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IDENTIFICATION *IN SILICO* AND CONFIRMATION VIA RT-qPCR OF CANDIDATE RESISTANCE GENES LINKED TO CERATCYSTIS WILT IN CACAO

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One strategy for the identification of candidate genes is the *in silico* detection of the genes present in the resistance linked genomic regions (QTLs). The present work locates and correlate *in silico* genes found in the previously region of mapped QTLs, selecting and testing candidate genes involved in the resistance of cacao to *C. cacaofunesta* by RT-qPCR. We found 156 genes identified in the LXD-GL3 region and 166 in the LXD-GL9 region were reduced to 17 and 19 candidate genes, respectively, and at least 2 genes (osmotin e thaumatin) are especially interesting. The level of osmotin transcription is generally low at the start of infection (6 hours after inoculation), increases slightly at 48 hai, and rapidly increases over the course of days (7 and 15 days after inoculation) in the two genotypes studied. The expression pattern of thaumatin in the susceptible CCN 51 genotype increased abruptly in the first days after infection (7 and 15 dai), while the resistant genotype TSH 1188 showed a smaller increase in 7 dai and 15 dai. The antifungal activity of these genes makes them candidates for genetic engineering in the production of disease resistant plants.

Key words: *Ceratocystis cacaofunesta*, QTLs, osmotin, thaumatin, differential expression.

Identificação *in silico* e confirmação via RT-qPCR de genes candidatos ligados a resistência à murcha-de-Ceratocystis em cacau. Uma estratégia para a identificação de genes candidatos é a detecção *in silico* dos genes presentes nas regiões genômicas ligadas à resistência (QTLs). O presente trabalho localiza e correlaciona genes encontrados *in silico* em regiões de QTLs previamente mapeados, selecionando e testando genes candidatos envolvidos na resistência do cacaueiro a *C. cacaofunesta* por RT-qPCR. Encontramos 156 genes identificados na região LXD-GL3 e 166 na região LXD-GL9, que foram reduzidos a 17 e 19 genes candidatos, respectivamente, e pelo menos 2 genes (osmotina e taumatina) são especialmente interessantes. O nível de transcrição de osmotina é geralmente baixo no início da infecção (6 horas após a inoculação), aumenta ligeiramente aos 48 hai e aumenta rapidamente ao longo dos dias (7 e 15 dias após a inoculação) nos dois genótipos estudados. O padrão de expressão da taumatina no genótipo suscetível CCN 51 aumentou abruptamente nos primeiros dias após a infecção (7 e 15 dai), enquanto o genótipo resistente TSH 1188 apresentou um aumento menor em 7 dai e 15 dai. A atividade antifúngica desses genes os torna candidatos à engenharia genética na produção de plantas resistentes a doenças.

Palavras-chave: *Ceratocystis cacaofunesta*, QTLs, osmotina, taumatina, expressão diferencial.

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Introduction

The genus Ceratocystis includes several fungal species in various parts of the world. In Brazil, there are reports of the existence of three species: *C. paradoxa*, attacking mainly monocotyledons, *C. cacaofunesta* causing great problems in cacao plantations (*Theobroma cacao*) (Bezerra, 1997), and *C. fimbriata*, which causes disease in many cultures of economic importance such as mango (*Mangifera indica*) and eucalyptus (*Eucalyptus* spp.). In Brazil in 2001 this fungus was reported causing great problems in cacao plantations, causing a disease known as Ceratocystis wilt (CW). Currently in the cacao region of the State of Bahia the disease is already widely disseminated and the deaths already add up to the equivalent of 24% of the plants, mainly those derived from the clone ICS-1, such as the variety Theobahia (ICS-1 x Sca-6) (Silva and Luz, 2000; Ram, Valle and Freitas 2004), that are used as a source of resistance to witches' broom disease.

Ceratocystis cacaofunesta is a pathogen of difficult control, causes irreversible damage to the vascular system of the plant and in a short time the plant dies, but the dead leaves remain adhered for a long time, passing the fungus to grow in the decomposing tissue where the sexed and asexual spores are formed (Silva, Paim and Castro, 2004; Tumura, De Pieri and Furtado, 2012). The use of resistant cacao genotypes is the most efficient method to control of the disease (Silva, Paim and Castro, 2004; Silva et al., 2012; Delgado and Suárez, 2003; Baker et al., 2003; Alarcon, 1994; Lawrence, Campêlo and Figueredo, 1991).

In recent years, researchers around the world have used genomic information to identify disease resistance genes in cocoa (Micheli, Guiltinan and Gramacho, 2010; Argout et al., 2011). It is necessary to identify a large number of genes, so as to be able to study defense mechanisms that contribute to the development of resistant genotypes (Vrain, 1999). The cacao tree has a large database of ESTs composed of 56 cDNA libraries, including three libraries of interaction between cocoa (Jaca resistant genotype) and *C. cacaofunesta* (Argout et al., 2008; Jones et al., 2002; Gesteira et al., 2003; Verica et al., 2004), in addition to its decoded genome V1 (https://cocoa-genome-hub.southgreen.fr/genome-browser) made from the Criollo clone B97 (Argout et al., 2011). One strategy for the identification of candidate genes is the *in silico* detection of the genes present in the resistance linked genomic regions (QTLs). Santos et al. (2012b) established the quantitative nature of the resistance to CW of cacao.

In this study, 2 QTLs were found linked to CW resistance in linkage groups (LG) 3 and 9 (6.9% and 8.6 % of the phenotypic variation, respectively) from a segregating F2 Sca-6 x ICS-1 population using SSR markers and EST-SSRs developed for this interaction (Santos et al., 2012a). The identification of genes already described in the literature as correlated to mechanisms of resistance in the QTL region may suggest the association of these genes to resistance to *C. cacaofunesta* in cacao, allowing the use of these genes for RT-qPCR studies and to confirm their possible association with this trait.

In this context, the present work was developed to locate and correlate in silico genes found in the region of mapped QTLs, selecting and testing candidate genes involved in the resistance of cacao to CW via RT-qPCR.

Materials and Methods

Identification of genes in the QTL region

The molecular markers located flanking or in peaks of QTLs for resistance to CW (Santos et al., 2012b) were used (Table 1) as an anchor point in the physical genome of cacao V1 (https://cocoa-genome-hub.southgreen.fr/genome-browser) (Argout et al., 2011). The genetic reference map of the cacao was used (Pugh et al., 2004), a search in the NCBI (http://www.ncbi.nlm.nih.gov/gquery/$Url) for the marker mTcCIR135 for the LXD-GL3 and the marker mTcCIR266 for the LXD-GL9 was performed. The sequence containing the marker was then searched for in the cocoa genome (http://cocoagendb.cirad.fr/gbrowse/cgi-bin/searching.pl). From the genome location, a search for 500 kb genes above and below the marker was made, totaling 1Mb per selected region (Figure 1).

The functional annotation of the genes was performed using the Blast2GO program (Gotz et al., 2008) using BlastN (E-value $\leq 1e^{-3}$) in the non-redundant database (NR) of the NCBI. The mapping of ontology terms (GOs) was performed according to

Table 1. QTLs identified by Santos et al. (2012b) for resistance to Ceratocystis wilt

QTL	Linkage group	Flanking markers	Peak marker of QTL	Position in CM in map (Pugh et al., 2004)
LXD-G3	3	mTcCIR254-mTcCIR128	mTcCIR135	78.63
LXD-G9	9	mTcCIR266-mTcCIR126	mTcCIR108	8.22

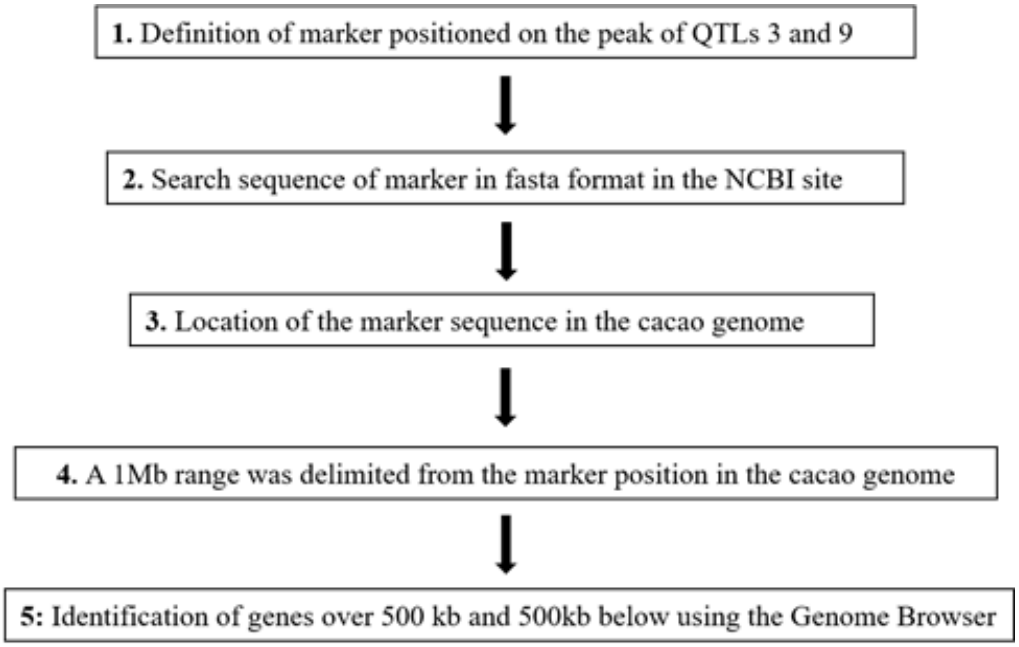

Figure 1. Schematic representation of the methodology used to identify genes close to the QTLs of LG 3 (Tc03) and LG 9 (Tc09).

Blast2GO instructions (http://www.blast2go.org/localgodb). Protein domains were mapped to Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan) using the parameters determined by the Blast2GO program.

Inoculation of *C. cacaofunesta* in cocoa seedlings

Seedlings of the clones TSH 1188 (resistant to *C. cacaofunesta*) and CCN 51 (susceptible to *C. cacaofunesta*) from *Theobroma cacao* L. were grown in the CEPEC/CEPLAC greenhouse on a previously sterilized substrate under natural light and relative humidity of 90 %. The susceptible clone CCN 51 and the resistant clone TSH 1188 were chosen based on evidence of their demonstrated responses to CW from field progeny trials (Silva et al., 2006; Sanches et al., 2008; Santos et al., 2012a). The inoculation was carried out in six-month-old seedlings through a scalpel incision above the first stem node, where 30 ì l of a suspension at 3.0 x 10^4 CFU/mL of the isolate Cf 20 were deposited with an automated pipette. A moistened cotton was then placed below the incision to form a wet chamber and the site was sealed with adherent plastic tape (screw-thread).

Control seedlings were inoculated with sterile water and submitted to the same growth conditions. Typical symptoms of the disease were observed in 7 days in the CCN 51 clone, and at 15 days the plants were dead. The TSH 1188 clone did not develop visible symptoms. Approximately 125 mm^3 of the tissue (stem) at the site where the inoculation was done were excised in 3 seedlings. Tissue stem from inoculated and non-inoculated (control) of CCN 51 and TSH 1188 plants were collected at 6, 48 hours of inoculation) (hai) and 7, 15 days after inoculation (dai), determined based on histopathological studies performed by Santos et al. (2013). The collected tissues were frozen in liquid nitrogen and stored at -80ºC until use.

Extraction of total RNA from cocoa stem tissues and cDNA synthesis

Total RNA was extracted using the RNAqueousTM kit according to the manufacturer's instructions (Ambion®). RNA concentrations were determined by the GeneQuant II RNA/DNA Calculator spectrophotometer (Pharmacia Biotech). The synthesis of the cDNA was performed using the total RNA treated with DNAse according to the recommendations of the manufacturer of the High Capacity RNA-to-cDNA kit (Applied Biosystems).

Primers used and RT-qPCR

The pairs of primers were designed using Primer Express 3.0 software (Applied Biosystems) and were based on sequences selected from the previously described cocoa cDNA libraries (Gesteira et al., 2007) and the endogenous reference genes for cocoa according to Pinheiro and Litholdo (2011) are shown in Table 2.

As a calibrator, the treatment not inoculated with a pool of the 3 times tested for both genotypes was used. All samples used in the assay (target gene, endogenous gene, calibrator and NTC) were amplified in experimental triplicates for each biological replicate. Expression analyzes were performed on model 7500 Fast Thermal cycler (Applied Biosystems) using SYBRgreen. The reaction consisted of 1ul of the cDNA, 0.3µl of each primer (F-R) and 5µl of qRGreen PCR SuperMix (Invitrogen) to a total volume 10ul of each reaction. The reaction conditions were 50° C for 20 min, 95° C for 10 min followed by 40 cycles of 95° C for 15 min, 58 °C for 1min. To generate a lower cycle Threshold (Ct) a test was performed to verify the cDNA concentration as well as a concentration test of the primers to generate a lower Ct, higher ÄRn and free of dimers. Before evaluating the expression profile of the gene, a test of the efficiency of the assay was performed, performing serial dilutions with 5 dilutions and 3 replicates, and the evaluation done by the slope indication of the standard curve and R^2. After the reaction the data were collected and stored in Software 7500 version 2.0.5. The results were normalized using DataAssist ™ software ver.3.01 (Life Technology).

Table 2. Primers used to evaluate the gene expression of cacao genotypes with primers sequence, amplification efficiency and R^2 obtained by RT-qPCR

Gene	Description Sequence (5'-3')	Efficiency	R^2
Taumatin	F:CGGAGCGTGAAAGAGGAAT R:ACCAGCTTCAAAACCACCAG	1,93	0,98
Osmotin	F:TGTTGCATCCTGGTACTGCT R:GGCTTTCTTGCACTGGTACAC	1,94	0,99
Tubulin*	F:TCCTCTTCCAGCCATCTCTC R:TCTCCTTGCTCATTCGGTCT	1,95	0,99
EF1α*	F:AGGTCCACCAACCTTGACTG R:TTGGGCTCGTTAATCTGGTC	1,98	0,99

* Endogenous reference genes for cocoa according to Pinheiro et al (2011).

Results and Discussion

Search and annotation of the genes found in the QTLs region

A search and annotation of the genes comprised between 1Mb from the QTLs identified by Santos et al. (2012b) in the cacao genome (Argout et al., 2011; http://cocoagendb.cirad.fr/gbrowse) is performed (Figure 1). QTLs are found in LG 3 (LXD-GL3) and in LG 9 (LXD-GL9). We found 156 probable genes for LXD-GL3 and 166 for LXD-GL9 using BlastN with moderate astringency (Astchul et al., 1997).

These genes were annotated for gene ontology (GO) (Ashburner et al., 2000), biochemical routes (KEEG) (Kanehisa et al., 2008) and conserved protein domains (Interproscan) (Hunter et al., 2009). The description of homologous genes and all individual annotations were manually inspected for their relevance in resistance to CW and selected genes that showed correlations with biological functions linked to biotic and abiotic stresses or that were important for resistance to diseases according to specialized literature.

Candidates genes for resistance were selected by searching for unigenes associated with the word "stress", where the 156 genes identified in the LXD-GL3 region and 166 in the LXD-GL9 region were reduced to 17 and 19 candidate genes, respectively. These genes are listed in Table 3. The specific terms used for the selection were: "stress response to abiotic stimulus" (GO: 0009628)"; "response to endogenous stimulus" (GO: 0009719); "response to extracellular stimulus" (GO: 0009991);" and "response to stress" (GO: 0006950).

Table 3. Candidate genes for Ceratocystis wilt resistance in the QTLs region

QTL	Unigene ID	Description Blast	Acess	Domains Interproscan	Description terms
LXD-GL3	Tc03:22999053..23000016	thaumatin-like protein	GO:0050832	1	defense response to fungus
	Tc03:23004824..23005876	osmotin-like protein	GO:0009817	6	defense response to fungus
	Tc03:23027163..23032865	RNA helicase	GO:0009651	1	response to salt stress
	Tc03:23108549..23111256	tubulin beta	GO:0009651	8	response to salt stress
	Tc03:23147754..23149975	glutathione peroxidase-l prot	GO:0006979	1	response to oxidative stress
	Tc03:23195032..23201524	glutamylcysteine synthetase	GO:0050832	1	defense response to fungus
	Tc03:23206263..23208820	Protein	GO:0071367	7	cellular response stimulus
	Tc03:23413483..23420621	beta-adaptin-like protein b	GO:0050690	4	regulation of defense
	Tc03:23421083..23427653	dna repair and transc factor	GO:0009636	1	response to toxin
	Tc03:23442911..23444152	zinc finger	GO:0009737	1	response to abscisic acid stimulus
	Tc03:23531067..23535825	big map kinase	GO:0009737	5	response to abscisic acid stimulus
	Tc03:23538562..23541706	heat shock protein 81-2	GO:0009414	5	response to water deprivation
	Tc03:23658247..23663956	respiratory burst oxidase	GO:0050832	1	defense response to fungus
	Tc03:23721620..23726477	calcium-dependent protein kinase	GO:0009651	7	response to salt stress
	Tc03:23759396..23760428	Protein	GO:0006952	5	defense response
	Tc03:23822312..23825122	6-phosphogluconate dehydrogenase	GO:0009749	10	response to glucose stimulus
	Tc03:23922317..23975326	bax inhibitor	GO:0006983	1	ER overload response
LXD-GL9	Tc09:721425..723705	enhancer of rudimentary	GO:0050832	1	defense response to fungus
	Tc09:728135..730454	glutathione peroxidase	GO:0080167	1	response to karrikin
	Tc09:769270..775070	btb poz domainprotein npy1-like	GO:0009416	4	response to light stimulus
	Tc09:831770..833752	cbl-interacting protein kinase	GO:0050832	10	defense response to fungus
	Tc09:897196..901975	Protein	GO:0009408	1	response to heat
	Tc09:940710..942098	acidic mammalian chitinase-like	GO:0009753	8	response to jasmonic acid stimulus
	Tc09:974742..975760	ap2 erf domain transcription factor	GO:0003700	1	transcription factor activity
	Tc09:983715..988221	aspartate aminotransferase	GO:0009409	1	response to cold
	Tc09:1078730..1080693	flotillin-like protein	GO:0071456	5	cellular response to hypoxia
	Tc09:1127016..1133091	protein topless	GO:0009733	1	response to auxin stimulus
	Tc09:1204374..1208993	protein far1-related sequence 6-like	GO:0009639	4	response to red or far red light
	Tc09:1104406..1107482	octicosapeptide phox domain protein	GO:000695	8	response to stress
	Tc09:1244231..1247337	h+-transp atp synthase-like protein	GO:0042742	3	defense response to bacterium
	Tc09:1256175..1257803	indole-3-acetic acid inducible 29	GO:0009733	1	response to auxin stimulus
	Tc09:1293802..1296902	l-ascorbate peroxidase	GO:0006979	1	response to oxidative stress
	Tc09:1391201..1396114	nitrate transporter	GO:0009611	3	response to wounding
	Tc09:1441437..1442156	low temp and salt responsive protein	GO:0050832	1	defense response to fungus
	Tc09:1619307..1621132	protein kinase	GO:0009414	1	response to water deprivation

Terms used for selection of candidate genes: response to abiotic stimulus (GO: 0009628); response to biotic stimulus (GO: 0009607); response to the endogenous stimulus (GO: 0009719); response to the extracellular stimulus (GO: 0009991); response to stress (GO: 0006950). The following terms have been omitted because of the low information content: biological process (GO: 0008150); biosynthesis process (GO: 0009058); connection (GO: 0005488); catalytic activity (GO: 0003824); cell process (GO: 0009987); DNA metabolic process (GO: 0006259); metabolic process (GO: 0008152). binding to proteins (GO: 000551).

Of the 17 genes selected for the LXD-GL3 region and the 19 genes for LXD-GL9, 8 and 5 genes respectively, were noted in the category "response to biotic factors" (GO: 0009607) and at least 2 of these 13 genes are especially interesting and were selected for confirmation of the differential expression via RT-qPCR. They are located on chromosome 3 at positions 22999053 to 23000016 and 23004824 to 23005876 (87.05% and 86% similarity; BLASTN E-value = $4.10E^{-87}$ and $1.06E^{-148}$, respectively) being coded for osmotin (osmotin-like protein) with 6 related protein domains in Interproscan and thaumatin (thaumatin like-protein) (Table 3). Both osmotins and thaumatins belong to the family of the Pathogenesis Related Proteins 5 (PR5) (Liu, Sturrock and Ekramoddoullah, 2010), implicated in acquired and induced systemic resistance (Dong, 2001). Members of PR5 family have shown antifungal action, including against fungi that attack cocoa (Resende et al., 2002, 2010).

It is important to note that these data do not prove a causal relationship between the genes identified and the probable genes involved in resistance to CW, but these genes are, however, good candidates for validation and differential gene expression experiments using RT-qPCR. Therefore, the study of the differential expression of genes in resistant and susceptible CW cacao clones under conditions of induction of expression by the pathogen will allow a better understanding of the plant-pathogen relationship, representing a valuable source of information that may be used in identification studies of resistance genes.

Expression of osmotin and thaumatin genes after fungal infection in cocoa

To study the role of PR5 osmotin and thaumatin in cacao, we analyzed the accumulation of transcripts in two contrasting genotypes for resistance to CW. CCN 51 is a highly susceptible and TSH 1188 highly resistant genotype (Silva et al., 2012a). The progress of the disease after inoculation of these genotypes with *C. cacaofunesta* at different inoculation times (6 and 48 hai and 7 and 15 dai) can be observed in Figure 2. It can be observed that the inoculated TSH 1188 seedlings showed no symptoms, whereas in CCN 51 it is possible to notice characteristic progressive wilt and disease from 7 dai (Figure 2).

PR5 may also promote the release of phytoalexin elicitors (Bol, Linthorst, and Cornelissen, 1990; Kuc, 1985; Neuenschwander et al., 1995) as well as induce the synthesis of phenolic compounds (Keen and Yoshikawa, 1983; Kurosaki, Amin and Nishi, 1986). It is important to note that in histological studies of the *C. cacaofunesta*-cacao interaction was found a high concentration of phenolic compounds in the genotypes elicited with the fungus (Santos et al., 2013), indicating that these proteins may be related to the defense process in this patosystem. Studies have already confirmed the action of thaumatins against *Phytophthora* spp., causal agent of the brown rot disease of cacao (Evans, 2007).

These two genes are present in the libraries constructed from the *C. cacaofunesta*-cacao interaction. (Argout et al., 2008). We monitor the levels of transcription of osmotin and thaumatin after infection by *C. cacaofunesta*. In both genes analyzed, a discrete increase in transcript levels in the first hours after inoculation (6 and 48 hai) and a large increase in later times (7 and 15 days) (Figure 3) were observed. Other factors that stimulate the expression of both thaumatin and osmotin are: abscisic acid (ABA), wounds, tobacco mosaic virus and ethylene (Singh, Makkar and Negi, 1989; Neale et al., 1990; Casas et al. (1992), which corroborates the expression levels of these genes in the initial times (6 and 48 hai) (Figure 3).

RT-qPCR analysis revealed that the level of osmotin transcription is generally low at the start of infection (6 hai), increases slightly at 48 hai, and rapidly increases

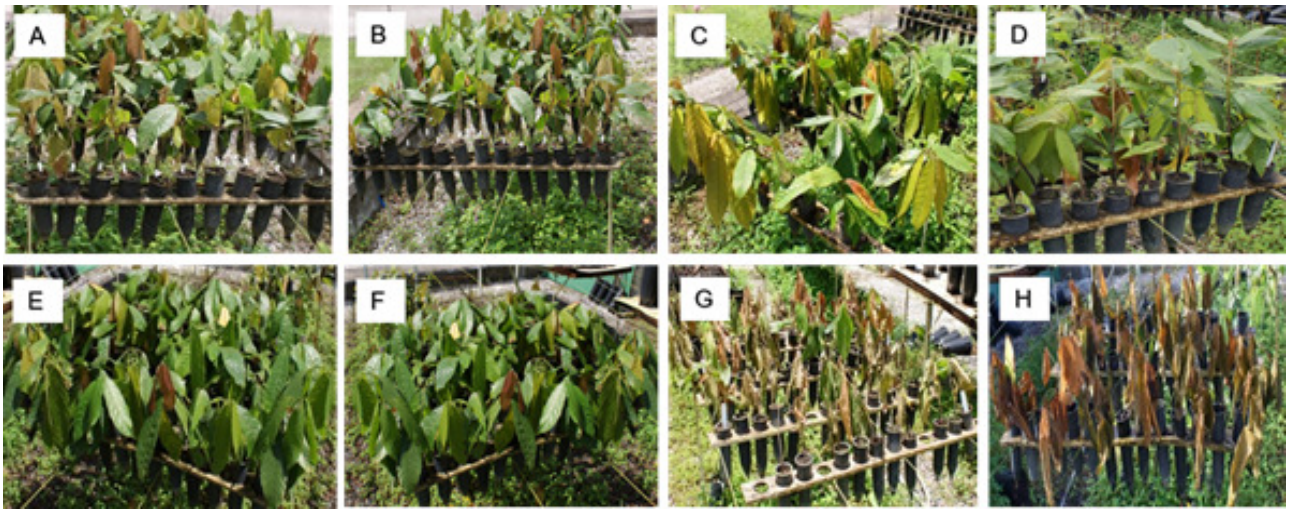

Figure 2. Cocoa seedlings inoculated with *Ceratocystis cacaofunesta*. A) TSH 1188 6 hai; B) TSH1188 48 hai; C) TSH 1188 7 dai; D) TSH 1188 15 dai; E) CCN 51 6 hai; F) 48 hai; G) CCN 51 7 dai; H) CCN 51 15 dai.

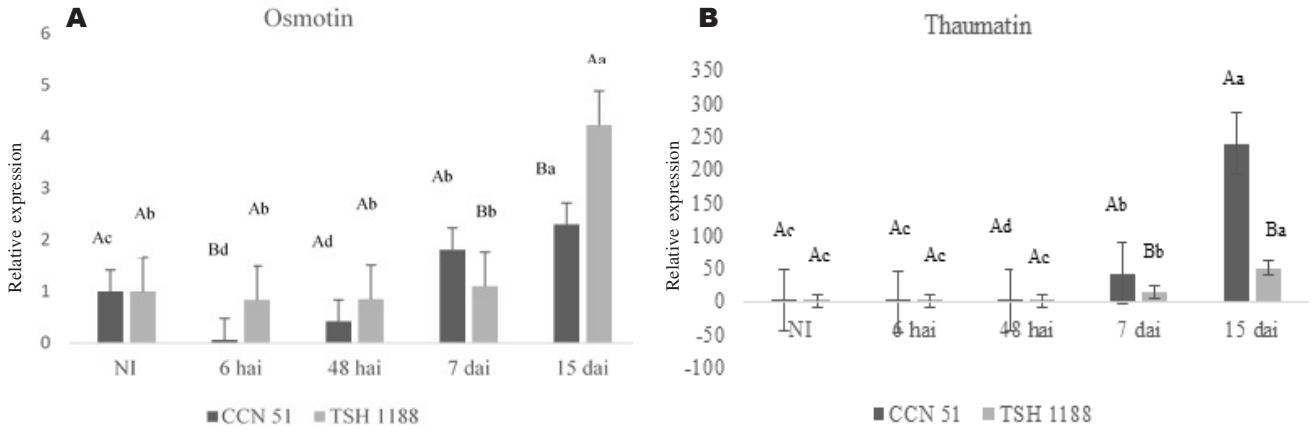

Figure 3. Relative expression based on RT-qPCR data from: **A**) the osmotin gene at 5 inoculation times (NI (control -not inoculated), 6 and 48 hai; 7and 15 dai) and **3B**) the thaumatin gene at 5 inoculation times (NI (control - not inoculated), 6 and 48 hai; 7and 15 dai) in CCN 51 and TSH 1188 of *C. cacaofunesta*-cacao interaction. The control, used as calibrator (for this reason is always 1), corresponds to the average of the expression values of Osmotin or Thaumatinin in 3 non-inoculated samples in each genotype (see also Methods section). The results are the arithmetical mean of the repetitions ± standard error. Different letters indicate significant statistical difference between samples by the Scott-Knott test ($P \leq 0.01$): lower case letters correspond to statistics between harvesting times for each genotype while upper case letters correspond to statistics between genotypes for each harvesting time. hai: hours after inoculation; dai: days after inoculation.

(approximately 4.5×) over the course of days (7 and 15 dai) in the two genotypes studied (Figure 3A).

Osmotin-like proteins (OLPs) are proteins of approximately 26 kDa belonging to the PR5 family, which have a high degree of homology to the thaumatin protein (Yun et al., 1997). The first osmotin was described in cultures of tobacco plant cells (*Nicotiana tabacum*) adapted to salinity conditions (Singh and Bracker, 1987). After the discovery of osmotin many studies have shown that its synthesis in plants is regulated by various hormonal and environmental signals including fungal infections, mechanical injury, salinity, desiccation, cold, ultraviolet radiation and hormones such as auxins, ethylene and abscisic acid (Noori and Sokhansanj, 2008). These proteins are indispensable molecules for plant resistance, since they are associated with the defense of plants to biotic and abiotic stresses.

Many studies describe the role of osmotin in the defense against pathogens. In plants of tobacco and tomato, it was discovered that osmotins are induced when the plants are in contact with pathogens of viral and fungal origin (Stintzi, Heitz and Prasad, 1993; Woloshuk and Meulenhoff, 1991), indicating a probable relation with the mechanisms of resistance to pathogenic attacks. There are reports in the literature that overexpression of these proteins in susceptible plants confers resistance to pathogens (Rajam et al., 2007).

In addition to defense against pathogens, there are many studies showing the protective action of osmotin to abiotic damage. Studies show the relationship of osmotin in stress protection caused by mechanical damage (Nelson, Salamini and Bartels, 1994), by cooling (Zhu, Chen and Li, 1995; Hong et al., 2003), and drought (Zhu, Chen and Li, 1995).

Thaumatin is also a protein of the PR5 group. In tobacco and other plant species, the proteins belonging to this group exhibit activity of inhibition of the growth of hyphae or spore germination of different fungi in vitro, probably through a mechanism of permeabilization of membranes (Stintzi, Heitz and Prasad, 1993). The expression pattern of thaumatin was different and contradicted the constitutive levels (Figure 3B). In the susceptible CCN 51 genotype, transcription levels increased abruptly in the first days after infection (7 dai- 43x and 15 dai - 245x), while the resistant genotype TSH 1188 showed a smaller increase in 7 dai (15x) and 15 dai (51x) (Figure 3B).

Anti-fungal properties of thaumatin were observed in Arabidopsis and other plants whose expression levels during fungal infection increased in both resistant and susceptible cultivars (Elkereamy and Guevara, 2011). The thaumatin protein induced expression under

conditions of low water potential. In this condition it accumulates about 15 times more than under normal water conditions (Singh, Makkar and Negi, 1989). It should be noted that one of the main symptoms of CW is the water deficit caused by fungus blockade in the xylem, which could explain the large increase in the expression of this gene in the susceptible CCN 51 genotype at 7 and 15 dai (Figure 3B). Other factors that stimulate the expression of both thaumatin and osmotin are: abscisic acid (ABA), wounds, tobacco mosaic virus and ethylene (Singh, Makkar and Negi, 1989; Neale et al., 1990; Casas et al., 1992).

The antifungal activity of these genes makes them candidates for genetic engineering in the production of disease resistant plants. Transgenic banana plants expressing the TLP (thaumatin-like protein) gene of rice challenged with Fusarium fungus presented higher resistance to *Fusarium oxysporum* sp. Cubense (race 4) in relation to non-inoculated plants (Mahdavi, Sariah and Maziah, 2012).

Conclusion

By means of the *in silico* analyzes presented this work it was possible to identify and select of two genes that are candidates for CW resistance. The identification and characterization of these genes enables the subsequent studies, such as analyzing the differential expression between plants grown in field and greenhouse, or prospecting natural variability using different genetic materials, thus enabling the identification and validation of polymorphisms for the development of molecular markers associated with CW resistance. The methodology used in this study allowed the identification of several genes with expression differential in response to CW resistance, opening new experimental possibilities for the characterization of function of these genes.

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Literature Cited

ALARCON, C. R. M. 1994. Determinacion de Resistencia de 250 Clones de Cacao de Origen Nacional al Ataque de Mal-de-Machete (*Ceratocystis fimbriata* Ellis & Halsted). Tesis de Grado. Universidad Agrária del Ecuador. Guayaquil, Ecuador. 125p.

ALTSCHUL, S. F. et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 17:3389-3402.

ARGOUT, X. et al. 2011. The genome of Theobroma cacao. Nature Genetics 43:101-108.

ARGOUT, X. et al. 2008. Towards the undestanding of the cacao transcriptome: Production and analysis of an exhaustive dataset of ESTs of *Theobroma cacao* L. generated from various tissues and under various conditions. BMC Genomics 9:512-523.

ASHBURNER, M. et al. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nature Genetics 6:25-29.

BAKER, C. J. et al. 2003. Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata.* Phytopathology, 93:1274-1284.

BEZERRA, J. L. 1997. *Ceratocystis fimbriata* causing death of budded cocoa seedlings in Bahia, Brazil. Incoped Newsletter 1:6-14.

BOL, J. F.; LINTHORST, H. J. M.; CORNELISSEN, B. J. C. 1990. Plant pathogenesis-related proteins induced by virus infection. Annual Review Phytopathology 28:113-38.

CASAS, A. M. et al. 1992. Expression of osmotin-like genes in the halophyte *Atriplex nummularia* L. Plant Physiol 99:329-337.

DELGADO, R.; SUÁREZ, C. 2003. Diferencias em agressividad entre aislamientos de Ceratocystis fimbriata de Ecuador y Brasil em cacao. In: XII Seminário Nacional de Sanidad Vegetal, 12. Latacunga, Ecuador. 8p.

DONG, X. 2001. Genetic dissection of systemic acquired resistance. Current Opinion in Plant Biology 4:309-14.

ELKEREAMY, A.; GUEVARA, D. 2011. Exploring the molecular and metabolic factors contributing to the adaptation of maize seedlings to nitrate limitation. Front Plant Science 2:49.

EVANS, H. C. 2007. Cacao diseases-the trilogy revisited. Phytopathology 97:89-95.

GESTEIRA, A. S. et al. 2003. Isolation and puriûcation of functional total RNA from different organs of cocoa tree during its interaction with the pathogen *Crinipellis perniciosa*. BioTechniques 35:494-500.

GESTEIRA, A. S. et al. 2007. Comparative analysis of expressed genes from cacao meristems infected by Moniliophthora perniciosa. Annals of Botany 100:129-140.

GÖTZ, S. et al. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Research 36:3420-3435.

HONG, S. W. et al. 2003. Arabidopsis hot mutants define multiple functions required for acclimation to high temperatures. Plant Physiology 132:757-767.

HERBERS, K. et al. 1996. Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell 87:78-98.

HUNTER, S. et al. 2009. InterPro: the integrative protein signature database. Nucleic Acids Research 57:875-897.

JONES, P. G. et al. 2002. Gene discovery and microarray analysis of cacao (*Theobroma cacao* L.) varieties. Planta 216:255-264.

KANEHISA, M. et al. 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Research 58:478-498.

KEEN, N. T.; YOSHIKAWA, M. 1983. 1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiology 71:460-465.

KUC, J. 1985. Increasing crop productivity and value by increasing disease resistance through non-genetic techniques. In: Symposium on Forest Potentials: Productivity and Value. Proceedings. 1985. Weyerhaeuser Co, Tacoma, Washington. pp.147-190.

KUROSAKI, F.; AMIN, M.; NISHI, A. 1986. Induction of phytoalexin production and accumulation of phenolic compounds in cultured carrot cells. Physiological and Molecular Plant Pathology 28:359-370.

LAWRENCE, J. S.; CAMPÊLO, A. M. F. L.; FIGUEIREDO, J. M. 1991. Enfermidades do cacaueiro. II - Doenças fúngicas que ocorrem nas folhas, ramos e tronco. Agrotrópica (Brasil) 3(1):1-14.

LIU, J. J.; STURROCK, R.; EKRAMODDOULLAH, A. K. 2010. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. Plant Cell Reports 29(5):419-436.

MAHDAVI, F.; SARIAH, M.; MAZIAH, M. 2012. Expression of rice thaumatin-like protein gene in transgenic banana plants enhances resistance to fusarium wilt. Biotechnology and Applied Biochemistry 166:1008-1019.

MICHELI, F.; GUILTINAN, M.; GRAMACHO, K. P. 2010. Functional genomics of cacao. Adv Bot Res 155:119-177.

NEALE, A. D. et al. 1990. Chitinase 1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2:673-684.

NELSON, D.; SALAMINI, F.; BARTELS, D. 1994. Abscisic acid promotes novel DNA-binding activity to a desiccation-related promoter of *Craterostigma plantagineum*. Plant Journal 5(4):451-458.

NEUENSCHWANDER, U. et al. 1995. Activation of plant disease resistance. Aspects of Applied Biology 42:217-225.

NOORI, S. A. S.; SOKHANSANJ, A. 2008. Wheat plants containing an osmotin gene show enhanced ability to produce roots at high NaCl concentration. Russian Journal of Plant Physiology 55:256-258.

PINHEIRO, T. T. ; LITHOLDO JR, C. G. 2011. Establishing references for gene expression analyses by RT-qPCR in *Theobroma cacao* tissues. GMR 10(4):3291-3305.

PUGH, T. et al. 2004. A new cacao linkage map based on codominant markers: development and integration of 201 new microsatellite markers. Theoretical and Applied Genetics 108:1151-1161.

RAJAM, M. V. et al. 2007. Thaumatin gene confers resistance to fungal pathogens as well as tolerance to abiotic stresses in transgenic tobacco plants. Biol Plant 51:135-141.

RAM, A.; VALLE, R. R. M.; FREITAS, D. B. 2004. Controle de cancro ou murcha de Ceratocystis do cacaueiro na Bahia, Brasil. Agrotrópica (Brasil) 16:111-114.

RESENDE, M. L. V. et al. 2002. Induction of resistance in cocoa against *Crinipellis perniciosa* and *Verticillium dahlia* by acibenzolar-S-methyl (ASM). Plant Pathology 51:621-628.

RESENDE, M. L. V. et al. 2010. Percepção, transdução e tradução de sinais para respostas de defesa contra patógenos em plantas. In: Simpósio de Biologia Molecular da Resistência de Plantas a Patógenos: aplicações no manejo integrado de fitodoenças, 1. Lavras, MG. pp.9-35.

SANCHES, C. L. G. et al. 2008. Assessment of resistance to *Ceratocystis cacaofunesta* in cacao genotypes. European Journal of Plant Pathology 122:517-528.

SANTOS, R. M. F. et al. 2012a. Identification, characterization and mapping of EST-derived SSRs from the cacao *Ceratocystis cacaofunesta* interaction Tree Genetics & Genomes 10:117.

SANTOS, R. M. F. et al. 2012b. Identification of QTLs linked to Ceratocystis wilt resistance in cacao. Molecular Breeding 4:30-38.

SANTOS, R. M. F. et al. 2013. Kinetics and histopathology of the Cacao - *Ceratocystis cacaofunesta* interaction. Tropical Plant Biology 6:37-45.

SILVA, S. D. V. M.; LUZ, E. D. M. N. 2000. *Ceratocystis fimbriata* em cacaueiros dasvariedades cultivadas na Bahia. Fitopatologia Brasileira 25:424.

SILVA, S. D. V. M.; PAIM, M. C. A.; CASTRO, W. M. 2004. Cacau Jaca resistente a *Ceratocystis fimbriata* na região cacaueira da Bahia, Brasil. Fitopatologia Brasileira 29:538-540.

SILVA, S. D. V. M et al. 2006. Indicações de resistência à murcha-de-ceratocystis em genótipos de cacaueiros no sul da Bahia, Brasil. In: Conferência Internacional de Pesquisas em Cacau, 15. San José, Costa Rica. pp.9-14.

SILVA, S. D. V. M. et al. 2012. Resistência de progênies de cacaueiro à murcha-de-Ceratocystis. Tropical Plant Pathology 37:191-195.

SINGH, N. K.; BRACKER, C. A. 1987. Characterization of osmotin and thumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiology 85(79):126-137.

SINGH, B.; MAKKAR, H. P. S.; NEGI, S. S. 1989. Rate and extent of digestion and potentially digestible dry matter and cell wall of various tree leaves. Journal of Dairy Science 72(12):3233-3239.

STINTZI, A.; HEITZ, T.; PRASAD, V. 1993. Plant Pathogenesis-Relsted proteins and their role in defense against pathogens. Biochimie 75:687-706.

TUMURA, K. G.; DE PIERI, C.; FURTADO, E. L. 2012. Murcha por Ceratocystis em eucalipto: avaliação de resistência e análise epidemiológica. Summa Phytopathologica 38(1):54-60.

VERICA, J. A. et al. 2004. Isolation of ESTs from cacao (*Theobroma cacao* L.) leaves treated with inducers of the defense response. Plant Cell Report 23:404-413.

VRAIN, T. C. 1999. Engineering natural and synthetic resistance for nematode management. Journal of Nematology 31:424-436.

WOLOSHUK, C. R.; MEULENHOFF, J. S. 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3:619-628.

YUN, D. J. et al. 1997. Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. Proceedings of the National Academy of Sciences 94:7082-7087.

ZHU, B.; CHEN, T. H. H.; LI, P. H. 1995. Expression of three osmotin-like protein genes in response to osmotic stress and funga1 infection in potato. Plant Mo1ecular Biology (in press). ●