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Article *in* Genetics and molecular research: GMR · July 2010

DOI: 10.4238/vol9-3gmr825 · Source: PubMed

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EVOLUTION AND TECHNOLOGY

Expression analysis of transcription factors from the interaction between cacao and *Moniliophthora perniciosa* (Tricholomataceae)

M.A. Lopes[1,2], B.T. Hora Junior[1], C.V. Dias[1], G.C. Santos[1], K.P. Gramacho[3], J.C.M. Cascardo[1], A.S. Gesteira[1,4] and F. Micheli[1,5]

[1]Laboratório de Genômica e Expressão Gênica,
Departamento de Genética e Biologia Molecular,
Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil
[2]Departamento de Ciências Biológicas,
Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brasil
[3]Comissão Executiva do Plano da Lavoura Cacaueira, Itabuna, BA, Brasil
[4]Embrapa Mandioca e Fruticultura Tropical, Cruz das Almas, BA, Brasil
[5]CIRAD, UMR DAP, Montpellier Cedex 5, France

Corresponding author: F. Micheli
E-mail: fabienne.micheli@cirad.fr

Genet. Mol. Res. 9 (3): 1279-1297 (2010)
Received March 18, 2010
Accepted May 10, 2010
Published July 6, 2010
DOI 10.4238/vol9-3gmr825

ABSTRACT. Cacao (*Theobroma cacao*) is one of the most important tropical crops; however, production is threatened by numerous pathogens, including the hemibiotrophic fungus *Moniliophthora perniciosa*, which causes witches' broom disease. To understand the mechanisms that lead to the development of this disease in cacao, we focused our attention on cacao transcription factors (TFs), which act as master regulators of cellular processes and are important for the fine-tuning of plant defense responses. We developed a macroarray with 88 TF cDNA from previously obtained cacao-*M. perniciosa* interaction libraries. Seventy-two TFs were found differentially expressed between the susceptible (Catongo) and resistant (TSH1188) genotypes and/or during the disease time course - from 24 h to 30 days after infection. Most of the differentially expressed TFs belonged to the bZIP, MYB

and WRKY families and presented opposite expression patterns in susceptible and resistant cacao-*M. perniciosa* interactions (i.e., up-regulated in Catongo and down-regulated in TSH1188). The results of the macroarray were confirmed for bZIP and WRKY TFs by real-time PCR. These differentially expressed TFs are good candidates for subsequent functional analysis as well as for plant engineering. Some of these TFs could also be localized on the cacao reference map related to witches' broom resistance, facilitating the breeding and selection of resistant cacao trees.

Key words: Macroarrays; RT-qPCR; Gene regulation; *Theobroma cacao*; Witches' broom disease

INTRODUCTION

Plant diseases caused by pathogens are a major threat to agriculture production worldwide. Cacao (*Theobroma cacao* L.) is one of the most important tropical crops but its production is threatened by numerous pathogens such as the hemibiotrophic fungus *Moniliophthora perniciosa*, which is responsible for witches' broom disease (Purdy and Schmidt, 1996). Witches' broom is one of the major cacao diseases in South America and the Caribbean Islands, destroying plantations and leading to important economic and social changes in areas of concern, such as the State of Bahia in Brazil (Andebrhan et al., 1999). Basidiospores infect meristematic tissues (shoots, flower cushions, single flowers, and developing fruits) and induce a range of symptoms depending on the organ infected and the developmental stage. Infection of apical meristems leads to hypertrophy and hyperplasia of the tissues, loss of apical dominance, and proliferation of axillary shoots, which results in the formation of abnormal stems called green brooms. In this biotrophic phase of the disease, the fungus is intercellular, whereas in the necrotrophic phase, the fungus becomes intracellular and causes necrosis and death of infected tissues distal from the original infection site, producing a dry broom (Ceita et al., 2007). Basidiocarp production and spore formation occur on infected necrotic tissue completing the infection cycle (Silva et al., 2002).

In order to understand the mechanisms that lead to the disease development in cacao, some molecular studies have been recently conducted, and the identification of candidate genes involved in cacao resistance to *M. perniciosa* initiated. In 2007, Leal et al. obtained two SSH libraries from shoot tips of susceptible (ICS 39) and resistant (CAB) cacao genotypes inoculated or not with *M. perniciosa*, and identified genes related to plant defense at different stages of the interaction. Gesteira et al. (2007) made two full-length cDNA libraries from susceptible (Catongo) and resistant (TSH1188) cacao genotypes challenged with *M. perniciosa*. A wide range of information generated from these different molecular studies on cacao offers an unprecedented opportunity to identify genes and regulatory networks that control the response of the defense of cacao to *M. perniciosa*, such as transcription factors (TFs) that act as master regulators of cellular processes and play critical roles in the regulation of gene expression during plant defense responses (Journot-Catalino et al., 2006). Members of several families of transcription factors such as bZIP, MYB, MYC, and WRKY have been able to bind to promoter elements of individual genes related to defense and regular expression (Jalali

et al., 2006). The identification and characterization of TFs may highlight the initial steps of transduction pathway leading to cacao resistance (or susceptibility) to *M. perniciosa*.

In this study, we developed a macroarray with 88 cDNAs identified as TFs in the libraries obtained by Gesteira et al. (2007). Seventy-two TFs were found differentially expressed between the susceptible (Catongo) and resistant (TSH1188) genotypes and/or during the disease time course - from 24 h after infection (hai) to 30 days after infection (dai). Most of the TFs differentially expressed belong to bZIP, MYB and WRKY families and presented opposite expression patterns in susceptible and resistant cacao-*M. perniciosa* interactions (i.e., up-regulated in Catongo and down-regulated in TSH1188). The results of the macroarray were confirmed for bZIP and WRKY TFs by real-time polymerase chain reaction (PCR).

MATERIAL AND METHODS

Plant material and fungus strain

Plantlets of *T. cacao* L. varieties Catongo (susceptible to *M. perniciosa*) and TSH1188 (resistant to *M. perniciosa)* were grown in the greenhouse at CEPEC/CEPLAC (Centro de Pesquisas da Comissão Executiva do Plano da Lavoura Cacaueira, Itabuna, BA, Brazil) under natural light and 90% relative humidity. Apical meristems of 4-week-old plantlets were inoculated by the spray method using a 10^5/mL basidiospore suspension from the *M. perniciosa* strain Cp 1441 CEPEC/CEPLAC. After inoculation, plantlets were acclimated for 24 h at 25 $\pm$ 2°C in a water-saturated atmosphere to allow *M. perniciosa* spore germination, penetration and consequently infection (Frias et al., 1995). A test of spore viability was made in a humid chamber (25°C) 24 h after inoculation and was compared with spore viability obtained before inoculation. Control plantlets were inoculated with sterile water and submitted to the same growing conditions as the inoculated ones. Expression of susceptibility was estimated 4 weeks after inoculation by detection of the Catongo plants with disease symptoms. Disease development was monitored on the growing plants for a period of 90 dai. Inoculated and non-inoculated apical meristems from Catongo and TSH1188 were harvested at 24, 48 and 72 hai, and 15 and 30 dai. Infected and uninfected resistant and susceptible apical meristems were harvested, frozen in liquid nitrogen and stored at -80°C.

Macroarray procedure

A total of 88 cDNAs were selected from two libraries providing, respectively, TSH1188 and Catongo challenged with *M. perniciosa* (Gesteira et al., 2007). For putative functional cDNA designation, sequences were compared with a public sequence database (http://www.ncbi.nlm.nih.gov) using BLASTX. The complete 88 inserts were amplified from the plasmid clones by PCR in a final volume of 50 µL containing 100 ng DNA, 0.2 µM of each primer (pDNR-F 5'-ATCAGTCGACGGTACCGGAC-3'; pDNR-R 5'-ACAGCTATGACCATGTTCAC-3'), 1X buffer, 0.4 mM dNTPs, 1 mM $MgCl_2$, and 2 U *Taq* DNA polymerase. A touchdown PCR was made under the following conditions: 5 min at 95°C followed by 10 cycles of 40 s at 94°C, 45 s at 65°C (decreasing of 0.2°C per cycle), 1 min and 20 s at 72°C, followed by 30 cycles of 40 s at 94°C, 45 s at 62°C, 1 min and 20 s at 72°C, and a final extension of 7 min at 72°C done in a Mastercycler Gradient (Eppendorf). Quality of PCR products was checked on 1% agarose gel

stained with ethidium bromide. The PCR products were organized on 96-well plates. To each well, 10 µL 0.8 M NaCl was added and the plates were held at 37°C for 15 min to denature DNA. Plasmid DNAs were printed on Hybond N^+ Nylon membranes (Amersham Pharmacia Biotech) using a 96-well replicator and linked to the membrane at 80°C for 2 h. Four replicates for each clone were spotted on each membrane.

Total RNA isolation

Total RNA was extracted from 24, 48, and 72 hai, and 15 and 30 dai samples (pools of three frozen meristems each) as described by Gesteira et al. (2003) with modifications according to Rodrigues et al. (2007), and then cleaned using the Rneasy Plant Mini kit as described by the manufacturer protocol (Qiagen). The RNA was treated with DNase (Fermentas), and the RNA purity and concentration were determined spectrophotometrically at 260 nm (Cary® 100 UV-Visible Spectrophotometer, Varian, Palo Alto, CA, USA). RNA was separated on 1% DEPC-treated agarose gel and stained with ethidium bromide to confirm RNA integrity. For macroarray probe preparation, total RNA from 24, 48, and 72 hai samples were joined in a unique pool named Pool 72 h).

Macroarray probe preparation

Because of the low amounts of RNA obtained from meristems, it was necessary to linearly amplify the RNA as described by Wang (2005). First-strand cDNAs were reverse transcribed from total RNA (about 200 ng) using 0.1875 µg/µL primer $dT_{(15)}$-T7 (5'-AAACGACG GCCAGTGAATTGTAATACGACTCACTATAGGCGCT$_{(15)}$-3'), 0.1875 µg/µL primer TS (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3') and 200 U RevertAid™ M-MuLV Reverse Transcriptase as described by the manufacturer instruction (Fermentas). To prevent RNA degradation during reverse transcription, 2 U RNase Out (Invitrogen) was used. Second-strand cDNA was obtained using 1X Advantage PCR Buffer, 0.8 M dNTPs, 1 U RNase H and 3 µL Advantage cDNA Polymerase Mix in a final volume of 200 µL, as described by the manufacturer instruction (Clontech). Double-strand cDNA was purified by adding 0.1 µg linear acrylamide (Ambion) and phenol-chloroform-isoamyl alcohol (25:24:1) to the reaction, and then transferred to Phase Lock Gel tubes (Eppendorf). The *in vitro* transcription was made using a RiboMAX Large Scale RNA Production System-T7 as described by the manufacturer instruction (Promega). The complementary RNA (cRNA) was cleaned with TRIzol® reagent (Invitrogen) and chloroform. Final cDNA synthesis from cRNA was obtained by reverse transcription using 2 µg/µL random hexamer primer (dN6) and the RevertAid™ M-MuLV Reverse Transcriptase, as described by the manufacturer instruction (Fermentas). cDNA quantification was realized on a GeneQuant Pro spectrophotometer (Biochrom). Concentrations of the cDNA from different plant samples were comprised between 1.1 and 1.5 µg/µL.

Hybridization conditions, data management and statistical analyses

The cDNA spotted on nylon membranes were hybridized with the cDNA probes labeled according to manufacturer instructions (Alkphos Direct™ Labelling kit, GE Healtcare) and following the procedure described by Li et al. (2006). Hybridization signals were detected

using CDP-star chemiluminescence (Amersham Pharmacia Biotech). The arrays were exposed to ECL Hyperfilm™ (Amersham Biosciences) for 2 h. The target signal intensities were scanned on a Kodak EDAS 290 imaging system. Quantification of signal intensity representing hybridized cDNA was performed using the BZScan program (Lopez et al., 2004). Each spot was first defined by automatic grid positioning over the array image, and then a manual checking and correction of positioning for each spot were performed. Intensity values were calculated by the Quant Fit Calculated algorithm, which: i) subtracts the local background for each spot and ii) determines qualitatively the non-confident spots (quality metric). The normalization was made by the global method as described by Yang et al. (2002) comparing each inoculated time with the control (non-inoculated one) for each genotype: a factor *c,* calculated separately using the intensity average of all the spots, was subtracted from the raw values. The genes were determined as differentially expressed if the fold change was greater than 2 or below -2. In order to identify temporal patterns of expression within the *M. perniciosa*-responsive transcripts, we subjected the filtered, normalized macroarray data points to Cluster package analysis (Eisen et al., 1998), which classifies the data into groups based on temporal expression patterns. Fold changes were supplied to complete hierarchical clustering algorithm and graphic results of clustering were visualized by the TreeView program.

Real-time quantitative PCR analyses

For real-time quantitative PCR (RT-qPCR), the cDNA was obtained by reverse transcription from total RNA using 0.5 µg/µL oligo(dT)$_{18}$ primer and the RevertAid™ M-MuLV Reverse Transcriptase as described by the manufacturer instruction (Fermentas). RT-qPCR analyses were used to follow the expression pattern of a selected number of genes at early stage of infection: two bZIP (RT42C09 and RT57A09) and one WRKY TFs (RT43H02). The β-tubulin gene (RT001C02), which did not show significant differential expression on the macroarray, was used as endogen for the RT-qPCR experiment. Specific primers were designed for each gene: RT42C09-F 5'-GCCCGATATAAGGGATGCTTT-3' and RT42C09-R5'-CAACAATGGCCATGTCATCATT-3' (amplified product of 100 bp, Tm = 80°C) for bZIP-RT42C09; RT57A09-F 5'-GGCCAAATTGAATGGGTTAAAG-3' and RT57A09-R 5'-CTTGAATGCAAACAATGGCAGTA-3' (amplified product of 100 bp, Tm = 77°C) for bZIP-RT57A09; RT43H02-F 5'-AACATAACCACGAGATGCCACTT-3' and RT43H02-R 5'-TTCAGTTGCTATCATCGCTTGTC-3' (amplified product of 100 bp, Tm = 78°C) for WRKY-RT43H02, and tubulin-F 5'-TGCAACCATGAGTGGTGTCA-3' and tubulin-R 5'-CTGGTCTCAGCAGCCAAAATG-3' for the endogen (amplified product of 193 bp, Tm = 85°C). The RT-qPCR analysis was made using SYBRGreen® (Invitrogen) for fluorescence detection during amplification. Assays were performed on an ABI PRISM 7500 Sequence Detection System (SDS) coupled to an ABI PRISM 7500 SDS software (Applied Biosystems) using standard settings. The 20-µL RT-PCR consisted of SYBR Green 1X (Applied Biosciences), PCR buffer 1X, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, Rox 0.0625X (Invitrogen), 0.2 pmol of each primer, 0.5 U Taq platinium (Invitrogen) and 100 ng single-stranded cDNA. The thermal cycling conditions were 94°C for 10 min, followed by 45 cycles of 94°C for 15 s and 60°C for 45 s. A dissociation analysis was conducted after each amplification to investigate primer dimer and hairpin formation. Melting temperature of the fragments was determined according to the manufacturer protocol (Applied Biosystems). No-template reactions were used

as negative controls. The results obtained with the Sequence Detection Software (Applied Biosystems) were transferred to Microsoft Excel for further analysis. Raw expression levels were calculated from the average of the triplicate ddCT (RQ) values. Non-inoculated plants (control) were used as a calibrator for both varieties and the experiment was conducted with three biological replicates, each one formed by a pool of three meristems. The BioEstat 5.0 program was used for statistical analyses.

RESULTS

Macroarray analyses

The expression analysis of cacao TFs possibly involved in plant response to *M. perniciosa* infection was performed by macroarray containing cDNAs identified on *T. cacao-M. perniciosa* interaction libraries (Gesteira et al., 2007). The macroarray was formed by 88 TF cDNAs, two constitutive cDNAs (α- and β-tubulin) and one negative control (λ DNA). The TF cDNA list is divided into 24 zinc finger proteins, 6 bZIP, 5 WD-40, 8 MYB, 5 bHLH, 3 WRKY, and 37 sequences belonging to other TF families. The cDNA length varied between 123 and 808 bp with an average of 386 bp (Supplementary material). The macroarrays were hybridized with probes from non-inoculated plants (control) or inoculated plants harvested at 24, 48 and 72 hai (joined in an unique cDNA pool named Pool 72 h) and at 15 and 30 dai on both resistant (TSH1188) and susceptible (Catongo) varieties. The differentially expressed genes were listed in Table 1.

From the 88 TF cDNAs analyzed, 72 showed a differential expression pattern. Sixty TF cDNAs were differentially expressed in Catongo: 18 were up-regulated, 41 down-regulated, and only one was up- and down-regulated through the disease time course (clone RT42C09; Table 1). Among the up-regulated TFs, only one was induced in the early hours (Pool 72 h), 3 at 15 dai, 7 in both Pool 72 h and 15 dai, and 7 in both 15 and 30 dai (Figure 1A). Among down-regulated TFs, one was repressed at Pool 72 h, 7 were repressed in both Pool 72 h and 15 dai, 20 at 15 dai, 5 at 30 dai, 5 in both 15 and 30 dai, and 3 in both Pool 72 h and 30 dai (Figure 1B).

In the susceptible variety, most of the TF family members potentially related to defense response of cacao to *M. perniciosa* infection were repressed from 24 hai to 30 dai (Figure 2). By analyzing all the significantly expressed genes in Catongo, four different regulatory patterns were observed in the hierarchical clustering (Figure 2). Cluster I corresponded to TFs with high expression at Pool 72 h followed by a decreasing expression at 15 and 30 dai (Figure 2B). This group included three MYB and one gene similar to CCR4-NOT transcription complex, which is known to be involved in plant defense (Sarowar et al., 2007; Table 1). Cluster II contained TFs repressed in control and at Pool 72 h followed by an increase of expression at 15 and 30 dai (Figure 2C). In this group, zinc finger proteins and TFs of the bHLH family were found (Table 1). Cluster III contained TFs slightly down-regulated at Pool 72 h and 15 dai, and then slightly up-regulated at 30 dai (Figure 2D). This cluster contained the largest number of TFs including the WRKY, bZIP, MYB, and zinc finger protein families considered as the main TF families regulating plant defense responses (Table 1). In cluster IV, TFs were slightly up-regulated in control then a decrease of expression intensity at Pool 72 h, an up-regulation of the genes at 15 dai, and finally a repression of the expression at 30 dai was observed (Figure 2E).

Table 1. Fold changes of the differentially expressed genes in comparison with the control (plants non-inoculated - NI) during cacao-*Moniliophthora perniciosa* interaction.

Clone	Gene name	E-value	Catongo				TSH1188			
			Pool 72 h x NI	15 dai x NI	30 dai x NI	Cluster	Pool 72 h x NI	15 dai x NI	30 dai x NI	Cluster
SP01A04	Zinc finger (C3HC4-type RING finger) *	2.10^{-10}	-2.83		-2.73	IV				
SP06B12	bZIP family transcription factor *	4.10^{-40}	-4.44		-4.35	IV				
SP06C09	BT2 (BTB and TAZ domain protein 2)	3.10^{-13}			-5.79	IV		-2.17		III
SP06E12	BTB/POZ (zinc finger)	4.10^{-19}		2.00		III		3.68		II
SP06F03	Transcription regulatory protein SNF2 *	2.10^{-28}			-5.59	IV				
SP06F06	MYB transcription factor	5.10^{-12}			-2.02	III	-4.64	-2.17		IV
SP06G09	Zinc finger (CCCH-type) family protein	3.10^{-10}		5.96	6.59	II	-4.28			IV
SP08A03	Zinc finger (C3HC4-type RING finger)	4.10^{-09}		5.12	5.48	II	-4.72	-2.25		IV
SP09B11	Putative transcription factor *	6.10^{-10}		2.74	4.65	IV				
SP14B01	MYB family transcription factor **	5.10^{-09}					-2.13	-1.53	2.06	IV
SP14D09	WREBP-1 myb family transcription factor	2.10^{-20}		-4.02		III		3.07		II
SP15E02	Transcription factor WRKY-type ♦	9.10^{-08}		-2.67		III	2.74	4.46		I
SP15F06	XS zinc finger domain-containing protein ♦	4.10^{-46}		-3.4		III	2.26	5.3		I
SP15G02	MYB family transcription factor ♦	3.10^{-17}		-2.07		III	3.17	4.85		I
SP16D03	bZIP transcription factor **	3.10^{-13}					3.78	4.66		I
SP21C11	Zinc finger protein	5.10^{-20}		-3.41		III	-5.33	2.33		II
SP33C04	Putative transcription activator	2.10^{-32}				IV	-4.98	-4.39		IV
SP34A09	TAFII15 transcription factor *	1.10^{-52}	-2.62			III				
SP35C04	Zinc finger (DNL-type)	9.10^{-27}	-2.43	-5.08		III	3.77	5.14	2.61	II
SP36D07	Basic helix-loop-helix (bHLH) *	8.10^{-23}		5.59	4.52	II				
SP37C08	Zinc finger (C2H2-type) protein (WIP3) *	3.10^{-23}	-3.89		-3.8	IV				
SP44B11	Probable zinc finger protein ♦	2.10^{-07}		-4.44		III	3.2	4.04		I
SP44H03	Zinc finger (C2H2-type) family protein ♦	6.10^{-39}	-1.92	-3.37		III	3.55	2.5		I
SP46B07	Polypeptide-associated complex NAC ♦	4.10^{-04}	-2.14	-3.99		III	2.77	4.33		I
SP46C08	Protein-associated phosphoprotein Dr1	8.10^{-51}		-4.44		III	3.2	4.04		II
SP47C07	Zinc finger (CCCH-type) family protein	1.10^{-33}			-3.61	IV	-2.12	4.8	-2.53	II
SP47E01	KH domain-NOVA, binding protein **	2.10^{-14}							-2.52	V
SP49G08	WD-40 repeat protein (MSI4) **	9.10^{-120}						-2.58		III
SP61D10	THUMP domain-containing protein ♦	9.10^{-61}		-4.13		III		3.32		I
SP61F10	Zinc finger (C3HC4-type RING finger) ♦	2.10^{-18}		-3.37		III	2.33			I
RT01D10	TCP family transcription factor ♦	3.10^{-30}		-3.76		III	2.79	4.47		I
RT01E10	Putative regulator of nonsense transcript ♦	1.10^{-62}		-3.23	-2.36	III	3.14	2.96		I
RT01D02	MYB family transcription factor	2.10^{-32}	5.16	4.6		I		-3.11		III
RT02F02	Transcription factor (E2F) ♦	4.10^{-28}		-4.01		III	2.28			I
RT04E12	WD-40 repeat family protein	9.10^{-46}		-3.63	-2.09	III	3.86			II

Continued next page

Table 1. Continued.

Clone	Gene name	E-value	Catongo				TSH1188			
			Pool 72 h x NI	15 dai x NI	30 dai x NI	Cluster	Pool 72 h x NI	15 dai x NI	30 dai x NI	Cluster
RT04G04	Zinc finger (C3HC4-type RING finger) ♦	3.10^{-16}	-2.13	-3.99		III	4.02	3.05		I
RT05C05	MYB-related *	5.10^{-31}	3.49	4.06		I				
RT06D01	PHD-type zinc finger protein	3.10^{-16}	3.74	4.32		I	-2.12	3.05		II
RT07C08	Putative ZF-HD homeobox protein	4.10^{-22}		4.2	4.34	II	-2.12		2.27	IV
RT08G05	(bHLH) family protein	2.10^{-40}			-3.39	IV	2.97		2.85	III
RT09D12	AP2-related transcription factor	2.10^{-18}		-3.07	-6.1	IV	2.3	2.17		II
RT09F03	WD-40 repeat protein (MSI3) ♦	2.10^{-70}	-2.23	-4.25		III	2.39	2.4		I
RT10C01	Zinc finger (AN1-like) family protein ♦	7.10^{-05}		-2.82		III	3.89	4.01		I
RT12G06	NAC domain protein NAC1	2.10^{-06}		-4.24		III		2.73		II
RT13F06	Transcription activator (GRL2)	4.10^{-04}	4.38	4.19		I			-2.52	V
RT14D05	Polypeptide-associated complex (NAC) **	5.10^{-23}					-2.71	5.72		II
RT15F07	AREB-like protein	3.10^{-46}		-2.25		III	2.77		2.29	III
RT15H07	Zinc finger protein-related *	2.10^{-05}	3.8			I				
RT16B04	Basic helix-loop-helix (bHLH) **	4.10^{-31}					3.19	-3.12		III
RT19A02	Transcriptional regulator-related ♦	9.10^{-49}		-2.51		III	3.3	3.31		I
RT19B02	MYB family transcription factor	6.10^{-11}	3.91	3.74		I	-2.12		2.88	IV
RT25D12	CCR4-NOT transcription complex protein	7.10^{-06}	3.62	3.63		I	-2.12	2.87	2.84	II
RT26F08	HB2 homeodomain protein	7.10^{-49}		2.07		III	-4.29	2.46		II
RT27G02	Transcription factor Hap5a **	6.10^{-12}					-4.98	-4.39		IV
RT28E04	ZF-HD homeobox family protein *	1.10^{-23}		5.16	5.26	II				
RT28E07	Putative transcription factor APFI	4.10^{-55}		-3.81		III	3.18	3.03		II
RT35D03	XH/XS domain-containing protein **	4.10^{-15}						-3.34		III
RT37A04	bZIP2 protein	7.10^{-05}		-2.59		III	2.0			III
RT37F09	ZF-HD homeobox protein	7.10^{-14}		-2.54	-2.49	III		-3.86		III
RT42C09	bZIP DNA-binding protein HBF-1 ♦	1.10^{-54}		-2.03	2.56	III	2.96	3.68		I
RT43H02	WRKY transcription factor ♦	3.10^{-27}		-4.29		III	2.48	4.86		I
RT44F09	Zinc finger (C2H2-type) family protein ♦	4.10^{-35}	-2.43	-4.11		III	2.83	2.25		I
RT46D05	MYB-related protein 1 **	6.10^{-18}					-2.47		2.32	IV
RT49A12	bZIP protein DPBF3 **	4.10^{-32}					-3.93	-3.34		IV
RT49D12	Zinc finger protein family-like	9.10^{-08}		4.54	4.50	II	-5.88	-3.29	-2.08	IV
RT50B08	Myc-like regulatory protein **	2.10^{-26}						-5.09		III
RT50F05	Transcription factor EREBP-like protein	8.10^{-13}		-2.58	-2.74	III	2.03	-3.24	2.03	III
RT50G06	Zinc finger (C3HC4-type RING finger)	1.10^{-11}		-2.6		III	2.72			II
RT50H06	(bHLH) family protein	5.10^{-40}		2.61		III	-2.71	4.23	2.33	II
RT51G11	Zinc finger (C3HC4-type RING finger)	9.10^{-22}	5.42	4.19		II		-3.7	1.12	II
RT55A06	WD-40 repeat family protein (LEUNIG) ♦	1.10^{-51}	-2.63	-4.53		III	2.58	3.25		I
RT57A09	bZIP transcription factor ATB2 ♦	2.10^{-26}		-4.86		III	3.25	3.69		I

dai: days after inoculation. *Genes differentially expressed only in Catongo. **Genes differentially expressed only in TSH1188. ♦Genes found in cluster I in TSH118 and cluster III in Catongo.

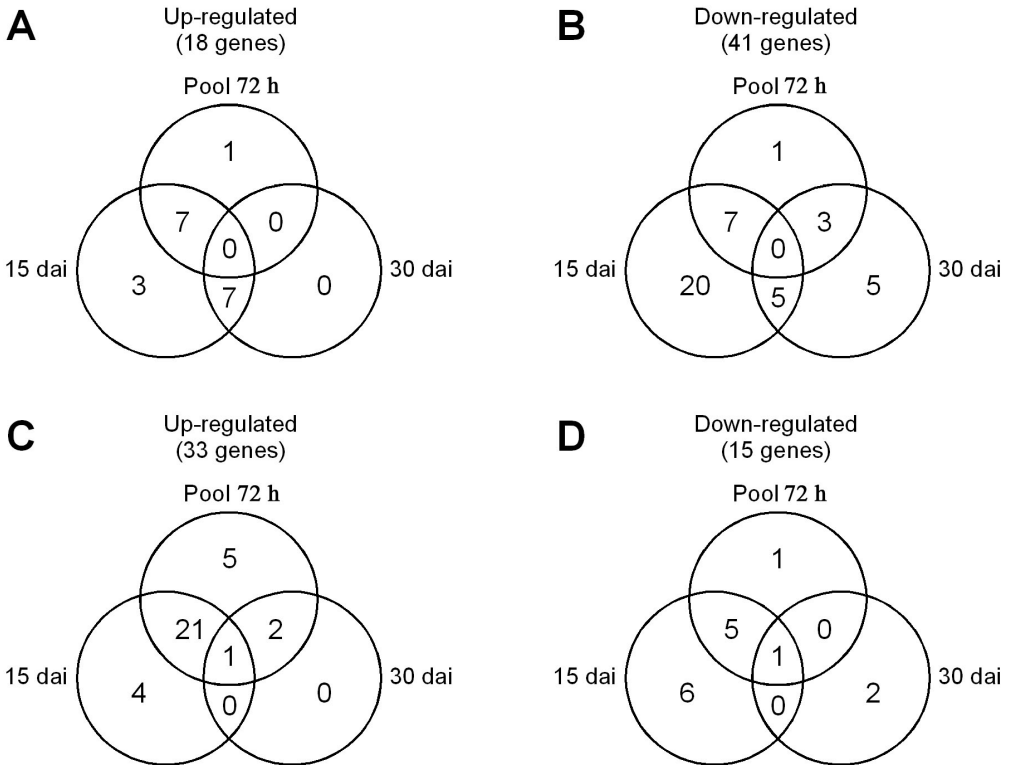

Figure 1. Venn diagram showing the distribution of the differentially expressed transcription factors of cacao challenged with *Moniliophthora perniciosa* between three measurement times after inoculation (Pool 72 h, and 15 and 30 days after infection, dai). **A.** Genes up-regulated in Catongo. **B.** Genes down-regulated in Catongo. **C.** Genes up-regulated in TSH1188. **D.** Genes down-regulated in TSH1188.

In TSH1188, 62 of the 88 TFs displayed change in the transcript level (Table 1): 33 were induced, 15 were repressed and 14 were up- and down-regulated through the disease time course. Among the up-regulated TFs, 5 were induced at Pool 72 h, 4 at 15 dai, 21 in both Pool 72 h and 15 dai, 2 in both Pool 72 h and 30 dai, and 1 common to all the harvesting times (Figure 1C). Among the down-regulated TFs, only one was repressed at Pool 72 h, 6 at 15 dai, 2 at 30 dai, 5 in both Pool 72 h and 15 dai, and 1 common to all the harvesting times (Figure 1D). In TSH1188, unlike Catongo, various members of TF families important to defense responses were up-regulated in the early hours after the infection by *M. perniciosa*. Five regulatory patterns of expression were observed in TSH1188 (Figure 3A). TFs with an increase of expression at Pool 72 h and 15 dai, followed by a decrease of expression at 30 dai (with the same intensity than control) were grouped in cluster I (Figure 3B). This cluster contained 21 TFs including WRKY, bZIPs, zinc finger proteins, and other TF families (Table 1). Cluster II contained 17 TFs with high repression at Pool 72 h, high up-regulation at 15 dai, followed by a slight expression decrease at 30 dai (Figure 3C). In this cluster, we found zinc finger proteins, WD-40, bHLH, and CCR4-NOT TFs (Table 1). In cluster III, we observed TFs with a down-regulation at 15 dai (Figure 3D). In cluster IV, TFs were highly expressed in control and at

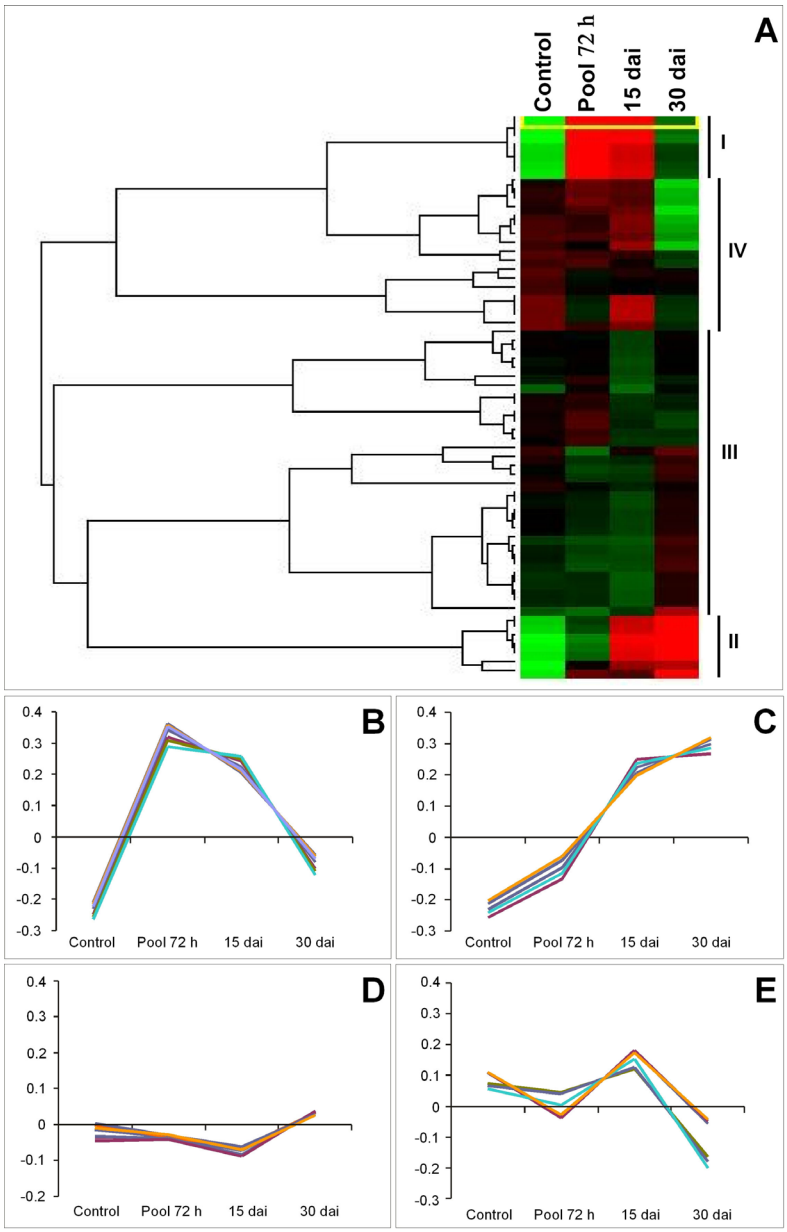

Figure 2. Hierarchical clustering illustrating groups of Catongo genes co-ordinately expressed in response to *Moniliophthora perniciosa* infection at Pool 72 h, and 15 and 30 days after infection (dai). **A.** Hierarchical clustering of 60 genes differentially expressed in Catongo variety. Each column corresponds to a measurement time (Pool 72 h, 15 or 30 dai) and each row corresponds to one gene. For each gene, the fold change of mRNA level in inoculated versus the corresponding control tissues is represented by red or green, indicating up-regulation or down-regulation, respectively. The differentially expressed genes were classified into four regulatory patterns (clusters), indicated by numbers I through IV. **B.** to **E.** Graphical representations of the four regulatory patterns. The y-axis represents spot intensity in logarithm value.

30 dai, and strongly repressed at Pool 72 h and 15 dai (Figure 3E). Finally, cluster V, containing only two genes, corresponded to TFs with high expression in control and at Pool 72 h, and repression of gene expression at 15 and 30 dai (Figure 3F).

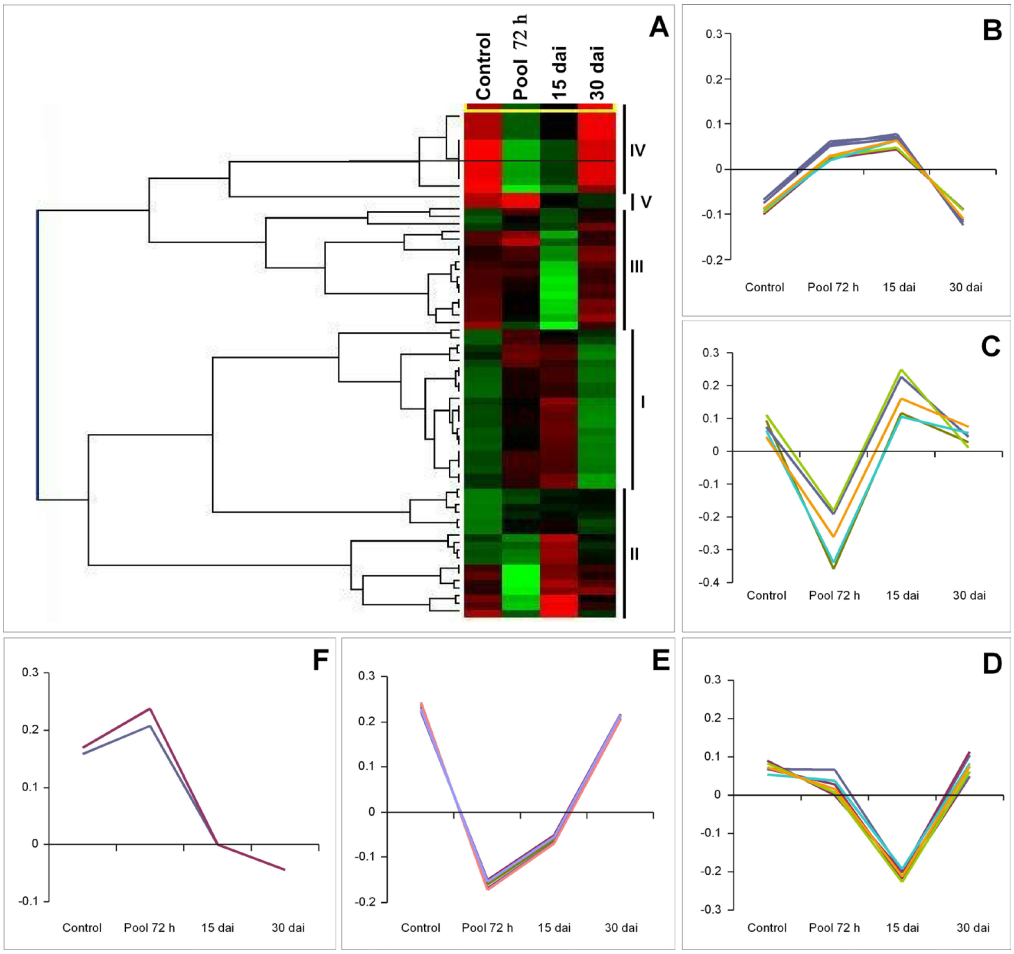

Figure 3. Hierarchical clustering illustrating groups of TSH1188 genes co-ordinately expressed in response to *Moniliophthora perniciosa* infection at Pool 72 h, and 15 and 30 days after infection (dai). **A.** Hierarchical clustering of 62 genes differentially expressed in TSH1188. Each column corresponds to a measurement time (Pool 72 h, 15 or 30 dai) and each row corresponds to one gene. For each gene, the fold change of mRNA level in inoculated versus the corresponding control tissues is represented by red or green, indicating up-regulation or down-regulation, respectively. The differentially expressed genes were classified into five regulatory patterns (clusters), indicated by numbers I through V. **B.** to **F.** Graphical representations of the five regulatory patterns. The y-axis represents spot intensity in logarithm value.

Among the 88 TFs, 10 showed a differential expression only in Catongo (Table 1; 1 asterik) while 11 were differentially expressed only in TSH1188 (Table 1; 2 asteriks). Among the 18 TFs up-regulated in Catongo, 5 were differentially expressed only in Catongo, one was up-regulated in both varieties, 7 were both repressed and induced, and 5 were down-regulated

in TSH1188. Conversely, from the 41 TFs down-regulated in Catongo, 5 were differentially expressed only in Catongo, 3 were down-regulated in both varieties, 2 were repressed and induced, and 31 were up-regulated in TSH1188. Among the TFs down-regulated in Catongo and up-regulated in TSH1188, some members of the main TF families in plant defense regulation seemed more interesting, such as MYB, zinc finger proteins and especially bZIP and WRKY, which can act together in plant defense signaling (Eulgem, 2005; Jalali et al., 2006).

Real-time PCR

Expression analysis by RT-qPCR was made on 24, 48 and 72 hai, and 15 dai samples in Catongo and TSH1188 for two bZIP and one WRKY TF (RT42C09, RT57A09 and RT43H02, respectively; Figure 4). On macroarrays, these TFs are grouped in cluster III in Catongo (showing down-regulation at 15 dai) and in cluster I in TSH1188 (showing up-regulation at Pool 72 h and 15 dai, with quite similar expression intensity in this two harvesting times) (Table 1, Figures 2 and 3). The RT-qPCR data showed that globally, for the three genes analyzed, the expression intensity is higher in TSH1188 than in Catongo (Figure 4). In TSH1188, for the bZIP-RT42C09, similar expression intensities were observed in the early hours of interaction (0.93, 0.88, 0.81 for 24, 48 and 72 h, respectively) while the expression increased at 15 dai (2.58 with a statistically significant difference in relation to the early hours of interaction). In Catongo, the highest intensity was observed at 48 hai (0.87), and the lowest expression intensity was observed at 15 dai (0.19) (Figure 4A). For bZIP-RT57A09, in TSH1188, the intensities were relatively high (1.19 at 15 dai to 2.8 at 24 hai) but no significant difference was observed between the four samples analyzed. In Catongo, no expression was detected at 24 hai, and similar intensities were observed at 48 hai, 72 hai and 15 dai (0.37, 0.38 and 0.55, respectively, without significant statistical differences) (Figure 4B). For the WRKY-RT43H02 gene, in TSH1188, the same intensity was observed between 24 hai (1.4) and 72 hai (1.4). However, a significant intensity difference was observed between 48 hai and 15 dai samples (0.92 and 1.67, respectively). In Catongo, the highest intensity was observed at 48 hai and 72 hai (0.45), and the lowest expression intensity was observed at 15 dai (0.08) (Figure 4C).

DISCUSSION

Global gene expression studies have become a highly valuable source for functional genomics. In particular, arrays- and qPCR-based studies have identified several TF types, such as WRKY, bZIP and MYB factors in different plant species (Eulgem, 2005; Jalali et al., 2006; Yang et al., 2009). Such TFs are known to participate in defense regulation processes in plants: to defeat pathogens, the plant has to regulate TFs in a timely manner after recognizing the pathogen in order to activate a flood of defense-related genes (Eulgem, 2005; Jalali et al., 2006). To understand the role of TFs in cacao, the present study aimed to identify defense-related TF genes whose expression is differentially regulated in cacao plants infected versus uninfected by *M. perniciosa*. Eighty-eight TF genes were selected from cacao-*M. perniciosa* interaction cDNA libraries (Gesteira et al., 2007); among them TFs belonging to bZIP, zinc finger proteins, MYB, and WRKY families were identified. In tobacco, TGA factors, which belong to a sub-family of bZIP TFs, have a major importance in salicylic acid (SA)-inducible

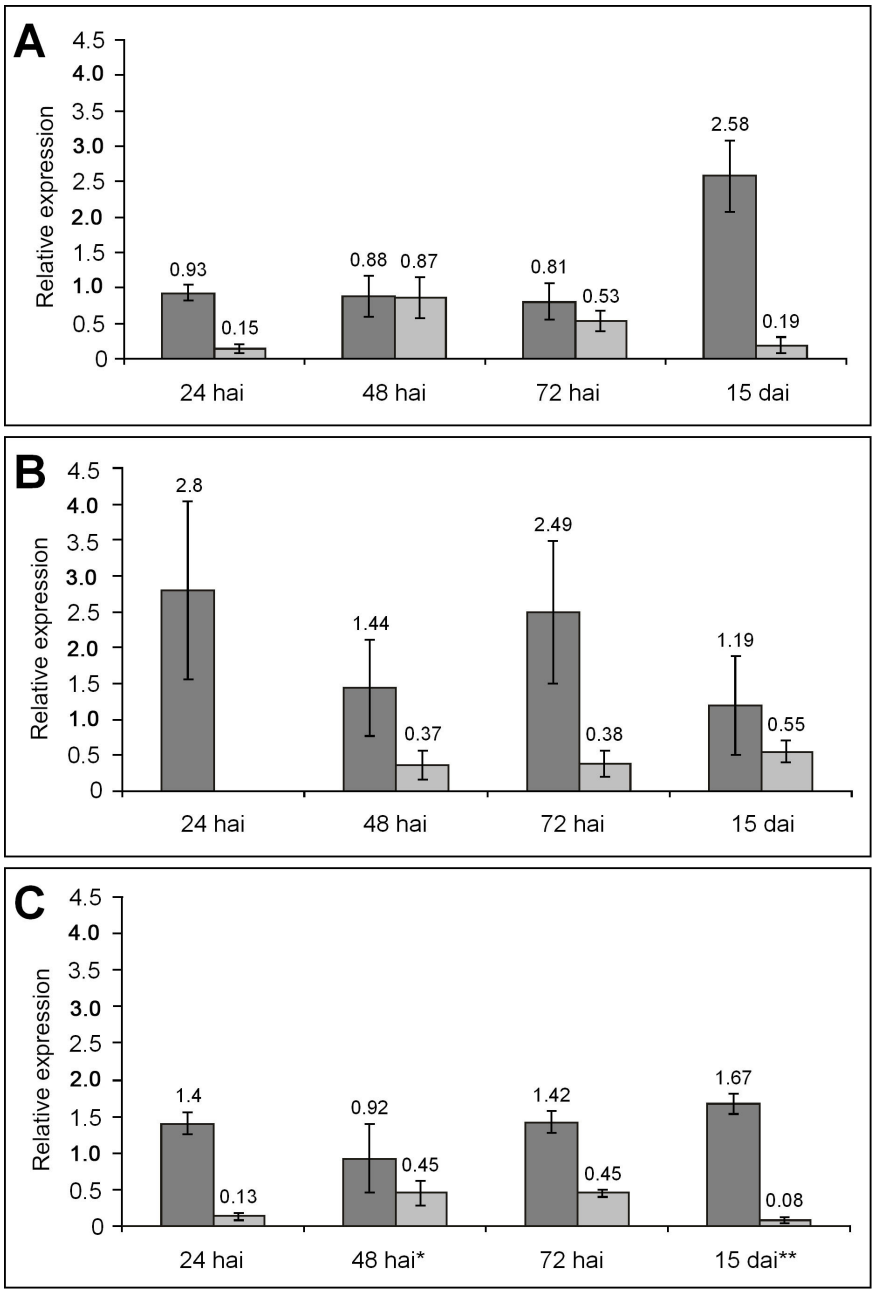

Figure 4. Expression of two different bZIP and one WRKY transcription factors by RT-qPCR in Catongo and TSH1188 cacao varieties. **A.** Relative expression level of bZIP-RT42C09. **B.** Relative expression level of bZIP-RT57A09. **C.** Relative expression level of WRKY-RT43H02. *Times analyzed only with two biological replicates in TSH1188. **Times analyzed only with two biological replicates in Catongo. Expression in TSH1188 and Catongo is shown in dark and light gray, respectively. hai = hours after infection; dai = days after infection.

gene expression (Thurow et al., 2005), while in *Arabidopsis* they play a role up-regulating the plant's systemic acquired resistance (SAR) (Eulgem, 2005). The bZIP TFs also have role in modulation of basal defense and cell death following infection by a pathogen: in *Arabidopsis*, the *bZIP10* gene is induced by reactive oxygen species inducing the hypersensitive response (HR) (Kaminaka et al., 2006). In *Arabidopsis*, several genes encoding MYB TFs are up-regulated after infection by pathogen, and act as a positive regulator of the HR (Raffaele et al., 2006). The MYB factors can bind to promoters of a variety of defense-associated genes but also to promoters of WRKY TFs (Dong et al., 2003; Eulgem, 2005). The WRKY proteins seem to play a greater transcriptional reprogramming role during a variety of immune responses. Functional studies involving loss and gain of function in *Arabidopsis* have shown that WRKY factors can act as both positive and negative regulators in the complex network of plant defense responses (Eulgem and Somssich, 2007). WRKY proteins bind to W-box elements present in promoters of several plant defense-related genes (Eulgem, 2005). These TFs appeared to be involved in regulation of basal defense and SAR, acting in regulation of the SA biosynthesis, and in regulation of the expression of NPR1, a central regulator of SA- and SAR-dependent defenses (Pandey and Somssich, 2009).

The cacao-*M. perniciosa* interaction TF macroarrays we developed here showed differential quantitative and qualitative gene expression between resistant and susceptible interactions and during the disease time course. The number of TFs induced in response to *M. perniciosa* was higher in the resistant cacao-*M. perniciosa* interaction (TSH1188) than in the susceptible one (Catongo): while 33 TFs were up-regulated in TSH1188 only 18 TFs were up-regulated in Catongo. Among the 33 TFs up-regulated in TSH1188, 21 were induced in the first 72 hai, while only seven of the 18 TFs up-regulated in Catongo were induced in the early hours of the infection. Among the TFs that present significant expression pattern differences between TSH1188 and Catongo were encountered members of the three large TF families involved in plant defense responses: MYB, bZIP and WRKY. It has been shown that MYB TFs are up-regulated in both varieties in the early hai: three and one MYB TFs were induced in Catongo and in TSH1188, respectively. In *Arabidopsis*, *AtMYB30* acted as a positive regulator of the HR and was induced in response to bacteria pathogens (Raffaele et al., 2006). In perennial crops, the possible involvement of MYB factors in defense was also reported. In grapevine and poplar, they were involved in the regulation of the proanthocyanidin (PA) biosynthesis, a derivative of flavonoids that contributes to plant defense mechanisms against biotic and abiotic stress (Mellway et al., 2009; Terrier et al., 2009). In poplar, the *MYB134* gene was induced by pathogen attack and abiotic stress, and transgenic poplar expressing constitutively *MYB134* showed dramatic increase in PA concentration (Mellway et al., 2009). In addition, MYB TFs and PA biosynthesis genes are induced after infection by pathogens (Mellway et al., 2009). In our study, two of the three WRKY TFs were induced in TSH1188 in the early hai and repressed in Catongo at 15 dai. The role of WRKY TFs in the transcriptional reprogramming of plant defense responses, shown in studies using plant models, was also confirmed in perennial plants. In *Coffea arabica*, *CaWRKY1* was induced in both compatible and incompatible interactions after 12 h of inoculation with *Hemileia vastatrix*, but the level of expression was higher in the incompatible interaction (Ganesh et al., 2006). The over-expression of grapevine *VvWRKY1* in tobacco led to reduced susceptibility to both necrotrophic and biotrophic fungus (Marchive et al., 2007), whereas the constitutive expression of grapevine *VvWRKY2* in tobacco plants reduced the susceptibility to *Botrytis cinerea*, *Pytium* spp and *Alternaria tenius*

(Mzid et al., 2007). In cacao, previous expression studies showed changes in expression level of the WRKY family member *TcWRKY1* during leaf development, after treatment with Nep1 and *Phytophthora*, and in response to mechanical wounding and defense inducers (Bailey et al., 2005a,b). In 2004, Borrone et al. isolated 16 cacao *WRKY* genes using degenerated primers. Four of these (*TcWRKY3*, *-10*, *-11*, *-14*) were successfully converted into molecular markers and used for mapping (Borrone et al., 2004). These markers were used in an assisted selection program and mapping of quantitative trait locus for resistance to frosty pod and black pod diseases (Brown et al., 2007; Schnell et al., 2007). In the present study, we identified 3 WRKY TFs, one of which, RT43H02, is homolog to *TcWRKY13* identified by Borrone et al. (2004) (data not shown). Because of the shortness of the sequences, it has not been possible to identify the homolog for the 2 other clones (SP15E02 and SP35E08). Further analysis of these three *WRKY* clones may allow their complete identification and correlation with WRKY clones already mapped (Borrone et al., 2004). Moreover, sequence analysis may allow marker identification and mapping on cacao population segregating for witches' broom disease (e.g., Sca6 x ICS1).

In the present study, MYB, bZIP and WRKY factors belonged to Catongo cluster III (TFs down-regulated at Pool 72 h and 15 dai, and then highly expressed at 30 dai) and to cluster I in TSH1188 (TFs with high expression intensity at Pool 72 h and 15 dai, followed by a slight decrease in intensity at 30 dai). Because they belonged to the same clusters both in resistant and susceptible interactions, it may be suggested that these TFs were co-regulated. In the literature, it has been described that WRKY, TGA and NPR1 TFs act coordinately for expression activation of PR1, a well-characterized marker of defense-response (Eulgem, 2005). WRKY and MYB factors are involved in regulation of the N-mediate resistance of the *Nicotiana tabacum* to tobacco mosaic virus (Liu et al., 2004), and in grapevine these factors are possibly involved in the regulation of the expression of the type I lipid transfer proteins (VvLTP1) involved in plant defense (Laquitaine et al., 2006). Thus, in cacao, it may be suggested that different TF families may be coordinately involved in defense response pathways against *M. perniciosa*.

For two TFs of the bZIP group (RT42C09 and RT57A09), we developed RT-qPCR experiments. The RT-qPCR results were correlated with the macroarray results, confirming them. Globally, these three genes were less expressed in Catongo than in TSH1188, which may be related to the down-regulation in Catongo observed on the macroarrays. It was also observed that the genes from cacao-*M. perniciosa*-resistant library (TSH1188/RT library; Gesteira et al., 2007) were more expressed in the TSH1188 tissues than in the Catongo ones, as expected. In TSH1188, the bZIP-RT42C09 expression was higher at 15 dai as observed in macroarray analysis (2.96 at Pool 72 h and 3.68 at 15 dai; Table 1). The WRKY-RT43H02 presented a relatively constant expression in TSH1188, as also observed on macroarrays (3.25 at Pool 72 h and 3.69 at 15 dai; Table 1). Only a few discrepancies were found between the two approaches: for the bZIP-RT57A09 clone in TSH1188, no difference of expression was observed between the 4 studied samples by RT-qPCR while on macroarrays the expression was higher at 15 dai. This can be explained by the level of sensitivity of the two methodological approaches: for the RT-qPCR experiment, primers and specific conditions of high stringency were used while in the macroarray experiment the stringency may be influenced by many more parameters. However, the use of a thematic macroarrays containing only TFs, which are generally lowly expressed, is more efficient than the use of arrays containing all kind of genes with a high expression level variability and containing highly expressed genes.

In this study, we observed differences in cacao TF expression profile between susceptible and resistant interactions and during disease time course. Because TFs act as main regulators of the cellular process, the TFs with differential expression identified here may be considered as good candidates for subsequent functional analysis as well as for plant engineering, in order to allow a better understanding of the signaling pathways that trigger the resistance and/or susceptibility processes in cacao in response to *M. perniciosa* (Gurr and Rushton, 2005a,b; Century et al., 2008). Some of these TFs can also be localized on the cacao reference map related to witches' broom disease resistance, helping breeding programs and resistant cacao tree selection.

ACKNOWLEDGMENTS

M.A. Lopes, C.V. Dias and G.C. Santos were supported by Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB). B.T. Hora Júnior was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Research supported by FAPESB, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the International Foundation for Science (IFS). We thank Claudia Fortes Ferreira (Embrapa) for critical reading the manuscript.

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Supplementary material

Clone	Length (bp)	Functional annotation	Species	E-value	Biological process
SP01A04	329	Zinc finger (C3HC4-type)	*Arabidopsis thaliana*	2.10^{-10}	Unknown
SP04H06	419	GAGA-motif binding transcriptional activator	*Populus trichocarpa*	1.10^{-15}	Development
SP05A03	285	WD-40 repeat family protein	*Arabidopsis thaliana*	1.10^{-28}	Unknown
SP05A11	332	Zinc-binding family protein	*Platanus x acerifolia*	3.10^{-06}	Unknown
SP06B12	440	bZIP family transcription factor	*Glycine max*	4.10^{-40}	Abiotic stress
SP06C09	185	BT2 (BTB and TAZ domain protein 2)	*Arabidopsis thaliana*	3.10^{-13}	Development
SP06E12	262	BTB/POZ (zinc-finger)	*Medicago truncatula*	4.10^{-19}	Unknown
SP06F03	284	Transcription regulatory protein SNF2	*Arabidopsis thaliana*	2.10^{-28}	Unknown
SP06F06	479	MYB transcription factor	*Glycine max*	5.10^{-12}	Abiotic stress
SP06G09	260	Zinc finger (CCCH-type)	*Arabidopsis thaliana*	3.10^{-10}	Unknown
SP08A03	343	Zinc finger (C3HC4-type)	*Arabidopsis thaliana*	4.10^{-09}	Unknown
SP08A07	265	Leucine zipper protein	*Gossypium hirsutum*	5.10^{-29}	Unknown
SP09B11	248	Putative transcription factor	*Oryza sativa*	6.10^{-10}	Unknown
SP09C06	272	Zinc finger protein	*Oryza sativa*	3.10^{-10}	Programmed cell death
SP09F08	260	KH domain-containing protein	*Arabidopsis thaliana*	4.10^{-11}	Unknown
SP14B01	535	MYB transcription factor	*Boechera divaricarpa*	5.10^{-09}	Unknown
SP14D09	467	WREBP-1 MYB family transcription factor	*Nicotiana tabacum*	2.10^{-20}	Phosphate starvation response
SP15E02	198	Transcription factor WRKY	*Glycine max*	9.10^{-08}	Abiotic stress
SP15F06	514	XS zinc finger domain-containing protein	*Arabidopsis thaliana*	4.10^{-46}	Unknown
SP15G02	330	MYB transcription factor	*Glycine max*	3.10^{-17}	Unknown
SP16D03	412	Putative ripening-related bZIP protein	*Vitis vinifera*	3.10^{-13}	Development
SP21C11	402	Putative zinc finger protein SHI	*Arabidopsis thaliana*	5.10^{-26}	Biosynthesis of gibberellins
SP33C04	397	Putative transcription activator	*Oryza sativa*	2.10^{-32}	Unknown
SP34A09	619	TAFII15 transcription factor	*Arabidopsis thaliana*	1.10^{-52}	Abiotic stress
SP35C04	489	Zinc finger (DNL type)	*Arabidopsis thaliana*	9.10^{-27}	Unknown
SP35E08	415	WRKY transcription factor 10	*Nicotiana tabacum*	6.10^{-10}	Unknown
SP36D07	437	Basic helix-loop-helix (bHLH) family protein	*Arabidopsis thaliana*	8.10^{-23}	Unknown
SP37C08	433	Zinc finger (C2H2 type) protein (WIP3)	*Arabidopsis thaliana*	3.10^{-26}	Unknown
SP41D03	318	SPL1 (squamosa promoter-binding protein)	*Arabidopsis thaliana*	4.10^{-33}	Unknown
SP44B11	420	Zinc finger protein	*Arabidopsis thaliana*	2.10^{-07}	Development
SP44H03	352	C2H2-type zinc finger	*Picea abies*	6.10^{-39}	Unknown
SP46A01	441	Zinc finger (CCCH-type)	*Arabidopsis thaliana*	1.10^{-14}	Unknown
SP46B07	302	Polypeptide-associated complex NAC	*Medicago truncatula*	4.10^{-04}	Unknown
SP46C08	418	Protein-associated phosphoprotein Dr1	*Arabidopsis thaliana*	8.10^{-51}	Unknown
SP47C07	404	Zinc finger (CCCH-type)	*Arabidopsis thaliana*	1.10^{-33}	Unknown
SP47E01	382	KH domain-NOVA, binding protein	*Medicago truncatula*	2.10^{-14}	Symbiosis Rhizobium
SP47E11	494	MYB transcription factor	*Arabidopsis thaliana*	1.10^{-25}	Unknown
SP49G08	808	WD-40 repeat protein (MSI4)	*Medicago truncatula*	9.10^{-120}	Unknown
SP61D10	443	THUMP domain-protein	*Arabidopsis thaliana*	9.10^{-61}	Unknown
SP61E06	376	NPR1/NIM1-interacting protein 1 (NIMIN-1)	*Arabidopsis thaliana*	1.10^{-04}	Unknown
SP61F10	224	Zinc finger (C3HC4-type RING finger)	*Arabidopsis thaliana*	2.10^{-18}	Unknown
RT01D10	536	TCP family transcription factor	*Arabidopsis thaliana*	3.10^{-30}	Morphogenesis
RT01E10	367	Putative regulator of nonsense transcript	*Oryza sativa*	1.10^{-62}	Unknown
RT02D02	509	MYB family transcription factor	*Arabidopsis thaliana*	2.10^{-32}	Unknown
RT02F02	469	Transcription factor (E2F)	*Chenopodium rubrum*	4.10^{-28}	Control of cell cycle
RT04E12	400	WD-40 repeat family protein	*Arabidopsis thaliana*	2.10^{-46}	Unknown
RT04G04	239	Zinc finger (C3HC4-type RING finger)	*Arabidopsis thaliana*	9.10^{-16}	Unknown
RT05C05	336	MYB-related	*Craterostigma plantagineum*	5.10^{-31}	Tolerance to stress
RT05H08	380	Putative leucine zipper protein	*Gossypium hirsutum*	8.10^{-18}	Unknown
RT06D01	489	PHD-type zinc finger protein	*Arabidopsis thaliana*	3.10^{-16}	Unknown
RT06F08	396	Transcription factor	*Vicia faba*	6.10^{-28}	Unknown
RT07C08	405	ZF-HD homeobox protein	*Flaveria bidentis*	4.10^{-22}	Unknown
RT08G05	405	bHLH family protein	*Arabidopsis thaliana*	2.10^{-40}	Development
RT08H01	168	PHD finger transcription factor	*Arabidopsis thaliana*	3.10^{-04}	Unknown
RT09D12	390	Ethylene responsive element binding protein 1	*Gossypium barbadense*	1.10^{-21}	Unknown
RT09F03	446	WD-40 repeat protein (MSI3)	*Gossypium hirsutum*	2.10^{-80}	Unknown
RT10C01	264	Zinc finger (AN1-like) family protein	*Brassica rapa*	7.10^{-05}	Unknown
RT13F06	449	Transcription activator (GRL2)	*Oryza sativa*	4.10^{-04}	Unknown
RT14D05	562	Polypeptide-associated complex (NAC)	*Arabidopsis thaliana*	5.10^{-23}	Development
RT15F07	458	AREB-like protein	*Lycopersicon esculentum*	3.10^{-46}	Unknown
RT15H07	210	Zinc finger protein-related	*Arabidopsis thaliana*	2.10^{-05}	Unknown

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Supplementary material. Continued.

Clone	Length (bp)	Functional annotation	Species	E-value	Biological process
RT16B04	518	Basic helix-loop-helix protein (bHLH)	*Sesamum indicum*	4.10^{-31}	Development
RT16G11	446	ATBPM1 (BTB-POZ and MATH domain 1)	*Arabidopsis thaliana*	2.10^{-62}	Unknown
RT19A02	461	Transcriptional regulator-related	*Arabidopsis thaliana*	9.10^{-49}	Unknown
RT19B02	446	MYB family transcription factor	*Arabidopsis thaliana*	6.10^{-11}	Unknown
RT25D12	318	CCR4 associated factor 1-related protein	*Capsicum annuum*	7.10^{-06}	Plant defense
RT26F08	440	HB2 homeodomain protein	*Populus_tremula_x_Populus_tremuloides*	3.10^{-47}	Unknown
RT27G02	477	Transcription factor Hap5a	*Medicago truncatula*	2.10^{-19}	Unknown
RT28C03	449	Zinc finger (B-box-type) family protein	*Arabidopsis thaliana*	6.10^{-36}	Unknown
RT28E04	415	ZF-HD homeobox family protein	*Arabidopsis thaliana*	1.10^{-23}	Development
RT28E07	394	Putative transcription factor APFI	*Oryza sativa*	2.10^{-55}	Unknown
RT35D03	246	XH/XS domain-containing protein	*Arabidopsis thaliana*	4.10^{-15}	Unknown
RT37A04	123	bZIP2 protein	*Petroselinum crispum*	7.10^{-05}	Unknown
RT37F09	132	ZF-HD homeobox protein	*Flaveria bidentis*	7.10^{-14}	Unknown
RT42C09	500	bZIP DNA-binding protein HBF-1	*Glycine max*	1.10^{-54}	Plant defense
RT43H02	448	WRKY transcription factor	*Glycine max*	3.10^{-27}	Plant defense
RT44F09	395	Zinc finger (C2H2-type) family protein	*Arabidopsis thaliana*	4.10^{-35}	Unknown
RT46D05	416	MYB-related protein	*Dendrobium* sp	6.10^{-18}	Unknown
RT49A12	470	bZIP protein DPBF3	*Malus x domestica*	4.10^{-32}	Unknown
RT49D12	244	Zinc finger protein family-like	*Arabidopsis thaliana*	9.10^{-08}	Unknown
RT50B08	416	Myc-like regulatory protein	*Citrus sinensis*	2.10^{-26}	Unknown
RT050F05	374	Transcription factor EREBP-like protein	*Gossypium barbadense*	8.10^{-13}	Unknown
RT50G06	395	Zinc finger (C3HC4-type RING finger)	*Arabidopsis thaliana*	1.10^{-11}	Unknown
RT50H06	437	bHLH family protein	*Nicotiana tabacum*	5.10^{-40}	Unknown
RT51G11	290	Zinc finger (C3HC4-type RING finger)	*Arabidopsis thaliana*	9.10^{-22}	Hydric stress
RT55A06	367	WD-40 repeat family protein (LEUNIG)	*Antirrhinum majus*	1.10^{-51}	Development
RT57A09	323	bZIP transcription factor ATB2	*Glycine max*	2.10^{-26}	Salt and freezing tolerance
RT59E10	478	Putative transcription factor	*Arabidopsis thaliana*	4.10^{-24}	Unknown