Successful pod infections by *Moniliophthora roreri* result in differential *Theobroma cacao* gene expression depending on the clone's level of tolerance

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SUMMARY

An understanding of the tolerance mechanisms of *Theobroma cacao* used against *Moniliophthora roreri*, the causal agent of frosty pod rot, is important for the generation of stable disease-tolerant clones. A comparative view was obtained of transcript populations of infected pods from two susceptible and two tolerant clones using RNA sequence (RNA-Seq) analysis. A total of 3009 transcripts showed differential expression among clones. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of differentially expressed genes indicated shifts in 152 different metabolic pathways between the tolerant and susceptible clones. Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) analyses of 36 genes verified the differential expression. Regression analysis validated a uniform progression in gene expression in association with infection levels and fungal loads in the susceptible clones. Expression patterns observed in the susceptible clones diverged in tolerant clones, with many genes showing higher expression at a low level of infection and fungal load. Principal coordinate analyses of real-time qRT-PCR data separated the gene expression patterns between susceptible and tolerant clones for pods showing malformation. Although some genes were constitutively differentially expressed between clones, most results suggested that defence responses were induced at low fungal load in the tolerant clones. Several elicitor-responsive genes were highly expressed in tolerant clones, suggesting rapid recognition of the pathogen and induction of defence genes. Expression patterns suggested that the jasmonic acid–ethylene- and/or salicylic acid-mediated defence pathways were activated in the tolerant clones, being enhanced by reduced brassinosteroid (BR) biosynthesis and catabolic inactivation of both BR and abscisic acids. Finally, several genes associated with hypersensitive response-like cell death were also induced in tolerant clones.

Keywords: differential transcriptome, frosty pod rot, hemibiotroph, plant defence, RNA-Seq, tolerance.

INTRODUCTION

Theobroma cacao L. (cacao) is a major cash crop in the tropics and the source of chocolate, one of the world's most popular foods. In addition, cacao-based agroforestry systems provide a promising means to address the challenges of deforestation by creating a habitat for biodiversity, whilst simultaneously providing a profitable crop for agricultural communities (Perfecto *et al.*, 1996). However, this crop is currently under serious threat from diseases (Bowers *et al.*, 2001). Frosty pod rot (FPR) disease, caused by *Moniliophthora roreri* (Evans *et al.*, 1978), occurs in all the major cacao-producing areas in the Western Hemisphere, other than Brazil (Evans, 2007; Phillips-Mora *et al.*, 2007). *Moniliophthora roreri* is a hemibiotrophic basidiomycete that attacks only the pod, causing both internal and external damage, resulting in yield losses of up to 80% (Hidalgo *et al.*, 2003). Chemical control of FPR has failed to deliver effective management (Krauss and Soberanis, 2001) and phytosanitation practices are often not employed because of their cost and limited impact. Improved planting materials, combined with better agronomic practices, are probably the most feasible strategy for long-term cost-effective management of FPR. Although there are no cacao clones resistant to *M. roreri*, tolerant clones have been developed (Phillips-Mora, 2010; Phillips-Mora *et al.*, 2005). A knowledge of the host–pathogen interaction at the molecular level should enhance the success of the cacao breeding programme.

The ability to conduct large-scale computational analysis in functional genomics has opened the door to the identification of large sets of genes involved in biological processes. Investigation at the gene network level rather than of individual genes is necessary to understand the complex biological processes occurring

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during plant–microorganism interactions. Despite its economic importance, very few molecular studies have been carried out to understand the interaction between cacao and *M. royeri*. FPR has two distinct stages: biotrophic and necrotrophic. During the biotrophic phase, pods may develop malformations, and then progress to the necrotrophic phase where rot and sporulation occur (Bailey *et al.*, 2013; Evans, 2007; Evans *et al.*, 2002). *Moniliophthora royeri* appears to delay the host response until late in the biotrophic phase (Bailey *et al.*, 2013), when it is too late for the plant defence to work.

The Tropical Agriculture Research and Higher Education Center of Costa Rica (CATIE) is distributing FPR-tolerant clones in Central America (Phillips-Mora, 2010). These clones are not immune to *M. royeri* as disease occurs at very low rates, sometimes resulting in sporulation. The stability of the tolerance mechanism being used is unknown. For this reason, the identification of differentially expressed genes between susceptible and tolerant cacao clones responding to FPR infections is essential. The objective of this study was to obtain and characterize a global comparative view of cacao transcripts responsive to *M. royeri* infection, and enriched for transcripts differentially expressed between susceptible and tolerant cacao clones through RNA sequence (RNA-Seq) analysis. Transcript populations of infected pods from two susceptible clones (Pound-7 and CATIE-1000) and two tolerant clones (CATIE-R7 and CATIE-R4) were obtained. An additional tolerant clone (UF-273) was used to verify the results of RNA-Seq analysis. The ultimate goal was to identify cacao genes of potential importance in the interaction with FPR that could be targeted during plant breeding or in the development of other disease management strategies.

RESULTS

Development of FPR disease in cacao clones

Hand pollinations were successful for all clones except CATIE-1000. Subsequently, a second hand pollination was of limited success for CATIE-1000, but these pods were not inoculated. Artificial inoculations of pods derived from hand pollinations were efficient at infecting pods of the susceptible clone Pound-7, of limited success in infecting pods of the tolerant clones UF-273 and CATIE-R7, and unsuccessful in infecting CATIE-R4. As a result, pods showing symptoms of *M. royeri* infection from natural field inoculum were also used. Pound-7 pods showed symptoms of malformation at 30 days after inoculation (DAI) (Fig. S1, see Supporting Information), whereas tolerant CATIE-R7 and UF-273 showed very few pods with FPR symptoms. By 75 DAI, necrotic and/or chlorotic symptoms were common in Pound-7 infected pods (see Fig. S1). By specifically looking for naturally infected pods in the field over 4 months, a comprehensive collection of material for each clone was established for the differential transcriptomic studies.

Estimation of fungal load (Fr)

Fr was estimated as the percentage (%) of the geometric mean of the expression levels of three *M. royeri* reference genes (Refs) relative to the expression of three cacao Refs (detailed in Experimental procedures). Fr varies greatly among different clones. Among the malformed and necrotic pods, the susceptible clones (Pound-7 and CATIE-1000) tended to have a higher Fr relative to the three tolerant clones (CATIE-R7, CATIE-R4 and UF-273) (Fig. S2, see Supporting Information). As Fr was calculated on the basis of Ref expression, it was validated on the basis of the DNA amount using the quantification of *M. royeri* actin and internal transcribed spacer (ITS) relative to cacao actin. The correlations between the *M. royeri* Fr using DNA-based actin and ITS levels and RNA-based Fr were 0.86 ($P < 0.0001$) and 0.90 ($P < 0.0001$), respectively, using 57 pods from all five clones (Fig. S3, see Supporting Information).

RNA-Seq analysis

The RNA-Seq analysis approach used samples with similar Fr of around 10% for two susceptible clones (Pound-7 and CATIE-1000) and two tolerant clones (CATIE-R7 and CATIE-R4). This level of Fr was chosen on the basis of previous experiments as a minimum level giving reasonable expectations of the detection of significant *M. royeri* transcripts, an aspect considered elsewhere (Bailey *et al.*, 2014). At 10% Fr, pods from susceptible clones were in the biotrophic phase of infection without chlorosis (Ch) or necrosis (Nc). Symptoms were poorly associated with Fr levels in the tolerant clones, and Ch or Nc was common at 10% Fr. The RNA-Seq analysis identified 25 984, 27 225, 25 257 and 27 158 possible cacao transcripts for the libraries of CATIE-R4, CATIE-R7, CATIE-1000 and Pound-7, respectively. After normalization, the base mean number of reads for each cacao Ref varied among clones as follows: *TcACP1* (1473–2105 reads), *TcEF1 α* (29 405–38 923 reads) and *TcACT* (25 644–40 230 reads). The geometric mean range for the three cacao Refs as calculated for each clone varied from 11 875 reads to 13 360 reads, a difference of only 12.5% or less between clones after normalization.

There were 3009 differentially expressed transcripts between at least one clone pairing based on an adjusted P value of ≤ 0.05 (Benjamini and Hochberg, 1995). The foremost number of reads per transcript ranged between seven and 782 937 (Excel file S1, see Supporting Information). There were 621 and 144 transcripts induced, and 1526 and 206 transcripts repressed, in CATIE-R4 relative to Pound-7 and CATIE-1000, respectively. Similarly, there were 641 and 89 transcripts induced, and 1302 and 79 transcripts repressed, in CATIE-R7 relative to Pound-7 and CATIE-1000, respectively. Among these, 391 and 1040 transcripts showed induction and repression, respectively, in more than one clone comparison. Only 18 transcripts were induced and 35 transcripts were repressed in a comparison of the two tolerant clones with

Fig. 1 Differential transcriptomes between tolerant *Theobroma cacao* clones CATIE-R4 and CATIE-R7 and susceptible cacao clones Pound-7 and CATIE-1000 in response to frosty pod rot caused by *Moniliophthora roreri*. Numbers of transcripts induced (A) and repressed (B) in the tolerant relative to the susceptible clones. Transcripts are RNA reads identified from RNA sequence (RNA-Seq) libraries aligned to the coding sequences of the cacao genome (Argout *et al.*, 2010). The differential expression ($P \leq 0.05$) level was determined using the count data of each gene with three replicates for each library.

the two susceptible clones in all combinations (Fig. 1). Among all the differential expression transcripts in the tolerant clones, 657 induced transcripts and 1138 repressed transcripts indicated proteins with known function. Thirty-six genes were selected for real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) analysis to confirm the RNA-Seq results. Gene ontology (GO) analysis of the four sets of genes differentially expressed between individual susceptible and tolerant clones showed substantial differences [see Fig. 2 and Excel file S2 (Supporting Information)]. Genes belonging to various GO categories responding to defence, biotic and abiotic stresses, heat and wounding were induced in larger numbers in the tolerant clones. There were 16–19 genes involved in the cell cycle that were repressed and one induced in the tolerant clones. Genes involved in the abscisic acid (ABA), jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) hormone signal pathways were induced in the tolerant clones, whereas genes of the brassinosteroid (BR)- and gibberellic acid (GA)-mediated pathways were repressed in the tolerant clones (Fig. 2). Similarly, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis involving the differentially expressed gene sets indicated a shift in 152 different metabolic pathways (Excel file S3, see Supporting Information). The top five KEGG pathways containing the largest numbers of induced genes in tolerant clones were plant hormone signal transduction, plant–pathogen interaction, biosynthesis of amino acids, starch and sucrose metabolism, and protein processing in endoplasmic reticulum. The top five KEGG pathways containing the largest numbers of repressed genes in tolerant clones were cell cycle, amino sugar and nucleotide sugar metabolism, plant hormone signal transduction, DNA replication and carbon metabolism. Shifts in expression were observed in key metabolic pathways involved in the disease response, including amino sugar and nucleotide sugar metabolism, steroid biosynthesis, plant hormone signal transduction, BR biosynthesis, glycolysis and the citrate cycle, plant–pathogen interactions, glycolysis/gluconeogenesis, galactose metabolism, and starch and sucrose metabolism (Fig. S4, see Supporting Information). Chitinase, phosphoenolpyruvate carboxylase and alcohol dehydrogenase were induced in the

tolerant clones, whereas malate dehydrogenase, fructose-1,6-bisphosphate, α -galactosidase and xylan-1,4- β -xylosidase were repressed in the tolerant clones. Genes involved in ABA-, SA- and GA-mediated signal transduction were induced in the tolerant clones, whereas genes involved in auxin-, cytokinin- and BR-mediated signal transduction were repressed in the tolerant clones. Various defence genes, including those associated with the hypersensitive response (HR), were induced in the tolerant clones.

Real-time qRT-PCR analysis of cacao gene expression in pods of susceptible and tolerant clones

Using data from the 12 RNA samples used for RNA-Seq analysis, 16 of the 18 genes selected on the basis of RNA-Seq results showed significant correlations ($P \leq 0.05$) between the RNA-Seq and real-time qRT-PCR results. Only four of the 18 previously studied genes showed significant correlations ($P \leq 0.05$) between RNA-Seq and real-time qRT-PCR results (Table S1, see Supporting Information). Fr for each pod is presented in Figs 3 and 4, together with the associated symptoms and gene expressions. A pattern of increased symptoms, as assessed by malformations, Ch and Nc, in association with increasing levels of Fr was observed for CATIE-1000 and Pound-7 pods (Fig. 4). The relationship between Fr and symptoms diverged greatly in tolerant clones. Based on the real-time qRT-PCR data of the artificially inoculated pods, almost all of the cacao genes selected were induced by *M. roreri* infection in both the susceptible and tolerant clones (Fig. 3). For some genes, temporal variation occurred in uninfected pods, but this was limited compared with *M. roreri*-induced variation. Variation in gene expression between clones for uninfected pods was limited with few exceptions (*TcChi4*, *TcAIL1* and *TcACO*) (Fig. 3).

Several patterns of gene expression were observed when comparing real-time qRT-PCR expression data with the Fr and pod symptoms (Fig. 4). The real-time qRT-PCR expression profiles for the susceptible clones showed relatively uniform progressions in gene expression for most genes. Similar progressions were less distinctive for the set of genes used in previous studies on abiotic and biotic stresses (Fig. 4). These progressive patterns diverged greatly when considering the

Fig. 2 Shift in the number of differentially expressed genes among key biological processes involved in the disease response between tolerant and susceptible *Theobroma cacao* clones in response to *Moniliophthora roreri* infection in pods. Differentially expressed genes were identified using RNA sequence (RNA-Seq) analysis between two tolerant clones CATIE-R4 and CATIE-R7 and two susceptible clones CATIE-1000 and Pound-7. Gene ontology analysis was carried out using the program Blast2GO (Conesa *et al.*, 2005). ABA, abscisic acid; BR, brassinosteroid; ET, ethylene; GA, gibberellic acid; HR, hypersensitive response; JA, jasmonic acid; PCD, programmed cell death; SA, salicylic acid.

tolerant clones. For example, *TcAmy*, *TcWAX2*, *TcADH2*, *TcPLP*, *TcLTP1*, *Tcqbda*, *TcGA2OX2*, *TcCNLS* and *TcOPR2* showed higher levels of expression in tolerant clones than in susceptible clones (Fig. 4). The expression of all the RNA-Seq-selected genes was induced as Fr and symptoms increased. When compared with the tolerant clones, in most pods, *TcCesA*, *TcHSP17.4*, *TcCYP89A2*, *TcWRKY*, *TcCesAG2* and *TcABCg39* were expressed at much lower levels in both susceptible clones when Fr and symptom levels were low. *TcPR10C* was expressed at lower levels in the tolerant clones

CATIE-R4 and UF-273 relative to both the susceptible clones and CATIE-R7 (Fig. 4). Differences occurred between the expression patterns of the susceptible clones. For example, *TcODC*, *TcChi1*, *TccrtZ* and *TcACO* were expressed at lower levels in the susceptible clone Pound-7 relative to the other clones (Fig. 4).

Multiple linear regression within clones was used to analyse the real-time qRT-PCR data to determine the relationships between the designated independent variables weight (Wt), Ch, Nc and Fr and the expression of individual cacao genes (dependent

Fig. 3 Relative expression of fungal reference genes (fungal load, Fr), symptoms of infection and expression of 36 *Theobroma cacao* genes analysed over 74 artificially infected and uninfected pods representing two susceptible clones (Pound-7 and CATIE-1000) and three tolerant clones (UF-273, CATIE-R7 and CATIE-R4). The expression levels for each of the three *Moniliophthora roreri* reference genes (Refs) in each sample were calculated as % cacao Ref(1–3) = $100 \times [(E)^{\Delta CT}]$, where E is the primer efficiency. Fr was estimated as the geometric mean of the expression levels of the three *M. roreri* Refs. Symptoms of pod infection: –3, uninfected; –1, malformed; +1, chlorotic; +3, necrotic. Gene expression is the $\log_{10}$ value of the relative mRNA expression (% cacao Ref) in each pod. Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) was conducted using RNA harvested from infected (+) and uninfected pods (C) at regular time intervals (0 and 30 days after inoculation).

variable). Significant ($P \leq 0.05$) linear regression models with the largest R^2 are presented for each of 36 cacao genes in Table 1. For CATIE-R4, seven genes showed highly significant models ($P \leq 0.001$), most including Wt. For CATIE-R7, 11 genes showed highly significant models ($P \leq 0.001$), most including Nc in combination with other variables. For CATIE-1000, 19 genes showed highly significant models ($P \leq 0.001$), with six models including both Ch and Nc. Pound-7 had 26 genes with highly significant models ($P \leq 0.001$), with Wt, Ch and Nc being components of 15, 15 and 18 models alone or in combination with other variables, respectively. For UF-273, there were six very highly significant models among both sets of genes, and 16 models including Ch alone or in combination with other variables.

Principal coordinate analysis (PCoA) was carried out using the real-time qRT-PCR data from 36 genes for the pods sampled from artificially inoculated plots and for field-collected samples from all five clones in various combinations (Fig. 5): (i) all 242 pods, highlighting different stages of infection; (ii) all 140 infected pods, highlighting five different clones; (iii) all 66 malformed and chlorotic pods, highlighting different clones; and (iv) all 74 necrotic pods, highlighting different clones. For each PCoA, the comparison of coordinates 1 and 2 is presented. The comparisons of coordinates 1 and 3 and coordinates 2 and 3 are presented in Fig. S5 (see Supporting Information). Uninfected pods clustered separately from infected pods. There was a gradual shift in position away from the uninfected pod cluster associated with increasing

Fig. 4 Relative expression of fungal reference genes (fungal load, Fr), symptoms of infection and expression of 36 cacao genes analysed over 140 infected pods representing two susceptible clones (Pound-7 and CATIE-1000) and three tolerant clones (UF-273, CATIE-R7 and CATIE-R4). The expression levels for each of the three *Moniliophthora roreri* reference genes (Refs) in each sample were calculated as $\% Theobroma cacao \text{ Ref}(1-3) = 100 \times [(E)^{\Delta CT}]$, where E is the primer efficiency. Fr was estimated as the geometric mean of the expression levels of the three *M. roreri* Refs. Symptoms of pod infection: -3, uninfected; -1, malformed; +1, chlorotic; +3, necrotic. Gene expression is the $\log_{10}$ value of the relative mRNA expression (% cacao Ref) in each pod. Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) was conducted using RNA from pods harvested from infected plots with different stages of infection.

Table 1 Linear regression analysis of cacao gene expression in relation to the designated independent variables pod weight (Wt), chlorosis (Ch), necrosis (Nc) and fungal load (Fr).

Gene	CATIE-R4			CATIE-R7			CATIE-1000			Pound-7			UF-273		
	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model
<i>TcARF13</i>				‡	0.28	Nc	†	0.26	Ch	‡	0.34	Ch × Nc	*	0.14	Fr
<i>TcOPR2</i>	‡	0.50	Wt × Ch	*	0.10	Nc	‡	0.84	Ch × Nc × Fr	‡	0.75	Wt × Ch	‡	0.37	Ch
<i>TcCNLS</i>	†	0.35	Nc × Fr				‡	0.69	Ch	*	0.16	Ch	*	0.19	Ch
<i>TcCesA</i>	†	0.37	Ch	‡	0.38	Nc	†	0.37	Nc	‡	0.84	Wt × Nc	†	0.35	Ch
<i>TcHSP17.4</i>	‡	0.57	Wt × Nc × Fr							‡	0.65	Nc	*	0.17	Fr
<i>TcCYP89A2</i>							†	0.29	Ch	‡	0.73	Wt × Ch × Fr	*	0.25	Fr
<i>TcGA2OX2</i>	*	0.13	Wt				‡	0.91	Wt × Nc	‡	0.89	Wt × Nc	†	0.35	Ch
<i>Tcqbda</i>	*	0.18	Ch				*	0.16	Fr	‡	0.41	Ch × Fr			
<i>TcLTP1</i>							‡	0.48	Nc × Fr	‡	0.77	Wt × Ch			
<i>TccrtZ</i>	*	0.13	Wt				‡	0.74	Wt × Ch	‡	0.92	Wt × Ch	‡	0.55	Ch × Fr
<i>TcWRKY</i>	‡	0.48	Wt × Nc × Fr	‡	0.41	Nc × Fr	‡	0.47	Ch	‡	0.73	Nc	†	0.35	Ch
<i>TcPLP</i>	‡	0.76	Wt × Ch × Nc	‡	0.82	Nc	‡	0.48	Nc	‡	0.77	Wt × Nc	‡	0.42	Ch
<i>TcCesAG2</i>	*	0.14	Ch				*	0.29	Wt × Ch	‡	0.86	Wt × Ch × Nc	†	0.24	Nc
<i>TcABCG39</i>	*	0.18	Wt				†	0.28	Nc	‡	0.33	Nc	*	0.14	Ch
<i>TcADH2</i>	‡	0.43	Wt × Nc	†	0.21	Nc	†			*	0.13	Fr			
<i>TcWAX2</i>				†	0.22	Wt	†	0.23	Ch	‡	0.54	Ch × Nc			
<i>TcAmy</i>										†	0.21	Ch			
<i>TcACO</i>	†	0.35	Ch × Nc	‡	0.45	Ch × Nc	‡	0.70	Ch × Nc	‡	0.63	Wt × Nc	‡	0.43	Ch
<i>TcODC</i>	†	0.31	Nc × Fr	‡	0.58	Fr	‡	0.78	Ch × Nc				‡	0.66	Fr
<i>TcCaff_CAB</i>				‡	0.23	Nc × Fr	‡	0.50	Ch	‡	0.79	Wt × Ch			
<i>TcChi4</i>	*	0.13	Wt	*	0.12	Fr	*	0.20	Ch	‡	0.43	Ch			
<i>TcChi1</i>	*	0.14	Fr	†	0.27	Wt	*	0.14	Ch	‡	0.52	Nc	*	0.19	Ch
<i>TcPR5</i>	†	0.35	Wt × Nc	‡	0.49	Nc × Fr	‡	0.83	Nc	‡	0.74	Nc	*	0.23	Ch
<i>TcP59</i>	†	0.39	Wt × Ch	‡	0.46	Nc × Fr	†	0.35	Ch	‡	0.59	Nc	†	0.40	Ch × Nc
<i>TcLOX3</i>	†	0.33	Wt × Ch	*	0.14	Ch	‡	0.94	Ch × Nc	‡	0.70	Nc			
<i>TcJMT3</i>	†	0.25	Nc	‡	0.46	Nc × Fr	‡	0.82	Ch	‡	0.87	Wt × Nc	‡	0.38	Ch
<i>TcOPR2</i>	†	0.42	Wt × Ch	‡	0.35	Fr	‡	0.77	Nc				†	0.33	Fr
<i>TcAIL1</i>										‡	0.49	Wt × Nc	†	0.34	Ch
<i>TcACC8</i>	‡	0.75	Ch	*	0.10	Fr	‡	0.52	Wt × Ch	‡	0.38	Ch			
<i>TcACS</i>	‡	0.49	Wt × Ch				†			†	0.32	Wt × Ch × Fr			
<i>TcKAOa</i>	†	0.36	Fr				‡	0.90	Ch × Nc	‡	0.87	Nc	†	0.27	Ch
<i>TcPR10C</i>							*	0.21	Fr				*	0.18	Fr
<i>TcPER1</i>	*	0.19	Fr	†	0.38	Ch × Fr	‡	0.57	Ch	†	0.29	Wt × Ch × Fr	†	0.24	Nc
<i>TcNPR1e</i>	†	0.30	Nc × Fr	*	0.14	Ch	‡	0.49	Ch × Nc × Fr	‡	0.53	Wt × Nc	*	0.14	Ch
<i>TcGHMPK</i>	†	0.32	Nc × Fr												
<i>TcPYL8</i>	†	0.35	Wt × Ch	‡	0.30	Nc	‡	0.53	Ch						

**P* ≤ 0.05.†*P* ≤ 0.01.‡*P* ≤ 0.001.R², largest when all included independent variables are significant.

symptoms of malformation to Ch to Nc (Fig. 5A). To some extent, PCoA of infected pods showed that the two susceptible clones were separated from the three tolerant clones along the upper quadrants (Fig. 5B). With limited exceptions, the malformed and chlorotic pods of the susceptible clones were separated from the malformed and chlorotic pods of the tolerant clones and were grouped towards the right quadrants (Fig. 5C). In contrast, the separation between susceptible and tolerant clones was not clearly evident for the necrotic pods (Fig. 5D). Similarly, Pearson's correlation analysis conducted within clones to compare the expression patterns of the individual 36 cacao genes showed that the correlation among the 18 RNA-Seq-selected genes was higher than for the set of 18 previously reported genes (Tables S2 and S3, see Supporting Information).

DISCUSSION

Yield losses caused by FPR for the five clones averaged 86%, 85%, 16%, 19% and 12% in a 5-year period for Pound-7, CATIE-1000, UF-273 T1, CATIE-R7 and CATIE-R4, respectively (Phillips-Mora *et al.*, 2013). In a previous study of disease progression in a susceptible cacao population, malformation was the most common symptom 30 days after artificial inoculation, and Nc and sporulation were most common 60 days after inoculation (Bailey *et al.*, 2013). This is in agreement with Evans (1981), who indicated that Nc and sporulation occurred 45 days or more after infection. In clone CATIE-R4, malformed pods often showed much lower pathogen levels than those detected in susceptible clones. In addition, Ch in the absence of Nc was common in the tolerant

Fig. 5 Principal components plot of the relative mRNA expression of the 36 genes selected on the basis of RNA sequence (RNA-Seq) analysis and previous reports. (A) Pods from all the clones compared among different stages of infection. (B) All the infected pods compared for different clones. (C) All the malformed and chlorotic pods compared among different clones. (D) All the necrotic pods compared among different clones. Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) was conducted using RNA from samples harvested from infected and uninfected pods of cacao.

clones, but rare in the susceptible clones. These results suggest that successful infections of tolerant clones often trigger responses leading to premature senescence, indicated by Ch at low Fr. The low Fr in the malformed and necrotic pods of tolerant clones also suggests an early response to infection compared with the susceptible clones.

Although differential transcript accumulation does not prove that a gene is directly involved in FPR tolerance or susceptibility, most of the transcriptional differences detected were related to pathways and processes associated with plant defence. The real-time qRT-PCR study of the 18 genes selected on the basis of the RNA-Seq results not only validated the RNA-Seq findings, but also generated a wealth of information to aid in understanding disease progression in susceptible and tolerant clones. The induction of these genes by *M. royeri* infection and the prediction of expression in relation to Fr and pod symptoms in susceptible clones suggest that they are probably involved in the general plant defence and stress response. The significant positive correlation among these genes and between their RNA-Seq and real-time qRT-PCR results indicates collective coordination of their expression in response to *M. royeri* infection.

The gene expression patterns diverged in tolerant clones, with many genes studied by real-time qRT-PCR showing elevated

expression regardless of the Fr or associated symptoms. Regression analysis further verified the close association of symptomatology and Fr with cacao gene expression in the susceptible compared with the tolerant clones. These results suggest that cacao defence responses shared by all five clones are induced at an earlier stage of infection in the tolerant clones.

PCoA showed that global gene expression patterns in pods of tolerant and susceptible clones differed in response to *M. royeri* infection. This differential response was more obvious in the malformed and chlorotic pods relative to necrotic pods. Most studies of plant defence responses are carried out over much shorter time periods after infection. The approach here was unique in that it characterized the interactions between *M. royeri* and tolerant cacao clones when the pathogen had overcome tolerance to cause disease. This is of critical importance as it has implications with regard to how the pathogen might adapt to the widespread use of tolerant clones. Going forward, it should be remembered that, with all the defence responses being discussed, the pathogen was able to overcome them in these specific *M. royeri*–cacao interactions.

Chitin is a polymer of *N*-acetyl-D-glucosamine, which is a major component of fungal cell walls and is an elicitor of plant defence responses (Boller, 1995). GO analysis showed that there were 11–15 chitin-responsive genes induced in infected pods of tolerant

relative to susceptible clones. Chitinases, enzymes that hydrolyse chitin, are induced by numerous abiotic and biotic factors. The resulting chitin fragments (chitooligosaccharides) elicit downstream defence response genes (Eckardt, 2008). Six chitinase genes were highly induced in the tolerant clones, whereas only one was induced in the susceptible clones. Real-time qRT-PCR analysis of *TcChi1* also showed low expression during infection in the susceptible clone Pound-7. Transgenic expression of *TcChi1* in cacao reduced *Colletotrichum gloeosporioides* severity (Maximova *et al.*, 2006), and a similar approach can be taken for the other genes identified here. Real-time qRT-PCR analysis of *TcCesA* and *TcWAX2* suggested early induction of cell wall and cuticle thickening genes in the pods of tolerant clones during infection. GO analysis showed that many fungal-, wounding-, biotic- and defence-responsive genes were induced in the tolerant clones, indicating an active defence. Pathway analysis of important events, such as plant hormone signal transduction and plant-pathogen interactions, also demonstrated important differences between the tolerant and susceptible lines.

Approximately eight to nine heat-responsive genes and 20 heat shock protein (HSP) genes were induced by *M. roreri* in the tolerant relative to the susceptible clones. Real-time qRT-PCR analysis of *TcHSP17.4* verified its early induction in tolerant clones. The induction of a large number of leucine-rich repeat (LRR) domain-containing proteins in the tolerant clones suggests a link to HSPs, as HSPs and their co-chaperones can bind to the LRR domain of *R* genes (Lu *et al.*, 2003; Vossen *et al.*, 2005). Although there is no verified *R* gene-mediated resistance to *M. roreri* in cacao, the induction of HSPs and LRR domain-containing proteins in the tolerant clones also attests to an active defence response. Real-time qRT-PCR analysis of putative plant LRR protein *TcCNLS* showed early induction in response to infection in the tolerant clones and may play a significant role in the tolerance to FPR. *TcCNLS*-related genes recognize specific pathogen-derived products and initiate a resistance response (Moffett *et al.*, 2002). Several genes induced in tolerant clones are related to transcription factors (TFs) that regulate other plant defence genes: NAC (NAM, ATAF1/2 and CUC2) domain proteins, *WRKY* TFs, disease resistance GRAS family genes (Mayrose *et al.*, 2006) and TGA genes (Ekengren *et al.*, 2003). The plant-specific NAC proteins constitute one of the largest TF families and are involved in the regulation of abiotic and biotic stress-responsive gene expression (Nakashima *et al.*, 2007; Oh *et al.*, 2005; Xia *et al.*, 2010). Some *WRKY* genes respond to pathogen attack and to the endogenous signal molecule SA (Eulgem and Somssich, 2007; Pandey and Somssich, 2009). *TcWRKY*, studied here, is at least partially induced early in infected tolerant clones. As SA-dependent defences are often triggered by biotrophic pathogens, *WRKY* genes may play an important role in the early biotrophic stage of FPR. The interacting ET and JA pathway might also be involved in FPR defence as basic helix-loop-helix TFs, responsible for the

suppression of jasmonate-mediated plant defence, were repressed in the tolerant clones (Song *et al.*, 2013). Although ET-responsive TFs and MYB TFs (Du *et al.*, 2009; Wang *et al.*, 2002) were induced in both tolerant and susceptible clones, early induction of ACC synthase (Tc01_g007030_1) occurred only in tolerant clones, and the real-time qRT-PCR results of *TcACO*, *TcACC8* and *TcACS* suggest enhanced ET biosynthesis in the infected pods of tolerant clones. The RNA-Seq results, as supported by the real-time qRT-PCR results, suggest that the initiation of FPR disease triggers transcriptional reprogramming in the cacao pods, and the two tolerant clones react more rapidly than susceptible clones.

KEGG pathway analysis showed that the putative plant-pathogen interaction pathway calcium-dependent protein kinase (*CDPK*) (Tc04_g018630) and respiratory burst oxidase (*Rboh*) (Tc08_g002020) genes were induced in tolerant clones, these genes being linked to HR-like cell death (Kobayashi *et al.*, 2007). The induction of these two genes, together with nitrate reductase (Tc08_g009960), suggests an early role in the Ch and Nc symptoms of infected pods. Similarly, the induction of an LRR receptor-like serine/threonine protein kinase FLS2 (Tc04_g019380) gene in the tolerant clones may suggest enhanced reactive oxygen species (ROS) production. GO analysis also indicated the induction of three to seven genes involved in the respiratory burst in tolerant clones. In order to keep ROS below a threshold level compatible with cellular metabolism, plants possess an array of enzymatic and nonenzymatic ROS-detoxifying mechanisms (De Gara *et al.*, 2003). Glutathione *S*-transferase (GST), induced in the tolerant clones, may play a role as a cellular protectant, preventing oxidative damage (Marrs, 1996). SA-mediated TF TGA (Tc00_g028220) and pathogenesis-related (PR) protein 1 (Tc02_g002400) were induced in tolerant clones. The real-time qRT-PCR results showed that the ABA receptor PYR/PYL family and protein phosphatase 2C were induced in tolerant clones, suggesting a link to stomatal closure. The repression of histidine-containing phosphotransfer protein (AHP) and cyclin D3 (CYCD3)-related genes in tolerant clones suggests reduced cell division. Genes associated with programmed cell death, developmental senescence and responses to pathogens are linked through complex genetic controls that are influenced by redox regulation and are probably more active in the tolerant clones.

Moniliophthora roreri infection causes a huge shift in plant hormone signalling pathways. Genes involved in ABA-, JA-, ET- and SA-mediated pathways were induced in the tolerant clones, whereas the BR and GA pathways were repressed. The induction of *TcGA2OX2*, involved in GA deactivation (Thomas *et al.*, 1999), in tolerant clones suggests repression of the GA pathway. The plant hormones ABA, JA and ET are involved in diverse plant processes, including the regulation of gene expression in response to abiotic and biotic stresses. ABA has been implicated in the enhancement of disease susceptibility in various plant species, and it was shown that exogenous ABA suppressed both basal and JA-ET-activated transcription of defence genes, whereas its removal caused the induction of basal and induced transcription

from JA–ET-responsive defence genes (Anderson *et al.*, 2004). The induction of the ABA 8'-hydroxylase 1 (Tc03_g030910), a key enzyme in the oxidative catabolism of ABA (Saito *et al.*, 2004), might result in a more active JA–ET response in tolerant clones. GO analysis of the differentially expressed genes showed that two to eight genes involved in JA- and ET-dependent systemic resistance were induced in the tolerant clones. Similarly, in the BR pathway, a *CYP734A1* (cytochrome P450, family 734 protein)-related gene possibly involved in castasterone and brassinolide inactivation (Meaney, 2005) was induced in tolerant clones. Conversely, various genes in the steroid biosynthesis pathway were repressed in the tolerant clones, leading to a possible blockage of BR biosynthesis, as suggested by GO analysis. BR is a beneficial phytohormone active against biotic and abiotic stresses. There have been many reports of exogenous BR applications enhancing disease resistance of crop plants (Ali *et al.*, 2013; Bajguz and Hayat, 2009). Conversely, some pathogens exploit BRs as virulence factors to suppress the SA-mediated defence pathway and cause disease (De Vleeschauwer *et al.*, 2012). Real-time qRT-PCR analysis of the *TcNPR1e* gene, which is an initial regulatory protein of the SA-mediated defence pathway, showed almost uniform expression in both tolerant and susceptible clones, but the downstream regulatory proteins TGA (Tc00_g028220) and PR1 (Tc02_g002400) were repressed in the susceptible clones.

There were also shifts in metabolic pathways. GO analysis indicated the induction of various cell death and senescence-related genes in the tolerant clones, whereas genes for photosystem I and II and the cell cycle were repressed. It seems that tolerant clones detect the biotrophic pathogen early and initiate programmed cell death, shutting down many regular metabolic activities. Shifts in other pathways, such as glucose and galactose metabolism and glycolysis, are common during plant infection (Bolton, 2009), and may be the result of hormonal shifts. *Moniliophthora roreri* appears to persist as a biotroph for a relatively long period, achieving a higher Fr in the susceptible clones, providing access to a much larger metabolic sink. Plant defence mechanisms against pathogens are a complex process involving many genes with direct and indirect roles. The list of these candidate genes could be further refined by analysis of the pathogen response and transcript accumulation in progeny derived via the crossing of these tolerant clones.

In conclusion, successful infection of tolerant cacao pods by *M. roreri* occurs occasionally, but results in a dissociation between progressive fungal colonization patterns and disease symptom development. Differentially regulated molecular mechanisms between tolerant and susceptible clones revealed that various elicitor-responsive genes are induced in tolerant clones, suggesting the early detection of the pathogen by the plant and the induction of the defence response at low Fr. Genes related to the HR response are probably induced early in tolerant clones. Genes associated with the plant hormone signal transduction pathways, such as SA-mediated TF TGA and PR protein 1, are induced in

tolerant clones. Genes involved in BR biosynthesis are repressed and a cytochrome P450 gene that inactivates BR is induced in tolerant clones. A possible repression of BR synthesis and enhanced catabolic inactivation of both BR and ABA in tolerant clones may enhance the JA–ET- and SA-mediated defence pathways. Genes involved in signal transduction of growth hormones, such as auxin and cytokinin, are repressed in the tolerant clones. Based on the induction of cell death-related genes and the repression of cell cycle genes and other primary metabolism genes, it is likely that the tolerant clones initiate pod senescence. The ability of *M. roreri* to occasionally overcome this defence response and cause the infection of tolerant clones highlights the importance of understanding the changing dynamics of FPR. A parallel study carried out on the differential *M. roreri* gene expression associated with successful pod infections of susceptible and tolerant clones (Bailey *et al.*, 2014) has provided some valuable information on the fungal ability to overcome such defence mechanisms in tolerant cacao clones. The information from these two studies will help further our understanding of the detailed mechanisms of FPR development and the development of a breeding strategy for stable tolerant clones.

EXPERIMENTAL PROCEDURES

Artificial inoculation and infection of cacao clones with *M. roreri*

Five different cacao clones were selected for study: three tolerant clones and two susceptible clones. The susceptible clones Pound-7 and CATIE-1000 (an offspring of Pound-12 × Catongo) and tolerant clones UF-273, CATIE-R7 (an offspring of UF-273 × CATIE-1000) and CATIE-R4 (an offspring of UF-273 × PA-169) were used. The cacao trees were located in a plot at the CATIE field station, La Lola, near Siquirres, Costa Rica. To obtain disease-free pods, 200 cacao flowers from each clone were hand pollinated and covered with plastic bags to prevent infection. When the pods were 8 weeks old (6–8 cm), 40 pods from each clone were artificially inoculated with the FPR pathogen *M. roreri* and 40 uninoculated pods were selected as controls. CATIE-1000 did not set sufficient pods after two pollination attempts, and was only used as uninfected pods. Pods were inoculated as described by Bailey *et al.* (2013). Inoculated pods and uninfected pods were assessed in the field for FPR symptoms, and six pods per treatment were harvested for RNA isolation at 15-day intervals until the pods reached maturity.

Harvesting of pods showing symptoms of FPR as a result of natural infection

In 2010, monthly visits (July to November) were made to the plots described above, and pods of each of the five clones showing symptoms of natural *M. roreri* infection were harvested. Symptomatology was the primary selection criterion and pods showing a range of symptoms were intentionally sought for each clone. Symptoms ranged from malformation

to Ch and Nc. The pods were transported back to the laboratory in paper bags inside a cool box without ice. Pods were measured (length, diameter and weight), photographed and then placed in liquid nitrogen for 10 min (whole or bigger pods were cut into sections). Pods were collected for RNA as described by Bailey *et al.* (2013).

DNA extraction from cacao pods and quantification of *M. royeri* DNA

Two hundred milligrams of lyophilized pod tissue were ground under liquid nitrogen and transferred to tubes containing 15 mL of 65 °C cetyltrimethylammonium bromide (CTAB) buffer [3% CTAB, 100 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, 20 mM ethylenediamine-tetraacetic acid (EDTA), pH 8, 1.4 M NaCl, 1% polyvinylpyrrolidone (PVP) 40 000, 0.2% 2-mercaptoethanol]. The samples were homogenized for 30–60 s using an Ultra-Turrax T25 (Janke-Kunkel, Staufen, Germany) and returned to the 65 °C bath for 30–60 min. Samples were centrifuged for 30 min at 25 000 *g* at 14 °C, and the supernatant was transferred to fresh tubes containing 12 mL of chloroform. Samples were gently mixed and centrifuged as described above. The aqueous layer was transferred to fresh tubes containing 5 mL of 7.5 M ammonium acetate, and 20 mL of 100% ethanol were added with mixing before being held on ice overnight. Samples were centrifuged for 50 min at 4 °C and 25 000 *g*. The supernatant was decanted, and the pellet was air-dried, resuspended in 600 µL of sterile double-distilled H₂O (ddH₂O) and transferred to a microcentrifuge tube. Samples were treated with 6 µL of RNase for 15 min at 37 °C prior to being extracted with 600 µL of chloroform. The aqueous layer was transferred to a fresh tube. After the addition of 200 µL of 7.5 M ammonium acetate and 900 µL of 100% ethanol, samples were held on ice for 2 h and centrifuged at 14 000 *g* for 10 min at 4 °C. After decanting the supernatant, the air-dried pellet was resuspended in 150 µL of ddH₂O. For real-time qPCR, each sample was further diluted to 1:39 with ddH₂O.

Real-time qPCR was carried out with the DNA samples using primers for cacao actin (*TcACT*), *M. royeri* actin (*MrACT*) and ITS (*MrITS*) (Table S4, see Supporting Information). The *M. royeri*-specific *MrITS* primers were designed using the ITS region of the 18S ribosomal RNA gene (GenBank: JF769489.1) and tested for specificity against *M. royeri* and cacao genomic DNA. The *M. royeri* DNA content for each sample was quantified against *MrAct* and *MrITS* as % *TcACT* = $100 \times [(E)^{\Delta C_T}]$, where $\Delta C_T = (C_{T \cdot TcAct}) - (C_{T \cdot MrAct} \text{ or } C_{T \cdot MrITS})$ and *E* is the primer efficiency. Correlations were carried out between *M. royeri* DNA content based on *MrAct* and *MrITS* and Fr.

RNA extraction

Pods were coarsely ground under liquid nitrogen. Approximately 1 cm³ of pod material was ground finely and transferred to a disposable 50-mL centrifuge tube containing 15 mL of 65 °C extraction buffer (Bailey *et al.*, 2005). Additional extraction methods were conducted as in Melnick *et al.* (2012). The cDNA was synthesized using the Invitrogen (Carlsbad, CA, USA) Superscript VILO kit, following the manufacturer's directions.

Expression analysis using RNA-Seq and real-time qRT-PCR

Procedures for real-time qRT-PCR analysis were as described by Bailey *et al.* (2006). Fr in each sample was calculated by comparing the expres-

sion of three *M. royeri* Refs (*MrACT*, *MrGADP* and *MrUBQ*) with the expression of three cacao Refs (*TcACT*, *TcACP* and *TcEF1α*) (see Table S4). The geometric mean ($C_{T \cdot Tc \text{ Ref}}$) was calculated for cycle times of three cacao Refs ($C_{T \cdot Tc \text{ Ref}(1-3)}$). ΔC_T was calculated for each *M. royeri* Ref [$\Delta C_{T \cdot Mr \text{ Ref}(1-3)} = (C_{T \cdot Tc \text{ Ref}}) - (C_{T \cdot Mr \text{ Ref}(1-3)})$]. The expression levels for each of the three *M. royeri* Refs in each sample were calculated as % cacao Ref(1–3) = $100 \times [(E)^{\Delta C_T}]$, where *E* is the primer efficiency. Fr was estimated as the geometric mean of the expression levels of the three *Mr* Refs. Based on these real-time qRT-PCR results (data not shown), 12 RNA samples showing similar Fr were selected for RNA sequence analysis. The 12 samples included three samples for each of two tolerant clones CATIE-R4 and CATIE-R7 and two susceptible clones CATIE-1000 and Pound-7. For the RNA-Seq analysis, cDNA was generated using a routine RNA library preparation TruSeq protocol developed by Illumina Technologies (San Diego, CA, USA). Using the kit, mRNA was first isolated from total RNA by performing a polyA selection step, followed by the construction of single-end sequencing libraries with an insert size of 160 bp. Single-end sequencing was performed on 12 samples using the Illumina HiSeq platform. Samples were multiplexed with unique six-mer barcodes, generating 720 170 543 filtered (for Illumina adapters/primers, and PhiX contamination) 1 × 50-bp read pairs. The sequences acquired by RNA-Seq were verified by comparison with the cacao genome (Argout *et al.*, 2010). RNA reads from RNA-Seq libraries ranging from 50 to 70 million reads in fastq format were aligned using the memory-efficient short-read aligner Bowtie-0.12.7 (Langmead *et al.*, 2009) to the coding sequences (CDS) of the cacao genome (<http://cocoagendb.cirad.fr/>). Tabulated raw counts of reads to each CDS were obtained from the Bowtie alignment. Estimation and statistical analysis of expression level using the count data of each gene with three replicates for each library were performed using the DEseq package (Anders, 2011) and R x64 2.15.2 program (<http://www.r-project.org/>). The KEGG Automatic Annotation Server (KAAS) was used to obtain KEGG Orthology and KEGG pathways involving the differentially expressed genes by BLAST comparisons against the manually curated KEGG GENES database (Moriya *et al.*, 2007). GO analysis was carried out using the program Blast2GO (Conesa *et al.*, 2005).

After RNA-Seq, genes were chosen for analysis by real-time qRT-PCR to verify RNA-Seq analysis. Eighteen cacao genes monitoring the plant response in tolerant/susceptible pods during infection (see Table S4) were chosen based on their putative function and to represent a range of differential expression values between tolerant and susceptible clones based on the RNA-Seq analysis. Gene expression was analysed across the entire set of pods collected for the five clones being studied. To obtain the relative transcript levels, the threshold cycle (C_T) values for all genes of interest ($C_{T \cdot GOI}$) were normalized to the geometric mean (Vandesompele *et al.*, 2002) C_T value of the three cacao Refs ($C_{T \cdot Tc \text{ Ref}}$) as $\Delta C_T = (C_{T \cdot Tc \text{ Ref}}) - (C_{T \cdot GOI})$. Expression levels for each cacao gene in each sample were calculated as % cacao Ref = $100 \times [(E)^{\Delta C_T}]$, where *E* is the primer efficiency.

Another set of 18 cacao genes related to plant stress response and hormone biosynthesis pathways was chosen for similar real-time qRT-PCR analysis across the full set of infected and uninfected pods for each of the five clones (see Table S4). This set of genes was derived from previously published results (Argout *et al.*, 2010; Bae *et al.*, 2009; Bailey *et al.*, 2006, 2013; Leal *et al.*, 2007; Verica *et al.*, 2004).

Statistical analysis

Relative expression data of cacao gene expression as % cacao Ref were log-transformed to linearize the data (Rieu and Powers, 2009). Linear regression analysis was carried out within each clone using PROC REG in SAS 9.2 (SAS Institute, Raleigh, NC, USA) to determine the relationship of the pod variables to the relative expression of an individual cacao gene. For regression, the predictor variables were pod weight, percentage of pod with Ch, percentage of pod with Nc and Fr, whereas the dependent variable was the relative expression of a cacao gene. Only significant models ($\alpha = 0.05$) are presented for each clone. A heat map of the relative expression of the individual cacao genes was created using TIGR MultiExperiment Viewer (MeV) (Saeed *et al.*, 2003). Correlation between Fr and the relative amount of *Mr-Act* and *Mr-ITS* DNA was calculated using the Pearson's correlation statistic (Fisher's *z* transformation) employing PROC CORR in SAS 9.2. Similarly, correlation between RNA-Seq and real-time qRT-PCR results was calculated using the Pearson's correlation statistic.

Correlation of cacao gene relative expression within a clone was calculated using the Pearson's correlation statistic (Fisher's *z* transformation) employing PROC CORR in SAS 9.2. Statistically significant correlations ($\alpha = 0.05$) within a clone are presented. Finally, in examining the relationship between cacao gene expression patterns amongst clones, PROC DISTANCE of SAS 9.2 was used to calculate the Euclidean distance between pairs of genes. PCoA was performed using GenALEx 6.1 with the distance matrix of gene expression as % cacao Refs and the option of adjusting covariance to visually represent trends in gene expression through low-dimensional projection of high-dimensional data (Katagiri and Glazebrook, 2009).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig S1 Disease assessment of pods artificially inoculated with *Moniliophthora roreri* spores.

Fig S2 Fungal load (Fr) of *Moniliophthora roreri* in infected pods.

Fig S3 Comparison of pathogen level estimation based on transcriptome and DNA amount.

Fig S4 Shift in the key metabolic pathways involved in the disease response between tolerant and susceptible cacao clones in response to *Moniliophthora roreri* infection in pods.

Fig S5 Principal components plot of the relative mRNA expression of the 36 genes selected on the basis of RNA sequence (RNA-Seq) analysis and previous reports.

Table S1 Correlation between RNA sequence (RNA-Seq) and real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) results for the 36 genes.

Table S2 Correlation matrix of the expression levels of the 18 cacao genes selected on the basis of RNA sequence (RNA-Seq) analysis.

Table S3 Correlation matrix of the expression levels of the 18 cacao genes selected on the basis of previously reported genes.

Table S4 Source, primers, estimated length and accession of all *Theobroma cacao* and *Moniliophthora roreri* genes used in real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) analysis.

Excel file S1 Differentially expressed cacao genes identified on the basis of RNA sequence (RNA-Seq) analysis.

Excel file S2 Gene ontology (GO) analysis of the four different sets of differentially expressed genes.

Excel file S3 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of the four different sets of differentially expressed genes.