TTAGTTAA	2nd intron	-	-	-	+	+	-	-	-	-	-	-
14	deletion	GAATTTAGA	5th intron	+	+	+	+	-	-	-	-	-	-	-
15	deletion	TA	5th intron	-	-	-	-	-	+	+	-	-	-	-
16	insertion	CTA	4th intron	-	-	-	-	-	-	+	-	-	-	-

+ : presence of INDEL ; - : absence of INDEL

Table 4

treatment		*CaWRKY1a*	*CaWRKY1b*	*Ubiquitin*
none		5,6E+04	3,3E+04	1,6E+09
rust (12 h)	mock-inoculated	6,6E+04	2,2E+04	1,0E+09
	susceptible	2,6E+05	1,5E+05	1,5E+09
	resistant	1,1E+06	1,3E+06	9,7E+08
SA (3h)	water-infiltrated	1,5E+05	1,3E+05	7,9E+08
	SA-infiltrated	4,4E+05	4,9E+05	9,0E+08
wounding (1 h)	control leaf	7,7E+04	6,9E+04	1,3E+09
	wounded leaf	2,8E+06	4,6E+06	5,5E+08