Differential gene expression by *Moniliophthora roreri* while overcoming cacao tolerance in the field

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SUMMARY

Frosty pod rot (FPR) of *Theobroma cacao* (cacao) is caused by the hemibiotrophic fungus *Moniliophthora roreri*. Cacao clones tolerant to FPR are being planted throughout Central America. To determine whether *M. roreri* shows a differential molecular response during successful infections of tolerant clones, we collected field-infected pods at all stages of symptomatology for two highly susceptible clones (Pound-7 and CATIE-1000) and three tolerant clones (UF-273, CATIE-R7 and CATIE-R4). Metabolite analysis was carried out on clones Pound-7, CATIE-1000, CATIE-R7 and CATIE-R4. As FPR progressed, the concentrations of sugars in pods dropped, whereas the levels of trehalose and mannitol increased. Associations between symptoms and fungal loads and some organic and amino acid concentrations varied depending on the clone. RNA-Seq analysis identified 873 *M. roreri* genes that were differentially expressed between clones, with the primary difference being whether the clone was susceptible or tolerant. Genes encoding transcription factors, heat shock proteins, transporters, enzymes modifying membranes or cell walls and metabolic enzymes, such as malate synthase and alternative oxidase, were differentially expressed. The differential expression between clones of 43 *M. roreri* genes was validated by real-time quantitative reverse transcription polymerase chain reaction. The expression profiles of some genes were similar in susceptible and tolerant clones (other than CATIE-R4) and varied with the biotrophic/necrotrophic shift. *Moniliophthora roreri* genes associated with stress metabolism and responses to heat shock and anoxia were induced early in tolerant clones, their expression profiles resembling that of the necrotrophic phase. *Moniliophthora roreri* stress response genes, induced during the infection of tolerant clones, may benefit the fungus in overcoming cacao defense mechanisms.

Keywords: disease tolerance, frosty pod rot, hemibiotroph, metabolite, *Moniliophthora roreri*, RNA-Seq, *Theobroma cacao*.

INTRODUCTION

Moniliophthora roreri H. C. Evans, Stalpers, Samson & Benny is the causal agent (Evans, 2007; Phillips-Mora and Wilkinson, 2007) of frosty pod rot (FPR) on *Theobroma cacao* L. (cacao), the source of chocolate (Wood and Lass, 2001). FPR occurs in most major cacao-producing areas in the western hemisphere (Evans, 2007; Phillips-Mora and Wilkinson, 2007). FPR is a major concern because of its devastating effects on yields and limited available control measures (Evans, 2007; Phillips-Mora and Wilkinson, 2007).

Moniliophthora roreri is a hemibiotroph. During the biotrophic phase, fruits develop malformations and progress to the necrotrophic phase in which sporulation occurs (Evans, 2007; Evans *et al.*, 2002; Phillips-Mora and Wilkinson, 2007). Recently, a working model for the *M. roreri* disease cycle in a highly susceptible interaction has been proposed (Bailey *et al.*, 2013). *Moniliophthora roreri* gene expression is repressed under the limited nutrient conditions of the biotrophic phase (Meinhardt *et al.*, 2006; Mondego *et al.*, 2008). The fungus spreads slowly and positions itself to exploit resources available when necrosis (Nc) is triggered. A shift in *M. roreri* gene expression occurs between the biotrophic and necrotrophic phases, and pod metabolites are rapidly metabolized by the pathogen during the necrotrophic phase (Bailey *et al.*, 2013).

A program in Central America, centered at CATIE in Costa Rica, distributes clones which show tolerance to FPR (Phillips-Mora, 2010). The clones used in this study have been under observation for FPR for more than 11 years (Phillips-Mora *et al.*, 2012). Losses to FPR have increased over the past 7 years in susceptible and tolerant clones (Phillips-Mora *et al.*, 2012). The molecular and metabolomic characterization of successful infections of tolerant clones is a step towards understanding how *M. roreri* might overcome tolerance.

Although cacao clones can have significant tolerance to *M. roreri*, they are not immune, and infections occur at low rates. We used clones associated with the CATIE breeding program to carry out this research. The first objective was to characterize changes in pod metabolite concentrations associated with suc-

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cessful *M. royeri* infections of susceptible and tolerant clones. The second objective was to characterize the molecular signature for *M. royeri* when it overcomes the defenses faced in tolerant cacao clones. Comparisons are made between two susceptible cacao clones (Pound-7 and CATIE-1000) and three tolerant clones (UF-273, CATIE-R7 and CATIE-R4). By understanding the *M. royeri* interaction with tolerant cacao clones, we hope to identify the mechanisms associated with overcoming cacao defense responses with implications relative to the sustainability of tolerance in the field. A parallel study is being carried out to identify changes in cacao gene expression caused by *M. royeri* infection in tolerant and susceptible clones (Ali *et al.*, 2014).

RESULTS

RNA-Seq analysis of susceptible and tolerant clones infected with *M. royeri*

RNA-Seq analysis was carried out on samples from naturally infected pods of two tolerant cacao clones (CATIE-R7 and CATIE-R4) and two susceptible clones (Pound-7 and CATIE-1000). The RNA-Seq samples had similar fungal loads (Fr) of around 10%. 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. The 10% level was chosen based on previous RNA-Seq experience as a minimum Fr yielding significant *M. royeri* transcripts. Pods for the susceptible clones Pound-7 and CATIE-1000 were asymptomatic. Symptoms varied for CATIE-R7 and CATIE-R4 (Appendix S1, Library Stats) for three reasons: (i) pods of tolerant clones with Fr of approximately 10% and no chlorosis (Ch) or Nc were rare; (ii) symptoms were poorly associated with Fr in tolerant clones; and (iii) the *M. royeri* genes of interest in pods of tolerant clones should be consistently expressed regardless of symptoms as they are expected to be

reactions to the host defenses. The *M. royeri* transcript levels in the RNA-Seq samples ranged from 0.31% to 2.42% of the total processed reads (Appendix S1, Library Stats, see Supporting Information). The mean percentages of *M. royeri* transcripts per clone in the libraries were 1.09%, 1.35%, 0.57% and 0.72% for Pound-7, CATIE-1000, CATIE-R7 and CATIE-R4, respectively. The normalized geometric mean range for the three *M. royeri* Refs for each clone varied from 603 reads to 872 reads, a difference of 1.45-fold (Appendix S1, Reference Genes). The *M. royeri* transcriptome consists of 17 920 genes (Appendix S2, RNA-Seq, see Supporting Information). Transcripts of 14 580 genes were detected, with 10 321 being detected by an average of five reads and 4712 being detected by an average of 25 reads in one or more clones. The secretome of *M. royeri* consists of 1535 proteins (Appendix S2, Secretome). An additional 92 proteins with signal peptides have two or more transmembrane domains and 124 proteins have glycosylphosphatidylinositol (GPI) anchors.

There were 863 transcripts differentially expressed ($P \leq 0.05$) between at least one clone pairing (Fig. 1, Appendix S2, Diff Expressed), 198 encoding secreted proteins (Appendix S2, Secretome). Twenty-three transcripts showed differences between the tolerant and susceptible clones in all combinations (Table 1, Appendix S2, Tol-Sus). There were 602 transcripts differentially expressed between Pound-7 and CATIE-R4, and 408 transcripts differentially expressed between CATIE-1000 and CATIE-R4. There were 126 transcripts differentially expressed between Pound-7 and CATIE-R7, and 83 transcripts differentially expressed between CATIE-1000 and CATIE-R7.

Principal coordinate analysis (PCoA) was carried out using RNA-Seq data for the top 200 expressed genes (Fig. 2A) and genes differentially expressed between CATIE-R4 (Fig. 2B), CATIE-R7 (Fig. 2C) and susceptible clones. The three plots showed similar patterns separating tolerant clones from susceptible clones. The largest amount of variation explained by an individual coordinate

Fig. 1 Venn diagrams showing the numbers of genes differentially expressed between susceptible clones Pound-7 and CATIE-1000 and tolerant clones CATIE-R7 and CATIE-R4.

Table 1 *Moniliophthora royeri* genes differentially expressed between tolerant (CATIE-R7 and CATIE-R4) and susceptible (CATIE-1000 and Pound-7) clones in all comparisons in the RNA-Seq analysis.

Accession number	Real-time qRT-PCR	Putative ID/function	GPI anchor		Mean normalized read/library				Significance (P value)			
			No	Yes	P7	C1000	R7	R4	P7-R4	P7-R7	C1000-R7	C1000-R4
evm.model.sctg_0149_0001.18	<i>MrH520</i> <i>MrEP14</i> <i>MrAAO</i> <i>MrEP22</i> <i>MrHS</i> <i>MrEP15</i>	Methyl transferase	x		3771.92	2036.95	22.34	36.22	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0022_0002.80		Heat shock protein hsp20			2.33	10.80	353.36	427.07	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0094_0001.8		Methyl transferase	x		710.53	428.46	7.87	16.25	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0223_0002.11		D-Amino acid oxidase			409.74	93.90	0.95	0.00	<0.001	<0.001	0.002	<0.001
evm.model.sctg_0003_0001.19		Hypothetical protein			8.89	3.18	793.25	736.65	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0005_0005.20		Amide-like NADH oxidoreductase			18.98	25.81	405.07	564.41	<0.001	<0.001	0.001	<0.001
evm.model.sctg_0238_0003.13		Small heat shock protein			0.85	1.83	278.02	390.12	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0099_0001.2		Small heat shock protein			5.39	5.32	185.62	298.80	<0.001	<0.001	<0.001	0.001
evm.model.sctg_0199_0001.1		Hypothetical protein	x		4060.35	3617.16	74.08	22.55	<0.001	<0.001	<0.001	<0.001
evm.model.sctg_0077_0001.32		Short-chain dehydrogenase			6.16	18.77	121.06	874.98	<0.001	<0.001	0.024	<0.001
evm.model.sctg_0017_0002.15	<i>MrCp450b</i>	Hypothetical protein		x	647.09	651.42	50.31	79.23	0.002	0.002	0.003	0.001
evm.model.sctg_0016_0007.7		Hypothetical protein	x		0.69	0.11	294.51	264.84	<0.001	0.002	0.001	<0.001
evm.model.sctg_0022_0002.72		Lactose permease			1.71	0.98	27.74	14.98	0.001	0.003	0.009	0.006
evm.model.sctg_0165_0001.3		C6 transcription factor			3.97	3.59	73.47	37.12	0.008	0.004	0.002	0.002
evm.model.sctg_0119_0004.2		Cytochrome p450			6.66	12.72	919.92	294.64	<0.001	0.005	0.009	<0.001
evm.model.sctg_0231_0001.5		Rhodocoxin reductase			241.14	230.04	46.19	54.04	0.002	0.007	0.016	0.020
evm.model.sctg_0109_0001.13		Hypothetical protein	x		171.22	193.00	27.80	0.98	<0.001	0.012	0.002	<0.001
evm.model.sctg_0197_0006.7		Siderophore iron transporter			37.99	31.16	349.24	355.03	<0.001	0.024	0.009	<0.001
evm.model.sctg_0037_0001.30		NADH dehydrogenase 1 α subcomplex			4.35	1.58	532.48	188.44	0.004	0.030	0.003	<0.001
evm.model.sctg_0416_0001.3		Hypothetical protein	x		1.81	1.02	88.50	38.95	0.016	0.031	0.008	0.003
evm.model.sctg_0005_0005.21	<i>MrCAT1</i> <i>MrEP23</i>	Glyoxylate dehydrogenase			7.08	5.49	55.25	33.93	0.011	0.031	0.011	0.007
evm.model.sctg_0250_0003.8		Mycelial catalase cat1	x		211.44	808.43	68.06	29.47	0.001	0.033	<0.001	<0.001
evm.model.sctg_0089_0002.31		Hypothetical protein			793.47	480.95	28.63	14.98	0.010	0.036	0.006	0.001

Real-time qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction.

Fig. 2 Principal coordinate analysis (PCoA) of RNA-Seq reads ($\log_{10}$) for three libraries for clones CATIE-R4, CATIE-R7, CATIE-1000 and Pound-7. (A) Comparisons using the top 200 highly expressed genes based on the mean number of reads averaged over all libraries. (B) Comparisons using genes differentially expressed between CATIE-R4 and susceptible clones CATIE-1000 and/or Pound-7. (C) Comparisons using reads for genes differentially expressed between CATIE-R7 and susceptible clones CATIE-1000 and/or Pound-7.

was 56.06% for coordinate 1 using the CATIE-R4 differentially expressed genes. The susceptible clones CATIE-1000 and Pound-7 were also separated using the CATIE-R7 differentially expressed genes.

Real-time quantitative Reverse Transcription Polymerase Chain Reaction (Real-time qRT-PCR) analysis of *M. royeri* gene expression in susceptible and tolerant cacao clones

Fr and symptoms

After RNA-Seq, 43 *M. royeri* genes showing differential expression between tolerant and susceptible clones (Appendix S2, primers used) were chosen for analysis by Real-time qRT-PCR to confirm expression in infected pods for tolerant cacao clones UF-273, CATIE-R7 and CATIE-R4 and susceptible clones Pound-7 and CATIE-1000. Thirty-eight of the 43 *M. royeri* genes studied showed significant correlation ($P \leq 0.05$) between RNA-Seq reads and the Real-time qRT-PCR results (Appendix S1, RNA-Seq and qRT-PCR Cor). *Fr* for each pod is presented in Fig. 3, together with symptoms. The use of *Fr*, an RNA-based estimate of relative fungal content, was also supported by the significant correlations between fungal DNA content and *Fr* using *MrAct* and *MrITS* as probes (Appendix S1, DNA-RNA real-time qRT-PCR Cor). Increasing *Fr* was associated with increasing Ch and Nc in clones CATIE-1000 and Pound-7 (Fig. 3). This pattern was apparent for clone UF-273, but not for CATIE-R7 and CATIE-R4 (Fig. 3). These observations were supported by the R^2 values obtained when *Fr* was regressed against pod weight (Wt), Ch and Nc (Table 2). The R^2 for the model containing Nc was highly significant ($P \leq 0.0001$) exceeding 0.6 for both CATIE-1000 and Pound-7, and for UF-273 ($P \leq 0.002$) with R^2 of 0.397. Nc was less effective in predicting *Fr* for CATIE-R7 ($R^2 = 0.172$) and CATIE-R4 ($R^2 = 0.206$).

Linear regression analysis: *M. royeri* gene expression

The independent variables Wt, Ch, Nc and *Fr* in singular and all combinations were used to predict *M. royeri* gene expression within a clone. The model with the largest R^2 , where all included independent variables were significant, is presented (Table 3). For Pound-7, 22 *M. royeri* genes had highly significant models ($P \leq 0.001$), with 29 models including Ch and Nc, and 14 models including *Fr*. CATIE-1000 had 31 highly significant models ($P \leq 0.001$), with 14 models including Ch and Nc, and 18 models including *Fr*. UF-273 had 11 highly significant models ($P \leq 0.001$), with no models including Ch and Nc, 11 models including Wt and *Fr*, and 13 models including *Fr*. CATIE-R7 had three highly significant models ($P \leq 0.0001$), with zero models including Ch and Nc, and 11 models including *Fr*. CATIE-R4 had 10 highly significant models ($P \leq 0.0001$), with two models including Ch and Nc, 17 models including the independent variable Wt, and 13 models including *Fr*.

PCoA of Real-time qRT-PCR throughout results

PCoA was also carried out using the Real-time qRT-PCR data for 114 pods from five clones (Fig. 4). Plot coordinates 1, 2 and 3

Fig. 3 Fungal load (A), pod symptoms (B) and *Moniliophthora royeri* gene expression levels (C) for 43 *M. royeri* genes analysed over 114 naturally infected pods representing two susceptible clones (Pound-7 and CATIE-1000) and three tolerant clones (UF-273, CATIE-R7 and CATIE-R4). Graphs show relative expression of *M. royeri* reference genes (Fr, % *Theobroma cacao* Ref) in each pod, % chlorosis (Ch) and % necrosis (Nc) for each pod, and $\log_{10}$ of *M. royeri* gene expression (%Pr) for 43 *M. royeri* genes for each pod.

explained 47.08%, 23.20% and 10.71% of the variability, respectively. The malformed green (MG) pods and Ch pods of the five clones are presented in the PCoA plots, together with the Nc pods. In most cases, the pods showing Nc shared a similar distribution regardless of clone. With limited exceptions, the MG pods of the tolerant clones separated from the MG pods of the susceptible clones. Comparing coordinates 1 and 2 (Fig. 4A), the MG pods for the susceptible clones grouped in the lower right quadrant. The MG pods for the tolerant clones were offset across the upper right quadrant towards the left quadrants. Comparing coordinates 1 and 3 (Fig. 4B), the MG pods of the susceptible clones grouped towards the far right of the coordinate 1 axis with the MG pods of the tolerant clones dispersed to the left. Interestingly, CATIE-R4 and UF-273 separated from one another in the comparison of

coordinates 1 and 3. Comparing coordinates 2 and 3 (Fig. 4C), MG pods of the susceptible clones grouped to the left of the coordinate 2 axis and the MG pods of the tolerant clones were dispersed across the plot to the right with groupings above and below the coordinate 3 axis.

Pearson's correlation analysis

Pearson's correlation analyses were conducted within clones comparing the expression patterns of 43 *M. royeri* genes analysed by Real-time qRT-PCR. A matrix of 19 *M. royeri* gene pairs is presented (Table 4). The susceptible clones displayed a pattern of significant correlation in most comparisons. Of 170 comparisons, Pound-7 had 130 significant correlations and CATIE-1000 had 156 significant correlations. The tolerant clones had 80, 67 and 91

Table 2 Linear regression analysis was carried out within each clone using *Moniliophthora roreri* fungal load (Fr) as the dependent variable with the predictor variables of pod weight (Wt), percentage chlorosis (Ch) and percentage necrosis (Nc).

Clone		Wt	Nc	Wt–Nc	Ch–Nc
CATIE-R4	R^2		0.206		
	P value		0.015		
CATIE-1000	R^2		0.822	0.849	0.859
	P value		<.0001	<.0001	<.0001
Pound-7	R^2		0.654		
	P value		<.0001		
CATIE-R7	R^2	0.146	0.172		
	P value	0.041	0.028		
UF-273	R^2	0.160	0.397		0.484
	P value	0.046	0.002		0.001

Linear regression analysis was carried out within each clone using PROC REG in SAS 9.2 (SAS Institute, Raleigh, NC, USA). All significant models ($\alpha \leq 0.05$) are presented for each clone for which all individual predictor variables within the regression model are also significant ($\alpha \leq 0.05$).

significant correlations for UF-273, CATIE-R7 and CATIE-R4, respectively. There were 44 *M. roreri* gene pairs with significant correlations for all clones (shaded outlined boxes), 31 *M. roreri* gene pairs with significant correlations for four clones (unshaded outlined boxes) and 36 comparisons were only significant in the two susceptible clones (shaded not outlined boxes).

Real-time qRT-PCR heat maps

Several patterns of expression were observed when the Real-time qRT-PCR data were compared with Fr and pod symptoms (Ch, Nc). Most genes showed a significant increase or decrease in expression associated with increasing symptoms and Fr levels that was consistent between susceptible clones (Fig. 3). The patterns diverged when considering the tolerant clones. For example, tolerant clones had a more consistent high level of expression of *MrMS*, *MrFR* and *MrAOX* than the susceptible clones (Fig. 5A, B). Interestingly, the expression of *MrMS*, *MrFR* and *MrAOX* was abnormally high in CATIE-R7 relative to the susceptible clones only when Fr was low. *MrCE8b*, *MrMGT* and *MrLAC1* showed an abrupt increase in expression as Fr increased in all clones, except CATIE-R4 (Fig. 5B, C). *MrAAO* and *MrTDAT* were highly expressed in susceptible clones when symptoms and Fr were low, but were almost always expressed at low levels in tolerant clones (Fig. 5D).

Metabolites

Metabolite concentrations were determined for a subset of pods (at least 20 per clone including five or six uninfected) for clones CATIE-R4, CATIE-R7, CATIE-1000 and Pound-7. Regression identified 13 and 10 metabolites with very significant regression models ($P \leq 0.0001$) for Pound-7 and CATIE-1000, respectively (Table 5). The highly significant models included one or more of the variables Ch, Nc or Fr. Pearson's correlation analysis iden-

tified close correlations between the concentrations of fructose, glucose, sucrose and malic, aconitic and shikimic acids that generally was consistent across all four clones (Table 6). A second group of metabolites showed a less consistent association across clones (oxoglutaric acid, isoleucine, glycine, alanine, valine and leucine). Mannitol concentrations were always correlated with fumaric acid, and trehalose was correlated with mannitol in three of four comparisons. The data are presented for the metabolites mannitol, fructose, fumaric acid, 2-oxoglutaric acid and alanine (Fig. 6). Mannitol and fumaric acid increased in concentration as disease progressed. Fructose increased in the controls from 30–60 days after pollination (DAP), but decreased as symptoms and Fr increased in infected pods. 2-Oxoglutaric acid occurred in high concentrations in Pound-7. Alanine concentrations increased to high levels in the tolerant clones, especially CATIE-R7.

DISCUSSION

Disease progression in tolerant and susceptible clones

Malformations occur during the biotrophic phase of *M. roreri* infection before progressing to the necrotrophic phase (Bailey *et al.*, 2013; Evans, 1981). Tolerant clones CATIE-R4 and CATIE-R7 show typical FPR symptoms, but Ch in the absence of Nc is also common (Fig. 3). Fr is largely predicted by Nc levels in CATIE-1000 and Pound-7, but there is a reduced association between Fr and symptoms in the tolerant clones (Table 2). In clone CATIE-R4, MG pods often have very low Fr compared with the susceptible clones (Fig. 3). Successful infections of tolerant clones may cause pods to prematurely senesce at low Fr.

Changes in metabolites as a result of *M. roreri* infection

Quantitative trait locus (QTL) mapping identified linkage groups associated with tolerance to FPR in crosses between UF273 and Pound-7 (Brown *et al.*, 2007), all originating from UF-273. Tolerant clones CATIE-R7 and CATIE-R4 share UF-273 as a parent. CATIE-R4 also has the tolerant clone PA-169 as a parent. Some *M. roreri* reactions differ between highly susceptible clones (see *MrCDF1* and *MrEP16*, Fig. 3) and may be a response to the environment encountered in each clone. Metabolites tend to follow similar patterns in all clones. Fructose levels decrease and mannitol levels increase late in infection in all clones. The most unusual observations are the correlations involving organic acids and regression models involving amino acids in CATIE-R4. The ability of the fungus to alter metabolite concentrations is largely dependent on the amount of fungus present and/or the stress it induces. Most of the infection-induced metabolite changes have been reported previously (Bailey *et al.*, 2013; Melnick *et al.*, 2012). The accumula-

Table 3 Linear regression analysis was carried out within each clone using *Moniliophthora royeri* gene expression as the dependent variable with the predictor variables of pod weight (Wt), percentage chlorosis (Ch), percentage necrosis (Nc) and fungal load (Fr).

EST	Pound-7			CATIE-1000			UF-273			CATIE-R7			CATIE-R4		
	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model
<i>MrMS</i>	***	0.48	Ch–Nc	***	0.72	Ch–Nc							*	0.17	Ch
<i>MrPDC</i>	**	0.33	Ch–Nc	*	0.17	Nc	*	0.21	Ch	*	0.16	Wt	*	0.14	Ch
<i>MrXFP5</i>	*	0.18	Ch	***	0.49	Ch–Nc							**	0.35	Ch
<i>MrACK</i>	**	0.43	Ch–Nc–Fr				**	0.29	Ch	***	0.44	Fr	*	0.22	Wt–Ch
<i>MrChs</i>	*	0.15	Ch–Fr	*	0.16	Ch	**	0.36	Ch	**	0.21	Fr	***	0.72	Wt–Ch–Nc–Fr
<i>MrHS20</i>	**	0.36	Ch–Nc–Fr	***	0.51	Ch–Nc				*	0.14	Wt	***	0.45	Wt–Ch
<i>MrGMCOR2</i>	**	0.38	Ch–Nc	***	0.53	Ch–Nc	**	0.29	Ch				***	0.45	Wt–Ch
<i>MrCp450e</i>	*	0.29	Ch–Nc	**	0.42	Ch–Nc									
<i>MrFR</i>	*	0.22	Ch							*	0.14	Wt			
<i>MrAOX</i>				**	0.27	Nc									
<i>MrC22SD</i>	*	0.25	Ch–Nc–Fr	*	0.13	Ch	***	0.53	Ch						
<i>MrACCoAS</i>	*	0.30	Ch–Nc	***	0.53	Ch–Nc							***	0.50	Ch–Nc–Fr
<i>MrGMCOR1</i>	*	0.24	Ch	***	0.54	Ch–Nc	*	0.27	Ch				*	0.21	Wt
<i>MrHS</i>	*	0.29	Ch–Nc–Fr	*	0.15	Wt									
<i>MrGH3</i>	***	0.47	Ch–Nc	***	0.66	Ch–Fr	***	0.51	Wt–Fr						
<i>MrMAG</i>	*	0.32	Ch				*	0.18	Wt				*	0.17	Wt
<i>MrCE8b</i>	***	0.80	Ch–Nc	***	0.67	Ch–Fr	***	0.62	Wt–Fr	***	0.45	Fr			
<i>MrMGT</i>	***	0.74	Ch–Nc	***	0.62	Ch–Fr	***	0.62	Wt–Fr	*	0.19	Fr	***	0.49	Wt
<i>MrTPP</i>	***	0.73	Ch–Nc	***	0.63	Wt–Fr	***	0.76	Wt–Fr	*	0.22	Fr	*	0.18	Wt
<i>MrLAC1</i>	***	0.61	Ch–Nc	***	0.69	Ch–Fr	*	0.35	Wt–Fr	**	0.35	Fr			
<i>MrLAC2</i>	***	0.52	Ch–Nc	***	0.62	Ch–Nc	*	0.21	Nc	**	0.31	Wt–Fr	**	0.27	Wt
<i>MrCP1</i>	***	0.61	Ch–Nc	***	0.65	Wt–Nc							*	0.29	Fr
<i>MrAAO</i>	***	0.60	Ch–Nc	***	0.88	Ch–Fr									
<i>MrEP15</i>	***	0.83	Ch–Nc	***	0.91	Ch–Fr	**	0.43	Wt–Fr	**	0.28	Fr	**	0.40	Wt–Fr
<i>MrEP20</i>	*	0.23	Nc–Fr	***	0.61	Ch–Fr	*	0.20	Wt						
<i>MrEP21</i>	***	0.67	Ch–Nc	***	0.66	Wt–Fr	**	0.29	Nc						
<i>MrTDAT</i>	***	0.56	Wt–Fr	***	0.60	Ch–Nc	*	0.25	Nc				***	0.47	Wt–Fr
<i>MrEP16</i>	**	0.25	Fr	***	0.69	Wt–Nc	***	0.57	Wt–Nc	***	0.39	Wt	*	0.16	Fr
<i>MrEP14</i>	***	0.74	Fr	***	0.79	Ch–Nc									
<i>MrEP17</i>				*	0.18	Nc–Fr							**	0.25	Fr
<i>MrID</i>	**	0.39	Ch–Nc	***	0.53	Ch–Nc							*	0.16	Fr
<i>MrEP22</i>	***	0.71	Wt–Fr	***	0.40	Fr	***	0.91	Fr				***	0.68	Ch–Fr
<i>MrNEP2</i>	*	0.21	Ch	***	0.56	Ch–Nc									
<i>MrCDF1</i>	**	0.32	Fr	***	0.47	Fr	***	0.53	Fr				***	0.53	Wt–Fr
<i>MrEP23</i>	**	0.35	Ch–Nc	***	0.56	Wt–Fr							*	0.14	Fr
<i>MrEP24</i>	***	0.77	Ch–Nc	***	0.50	Ch–Nc	***	0.66	Wt–Ch–Fr	*	0.19	Fr	***	0.45	Wt–Nc
<i>MrEP18</i>	***	0.65	Ch–Nc	***	0.66	Ch–Fr	**	0.45	Wt–Nc	*	0.22	Fr	*	0.20	Wt
<i>MrGEGH16</i>	***	0.64	Ch–Nc–Fr				*	0.24	Ch–Fr	**	0.33	Wt	*	0.26	Wt–Nc
<i>MeHYP1</i>	***	0.50	Ch–Nc–Fr	***	0.65	Ch–Nc	**	0.47	Wt*–Ch–Fr						
<i>MrEP25</i>	***	0.60	Ch–Nc	**	0.39	Ch–Fr	***	0.60	Wt–Ch–Fr				**	0.25	Wt
<i>MrEP26</i>	***	0.64	Ch–Nc	***	0.65	Ch–Fr	*	0.24	Nc	*	0.21	Wt–Fr			
<i>MrEP19</i>	**	0.52	Ch–Fr	***	0.70	Wt–Fr	***	0.62	Wt–Fr				*	0.22	Fr
<i>MrEP27</i>	***	0.66	Ch–Nc–Fr	***	0.43	Ch–Fr	*	0.24	Wt				***	0.46	Wt–Fr

Linear regression analysis was carried out within each clone using PROC REG in SAS 9.2 (SAS Institute, Raleigh, NC, USA). The most significant models ($\alpha \leq 0.05$) are presented for each clone for which all individual predictor variables within the regression model are also significant ($\alpha \leq 0.05$). Within columns the shaded areas indicate very highly significant models ($\alpha \leq 0.001$). *, **, *** represent significant ($\alpha \leq 0.05$), highly significant ($\alpha \leq 0.01$) and very highly significant ($\alpha \leq 0.001$), respectively. EST, expressed sequence tag.

tion of alanine in the tolerant clones may be a result of the onset of anoxia (Menegus *et al.*, 1989).

Changes in *M. royeri* gene expression in susceptible and tolerant clones

The Real-time qRT-PCR data suggest an orderly disease progression in the susceptible clones CATIE 1000 and Pound 7 (Fig. 3) in association with changes in *M. royeri* gene expression (Fig. 3). In many cases, regression analysis verifies the association between

symptoms, Fr and *M. royeri* gene expression in the susceptible clones (Table 3), supporting previous results linking changes in *M. royeri* gene expression with the biotroph/necrotroph shift (Bailey *et al.*, 2013). *MrMS* expression in susceptible clones is predicted by regression in models including Ch and Nc. *MrMS* (a putative malate synthase) is associated with the glyoxylate pathway used by fungi to meet carbon requirements, bypassing steps in the citric acid cycle. The glyoxylate cycle is induced during the shift from biotrophy to necrotrophy (Bailey *et al.*, 2013) and

Fig. 4 Principal coordinate analysis (PCoA) of real-time quantitative reverse transcription polymerase chain reaction expression levels for 43 *M. royeri* genes analysed over 114 naturally infected pods representing susceptible clones Pound-7 and CATIE-1000 and tolerant clones UF-273, CATIE-R7 and CATIE-R4. (A) Comparisons between coordinates 1 and 2. (B) Comparisons between coordinates 1 and 3. (C) Comparisons between coordinates 2 and 3. Colours represent Pound-7 (purple), CATIE-1000 (red), UF-273 (blue), CATIE-R7 (yellow), and CATIE-R4 (green). Shapes represent malformed green pods (squares), yellow pods with no necrosis (triangles) and necrotic pods (diamonds).

can be a critical virulence factor in some interactions (Dunn *et al.*, 2009). The models for *MrMS* are of reduced significance ($R^2 = 0.17$) for CATIE-R4, and insignificant for CATIE-R7 and UF-273. These results suggest a disruption in the ordered disease progression when *M. royeri* attempts to survive the pod environment of tolerant clones.

Tolerant clones are clearly separated from susceptible clones using the global gene expression patterns of the 12 RNA-Seq datasets. The plot derived from genes differentially expressed between CATIE-R4 and susceptible clones separates CATIE-R4 from the susceptible clones, with CATIE-R7 tending towards the same separation. The plot derived from the differentially expressed genes of CATIE-R7 closely groups the tolerant clones and separates the susceptible clones from one another. The genes differentially expressed between CATIE-R7 and the susceptible clones may represent genes uniformly differentially expressed by the tolerant clones, possibly derived from UF-273, a parent they share.

PCoA comparing individual pod samples across clones suggests that *M. royeri* behaves differently in MG pods of tolerant clones relative to responses in susceptible clones. In tolerant clones, *M. royeri* expression is often typical of the necrotrophic stage, despite limited tissue Nc/Ch (Fig. 3). The correlation analysis verifies the dissociation of the ordered progression of gene expression during the infection process in tolerant clones. Among 19 genes, there are two groups of tightly co-regulated genes. The first group includes *MrMS*, *MrPDC*, *MrXFP5*, *MrACK*, *MrChs*, *MrHS20*, *MrGMCOR2*, *MrCp450e*, *MrFr*, *MrAOX* and *MrC22SD*, which are highly expressed regardless of symptom or Fr in the tolerant clones. A relationship between *MrMS*, *MrPDC*, *MrXFP5* and *MrACK* has already been proposed (Bailey *et al.*, 2013). *MrXFP5* (a putative D-xylulose 5-phosphate phosphoketolase) may serve to reverse the flow of acetate kinase (*MrACK*) towards acetate feeding into the glyoxylate pathway. *MrPDC* (a putative pyruvate decarboxylase) is potentially involved in the production of acetyl-CoA through the pyruvate–acetaldehyde–acetate pathway, also contributing to the glyoxylate cycle (Strijbis and Distel, 2010). The second group includes *MrGH3*, *MrCE8b*, *MrMGT*, *MrTPP*, *MrLAC1*, *MrCP1* and *MrLAC2*. These genes show a pattern of expression closer to that observed in susceptible clones, being induced as symptoms and Fr increase. These genes encode secreted proteins suggesting changes in the fungal cell wall and in nutrient acquisition strategies. In some comparisons between these two groups, genes show coordinated expression patterns in susceptible clones not found in tolerant clones. Correlation coefficients between *MrMS* and *MrLAC1* are significant ($P \leq 0.001$) for Pound-7 and CATIE-1000, but not in the tolerant clones. The differential expression patterns of these groups (Fig. 4) probably contribute to the patterns seen in the PCoA plots separating the MG pods of the susceptible and tolerant clones.

Relating RNA-Seq analysis to Real-time qRT-PCR analysis

The RNA-Seq analysis identified over 800 *M. royeri* genes as differentially expressed between clones (Appendix S2, Induced). The Real-time qRT-PCR analysis indicates that the expression of most genes shifts between MG and Nc pods in the susceptible clones

Table 4 Pearson's correlation coefficient (Fisher's Z transformation) was calculated between combinations of the 19 *Moniliophthora royeri* expressed sequence tags (ESTs) within clones to determine the relationship between their expression.

Clone	MHPDC	MIXFP5	MIACK	MIChs	MHS20	MIGMCR2	MICp450e	MFD	MIAOX	MIC22SD	MHS	MIGH3	MIMAG	MICE8b	MMGT	MITPP	MLAC1	MICP1
Pound-7																		
MIMS	0.74	0.87		0.62	0.88	0.80	0.58	0.75	0.76	0.70	0.69	0.74		0.61	0.62	0.66	0.60	0.73
MPPDC		0.89	0.68	0.62	0.77	0.92	0.49	0.83	0.83	0.81	0.60	0.56		0.54	0.57	0.51	0.48	0.76
MIXFP5			0.65	0.66	0.84	0.93	0.62	0.86	0.88	0.85	0.69	0.61		0.49	0.51	0.51	0.44	0.68
MIACK				0.72	0.54	0.65		0.72	0.52	0.79	0.60							
MIChs				0.77	0.77	0.73		0.71	0.58	0.71	0.84	0.68						
MHS20						0.81		0.84	0.82	0.78	0.88	0.74	0.44				0.55	0.70
MIGMCR2								0.80	0.85	0.81	0.68	0.67	0.48				0.53	0.83
MICp450e							0.55				0.68	0.43					0.58	
MFD									0.85	0.83	0.76	0.45					0.61	0.63
MIAOX										0.75	0.70	0.52					0.46	0.62
MIC22SD											0.74	0.46					0.56	0.50
MHS												0.72					0.85	0.81
MIGH3													0.42				0.78	
MIMAG														0.55			0.60	0.67
MICE8b															0.98		0.93	0.81
MMGT																0.84	0.94	0.88
MITPP																	0.80	0.69
MLAC1																		0.86
CATE-1000																		
MIMS	0.72	0.88		0.67	0.89	0.92	0.77	0.68	0.72	0.71	0.74	0.83	0.42	0.79	0.83	0.79	0.79	0.85
MPPDC		0.88	0.64	0.80	0.72	0.86	0.73	0.90	0.87	0.75	0.71	0.52	0.56	0.50	0.70	0.64	0.44	0.64
MIXFP5			0.57	0.84	0.84	0.95	0.80	0.83	0.89	0.80	0.68	0.77	0.51	0.72	0.85	0.76	0.69	0.81
MIACK				0.72	0.46	0.51		0.65	0.52	0.66	0.56							
MIChs					0.81	0.80	0.68	0.74	0.70	0.79	0.67	0.57	0.55	0.53	0.67	0.49	0.49	0.71
MHS20							0.61	0.59	0.66	0.76	0.75	0.83	0.60	0.82	0.88	0.74	0.83	0.88
MIGMCR2								0.77	0.87	0.80	0.74	0.79	0.53	0.75	0.88	0.79	0.76	0.84
MICp450e							0.80				0.65	0.60		0.48	0.60	0.59	0.45	
MFD								0.81	0.73	0.71	0.65	0.47			0.56	0.56	0.55	0.51
MIAOX									0.87	0.76	0.64	0.63	0.45	0.58	0.74	0.69	0.53	0.68
MIC22SD											0.82			0.51	0.64	0.56	0.53	0.62
MHS												0.50		0.45	0.53	0.54	0.53	0.55
MIGH3													0.45	0.88	0.89	0.81	0.85	0.80
MIMAG														0.53	0.65	0.51	0.49	0.67
MICE8b															0.94	0.82	0.90	0.87
MMGT																0.86	0.88	0.90
MITPP																	0.77	0.82
MLAC1																		0.84
UF-273																		
MIMS	0.69	0.86	0.48		0.72	0.77	0.85	0.68	0.74		0.78							
MPPDC		0.78	0.86	0.75	0.81	0.91	0.89	0.74	0.67	0.50	0.82							
MIXFP5			0.64	0.64	0.81	0.81	0.80	0.57	0.67		0.85							
MIACK				0.89	0.75	0.79	0.70	0.58			0.85							
MIChs					0.71	0.77	0.63			0.48	0.69							
MHS20						0.86	0.82	0.62	0.54		0.93							
MIGMCR2							0.87	0.73	0.59	0.57	0.84							
MICp450e								0.76			0.87							
MFD									0.76		0.87							
MIAOX										0.49	0.58							
MIC22SD											0.61							
MHS																		
MIGH3																		
MIMAG																		
MICE8b																		
MMGT																		
MITPP																		
MLAC1																		

Table 4 Continued.

Clone	MpDC	MxXP5	MiACK	MiChs	MiHS20	MiGMCOR2	Micp450e	MiFD	MiAOX	MiC22SD	MiHS	MiGH3	MiMAG	MiCE8b	MiMGT	MiTPP	MiLAC1	MiCP1
CATIE-R7																		
MiMS	0.64	0.78		0.58	0.79	0.84	0.66	0.67	0.49									0.43
MpDC		0.58	0.68	0.72	0.62	0.73		0.64	0.73									
MiXP5				0.54	0.78	0.93	0.91	0.79	0.52	0.51			0.45					0.49
MiACK				0.71					0.51				0.45					
MiChs					0.76	0.71		0.72	0.77									
MiHS20						0.86	0.61	0.73	0.53									
MiGMCOR2							0.81	0.85	0.64				0.46					0.53
Micp450e													0.47					0.47
MiFD								0.76					0.52					0.45
MiAOX									0.71				0.57					
MiC22SD												0.65						
MiHS																		
MiGH3																		
MiMAG																		
MiCE8b																		
MiMGT																		
MiTPP																		
MiLAC1																		
CATIE-R4																		
MiMS	0.84	0.83	0.61	0.74	0.76	0.84	0.42	0.64	0.63	0.63	0.71	0.29						0.27
MpDC		0.88	0.65	0.72	0.80	0.87	0.52	0.85	0.79	0.72	0.80	0.47						0.34
MiXP5				0.79	0.84	0.87	0.51	0.72	0.67	0.76	0.77							
MiACK				0.66	0.55	0.55		0.50	0.79	0.72	0.62							
MiChs					0.90	0.88	0.45	0.70	0.51	0.59	0.70							
MiHS20						0.92	0.56	0.83	0.52	0.60	0.73							
MiGMCOR2							0.53	0.82	0.60	0.60	0.71							0.54
Micp450e																		
MiFD								0.46										
MiAOX									0.61	0.58	0.76	0.41						0.44
MiC22SD										0.80	0.64	0.52						
MiHS											0.81							
MiGH3																		
MiMAG																		
MiCE8b																		
MiMGT																		
MiTPP																		
MiLAC1																		

Correlations between ESTs significant for all clones are within shaded outlined boxes. Correlations between ESTs significant for four clones are within unshaded outlined boxes. Numbers with shaded boxes that are not outlined represent EST comparisons in which correlations were significant only for the susceptible clones Pound-7 and CATIE-1000.

Fig. 5 *Moniliophthora royeri* gene expression levels for *MrMS* and *MrFR* (A), *MrAOX* and *MrLAC1* (B), *MrCE8b* and *MrMGT* (C) and *MrAAO* and *MrTDAT* (D) analysed over 114 naturally infected pods representing susceptible clones Pound-7 and CATIE-1000 and tolerant clones UF-273, CATIE-R7 and CATIE-R4. Graphs also show % chlorosis and % necrosis (E) and relative expression for *M. royeri* reference genes (Fr, %Pr) (F).

(Fig. 3). This association often results in highly significant predictive models dependent on Wt, Ch, Nc and/or Fr. The coordinated expression among these transcripts (Pearson's correlations) and between these transcripts, Ch, Nc and Fr (regression analysis) is often disrupted in tolerant clones.

Twenty-three genes (Table 1) showed differential expression between individual tolerant and susceptible clones in all four RNA-Seq comparisons. The putative functions of the 14 genes up-regulated in tolerant clones suggest an elite class of stress-responsive genes, including three small heat shock proteins (sHsps), a sugar transporter, a C6 transcription factor, a siderophore iron transporter, five reductases and three genes with no putative function. Consistently down-regulated genes include two *ich1*-like methyl transferases, a glycine/*b*-amino acid oxidase, a cellobiose dehydrogenase, an AIFL-like pyridine nucleotide-disulphide oxidoreductase, a catalase and three genes of unknown function. These genes are members of a larger group of related genes that show differential expression between clones (Appendix S2).

Differential gene expression between clones

Potential effector proteins

Effector proteins modify host responses to infection and promote the growth of the microbe (Rafiqi *et al.*, 2012). Effectors are generally secreted proteins functioning in the apoplast or taken up by the host cell, possibly reaching the nucleus (Rafiqi *et al.*, 2012). Effectors are often small (small secreted proteins, SSPs) and cysteine rich (Rafiqi *et al.*, 2012). Six hundred and thirty-seven of the secreted proteins are 300 amino acids or less in length, 126 having at least eight cysteines (Appendix S2, Secretome). Ninety SSPs are differentially expressed between clones, 44 having no putative function, and 29 having eight or more cysteines. There are a large number of hydrophobins (at least 38) in the *M. royeri* transcriptome, (Wosten, 2001), which may function as effectors (de Wit *et al.*, 2009). Seven hydrophobins are differentially expressed between clones (see *MrHYP1* in Fig. 3). Other potential effectors showing differential expression include pathogenesis-

Table 5 Linear regression analysis was carried out within each clone using metabolite concentration (ng/mg dry weight) as the dependent variable with the predictor variables of pod weight (Wt), percentage chlorosis (Ch), percentage necrosis (Nc) and fungal load (Fr).

Metabolite	Pound-7			CATIE-1000			CATIE-R7			CATIE-R4		
	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model	Sign	R ²	Model
Mannitol	***	0.75	Fr	***	0.86	Fr	***	0.82	Fr	***	0.61	Wt–Nc
Fructose	***	0.88	Wt–Ch–Nc	***	0.80	Nc	**	0.44	Wt–Fr	**	0.47	Wt–Nc
Glucose	***	0.84	Wt–Ch–Nc	***	0.70	Nc	**	0.43	Wt–Fr	**	0.45	Wt–Nc
Sucrose	***	0.80	Nc	***	0.69	Nc	***	0.48	Wt–Fr	***	0.61	Wt–Nc
Maltose	***	0.64	Wt–Nc				**	0.28	Wt			
Trehalose	***	0.85	Nc	***	0.60	Fr	***	0.49	Nc	***	0.44	Wt
Ribose	***	0.51	Wt–Nc	*	0.29	Nc	*	0.22	Fr	*	0.23	Wt
Pyruvic							*	0.19	Wt			
Malonic	***	0.54	Nc–Fr	***	0.52	Nc	***	0.49	Wt–Ch–Nc	**	0.37	Nc
Glyceric	**	0.35	Fr				*	0.15	Wt	**	0.44	Wt–Nc
Fumaric	***	0.79	Ch–Nc–Fr	**	0.34	Fr	***	0.69	Fr	**	0.39	Fr
Succinic	*	0.20	Fr							*	0.16	Nc
Maleic										**	0.30	Fr
Malic	***	0.58	Nc	***	0.73	Nc	***	0.57	Wt–Fr			
Adipic	*	0.19	Ch	*	0.21	Ch						
2-Oxoglutaric	*	0.16	Ch	***	0.50	Nc	*	0.23	Fr	*	0.20	Ch
Quinic				*	0.20	Fr						
Aconitate	***	0.79	Wt–Nc	*	0.26	Nc	*	0.30	Wt–Fr	**	0.35	Wt
Shikimic	***	0.85	Wt–Nc	***	0.82	Wt–Nc	**	0.33	Wt–Fr			
Citric	**	0.46	Wt–Nc	***	0.53	Nc	**	0.26	Wt	*	0.25	Nc
Serine	*	0.20	Nc	**	0.41	Nc	*	0.16	Nc	*	0.24	Nc
Threonine										*	0.24	Nc
glutamine	***	0.66	Nr–Fr	**	0.35	Nc	*	0.19	Fr			
Phenylalanine							*	0.29	Nc–Fr			
Isoleucine	*	0.22	Fr							***	0.67	Wt–Ch–Fr
Glycine	*	0.19	Ch	*	0.23	Nc				***	0.55	Wt–Nc
Proline				**	0.47	Ch–Nc				*	0.17	Nc
Alanine							**	0.33	Fr	***	0.47	Fr
Valine												
Leucine										*	0.15	Ch

Linear regression analysis was carried out within each clone using PROC REG in SAS 9.2 (SAS Institute, Raleigh, NC, USA). The most significant models ($\alpha \leq 0.05$) are presented for each metabolite for each clone for which all individual predictor variables within the regression model are also significant ($\alpha \leq 0.05$). Within columns, the shaded areas indicate very highly significant models ($\alpha \leq 0.001$). *, **, *** represent significant ($\alpha \leq 0.05$), highly significant ($\alpha \leq 0.01$) and very highly significant ($\alpha \leq 0.001$), respectively.

related-1 (PR-1)-like proteins (Teixeira *et al.*, 2012), ceratoplatins (Barsottini *et al.*, 2013; see *MrCP1* in Fig. 3), and necrosis and ethylene-inducing peptide 1 (NEP1)-like proteins (de Jonge and Thomma, 2009; see *MrNEP2* in Fig. 3).

Transcription factors

Several putative transcription factors (Appendix S2, Transcription) are up-regulated in the tolerant clones: HMG domain, BZip domain, C2H2 zinc finger and C6 transcription factors (Shelest, 2008). At least four members of the HMG domain MATA-type transcription factor family show induction in the tolerant clones. In the Real-time qRT-PCR analysis, *MrHMGTF*, a MATA-type transcription factor, shows partial induction in the tolerant clones prior to pathogen buildup. *MrHMGTF* is induced during the *M. royeri* biotrophic/necrotrophic shift (Bailey *et al.*, 2013). Four genes encoding C6 transcription factors show induction in the tolerant clones. In the future, it may be possible to connect the differential expression patterns of C6 transcription factors (zinc finger

proteins) with the specific genes and processes they regulate (MacPherson *et al.*, 2006).

sHsps

Three sHsps are induced in tolerant clones and six sHsps are induced in CATIE-R4 (Appendix S2, sHsp). *MrHS20* and *MrHS* are highly expressed in the tolerant clones, expressed at low levels in CATIE-1000 and increase in Pound-7 late in infection (Fig. 3). sHsps are generally active as large oligomers, preventing protein aggregation and aiding refolding (Haslbeck *et al.*, 2005). Mammalian sHsps protect cells from oxidative stress, among other processes (Haslbeck *et al.*, 2005). Less is known about fungal sHsps, although they are induced by heat and various stresses (Chagué *et al.*, 2006).

Alternative oxidase

An alternative oxidase gene (*MrAOX*) is induced early in tolerant clones (Fig. 3). AOX is a mitochondrial electron transport

[illegible]

Table 6 Continued.

	Fructose	Glucose	Sucrose	Trehalose	Malonic	Fumaric	Succinic	Malic	2-Oxoglutaric	Aconitate	Shikimic	Serine	Threonine	Isoleucine	Glycine	Proline	Alanine	Valine	Leucine
Succinic																			
Malic		≥ 0.05	≥ 0.41							0.48	0.66			0.50	0.48				0.41
2-Oxoglutaric		≥ 0.01	≥ 0.52									0.49							
Aconitate		≥ 0.001	≥ 0.62								0.62		-0.42		-0.43				-0.49
Shikimic		≥ 0.0001	≥ 0.71										0.67	0.56		0.53	0.62		0.64
Serine														0.60		0.50	0.70		0.74
Threonine															0.43		0.63		0.86
Isoleucine															0.72	0.60		0.86	0.54
Glycine																	0.48	0.63	0.54
Proline																			0.74
Alanine																			
Valine																			
CATIE-R4																			
Mannitol				0.86	0.98	0.50								0.58	0.56	0.45	0.82		0.69
Fructose	0.94		0.61					0.65			0.95								
Glucose			0.68					0.70			0.89		-0.50						
Sucrose								0.72			0.54								
Trehalose					0.75									0.49			0.75		0.53
Malonic						0.52							0.45	0.59	0.61	0.48	0.79		0.69
Fumaric									0.74			0.63	0.82	0.92	0.89	0.88	0.81	0.71	0.88
Succinic							0.77		0.94			0.82	0.86	0.77	0.80	0.81	0.45	0.88	0.67
Malic										0.73	0.71								
2-Oxoglutaric		≥ 0.05	≥ 0.44									0.83	0.84	0.75	0.77	0.79		0.76	0.57
Aconitate		≥ 0.01	≥ 0.56																
Shikimic		≥ 0.001	≥ 0.61								0.93								
Serine		≥ 0.0001	≥ 0.74																
Threonine													0.86	0.70	0.74	0.77		0.74	0.59
Isoleucine														0.90	0.93	0.94	0.65	0.82	0.83
Glycine															0.97		0.85	0.73	0.92
Proline																0.93	0.81	0.76	0.91
Alanine																	0.75	0.82	0.92
Valine																		0.50	0.91
																			0.75

Correlations between expressed sequence tags (ESTs) were significant for all clones for which numbers are within outlined boxes. Numbers with shaded boxes represent metabolite comparisons with highly significant ($P \leq 0.001$) correlations.

Fig. 6 Graphs showing pod weight (g), symptoms (chlorosis and necrosis), fungal load (Fr), and mannitol, fructose, fumaric acid, 2-oxoglutaric acid and alanine concentrations (ng/mg dry weight) for pods of Pound-7, CATIE-1000, CATIE-R7 and CATIE-R4.

alternative oxidase insensitive to nitric oxide and can be induced by reactive oxygen (Joseph-Horne *et al.*, 2001). Nitric oxide is a component of plant defense, including the hypersensitive response (Joseph-Horne *et al.*, 2001). Thomazella *et al.* (2012) observed elevated AOX transcripts in the biotrophic phase of *Moniliophthora perniciosa*, but *MrAOX* is induced in the necrotrophic phase of FPR.

Transporters

A large number of transporter-like genes are differentially expressed: carboxylate and sugar transporters, peptide and amino acid permeases, and drug transporters [ATP-binding cassette (ABC) and major facilitator superfamily (MFS) type] (Appendix S2, Transporters). Carboxylate/monocarboxylate transporters are

required for the use of alternative carbon sources, such as acetate, lactate and pyruvate, as substrates for carbon assimilation (Casal *et al.*, 2008). An extracellular invertase gene is expressed in both tolerant and susceptible clones. The combination of extracellular invertases with hexose transporters has been observed in other hemibiotrophic fungi as an efficient way of using host sucrose (Münch *et al.*, 2008). Increased symptoms of FPR are associated with decreased sucrose and fructose levels.

Peptide/amino acid transporters/permeases show differential regulation between susceptible and tolerant clones in the RNA-Seq analysis. Substrate specificities of peptide/amino acid transporters/permeases vary (Wipf *et al.*, 2002). The pathogen is probably secreting peptidases/proteases (Appendix S2, Peptide Digestion), targeting plant peptide degradation.

At least eight MFS-multidrug-resistance transporters are differentially expressed between tolerant and susceptible clones. An ABC transporter is also induced in tolerant clones. Members of ABC and MFS transporter families can be critical virulence factors via the management of fungal metabolites toxic to plants and through the efflux of antifungal plant metabolites (Del Sorbo *et al.*, 2000).

Reductases

Three glucose-methanol-choline (GMC)-oxidoreductases are induced in the tolerant clones (Appendix S2, Reduction Oxidation). GMC-oxidoreductases catalyse a diverse range of reactions (Zámocký *et al.*, 2004). *MrGMCOR2* shows close correlation with the *MrMS/MrXFP* expression group, and expression in tolerant clones is elevated at low Fr. A group of cytochrome p450 monooxygenases is differentially expressed between tolerant and susceptible clones. Cytochrome p450 monooxygenases participate in sterol biosynthesis, degradation of lignin and breakdown of environmental contaminants (van den Brink *et al.*, 1998), processes probably altered in the interaction between tolerant and susceptible clones.

Many short dehydrogenase/reductases (SDR) and other reductases are differentially expressed. SDRs are involved in the synthesis and breakdown of metabolites, such as fatty acids, amino acids, polyketides, sugars and steroids/sterols (Mannhaupt *et al.*, 2003). Mannitol-1-phosphate dehydrogenase (up-regulated in tolerant clones) converts fructose-6-phosphate to mannitol-1-phosphate, leading to mannitol production (Solomon *et al.*, 2007). Mannitol accumulates with the onset of Nc.

Up-regulation of malate dehydrogenase in the tolerant clones may be related to a shift between the tricarboxylic acid (TCA) and glyoxylate cycles (Lorenz and Fink, 2001). Two unique reductases, a ferric-chelate reductase and a fumarate reductase, are induced in the tolerant clones. Microorganisms secrete siderophores which chelate ferric iron prior to transport (Lesuisse *et al.*, 1990). A putative MFS-type siderophore iron transporter is differentially expressed in the tolerant clones (RNA-Seq). Some microorganisms reduce the iron to Fe(II) using ferric-chelate reductase once the iron enters the cell, resulting in siderophore release. Real-time qRT-PCR analysis verifies that the putative fumarate reductase gene is expressed at higher levels in tolerant clones (Fig. 5). Fumarate reductase catalyses the conversion of fumarate to succinate. Enomoto *et al.* (2002) have suggested that fumarate reductase is required under anaerobic conditions to re-oxidize the reduced form of nicotinamide adenine dinucleotide (NADH), a possible link to the observed fumaric acid accumulation and differential expression of *MrAOX* in the tolerant clones.

Modifications of fungal and plant membranes and cell walls

Genes required for the synthesis of ergosterols are induced in CATIE-R4 in the RNA-Seq analysis (Appendix S2, Membrane and

Cell Wall): sterol C-14 reductase, C-4 methyl sterol oxidase, sterol C-24 methyltransferase, sterol C-8 isomerase, sterol C-5 desaturase and sterol C-22 desaturase (Lees *et al.*, 1995). Sterols, components of plant and fungal membranes, are important in fungal development and have been implicated in stress responses. Swan and Watson (1998) have observed that high ergosterol concentrations correlate with increased viability after exposure to heat and ethanol in *Saccharomyces cerevisiae*.

In CATIE-R4, three hydrophobin genes are up-regulated and two are down-regulated. Hydrophobins, wall structural proteins, mediate interactions with the environment (Linder *et al.*, 2005). Regression models tested for *MrHYP1* are significant for the susceptible clones and UF-273, but not for CATIE-R4 and CATIE-R7 (Table 3). *MrHYP1* is expressed at low levels early in infection in the susceptible clones and UF-273, and at higher levels in CATIE-R7 and CATIE-R4, suggesting a change in wall structure.

Chitin and chitosan are fungal cell wall components (El Gueddari *et al.*, 2002). Putative chitin synthases and deacetylases show increased expression in tolerant clones (RNA-Seq). Real-time qRT-PCR verifies that tolerant clones show high expression of putative chitin synthase *MrChs* (Fig. 3). El Gueddari *et al.* (2002) have suggested that fungi modify the chitin in their walls to chitosan to limit the activity of plant chitinases, thus reducing plant defense. The fungus may also down-regulate its own chitinases, as four *M. royeri* type II chitinases are down-regulated in CATIE-R4.

Several laccases are differentially expressed in CATIE-R4 (RNA-Seq). In CATIE-R4 (Real-time qRT-PCR), *MrLAC1* (Fig. 5) fails to show the typical shift in expression observed in other clones in association with the biotroph/necrotroph shift. Laccases are involved in lignin breakdown by the fungus, but also function in the deposition of pigments in the fungal wall (Singh and Chen, 2008). A secreted glucose oxidase is also induced in the tolerant clones (Appendix S2, Reduction and Oxidation), but other enzymes supporting lignin degradation are not differentially expressed (Hatakka, 1994).

Although *M. royeri* genes targeting pectin, cellulose and xylan show differential expression between tolerant and susceptible clones in the RNA-Seq analysis, some members are up-regulated and others are down-regulated. Complex stage-specific regulation of genes targeting plant cell walls during the biotroph/necrotroph shift has also been observed in hemibiotrophic *Colletotrichum* species (Gan *et al.*, 2013). 1,4- β -D-Glucan cellobiohydrolase cleaves cellobiosyl units from the nonreducing end of cellulose chains (Zeilinger *et al.*, 1998). Two putative 1,4- β -D-glucan cellobiohydrolases are up-regulated early in the infection of CATIE-R4 and a third is down-regulated. A putative xylanase is up-regulated and two xylanases are down-regulated. Xylans are hemicelluloses found in the plant cell wall (Polizeli *et al.*, 2005). Genes targeting other hemicelluloses (mannans, glucans, arabinans and galactans) show differential expression between

clones. Genes encoding enzymes targeting pectin (D-galacturonic acid reductase, endo- and exo-polygalacturonase, and pectin methylesterase) show differential expression depending on the clone. Pectin is a major component of cacao pods (Adomako, 1972). Real-time qRT-PCR verifies that *MrCE8b* (putative pectin methylesterase) and *MrGH3* (putative β -glucosidase) are differentially expressed. *MrCE8b* is induced during the shift from biotroph to necrotroph in all clones except CATIE-R4.

CONCLUSIONS

Successful infections of tolerant clones by *M. royeri* result in a disassociation between typical progressive fungal colonization patterns, disease symptom development and fungal gene expression as observed during infections of susceptible clones. In tolerant clones, disease symptoms occur at low Fr and include Ch in the absence of Nc, a rare occurrence in susceptible clones. These reactions leave *M. royeri* in a poor position to exploit nutrients released as tissues senesce. Pod metabolites change as disease progresses, but few changes are associated with tolerance. Infection of tolerant clones elicits specific *M. royeri* responses to the unfavourable environment, including changes in metabolism and the induction of fungal stress responses designed to mitigate plant defense mechanisms. The *M. royeri* genes differentially expressed between tolerant and susceptible clones are candidates for functional analysis with regard to their contribution to the overcoming of cacao defense mechanisms by *M. royeri*.

EXPERIMENTAL PROCEDURES

Artificial inoculation of five clones of cocoa with *M. royeri*

Three tolerant clones and two susceptible clones were selected for study. The susceptible clones were Pound-7 and CATIE-1000. The tolerant clones were UF-273 type 1, CATIE-R7 (an offspring of UF-273 $\times$ CATIE-1000) and CATIE-R4 (an offspring of UF-273 $\times$ PA-169). The trees were located at the CATIE field station, La Lola, near Siquirres, Costa Rica. Eighty pods for each clone were hand pollinated in 2009/2010 with 40 pods being artificially inoculated at 2 months of age (Bailey *et al.*, 2013). Artificial inoculation was inefficient for the tolerant clones. As a result, only uninoculated pods harvested at 60 and 90 DAP (five to six pods per clone per time point) were used.

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

In 2010, monthly visits (July to November) were made to the clonal plots described above and pods of five clones (CATIE-1000, Pound-7, UF-273 type 1, CATIE-R7 and CATIE-R4) showing symptoms of infection were harvested. Pods showing a range of symptoms (malformed, chlorotic, malformed and chlorotic, and necrotic) were harvested for each clone. The

Pods were transported to the laboratory, weighed, photographed and placed in liquid nitrogen for 10 min. The pods were lyophilized for 48–72 h and vacuum sealed in plastic bags for shipping to Beltsville, MD, USA.

RNA extraction

Pods were coarsely ground under liquid nitrogen, and approximately 1 cm³ of sample was used for processing. The pod material was ground finely and transferred to a 50-mL centrifuge tube containing 15 mL of 65 °C extraction buffer (Bailey *et al.*, 2005). Additional extraction methods were conducted as in Bailey *et al.* (2013). cDNA was synthesized using the Invitrogen Superscript VILO kit (Carlsbad, CA, USA), following the manufacturer's directions.

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

Procedures for Real-time qRT-PCR and analysis are as described by Bailey *et al.* (2013). The amount of *M. royeri* Ref transcript, a proxy for Fr in each sample, was estimated relative to the expression of three cacao Refs (Pr). Fr in each sample was calculated by comparing the expression of three *M. royeri* Refs (*MrACT*, *MrGADPH* and *MrUBQ*) with the expression of three cacao Refs (*TcACT*, *TcACP* and *TcEF1 α*). The geometric mean (Vandesompele *et al.*, 2002) (C_{T+Pr}) was calculated for the cycle times of three cacao Refs. ΔC_T was calculated for each *M. royeri* Ref [$\Delta C_T = (C_{T+Pr}) - (C_{T+Mr Fr(1-3)})$]. The expression levels for each of the three *M. royeri* Refs in each sample were calculated as %Pr = $100 \times [(E)^{\Delta C_T}]$, where E is the primer efficiency for each *M. royeri* Ref. Fr was estimated as the geometric mean of the expression levels of the three *M. royeri* Refs (%Pr).

RNA samples from infected pods of susceptible (Pound-7 and CATIE-1000) and tolerant (CATIE-R4 and CATIE-R7) clones were selected for RNA-Seq analysis carried out by the National Center for Genome Resources (Santa Fe, NM, USA). There were three replicate samples per clone. The cDNA was generated using an RNA library preparation TruSeq protocol developed by Illumina Technologies (San Diego, CA, USA). The mRNA was first isolated from total RNA by polyA selection, and single-end sequencing libraries were constructed with an insert size of 160 bp. Single-end sequencing was performed using the Illumina HiSeq platform. Samples were multiplexed with six-mer barcodes, generating 720 170 543 filtered 1 $\times$ 50-bp read pairs. The sequences were verified with regard to source by comparison with the *M. royeri* genome [National Center for Biotechnology Information (NCBI) BioProject PRJNA213737; <http://www.ncbi.nlm.nih.gov/bioproject/213737>] and the *T. cacao* genome (<http://cocoagendb.cirad.fr/>). RNA reads from RNA-Seq libraries, ranging from 50 to 70 million reads in fastq format, were aligned using memory-efficient short-read aligner Bowtie-0.12.7 (Langmead *et al.*, 2009) to the coding sequences of the *M. royeri* genome and the coding sequences of the *T. cacao* genome (Argout *et al.*, 2010). Tabulated raw counts of reads to each CDS were obtained from the Bowtie alignment. Estimation and statistical analysis of the expression level using gene count data with three replicates for each library were performed using the DESeq package (Anders and Huber, 2010) and R $\times$ 64 2.15.2 program (<http://www.r-project.org/>).

Moniliophthora royeri genes (43) were chosen for analysis by Real-time qRT-PCR across all infected pods based on their differential expression

patterns, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis (Appendix S3, see Supporting Information) and putative ID (Appendix S2). The *M. royeri* genes (Appendix S2, primers used) were chosen based on the putative function and differential expression between tolerant and susceptible clones. To obtain relative transcript levels, the threshold cycle (C_T) values for the genes of interest (C_{T-GOI}) were normalized to the C_T values (geometric mean) of the three *M. royeri* Refs (*MrACT*, *MrGADPH* and *MrUBQ*, C_{T-Ref}) for each sample [$\Delta C_T = (C_{T-Ref}) - (C_{T-GOI})$]. The expression levels for each *M. royeri* gene in each sample were calculated as %Fr = $100 \times [(E)^{-\Delta C_T}]$, where E is the primer efficiency.

Metabolite analysis

Metabolites were measured for healthy pods at 60 and 90 DAP (three to four pods per clone per harvest time) and for at least 15 naturally infected pods for clones Pound-7, CATIE-1000, CATIE-R7 and CATIE-R4. Wt was used as a proxy for age in the naturally infected pods by comparison with the healthy pods of known age. Infected pods showing extremely low (Fr < 0.03%Pr) or high (Fr > 11.00%Pr) Fr were not included. Extractions were conducted using the methodology in Melnick *et al.* (2012). Freeze-dried tissue was ground to a powder (50 mg dry weight) in liquid nitrogen and extracted at 4 °C with 1.4 mL methanol in a ground glass homogenizer. A mixture of 45 µL of internal standards, containing 62.5 nmol of α -aminobutyric acid and 26 µmol of ribitol, was injected prior to homogenization. The homogenates were collected, incubated at 70 °C in an H₂O bath for 15 min, and diluted with an equal volume of deionized H₂O. The aqueous methanol extracts were centrifuged for 15 min at 5800 g. The supernatants were transferred to fresh tubes and stored for up to 2 weeks at 20 °C. Amino acids, organic acids and soluble carbohydrates were quantified by gas chromatography coupled to mass spectrometry using known concentrations of chemical standards.

Statistical analysis

Relative *M. royeri* gene expression (%Fr and Fr expressed as %Pr) and metabolite dry weight (ng/mg) 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). Predictor variables were Wt, %Ch, %Nc and relative *M. royeri* Ref expression (Fr), and the dependent variable was the relative expression of a specific *M. royeri* gene. The most highly significant models (P -adjusted ≤ 0.05), where all included predictors are significant ($P \leq 0.05$), are presented. A heat map of the relative expression of the individual *M. royeri* genes was created using TIGR MultiExperiment Viewer (MeV) (Saeed *et al.*, 2003). Fr (%Pr) was also regressed against Wt, %Ch and %Nc.

Correlations within a clone between the expression of the 43 *M. royeri* genes ($\log_{10}$ %Fr) and between metabolite concentrations ($\log_{10}$ dry weight) were calculated using Pearson's correlation statistic (Fisher's Z transformation) employing PROC CORR in SAS 9.2. In examining the relationship between *M. royeri* gene expression patterns among clones, PROC DISTANCE of SAS 9.2 was used to calculate the Euclidean distance between gene pairs. PCoA was performed using GenAlEx 6.1 with the distance matrix of gene expression as $\log_{10}$ %Fr and the option of adjusting the covariance to represent trends in gene expression through

low-dimensional projection of high-dimensional data (Katagari and Glazebrook, 2009).

ACKNOWLEDGEMENTS

This work was funded by the US Department of Agriculture Agricultural Research Service (USDA-ARS). References to a company and/or product by the USDA are only for the purposes of information and do not imply approval or recommendation of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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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.

Appendix S1 Library statistics and correlations.

Appendix S2 RNA-Seq analysis.

Appendix S3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.