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ORIGINAL PAPER

The interaction of *Theobroma cacao* and *Moniliophthora perniciosa*, the causal agent of witches' broom disease, during parthenocarpy

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Abstract Witches' broom disease of *Theobroma cacao* L. is caused by the hemibiotrophic basidiomycete *Moniliophthora perniciosa*. Infection of flower cushions by *M. perniciosa* results in parthenocarpy. Healthy and parthenocarpic immature cacao pods were obtained from seven cacao clones. Microscopic observations of parthenocarpic pods from two clones confirmed that fruits lack viable seed. Septate mycelia colonized parthenocarpic pods, but were absent from healthy pods. Parthenocarpic pods had increased concentrations of leucine, methionine, serine, phenylalanine, and valine. Major transport metabolites sucrose and asparagine were decreased by 63 and 40 %, respectively, during parthenocarpy. *M. perniciosa* expressed sequence tags (ESTs) related to detoxification (*MpSOD2* and *MpCTA1*) and nutrient acquisition (*MpAS*, *MpAK*, *MpATG8*, *MpPLY*, and *MpPME*) were induced in parthenocarpic pods. Most *M. perniciosa* ESTs related to plant hormone biosynthesis were repressed (*MpGAox*, *MpCPS*, *MpDES*, *MpGGPPS*, and *MpCAO*) in parthenocarpic pods. RT-qPCR analysis was conducted for 54 defense-related cacao ESTs and 93 hormone-related cacao ESTs. Specific cacao ESTs related to plant defense were induced (TcPR5, TcChi4, TcThau-ICS) while others were repressed (*TcPR1*, *TcPR6*, *TcP12*, and *TcChiB*). Cacao ESTs related to GA biosynthesis (*TcGA20OX1B*) were repressed in parthenocarpic pods. Cacao ESTs putatively related to maintaining cytokinin (*TcCKX3* and *TcCKX5*) and IAA (*TcGH3.17a*, *TcGH3.1*, *TcARF18*) homeostasis were induced in parthenocarpic pods, suggesting an attempt to regulate cytokinin and auxin concentrations. In conclusion, *M. perniciosa* expresses specific sets of transcripts targeting nutrient acquisition and survival while altering the host physiology without causing significant necrosis resulting in parthenocarpy. Only a general host defense response is elicited.

Keywords Parthenocarpy · Cacao · *Moniliophthora perniciosa* · Witches' broom · Plant–microbe interaction

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Introduction

Theobroma cacao L. is an understory tropical tree with seeds that are processed into cocoa products. Throughout Central and South America, cacao production has been severely limited by fungal diseases (Hebbar 2007). Witches' broom, caused by the hemibiotrophic agaric basidiomycete *Moniliophthora perniciosa* (Aime and Phillips-Mora 2005), occurs in all major cacao-producing areas in South America (Meinhardt et al. 2008). Introduction of witches' broom to Bahia, Brazil caused the country to fall from the third largest cocoa producer in the world to having to import beans for production (Meinhardt et al. 2008). In Latin America, most cacao is produced on family farms of about 20–30 ha

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(Hebbar 2007). Witches' broom can be devastating, as farmers may not have the resources for control measures such as phytosanitation and fungicides. Resistant clones offer cost-effective means to manage disease, but breeding programs are lengthy and identifying and distributing resistant materials to farmers is difficult.

Basidiocarps of *M. perniciosa* are produced on necrotic tissues in the canopy (Aime and Phillips-Mora 2005). Basidiospores infect meristematic tissue causing symptoms in stems, flower cushions, and pods (fruits). The most researched symptoms of *M. perniciosa* infection are branch brooms, although flower cushions (Fig. 1a) are also infected. Infection of flower cushions results in the production of vegetative shoots (Fig. 1b) and the formation of parthenocarpic pods (Fig. 1c–f) (Meinhardt et al. 2008). In brooms, the biotrophic phase is associated with monokaryotic hyphae, while dikaryotic hyphae are associated with the necrotrophic phase (Meinhardt et al. 2008). Elucidation of *M. perniciosa* pathogenicity genes and modes of attack are still poorly understood, and *M. perniciosa* research has focused on mycelia grown under laboratory conditions or stem infections (Leal et al. 2010; Lima et al. 2009). Little is known about the physiology of parthenocarpic pods (Fig. 1c, d). Parthenocarpic pods remain green and distorted during the early biotrophic phases of infection (Fig. 1e) but become necrotic after 1 to 2 months (Fig. 1f) (Meinhardt et al. 2008). Parthenocarpic pods are an often overlooked source of inoculum. Basidiocarps often develop on them.

Infection of cacao stems with *M. perniciosa* alters the plant hormone balance resulting in hypertrophy and hyperplasia (Kilaru et al. 2007; Scarpari et al. 2005). Although genes for phytohormone production occur in the *M. perniciosa* genome (Mondego et al. 2008), it is not known if *M. perniciosa*-produced hormones are involved in pathogenicity or if the fungus instead alters the concentration of plant produced hormones. The objectives of this work were to characterize the differences between normal pods and *M. perniciosa*-induced parthenocarpic pods including (1) microscopic observation of tissue; (2) differences in metabolite levels including organic acids, amino acids, and carbohydrates; (3) differences in the expression of *M. perniciosa* expressed sequence tags (MpESTs) associated with detoxification, metabolism, and host cell degradation; (4) differences in the expression of cacao ESTs (TcESTs) associated with plant defense and general stress response; and (5) differences in expression of MpESTs and TcESTs putatively involved in hormones production or action. Overall, a better understanding of the molecular changes that occur during *M. perniciosa*-induced parthenocarpy and how these compare to broom infections could be utilized in breeding superior cacao clones. Together this information will help us understand how *M. perniciosa* alters cacao processes during parthenocarpy, which could lead to better management of witches' broom disease by reducing parthenocarpic pods and therefore inoculum.

Materials and methods

Pod material

Young cacao pods (1 to 2 months) were collected from seven clones, Comun, EQX-107, FB-44, HW-104, Joventina32, NO-55, and OZ100 at the Mars Center for Cocoa Science in Itajuipe, Bahia, Brazil. The clones used are farmers' selections from Bahia, Brazil, expect EQX-107, which is from Ecuador. All clones were susceptible to branch and flower cushion infections of *M. perniciosa* and were chosen to provide a measure of the diversity of susceptible *M. perniciosa*/cacao interactions present in the germplasm collection of the Mars Center for Cocoa Science. Equal numbers of healthy fertilized pods and parthenocarpic pods induced by natural infection with *M. perniciosa* were obtained from each clone. Pods were harvested from clonally propagated trees planted in multi-tree blocks in the germplasm collection at the Mars Center for Cocoa Science. Parthenocarpic pods were chosen by their distorted shape. Healthy pods were chosen from uninfected flower cushions in the same multi-tree blocks. Six replicate pods were obtained at each clone/infection combination, with the exception of NO-55with four pods per treatment and FB-44 with five pods per treatment. All parthenocarpic pods were in the green biotrophic phase of the disease cycle. Pod width, length, and weight were recorded. Pods were frozen in liquid nitrogen, lyophilized, and vacuum sealed before shipping to the USDA-ARS-SPCL facility in Beltsville, MD, USA under USDA-APHIS-PPQ permit P526P-11-00852. All pods were stored at −80°C until use.

Lyophilized pods were dissected and observed microscopically. Cross sections of four Comun and four NO-55 pods were observed for the presence or absence of seeds and for disease symptoms. Small sections of husk, receptacle, and pulp tissues were stained with lactophenol cotton blue and observed for the presence of fungal hyphae.

M. perniciosa cultures

M. perniciosa mycelia was grown in 100 ml potato dextrose broth (PDB) in four replicates in 250 ml flasks for 2 weeks, PDB was replaced with MIN media glucose (Srinivasan et al. 1992), and the mycelium was grown another 2 weeks to simulate growth in a low nutrient environment, such as in plant intercellular spaces. Another set of four replicates were grown in PDB for 4 weeks. Fed and starved mycelia was collected by centrifugation at 3,000×*g* for 8 min. Mycelia was washed twice by mixing with 50 ml of sterile distilled

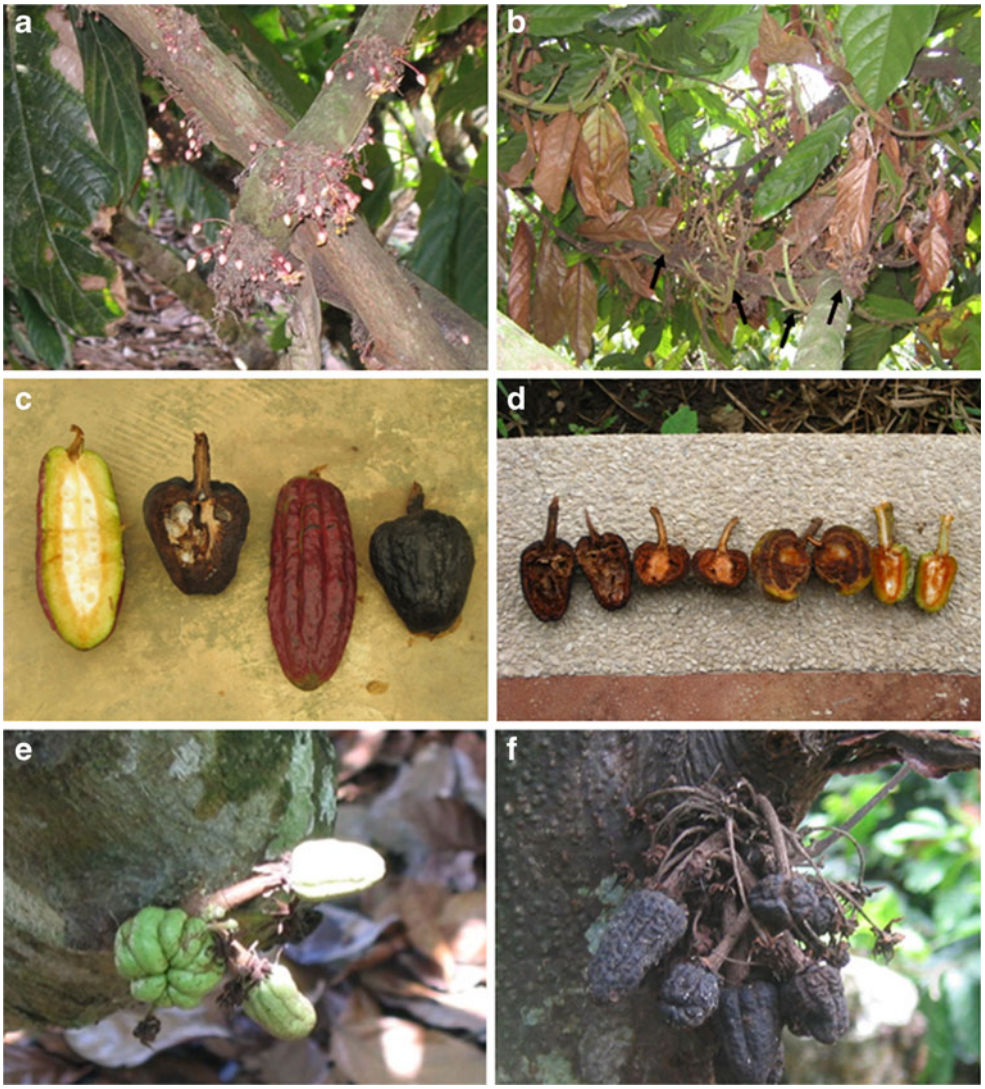

Fig. 1 Different stages in the development of witches' broom of cacao caused by *M. perniciosa*. **a** Healthy cacao branches bearing cauliflorous flower cushions. **b** The necrotrophic stage of branch infection, more commonly known as dry brooms. *Black arrows* show *M. perniciosa*-infected flower cushions with vegetative brooms in place of flowers. **c** Cross sections of cacao pods of similar age. The red ovular pod is healthy. The distorted dark brown necrotic pod is infected with *M. perniciosa*. **d** Cross sections of the differing stages in the development, with the last fully necrosed stage on the far left and the early green parthenocarpic pod on the far right. **e** The early biotrophic of parthenocarpic pods caused by infection with *M. perniciosa*. **f** Mummified parthenocarpic pods common in the later necrotrophic stage of disease

water, followed by centrifugation at 3,000×*g* for 8 min (Beckman Coulter Avanti J-20 XP, Palo Alto, CA, USA). Mycelia were flash frozen and stored at −20°C.

Metabolite measurements

Metabolite concentrations were measured for Comun and Joventina32 pods. Four healthy and four parthenocarpic pods for each of the two clones were used for the analysis. Freeze-dried pod tissue was ground to a fine powder in liquid nitrogen. The pulverized tissue (50 mg DW) was extracted at 4°C with 1.4 ml methanol in a ground glass tissue homogenizer. Ribitol (Sigma-Aldrich, St. Louis, MO, USA) added during extraction functioned as the internal standard. The homogenates were collected, incubated at 70°C in a H_2O bath for 15 min, and diluted with an equal volume of deionized H_2O. The aqueous methanol extracts were centrifuged for 15 min at 5,800×*g*. Supernatants were transferred to fresh tubes and stored for up to 2 weeks at −20°C.

A 1-ml aliquot of the aqueous methanol extract was evaporated to dryness under a stream of N_2 at 37°C. Dried samples were resuspended in 0.1 ml of 20 mM HCl prior to amino acid analysis. The resuspended extracts were filtered by centrifugation using a 0.22-μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA, USA) and stored at −20°C for up to 1 month. Samples and standards were derivatized in a total volume of 100 μl using the pre-column AccQ-Fluor Ultra kit (Waters Corp., Milford, MA, USA) according to the manufacturer's instructions. Separations were performed at 55°C on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system equipped with a 2.1×100-mm AccQ-Tag Ultra column for amino acid analysis. The column was eluted at 0.7 ml min^{-1} using mixtures of AccQ-Tag Ultra eluents A1 and B as described by the manufacturer. Individual peaks were detected by absorbance at 260 nm using a sampling rate of

10 datapoints s^{-1}. Detector output was monitored using Empower2 Plus software from Waters designed for Acquity UPLC systems. Standard curves were prepared using a mixture of amino acids (Amino acid Standard H WAT088122) that was purchased from the instrument's manufacturer (Waters Corp.).

Organic acids and soluble carbohydrates were measured by gas chromatography coupled to mass spectrometry as described by Roessner et al. (2001). For these analyses, 20 μl of each aqueous methanol extract was transferred to a 1-ml reactivial and dried overnight in a desiccator under vacuum. Dried samples and appropriate organic acid and soluble carbohydrate standards were dissolved in 100 μl of pyridine containing 2 mg of methoxyamine and were incubated for 90 min at 30 °C with continuous shaking. Organic acid standards were pyruvate, glycerate, fumarate, succinate, malate, 2-oxoglutarate, shikimate, aconitate, and citrate (Sigma-Aldrich), and soluble carbohydrate standards were sucrose, glucose, fructose, raffinose, *myo*-inositol, maltose, trehalose, and dulcitol (galactitol) (Sigma-Aldrich). Subsequently, 100 μl of *N*-methyl-*N*-(trimethylsilyl) fluoracetamide was added and incubated for 30 min at 37°C. Derivatized samples were separated by gas chromatography, and the resultant ions were detected with a mass selective detector (model 7125, Agilent technologies, Wilmington, DE, USA). Separations were performed with a Supelco SPB-50 column (30 m×0.25 mm, Sigma-Aldrich, St. Louis, MO, USA) using high-purity helium as a carrier gas at 1.2 ml min^{-1}. Total ion chromatograms were quantified using peak identification and calibration parameters within the Agilent MSD Chemstation Software program. Independent standard curves were prepared for each set of extractions with known mixtures of either organic acids or soluble carbohydrates.

RNA extraction

Tissues were broken up and coarsely ground in liquid nitrogen. RNA was extracted from a 1 cm^3 of ground pods following modifications to Bailey et al. (2005). Ground tissue was placed in 15 ml of 65°C extraction buffer. Each sample was homogenized in extraction buffer using a Janke & Kunkel Ultra Turrax T25 (IKA, Staufen, Germany) for 30–60 s. An additional 15 ml of extraction buffer was homogenized to rinse residual from the probe. The two homogenizations were combined in a 30-ml Oak Ridge tube. After 1 h incubation at 65°C, samples were centrifuged at 10°C and 31,000×*g* for 20 min. The supernatant was extracted in 12 ml of chloroform. The aqueous layer was combined with 8 ml 8.0 M DEPC-treated lithium chloride and incubated overnight at 4°C. Samples were centrifuged at 4°C and 31,000×*g* for 30 min and cleaned with an on column DNase digestion (Qiagen, Valencia, CA, USA). Synthesis of cDNA was done with the Invitrogen Superscript VILO kit (Carlsbad, CA, USA) using 4.5 μg of RNA following manufacturer's instructions.

Expression analysis using RT-qPCR

RT-qPCR analysis was conducted following Bailey et al. (2006), using the Brilliant III® SYBR® Green Q-PCR Master Mix (Agilent, Santa Clara, CA, USA). Primer sequences and sources and accessions of cacao ESTs (TcESTs) (Bae et al. 2008, 2009; Bailey et al. 2005, 2006; Argout et al. 2011; Leal et al. 2007; Verica et al. 2004) are included in Appendix 1, and *M. perniciosa* (MpESTs) (Leal et al. 2010; Mondego et al. 2008; Pungartnik et al. 2009; Santos et al. 2008; Zaparoli et al. 2009) are included in Appendix 2. RT-qPCR using primers for TcESTs related to defense and stress response and was conducted across all clones. RT-qPCR using primers for MpESTs was conducted across all clones and *M. perniciosa* mycelia. RT-qPCR using primers for TcESTs for hormone production and action was conducted across three clones (Comun, Joventina-32, and NO-55) that showed differential expression patterns for some stress related TcESTs (Tables 4 and 5). Genes of interest cycle time values ($C_{T\ GOI}$) were normalize to C_T values of actin (C_T act), using *TcAct* for TcESTs and *MpAct* for MpESTs ($\Delta C_T = C_T\ \text{act} - C_{T\ GOI}$). Relative transcript levels were calculated for each EST for each sample with respect to cacao or *M. perniciosa* actin transcript levels (%TcAct = $100\times(E^{\Delta C\tau})$, %MpAct = $100 \times (E^{\Delta C\tau})$) (Pfaffl 2001).

Statistical analysis

Relative expression of ESTs were LOG-transformed to linearize the data (Rieu and Powers 2009) and analyzed for significance using PROC MIXED (α=0.05) followed by Tukey testing in SAS 9.2 (SAS Institute Inc., Raleigh, NC, USA). To assess the relationships of EST expression patterns, PROC DISTANCE of SAS 9.2 was use to calculate Euclidean distance between pairs of genes. Fold change (Hirai et al. 2004) between relative expression in parthenocarpic pods and mean relative expression in healthy pods for TcESTs or mean relative expression in culture grown mycelia for MpESTs was used as: %*TcACT* for each parthenocarpic pod/mean %*TcACT* for healthy pods or %*MpACT* for each parthenocarpic pod/mean %*MpAct* for culture grown mycelia. The distance matrix of fold change values was used to perform principle coordinates analysis (PCoA) with GenAlEx 6.1 with the option of adjusting covariance to visually represent trends in EST expression through low-dimensional projection of high-dimensional data (Katagari and Glazebrook 2009). Metabolite data were analyzed using PROC GLM in SAS 9.2 followed by Tukey testing with a 95 % confidence interval.

Results

Microscopic observations of parthenocarpic pods

Young healthy and parthenocarpic pods from seven cacao clones Comun, EQX-107, FB-44, HW-104, Joventina-32, NO-55, and OZ100 were harvested from the field under natural infection conditions from the germplasm collection of the Mars Center for Cocoa Science located in Itajuiipe, Bahia, Brazil. Pod width and weight did not differ between healthy and parthenocarpic pods, but parthenocarpic pods were 20 mm shorter (Table 1). Cross sections were taken of lyophilized Comun and NO-55 pods and observed microscopically. Seed development occurred in all healthy pods from clones Comun and N0-55 (Fig. 2a). Parthenocarpic pods from clones Comun and N0-55 lacked ovules and seeds (Fig. 2b) or had wilted ovules (Fig. 2c). Some browning was observed in the receptacles of all Comun parthenocarpic pods (Fig. 2e, f) but not in receptacles of parthenocarpic NO-55 pods. Pod sections were stained with lactophenol cotton blue and observed. Mycelia were not observed in the receptacle, husk (Fig. 2g), or pulp (2 H) of healthy pods, but were observed in these tissues in parthenocarpic pods (Fig. 2i–l). Mycelia appeared to grow intercellular (Fig. 2i) and were detected throughout all sampled pod sections from clones Comun and NO-55.

Changes in carbohydrates, soluble amino acids, and organic acids

Carbohydrate, organic acid, and soluble amino acid concentrations were measured in pods of cacao clones Comun and Joventina32 to determine changes due to infection and parthenocarpy. There was a 63 % decrease in sucrose in parthenocarpic pods, regardless of clone (Table 2). There were no differences in the organic acids tested between clones or because of parthenocarpy (data not presented). The major amino acid detected was ASN, averaging 12,063.6 μg/g dry weight in healthy pods. Total soluble amino acids decreased by 28 % during infection. Parthenocarpic pods had 40 % less ASN (Table 2). SER, THR, MET, VAL, LEU, and PHE concentration increased in parthenocarpic pods by 45, 24, 28, 64, 63, and 22 %, respectively (Table 2).

Table 1 Mean width, length, and weight of healthy and *M. perniciosa*-infected parthenocarpic pods of seven cacao clones collected from the field in Bahia, Brazil

	Width (mm)	Length (mm)	Weight (g)
Infection status			
Healthy	20.4 A	62.1 A	15.0 A
Infected	20.9 A	42.1 B	12.1 A
Cacao clones			
Comun	20.3 AB	47.9 B	12.1 B
EQX-107	17.1 B	51.0 B	9.2 B
FB-44	21.2 AB	52.6 B	13.7 AB
HW-104	21.4 AB	51.8 B	13.7 B
Joventina32	24.2 A	67.0 A	22.9 A
NO-55	18.9 B	41.4 B	8.4 B
OZ100	21.5 AB	52.7 B	15.0 AB

Different letters within columns indicates significant differences (Tukey, α=0.05) in pod size

Expression levels of ESTs

Expression levels of MpESTs in parthenocarpic pods infected with M. perniciosa

RT-qPCR was conducted to determine the expression of MpESTs putatively related to pathogenicity and phytohormone biosynthesis (Appendix 2) during the development of parthenocarpic pods. Starved *M. perniciosa* mycelia grown in MIN were hollowed balls with a dark appearance compared to fed mycelia grown in PDB (data not presented). Expression of 31 MpESTs in all parthenocarpic pods confirmed the presence of *M. perniciosa* (Table 3). Expression of MpESTs in response to pod infection was compared to expression levels in *M. perniciosa* mycelia grown in liquid culture (Table 3), as healthy pods lacked *M. perniciosa*.

MpSOD2, *MpAK*, and *MpPME* were more highly expressed in parthenocarpic pods than in culture grown mycelia, regardless of growth medium (Table 3). *MpCTA1* was more highly expressed in parthenocarpic pods than in mycelia grown in MIN media glucose (starved), while *MpATG8*, *MpAS*, *MpPLY*, and *MpDOPA* were more highly expressed in parthenocarpic pods than in mycelia grown in PDB (fed) (Table 3). Of these MpESTs, *MpATG8*, *MpPLY*, and *MpDOPA* were also induced in starved mycelia compared to mycelia grown in a high nutrient environment. *MpSOD2* expression was not detected in starved mycelium (Table 3). Expression of *MpPAL* in parthenocarpic pods was lower than fed mycelia, but higher than starved mycelia. *MpYOR1*, *MpPER*, *MpGAox*, *MpCPS*, *MpDES*, *MpGGPPS*, and *MpCAO* were more highly expressed in both fed and starved mycelia than in cacao tissue (Table 3). *MpCP1*, *MpNTH1a*, *MpNTH1b*, *MpTSP*, and *MpP450* were not differentially expressed from mycelia (Table 3).

PCoA was used to graphically visualize trends in expression data. PCoA visualized the expression patterns of each MpEST in parthenocarpic pods based on fold change in expression relative to expression by *M. perniciosa* in liquid culture (Fig. 3). Fifty-three percent of the variance is explained by coordinate 1, 13.1 % of the variance is explained by coordinate 2, and 12.9 % of the variance is explain by coordinate 3 (data not presented). MpESTs with

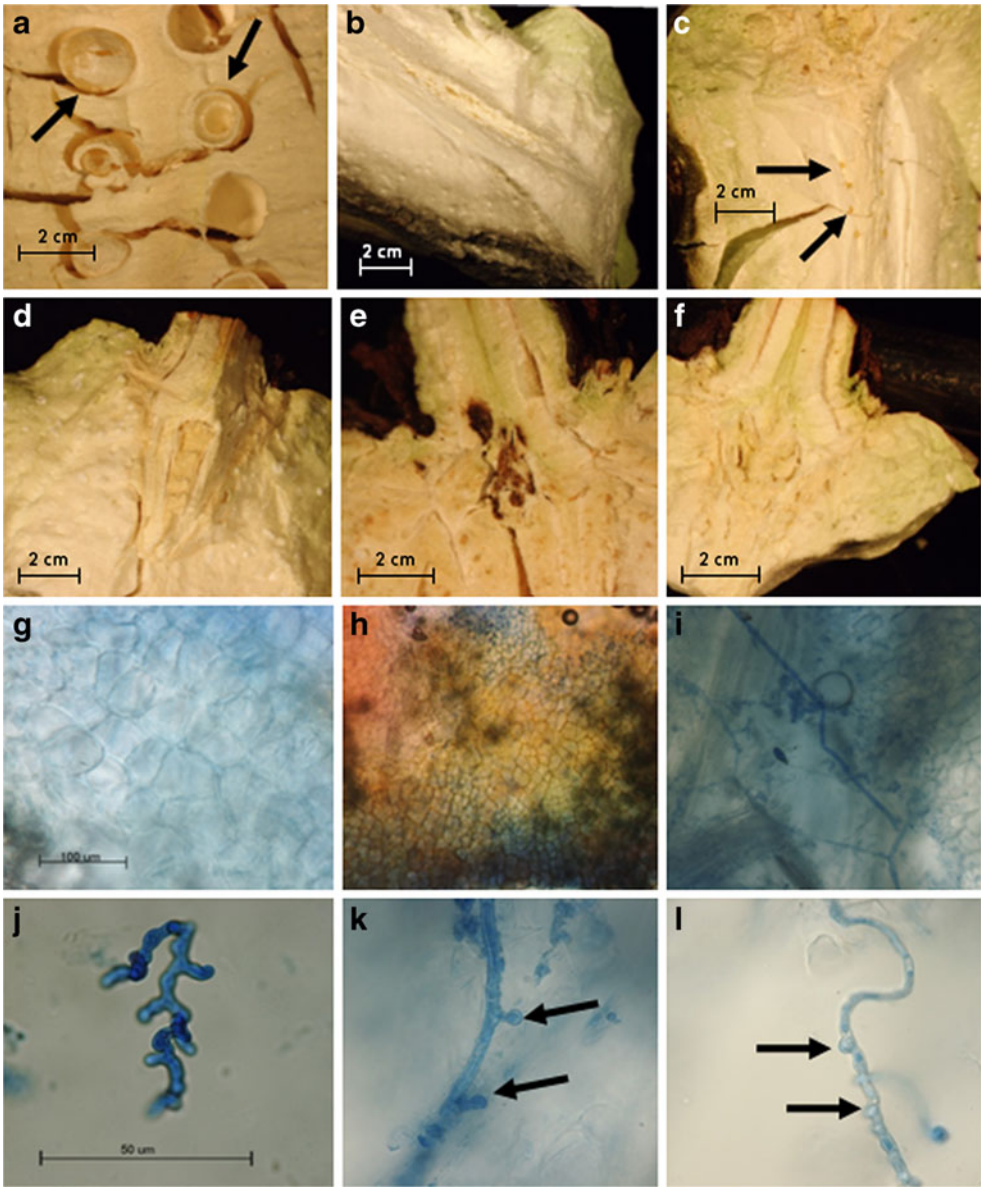

Fig. 2 Microscopic analysis of parthenocarpic cacao pods infected with *M. perniciosa*. **a** Healthy cacao pods with developing seeds (*black arrows*). **b** Parthenocarpic pods lacking fertilized ovules and seeds. **c** Parthenocarpic pods with shriveled wilted ovules (*black arrows*) but lacking seed development. **d** Receptacle of a healthy cacao pod. **e**, **f** Receptacle of parthenocarpic cacao pod infected with *M. perniciosa* with necrosis. **g** Cross section of the husk of a healthy cacao pod without septate hyphae. **h** Cross section of the pulp of a healthy cacao pod without septate hyphae. Cross section of the receptacle (**i**) and pulp (**j**) of a parthenocarpic cacao pod infected with *M. perniciosa* colonized with septate hyphae. **k**, **l** Septate mycelia colonizing a parthenocarpic pod infected with *M. perniciosa*. *Black arrows* indicate swellings and protrusion commonly occurring in *M. perniciosa* during the conversion from the biotrophic to necrotrophic stage of *M. perniciosa* (Meinhardt et al. 2006)

repressed expression in parthenocarpic pods cluster in the bottom right quadrant (Fig. 3). MpESTs with similar expression in clones and liquid culture cluster in the middle of the graph, while all MpESTs with higher expression in parthenocarpic pods than *M. perniciosa* mycelia cluster in the left quadrants (Fig. 3). *MpATG8*, *MpPME*, *MpSOD2*, and *MpPLY* cluster together as MpESTs with highly induced expression during pod infection (Fig. 3).

Expression of TcESTs putatively related to stress and disease response in parthenocarpic pods

RT-qPCR was conducted using 53 TcESTs putatively related to cacao response to abiotic and biotic stresses (Appendix 1) to determine whether they were differentially expressed in parthenocarpic pods. Expression of two of the 53 TcESTs did not differ in parthenocarpy or between the seven clones, seven TcESTs differed between clones, and ten were significant in only one or two clones (Appendix 3). *TcThau-ICS*, *TcLRP-ICS*, *TcLRP.2*-CAB, *TcUSP-CAB*, *TcChi4*, *TcODC*, *TcPR5*, *TcGT*, *TcGST*, *TcORFX*, and *TcSPMS* were induced in parthenocarpic pods, regardless of clone (Table 4). *TcChi4* and *TcPR5* were highly induced at 7.6-fold and 7.2-fold, respectively. *TcPR1*, *TcPR6*, *TcP12*, *TcChiB*, *TcCaff-CAB*, *TcLOX*, and *TcEIG.7-CAB* were repressed in parthenocarpic pods regardless of clone (Table 4). The altered expression of many TcESTs in parthenocarpic pods was dependent on the clone studied (Table 5). Expression of *TcAr-CAB*, *TcNAM-ICS*, *TcPER-1*, *TcP59*, *TcADC*, *TcABC-T*, *TcAOC*, *TcMAPK3*, *TcMKK4*, *TcNR*, *TcChi1*, and *TcNPR1e* was induced during parthenocarpy, but not in all seven cacao clones (Table 5).

Table 2 Metabolite measurement of healthy and *M. perniciosa*-infected parthenocarpic pods of Comun and Joventina32

	Mean (μg/g)		Significance[a]	Mean (μg/g)	
	Healthy	Infected		Comun	Joventina32
Carbohydrates					
Fructose	14,011.9	13,077.1		8,527.9	18,561.1
Glucose	2,766.4	2,568.9		1,712.4	3,622.9
myo-Inositol	13,930.1	11,235.4		12,471.0	12,694.5
Sucrose	86,347.5	29,911.4	*	67,344.7	48,914.2
Maltose	15.2	3.6		13.8	5.0
Trehalose	3.7	4.0		4.1	3.6
Raffinose	629.9	200.8		484.3	346.4
Amino acids					
HIS	32.9	42.1		40.6	34.4
ASN	12,063.6	7,200.0	*	9,776.3	9,487.3
SER	113.8	165.5	*	142.3	137.0
GLY	47.0	25.9		38.1	34.9
ASP	1,058.6	1,316.9		1,212.8	1,162.6
GLU	899.9	1,137.9		1,014.0	1,023.8
THR	108.9	134.6	*	126.3	117.2
ALA	91.2	105.3		100.4	96.1
GAGA	24.7	26.9		25.5	26.2
PRO	123.4	133.5		129.0	127.9
CYS	38.7	62.2		50.3	50.7
LYS	128.0	95.2		114.9	108.3
TYR	1,417.0	822.1		1,063.2	1,175.9
MET	3.1	3.9	*	3.8	3.2
VAL	109.5	179.7	**	148.1	141.1
ILE	221.2	193.6		218.8	196.0
LEU	82.3	134.0	**	115.0	101.2
PHE	28.0	34.3	**	32.5	29.8

Carbohydrate and amino acid concentration are presented as micrograms per gram dry weight tissue sampled

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ for four replicate pods per treatment/clone combination

[a]Significance as indicated by Tukey analysis (α=0.05)

The PCoA plot of stress-responsive TcEST expression data shows clustering of TcESTs based upon their patterns of induction/repression as verified by ANOVA (Fig. 4). Coordinate 1 explains 47.3 % of the variance, coordinate 2 explains 13.8 %, and coordinate 3 explains 11.3 % (data not shown). Nearly all TcESTs in the upper left-hand quadrant are repressed or not altered in parthenocarpic pods. TcESTs induced in parthenocarpic pods cluster in the lower two quadrants, with only the two TcESTs with the highest induction in parthenocarpic pods, *TcPR5* and *TcChi4*, being in the upper right-hand quadrant (Fig. 4).

Expression of TcESTs putatively related to hormone biosynthesis and action in parthenocarpic pods

Three clones, Comun, Joventina32, and N0-55, were used to determine the difference in expression of 93 TcESTs with sequences related to genes involved in hormone biosynthesis and action (Appendix 1). The expression of 28 TcESTs was not different between clones or due to parthenocarpy (Appendix 3). Thirty TcESTs differed only between Comun, Joventina32, and N0-55 pods (Appendix 3). The expression of 11 hormone-related TcEST was induced in parthenocarpic pods and four were repressed (Table 6). TcESTs putatively involved in regulation of auxin (*TcGH3.17a*, *TcARF18*, and *TcAIP10A5*) and cytokinin (*TcCKX3*, *TcCKX5*, *TcMK*, and *TcGHMPK*) levels were induced (Table 6). Cytokinin-related *TcCKX5* was 9.9-fold higher and *TcCKX3* was 26.2-fold higher in parthenocarpic pods. Gibberellin-related *TcGA20OX1B* was 3.4-fold repressed in parthenocarpic pods, respectively. Ethylene-related *TcAIL1* was 6.1-fold repressed in parthenocarpic pods, while auxin-related *TcAIP10A5* was 3.3-fold induced in parthenocarpic pods.

The interaction between infection and clone was significant for ten TcESTs associated with hormone biosynthesis and action (Table 7). Expression of *TcNPR1a*, *TcNPR1b*, and *TcNPR1c* was induced in parthenocarpic pods, but this response was clone specific (Table 7). Expression of *TcNPR1a* was induced in parthenocarpic pods from Comun

Table 3 Significant individual treatment effects on the expression of MpESTs in *M. perniciosa*-infected parthenocarpic pods from seven cacao clones and liquid culture grown mycelia grown in starved condition in MIN media glucose or in high nutrient conditions in PDB

MrEST[a]	*p* value	*M. perniciosa* mycelia		Cacao clones						
		Fed	Starved	Comun	EQX-107	FB-44	HW-104	Joventina32	NO-55	OZ100
Antioxidative stress										
MpSOD2	****	−1.49 E	N.d.	−0.70 D	0.01 AB	−0.18 ABC	−0.48 CD	0.15 A	−0.17 ABC	−0.28 BC
MpYOR1	****	1.28 AB	1.41 A	0.18 CD	0.07 CD	0.51 BC	0.12 CD	−0.35 D	0.41 CD	0.16 CD
MpATG8	****	0.50 B	2.03 A	1.79 A	1.94 A	2.07 A	1.93 A	1.55 A	2.11 A	1.89 A
MpPER	****	0.94 A	0.53 B	−0.57 CD	−0.64 CD	−0.48 CD	−0.37 CD	−0.33 C	−0.52 CD	−0.67 D
MpSNQ2	***	−0.23 ABC	−0.49 BC	−0.22 AB	−0.17 AB	0.14 A	−0.30 ABC	−0.71 C	−0.20 AB	−0.38 ABC
Metabolism										
MpAS	****	−0.68 C	0.09 AB	−0.23 B	−0.08 B	0.04 AB	−0.10 B	0.23 A	−0.05 AB	−0.11 B
MpAK	****	−0.84 B	−2.00 C	−0.26 A	−0.27 A	−0.20 A	−0.27 A	−0.29 A	−0.23 A	−0.21 A
MpCTA1	****	1.16 C	−1.23 D	1.20 BC	1.66 ABC	2.01 A	1.91 A	2.14 A	1.91 AB	1.84 AB
MpCP1	–	−1.86 A	−1.76 A	−1.45 A	−1.18 A	−1.32 A	−1.03 A	−2.39 A	−1.07 A	−1.31 A
MpGAPDH	***	2.59 A	2.57 A	2.16 B	2.04 B	2.22 AB	2.12 B	2.23 AB	2.24 AB	2.29 AB
MpPAL	****	0.76 A	−1.79 E	−0.24 D	0.02 CD	0.01 CD	0.10 BC	0.32 B	0.08 BCD	−0.11 CD
MpPLY	****	−1.60 C	−0.74 AB	−0.59 AB	−0.50 AB	−0.50 AB	−0.50 AB	−0.40 A	−0.48 AB	−0.81 B
MpPME	****	−1.44 D	−0.77 C	−0.08 AB	−0.04 AB	0.08 A	−0.11 AB	−0.10 AB	−0.24 AB	−0.25 B
MpTSP	***	−0.94 AB	−1.41 AB	−0.70 A	−0.65 A	−1.10 AB	−2.29 B	−1.44 AB	−0.48 A	−0.47 A
MpNHT1a	*	0.70 AB	−0.16 ABC	−0.24 ABC	0.98 A	−0.11 ABC	−1.04 C	−0.12 ABC	0.38 ABC	−0.67 BC
MpNTH1b	–	0.70 A	0.58 A	1.47 A	1.55 A	0.97 A	1.25 A	0.98 A	1.59 A	1.53 A
MpP450	–	0.96 A	−1.14 A	−0.02 A	0.66 A	−0.24 A	0.09 A	−0.16 A	1.36 A	0.14 A
Gibberellin biosynthesis										
MpGAox	****	−0.69 A	−1.28 B	−2.58 D	−2.39 CD	−1.97 C	−2.52 D	−2.56 D	−2.26 CD	−2.41 D
MpCPS	****	0.16 A	0.50 A	−1.93 BCD	−2.07 CD	−1.67 B	−2.21 D	−2.03 CD	−1.75 BC	−1.91 BC
MpDES	****	−0.89 A	−1.07 A	−2.33 C	−2.37 C	−1.85 B	−2.15 BC	−2.36 C	−1.86 B	−2.20 BC
MpGGPPS	****	−0.61 B	2.00 A	−2.75 C	−2.80 C	−1.70 BC	−2.38 C	−2.77 C	−2.56 C	−1.76 BC
IAA biosynthesis										
MpDOPA	****	−0.06 B	0.53 A	0.64 A	0.58 A	0.78 A	0.66 A	0.73 A	0.54 A	0.56 A
MpCAO	****	0.18 A	0.17 A	−0.85 C	−0.72 BC	−0.44 B	−0.85 C	−0.58 BC	−0.80 BC	−0.75 BC

Data are presented as LOG_{10} %*MpACT*. Different letters within rows indicate significant differences (Tukey, α=0.05) between expression in parthenocarpic pods of different clones and *M. perniciosa* mycelia. Six replicate pods were used for each clone/infection combination, with the exception of NO-55with four pods/treatment and FB-44 with five pods per treatment

N.d. EST in which expression was not detected

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ (p values from ANOVA)

[a] MpESTs were chosen by their reactivity to oxidative stress, reactivity during *M. perniciosa* meristematic infection, role in phytohormone production, cell wall degradation, and basidiocarps formation (see Appendix 2)

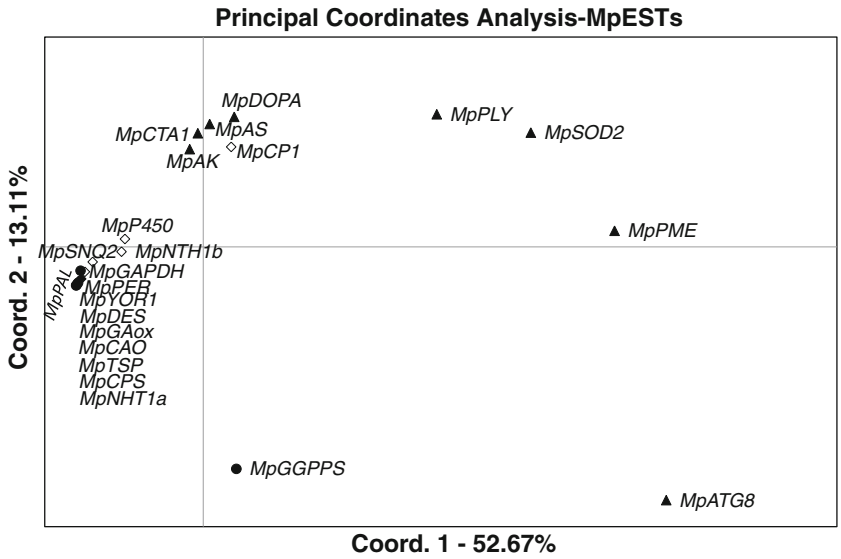

Fig. 3 Principle coordinate analysis plot of the relative expression of MpESTs. The first axis explains 56.6 % of the variance in the data, while the second axis explains 16.9 % of the variance. The *triangle symbol* indicates MpESTs with induced expression in parthenocarpic pods, the *circle* indicates MpESTs with repressed expression in parthenocarpic pods, and the *diamond* indicated MpEST expression that was not altered in parthenocarpic pods

and Joventina32 by 1.2 and 1.6-fold, respectively. Expression of *TcNPR1b* and *TcNPR1c* was induced in parthenocarpic NO-55 pods by 7.0 and 7.4-fold, respectively.

PCoA was used to compare expression patterns for TcESTs putatively related to hormone biosynthesis and action (Fig. 5). Thirty-eight percent of the variance was explained by coordinate 1, 25.4 % by coordinate 2, and 13.9 % by coordinate 3. There was no obvious grouping of TcESTs involved in the pathway of a single hormone. TcESTs with repressed expression in parthenocarpic pods cluster in the lower right-hand quadrant (Fig. 5). TcESTs in the left quadrants are either not affect by *M. perniciosa* or have increased expression (Fig. 4). The two TcESTs with the highest relative expression in parthenocarpic pods, *TcCKX5* and *TcCKX3*, cluster in the lower right-hand quadrant (Fig. 5).

Discussion

Visual observations confirm *M. perniciosa* colonization of parthenocarpic pods

A better understanding of *M. perniciosa*-induced parthenocarpic pod formation, an overlooked source of inoculum, may lead to new management approaches for witches' broom disease by emphasizing the importance of removing infected flower cushions. Additionally, a better understanding of the differences between cushion and branch infections can lead to improved cacao clones, with resistance to both types of infections. Although accepted in the literature (Meinhardt et al. 2008), published data demonstrating the absence of seeds in "parthenocarpic pods" arising from *M. perniciosa*-infected flowers cushions are limited. Septate mycelia (Fig. 2i–l) were visualized in Comun and NO-55 parthenocarpic pods (Fig. 2b, c). The absence of seeds in parthenocarpic pods likely contributes to their reduced length (Table 1).

Parthenocarpic pods have reduced levels of transport metabolites

Sucrose, the major compound for carbohydrate transport (Peretó et al. 1988), was the only carbohydrate altered by infection. The lack of fertile seeds may have disrupted the source sink relationship between the leaves/stems and parthenocarpic pods, decreasing sucrose levels. In *Pisum sativum*, GA biosynthesis activates the movement of sucrose into ovaries (Peretó et al. 1988). Parthenocarpy repressed the expression of *TcGA20OX1B* putatively related to GA-20 oxidase, in clones Comun, Joventina32, and NO-55 the initial step in GA biosynthesis (Lange et al. 1994) (Table 6), suggesting repression of GA biosynthesis. The lack of pollination may have limited GA biosynthesis, which in turn limited sucrose unloading to pods. There was also a decrease in the major transport metabolite asparagine (Lea et al. 2007) in parthenocarpic pods, also possibly from disruption of the source sink relationship by *M. perniciosa*. These findings are in direct contrast to findings in *M. perniciosa* brooms in which both sucrose and asparagine levels increased (Scarpari et al. 2005). An alternative explanation for reduced sucrose and asparagine levels is consumption by *M. perniciosa.* Sucrose is important in the switch from biotrophic to necrotrophic stage of infection for *M. perniciosa* (Scarpari et al. 2005), while asparagine is a good source of nitrogen for *M. perniciosa* growth (Lindberg and Molin 1949).

Concentrations of serine, threonine, methionine, valine, leucine, and phenylalanine were increased in Comun and Joventina32 parthenocarpic pods (Table 2). In higher plants, serine is derived from 3-P glycerate, and leucine and valine are derived from pyruvate during glycolysis (Sharma and Dietz 2006). Aspartate is derived from

Table 4 Significant individual treatment effects (infection status and clone) on the expression of TcESTs related to defense and general stress response in healthy and *M. perniciosa*-infected parthenocarpic pods from seven cacao clones presented as LOG_{10} %*TcACT*

EST ID[a]	Pub	Infection status			Cacao clones							
		p value	Healthy	Infected	*p* value	Comun	EQX-107	FB-44	HW-104	Joventina32	NO-55	OZ100
M. perniciosa in brooms												
TcCaff-CAB	*Mp+*	*	0.76 A	0.51 B	****	0.85 B	0.45 BC	0.70 B	1.45 A	−0.11 C	0.05 C	0.78 B
TcEIG.7-CAB	*Mp+*	*	1.29 A	1.12 B	****	1.18 A	1.46 A	0.65 B	1.44 A	1.28 A	1.37 A	1.09 A
TcThau-ICS	*Mp+*	****	1.89 B	2.33 A	****	1.92 D	2.45 A	2.19 ABC	2.01 CD	2.15 BC	2.34 AB	1.90 D
TcLRP-ICS	*Mp+*	**	1.53 B	1.65 A	*	1.68 A	1.67 AB	1.62 AB	1.53 AB	1.60 AB	1.42 B	1.60 AB
TcUSP-CAB	*Mp+*	****	1.12 B	1.30 A	**	1.16 B	1.19 AB	1.12 B	1.15 B	1.35 A	1.27 AB	1.24 AB
TcLRP.2-CAB	*Mp+*	****	1.29 B	1.51 A	****	1.49 A	1.23 B	1.37 AB	1.56 A	1.42 AB	1.51 A	1.27 B
Trichoderma spp.												
TcP12	T−	***	−0.13 A	−0.43 B	****	−0.32 BC	−0.71 C	−0.53 BC	0.19 A	−0.15 AB	−0.59 BC	−0.14 AB
TcGST	T+	****	0.96 B	1.17 A	****	0.94 BC	0.89 C	0.91 C	1.22 A	1.13 AB	1.15 AB	1.16 A
Drought												
TcChiB	D+T+	**	2.09 A	2.02 B		2.08 A	2.05 A	2.03 A	2.06 A	2.04 A	2.13 A	2.00 A
TcLOX	D+	**	−3.66 A	−4.45 B		−4.07 A	−3.91 A	−3.92 A	−4.25 A	−4.59 A	−3.76 A	−3.91 A
TcODC	D+T+	***	−0.49 B	−0.23 A	*	−0.29 AB	−0.56 B	−0.41 AB	−0.35 AB	−0.40 AB	0.01 A	−0.43 AB
TcPR5	D+	****	−1.34 B	−0.48 A	**	−0.78 AB	−1.30 C	−0.74 AB	−0.92 ABC	−1.00 ABC	−0.44 A	−1.02 BC
TcSPMS	D+	**	0.66 B	0.77 A	****	0.67 BC	0.62 BC	0.63 BC	0.82 B	0.65 BC	1.28 A	0.50 C
Defense and general stress												
TcPR1		****	0.38 A	0.008 B	***	0.30 ABC	−0.06 CD	0.05 BCD	0.20 ABCD	0.33 AB	−0.07 D	0.38 A
TcPR6		**	1.79 A	1.66 B	****	1.96 AB	1.63 C	1.72 BC	2.08 A	1.53 C	1.54 C	1.55 C
TcChi4		****	0.29 B	1.17 A	****	0.69 B	0.65 B	0.93 AB	0.25 C	0.59 B	1.23 A	0.76 B
TcGT		****	1.34 B	1.52 A	****	1.21 B	1.49 A	1.50 A	1.15 B	1.52 A	1.53 A	1.64 A
TcORFX	W+T+	****	0.72 B	0.96 A	****	0.67 B	0.73 B	0.81 AB	0.59 B	1.05 A	1.05 A	1.04 A

Different letters within rows indicate significant differences between parthenocarpic and healthy pods or differing clones through Tukey analysis (α=0.05). Six replicate pods were used for each clone/infection combination, with the exception of NO-55with four pods per treatment and FB-44 with five pods per treatment

Mp+ induction by *M. perniciosa* in Leal et al. (2007), *T−* repression, *T+* induction, *T~* unchanged by *Trichoderma* spp. in Bailey et al. (2006), *D+* induction by drought in Bae et al. (2008), *D+T+* induction by drought and *Trichoderma* in Bae et al. (2008), *W+* induction during wounding from Bailey et al. (2005)

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ (*p* values from ANOVA)

[a] TcESTs were chosen by their reactivity to *M. perniciosa* meristematic infection, *M. roreri* pod infections, *Trichoderma* colonization, and stress responses such as drought. Primers are in Appendix 1

Table 5 Significant effects of treatment interactions (infection status×clone) on cacao TcEST expression in healthy and *M. perniciosa*-infected parthenocarpic pods from seven cacao clones presented as LOG_{10} *%TcACT*

EST[a]	Infection	*p* value	Published reaction	Comun		EQX-107		FB-44		HW-104		Joventina32		NO-55		OZ100	
				Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.
M. perniciosa in brooms																	
TcAr-CAB	Healthy	*	*Mp*+	1.00 A	b	1.32 A	a	1.05 A	b	1.26 A	a	1.18 A	a	1.04 A	a	0.97 A	b
	Infected			1.31 A	a	1.42 A	a	1.50 A	a	1.16 A	a	1.21 A	a	1.19 A	a	1.33 A	a
TcNAM-ICS	Healthy	**	*Mp*+	1.71 AB	a	1.83 AB	b	1.77 AB	a	1.41 B	b	1.46 B	b	1.94 A	a	1.72 AB	a
	Infected			1.80 BC	a	2.11 A	a	1.86 ABC	a	1.68 C	a	1.97 AB	a	1.75 BC	a	1.81 BC	a
Trichoderma spp.																	
TcPER-1	Healthy	**	T+	2.02 AB	b	2.33 A	a	1.75 B	b	2.22 A	a	2.12 A	b	2.21 A	b	2.03 AB	a
	Infected			2.41 AB	a	2.29 AB	a	2.23 ABC	a	2.19 AB	a	2.26 ABC	a	2.54 A	a	2.11 B	a
TcP59	Healthy	*	T+	−2.94 C	a	−1.91 A	a	−2.48 ABC	b	−2.88 BC	b	−2.60 ABC	b	−2.12 AB	b	−2.57 ABC	b
	Infected			−2.49 C	a	−1.58 AB	a	−2.13 BC	a	−2.23 C	a	−1.50 A	a	−1.22 A	a	−1.60 AB	a
Drought																	
TcADC	Healthy	**	D+	1.35 AB	b	1.34 AB	a	1.18 AB	b	1.45 A	a	1.07 B	b	1.46 A	a	1.13 B	b
	Infected			1.78 A	a	1.47 B	a	1.35 BC	a	1.46 BC	a	1.35 B	a	1.50 B	a	1.43 B	a
TcABC-T	Healthy	*	D−	0.10 AB	b	−0.19 B	a	−0.11 BC	b	−0.12 B	b	−0.12 B	b	0.02 AB	b	0.40 A	b
	Infected			0.79 AB	a	−0.17 C	a	0.57 AB	a	0.39 BC	a	0.53 AB	a	1.21 A	a	0.87 AB	a
TcAOC	Healthy	**	D−	0.84 A	a	−1.30 C	b	−0.03 AB	b	−0.77 BC	a	−0.63 BC	a	−0.38 BC	a	−0.76 BC	a
	Infected			0.45 AB	a	−0.11 BC	a	0.69 A	a	−0.60 CD	a	−0.64 CD	a	−0.44 CD	a	−0.83 D	a
TcMAPK3	Healthy	*	D+T+	0.28 B	b	0.41 AB	a	0.28 B	b	0.36 B	b	0.75 A	a	0.37 AB	b	0.50 AB	a
	Infected			0.87 AB	a	0.77 AB	a	0.56 B	a	0.84 AB	a	0.88 AB	a	0.97 A	a	0.78 AB	a
TcMKK4	Healthy	***	D−T+	1.41 A	a	0.89 B	b	0.88 B	b	1.34 A	b	1.07 B	a	1.02 B	b	0.98 B	b
	Infected			1.21 A	a	1.49 A	a	1.15 A	a	1.55 A	a	1.22 A	a	1.57 A	a	1.43 A	a
TcNR	Healthy	*	D+T+	0.12 AB	b	−0.72 D	a	−0.23 BC	a	0.31 A	a	−0.33 CD	b	−0.27 BCD	b	−0.28 BCD	a
	Infected			0.77 A	a	−0.31 B	a	0.06 B	a	0.09 B	a	0.005 B	a	0.24 AB	a	0.05 B	a
Defense and general stress																	
TcPR4	Healthy	**		0.56 AB	a	0.58 AB	a	−0.22 C	a	0.08 BC	a	0.31 ABC	a	0.78 A	a	0.51 AB	a
	Infected			−0.15 A	b	0.05 A	b	0.22 A	a	0.24 A	a	0.36 A	a	0.20 A	a	−0.15 A	b
TcGluc	Healthy	*	W+	1.18 A	a	0.72 B	a	0.75 B	a	1.10 AB	a	1.15 A	a	1.05 AB	a	0.79 AB	a
	Infected			0.70 A	b	0.61 A	a	0.50 A	b	0.81 A	b	0.44 A	b	0.58 A	a	0.68 A	a
TcChi1	Healthy	****		0.41 D	b	0.73 CD	b	1.54 A	a	1.45 AB	a	1.87 A	a	1.53 ABC	a	0.86 BCD	b
	Infected			1.46 A	a	1.32 A	a	1.29 A	a	1.24 A	a	1.53 A	a	1.45 A	a	1.45 A	a

Table 5 (continued)

EST[a]	Infection	*p* value	Published reaction	Comun		EQX-107		FB-44		HW-104		Joventina32		NO-55		OZ100	
				Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.	Mean	Sep.
TcNPR1e	Healthy	*		0.53 BC	b	0.50 BC	b	0.63 AB	b	0.80 AB	b	−0.12 D	b	1.01 A	b	0.15 CD	a
	Infected			0.99 AB	a	0.78 B	a	1.00 AB	a	1.10 AB	a	0.31 C	a	1.33 A	a	−0.002 C	a

Different letters between rows indicate differences between parthenocarpic and healthy pods within clones and different letters between columns indicate differences between clones for healthy or *M. perniciosa*-infected pods through Tukey analysis (α=0.05). Six replicate pods were used for each clone/infection combination, with the exception of NO-55with four pods per treatment and FB-44 with five pods per treatment

Mp+ induction by *M. perniciosa* in Leal et al. (2007), *T* repression, *T+* induction, *T~* unchanged by *Trichoderma* spp. in Bailey et al. (2006), *D+* induction by drought in Bae et al. (2008), *D+T+* induction by drought and *Trichoderma* in Bae et al. (2008), *W+* induction during wounding from Bailey et al. (2005)

*$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$, ****$p\leq0.0001$ (*p* value of the infection×genotype interaction term from the two-way ANOVA)

[a] TcESTs were chosen by their reactivity to *M. perniciosa* meristematic infection, *M. roreri* pod infections, *Trichoderma* colonization, and stress responses such as drought. Primers are in Appendix 1

oxaloacetate during the TCA cycle, which is then used to synthesize threonine and methionine (Sharma and Dietz 2006). Accumulation of these amino acids is indicative of increased cellular respiration (Sharma and Dietz 2006). Increased concentrations of amino acids may also be due to plant defense. Grapes treated with the SA analog benzothiadiazole had increased levels of valine, leucine, and phenylalanine (Iriti et al. 2005). Phenylalanine activates defense as infection triggers its conversion into *trans*-cinnamic acid by phenylalanine ammonia lyase to begin the phenylpropanoid synthesis, while valine and leucine are a source of phytoalexins known as phytoanticipins (Iriti et al. 2005).

Increased expression of MpESTs related to oxidative stress and nutrient acquisition in parthenocarpic pods

The colonization of parthenocarpic pods by *M. perniciosa* was confirmed by the detection of 23 MpESTs (Table 3). Anti-ROS defense-related *MpSOD2* (putative superoxide dismutase) and *MpCTA1* (putative catalase) were induced in parthenocarpic pods when compared to fed mycelia (Table 3). Induction of autophagy-related protein 8 (*MpATG8*) in parthenocarpic pods suggests that a starvation-like response during the biotrophic phase of infection (Pungartnik et al. 2009) activates autophagy to degrade portions of *M. perniciosa* cells to preserve essential cellular functions (Levine and Klionsky 2004). Deletion of the homolog *Atg8* in the rice pathogen *Magnaporthe grisea* prevented appresorium penetration and thus pathogenicity (Veneault-Fourrey et al. 2006).

MpAs, *MpAK*, *MpPLY*, and *MpPME* were induced in parthenocarpic pods during the biotrophic stage of infection (Table 3) when compared to fed mycelia and during the interaction of *M. perniciosa* with cacao apical meristems (Leal et al. 2010). *MpAs* encodes a putative arlysulfatase (AS) to break down plant organic esters into usable sulfate (Goodwin et al. 2000), suggesting that parthenocarpic pods are a sulfur-deprived environment requiring increased AS levels. *MpAS* is also induced in starved mycelia relative to fed mycelia (Table 3), further supporting induction due to nutrient depletion. *MpAK* putatively encodes an aldo-keto reductase (AK). AK are members of a NAD(P)(H)-dependent oxidoreductase superfamily (Jez and Penning 2001) with many functions. Aldo-keto reductase is induced during lignin degradation by the basidiomycete *Phanerochaete chrysosporium* (Matsuzaki et al. 2008), suggesting that *MpAK* is involved in the breakdown of woody tissue. *MpAK* is not induced in starved mycelium (Table 3), suggesting that the host tissue is required to induce this MpEST. Cacao pulp is composed of 1–1.5 % pectin (Schwan 1998; Pettipher 1986). The

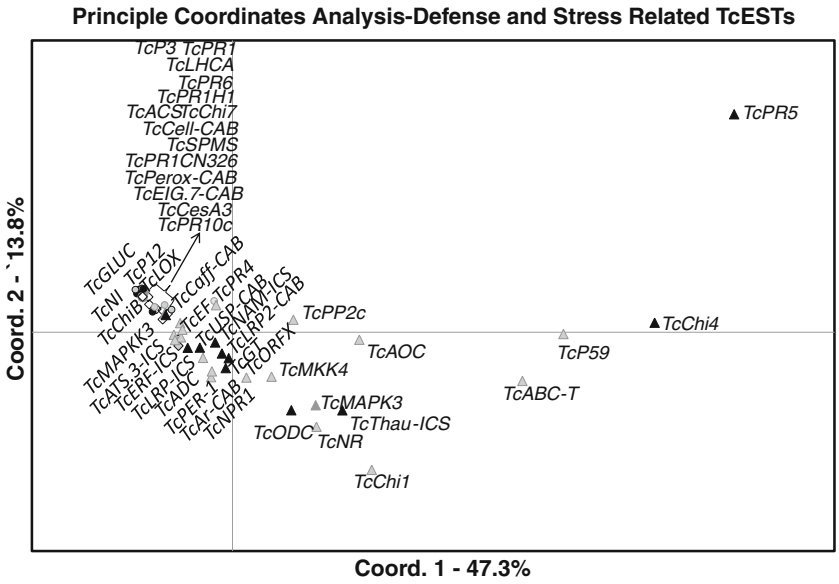

Fig. 4 Principle coordinate analysis plot of the relative expression of TcESTs related to defense and general stress response. The first axis explains 47.3 % of the variance in the data, while the second axis explains 13.8 % of the variance. The *black triangle* symbol indicates *TcESTs* with induced in parthenocarpic pods regardless of clone; the *gray triangle* indicates TcESTs induced in parthenocarpic pods, but only in specific clones; the *black triangle* indicates TcESTs repressed in parthenocarpic pod regardless of clone; the *gray triangle* indicates TcESTs repressed in parthenocarpic pods, but only in specific clones; and the *open diamond* indicates TcESTs without altered expression

increased expression of pectate lyase (*MpPLY*) and pectin methylesterase (*MpPME*) in parthenocarpic pods likely supports nutrient acquisition by *Mp*. Induction of *MpPME* in starved mycelium suggests that a low nutrient environment might induce expression of *MpPME*.

Table 6 Significant individual treatment effects (infection status and clone) on the expression of TcESTs related to hormone action and biosynthesis in healthy and *M. perniciosa*-infected parthenocarpic pods from three cacao clones presented as LOG_{10}% *TcACT*

EST[a]	*p* value	Infection status		*p* value	Cacao clones		
		Healthy	Infected		Comun	Joventina32	NO-55
Abscisic acid							
TcAREB5.1	*	−1.90 A[c]	−2.35 B		−2.01 A	−2.14 A	−2.21 A
TcNCED6	*	−2.50 B	−1.98 A		−2.10 A	−2.36 A	−2.32 A
Auxin							
TcGH3.17a	*	−2.18 B	−1.90 A	***	−1.73 A	−2.48 B	−1.90 A
TcARF18	*	−0.20 B	0.12 A	***	0.20 A	−0.44 B	0.15 A
TcAIP10A5	***	−1.66 B	−1.14 A	***	−1.01 A	−1.48 B	−1.88 B
Cytokinin							
TcCKX5	****	0.72 B	1.72 A	**	1.50 A	0.90 B	1.14 AB
TcCKX3	****	−2.14 B	−0.72 A		−1.55 A	−1.58 A	−1.23 A
Tc_crtXa	****	1.50 A	1.19 B	****	0.81 B	1.65 A	1.68 A
TcMK	**	0.54 B	0.69 A	**	0.74 A	0.53 B	0.54 B
TcGHMPK	***	0.94 B	1.13 A		1.05 A	0.98 A	1.07 A
Gibberellins							
TcGA20OX1B	**	−1.30 A	−1.83 B		−1.38 A	−1.76 A	−1.49 A
Jasmonic acid							
TcLOX1	****	1.38 B	1.91 A	****	1.59 B	1.46 B	1.91 A
TcJMT1	*	−3.56 B	−2.90 A		−2.88 A	−3.60 A	−3.30 A
Ethylene							
TcAIL1	**	−1.03 A	−1.82 B		−1.24 A	−1.71 A	−1.28 A
TcSAMS1	*	2.22 B	2.39 A	****	2.55 A	1.94 B	2.44 A

Different letters within rows indicate significant differences between parthenocarpic and healthy pods and differing clones through Tukey analysis (α=0.05). Six replicate pods were used for Comun and Joventina32, while four replicate pods were used for NO-55

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ (*p* values from ANOVA)

[a]Key hormones in terms of biosynthesis, action, and response were chosen to design primers. Primers and description are in Appendix 1

Table 7 Significant effects of treatment interactions (infection status×clone) on the expression of TcESTs related to hormone action and biosynthesis in healthy and *M. perniciosa*-infected parthenocarpic pods from three cacao clones presented as LOG_{10} %*TcACT*

EST[a]	*p* value	Infection	Comun		Joventina32		NO-55	
			Mean	Sep.	Mean	Sep.	Mean	Sep.
Abscisic acid								
TcABA8ox1	****	Healthy	0.07 A	b	0.31 A	a	−0.02 A	b
		Infected	1.29 A	a	0.28 B	a	1.26 A	a
Auxin								
TcGH3.1	*	Healthy	1.92 A	b	1.20 AB	b	0.47 B	b
		Infected	2.69 A	a	2.08 B	a	2.04 B	a
TcIAA14	*	Healthy	−1.83 A	b	−1.89 A	b	−2.18 B	a
		Infected	−1.35 A	a	−1.67 B	a	−2.14 C	a
Cytokinin								
TcCK-N-GT1	*	Healthy	0.49 B	a	0.90 A	a	0.24 B	a
		Infected	−0.28 A	b	−0.32 A	b	−0.36 A	b
TcCK-O-GT2	*	Healthy	−0.44 A	a	−1.32 B	b	−0.47 A	b
		Infected	−0.33 AB	a	−0.58 B	a	−0.19 A	a
Jasmonic acid								
TcAOS1	**	Healthy	1.43 A	a	0.36 B	a	1.43 A	a
		Infected	0.72 A	b	0.74 A	a	0.58 A	b
TcOPR1	****	Healthy	0.19 A	a	0.33 A	a	0.26 A	b
		Infected	0.08 B	b	−0.09 B	b	0.51 A	a
NPR1								
TcNPR1a	*	Healthy	0.85 A	b	0.48 B	b	1.04 A	a
		Infected	1.01 A	a	0.78 B	a	1.06 A	a
TcNPR1b	*	Healthy	−0.77 A	a	−1.33 B	a	−1.25 B	b
		Infected	−0.71 AB	a	−1.00 B	a	−0.40 A	a
TcNPR1c	**	Healthy	1.38 A	a	1.10 B	a	0.86 B	b
		Infected	1.51 AB	a	1.25 B	a	1.73 A	a

Different letters within rows indicate differences between parthenocarpic and healthy pods within clones, and different letters within columns indicate differences between clones for healthy or *M. perniciosa*-infected pods through Tukey analysis (α=0.05). Six replicate pods were used for each clone/infection combination, with the exception of NO-55with four pods per treatment

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ (p value of the infection×genotype interaction term from the two-way ANOVA)

[a] Key hormones in terms of biosynthesis, action, and response were chosen to design primers. Primers and description are in Appendix 1

Expression of TcESTs putatively involved in host defense

Expression of defense-related TcESTs is similar to that of a general defense response

The expression pattern for nine of the 11 TcESTs previously studied during endophytic colonization of cacao by *Trichoderma* spp. (Bailey et al. 2006) had the same pattern of induction/repression in parthenocarpic pods (Tables 4 and 5). *TcP59*, a putative nectarin V-glucose oxidase involved in hydrogen peroxide generation (Carter and Thornburg 2004), was highly induced in cacao seedlings colonized by *Trichoderma* spp. (Bailey et al. 2006) and in parthenocarpic pods. The association between drought response and the response to *M. perniciosa* in parthenocarpic fruit was less consistent with only seven of the 16 TcESTs having the same expression pattern (Tables 4 and 5). Expression of *TcPR5*, putatively related to osmotin, was highly induced in seedlings by drought (Bae et al. 2008) and by *M. perniciosa*-induced parthenocarpy.

The reaction to *M. perniciosa* differs between parthenocarpic pods and brooms, as only six of the 13 TcESTs induced in *M. perniciosa*-infected brooms (Leal et al. 2007) were induced in parthenocarpic pods. *TcThau-ICS*, *TcUSP-CAB*, *TcLRP-ICS*, and *TcLRP2.CAB* are related to the general defense response activated by abiotic and biotic stresses (Leal et al. 2007). Thaumatin (*TcThau-ICS*) adapts cells to osmotic stress and defense (Richardson et al. 1987). *TcUSP-ICS* is related to universal stress protein, induced by nutritional deficiency and osmotic stress (Kerk et al. 2003). *TcLRP-ICS* and *TcLRP2.CAB* are related to leucine-rich

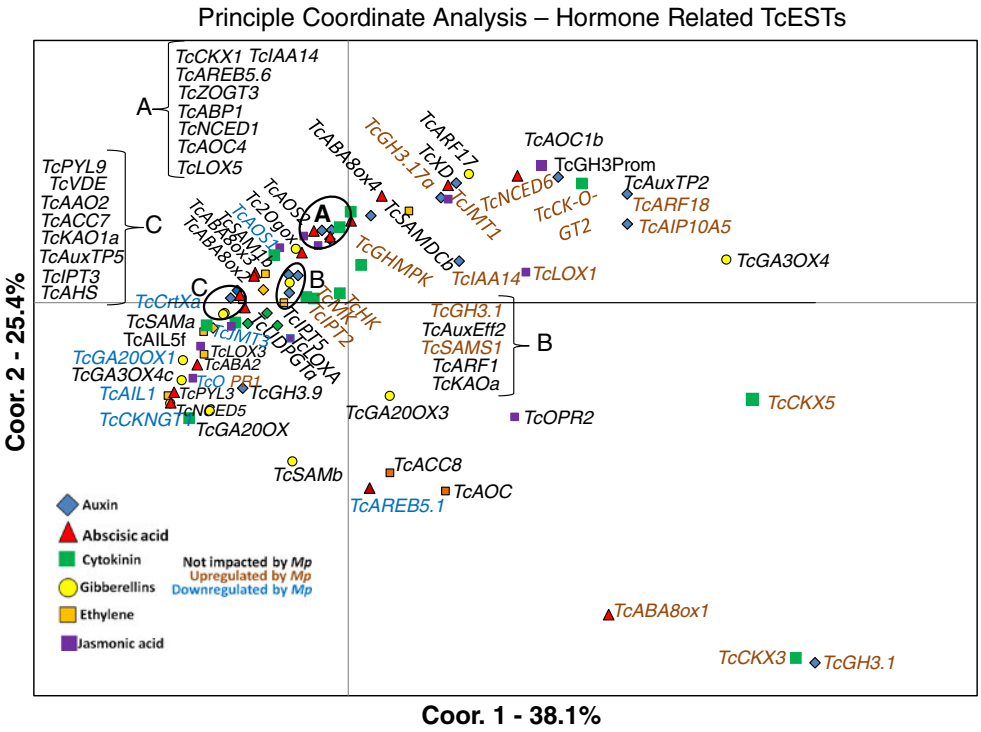

Fig. 5 Principle coordinate analysis plot of the relative expression of TcESTs related to hormone action and biosynthesis and their altered expression in parthenocarpic cacao pods infected by *M. perniciosa*. The first axis explains 38.1 % of the variance in the data, while the second axis explains 25.4 % of the variance. The graph has *color-coded symbols* to indicate hormone pathway, while the *color* of the TcESTs name indicates induced or repressed expression in response to *M. perniciosa*-induced parthenocarpy

protein membrane receptors involved in signal transduction of the brassinosteroid pathway (Li and Chory 1997) and as resistance genes (Dangl and Jones 2001).

Endophytes, microbes which colonize plants without causing disease, are common cacao inhabitants (Bailey et al. 2006). Endophytic *Trichoderma* alters the expression of stress TcESTs during colonization of cacao, but the response is weak (Bailey et al. 2006). A similar weak response occurs during to the susceptible interaction between cacao stems and *M. perniciosa (*Leal et al. 2007). It is hypothesized that pathogenic *Moniliophthora* spp. arose from an endophytic ancestor (Evans et al. 2003), which is supported by their close phylogenetic relationship with an endophytic *Moniliophthora* sp. inhabiting grass (Aime and Phillips-Mora 2005). *M. perniciosa* may exploit its recognition as an endophyte during the biotrophic stage of cacao infection, resulting in a weak defense response, as seen by the generally small fold changes in TcESTs expressed in parthenocarpic pods (Table 5).

Expression of TcESTs associated with JA/SA suggests complex regulation

Kilaru et al. (2007) and Chaves and Gianfagna (2006) both demonstrated production of SA by *M. perniciosa*, suggesting that SA may function in *M. perniciosa* pathogenesis rather than host resistance. Most TcESTs with putative PR protein functions (Appendix 1) were either non-responsive or were repressed in parthenocarpic pods (Fig. 4), suggesting that SA accumulation may not occur until the necrotrophic stage, as seen in other cacao tissues (Chaves and Gianfagna 2006).

Two of 15 TcESTs putatively related to the jasmonic acid response are induced in parthenocarpic pods from clones Comun, Joventina32, and NO-55 (Table 6). A putative jasmonic acid carboxyl methyltransferase *TcJMT* and a putative lipoxygenase *TcLOX1* (Seo et al. 2001) were induced by *M. perniciosa*. Methyl jasmonate can induce *PR5* transcripts (Xu et al. 1994), which could explain the 7.2-fold induction of *TcPR5* in parthenocarpic cacao pods (Table 4). These results suggest a limited JA response to *M. perniciosa*, as proposed in *M. perniciosa*-infected stems (Chaves and Gianfagna 2006)

NPR1-related genes function in the signal transduction of SA in many plants (Cao et al. 1997) and in cross talk with the jasmonic acid pathway through repression of JA during SA induction (Spoel et al. 2003). To obtain a clearer picture of the regulation of NPR1 in parthenocarpic pods, primers were designed for five separate NPR1 homologs. *TcNPR1d* was not induced by *M. perniciosa* during parthenocarpy (Table 3). Gene replacement studies in *Arabidopsis* with cacao *NPR1* (our *TcNPR1d*) confirmed that *TcNPR1d* encodes a functional NPR1 protein (Shi et al. 2010). Expression of *TcNPR1e* was induced in all clones except OZ100 (Table 5). The expression of *TcNPR1a* was induced in parthenocarpic Comun and Joventina-32 pods, but not NO-55, while the expression of *TcNPR1b* and *TcNPR1c* was strongly induced in parthenocarpic NO-55 pods, but not in Comun or Joventina32. These results suggest a complex regulation of cacao *NPR1* homologs between clones during *M. perniciosa* infection.

The interaction between *M. perniciosa* and cacao produced phytohormones during parthenocarpy

MpEST expression putatively involved in GA and IAA synthesis is generally repressed in parthenocarpic pods

M. perniciosa possesses putative biosynthetic genes for GA and IAA production (Mondego et al. 2008). *MpCPS* (Tudzynski et al. 1998), *MpDES*, *MpGAox* (Tudzynski et al. 2003), and *MpGGPPS* (Mende et al. 1997) putatively catalyze steps in the fungal GA biosynthesis pathway. The expression of the GA-related MpESTs was strongly repressed (10–100-fold) in parthenocarpic pods (Table 3). The expression of IAA-associated *MpCAO* was repressed while *MpDOPA* was 5-fold higher in parthenocarpic pods. Tryptophan decarboxylase (putative *MpDOPA*) coverts tryptophan to tryptamine at the beginning of IAA biosynthesis (Bartel 1997) but may also be used to synthesize melanin in basidiocarps (Hegnauer et al. 1985). Expression of *MpDOPA* in starved mycelia (Table 3) suggests that starvation may induce melanin production. Additionally, starved *M. perniciosa* mycelia had a darkened appearance not present in fed mycelium. The repression of MpESTs related to hormone biosynthesis verifies their differential regulation during parthenocarpy but leaves open the possibility that these MpESTs could be induced earlier in the interaction leading to parthenocarpy.

Expression of TcESTs putatively involved in hormones suggests a hormone imbalance in parthenocarpic pods

The publication of the cacao genome by Argout et al. (2011) allowed us to test the impact of *M. perniciosa* infection and parthenocarpy on the expression of TcESTs relating to hormone action and biosynthesis. ANOVA and PCoA indicate that TcESTs involved in regulation of cytokinin (*TcCKX5* and *TcCKX3*), auxin (*TcGH3.1*, *TcAIP10A5*, *TcARF18*), and ABA (*TcABA8ox1*) are induced, while those involved in gibberellin synthesis (*TcGA20OX1B*) were repressed in Comun, Joventina32, and NO-55 pods (Tables 6 and 7). The observed expression patterns among TcESTs related to different hormones suggest that altered regulation of multiple hormones occurs during parthenocarpy.

The expression of a few TcESTs relating to abscisic acid was altered by *M. perniciosa*. The expression of a putative abscisic acid-insensitive 5-like protein 1 (*TcAREB5.1*) was repressed (Table 6). In *Arabidopsis*, *abscisic acid-insensitive 5* (*AtABI5*) is a leucine zipper transcription factor that regulates genes during late embryogenesis (Finkelstein and Lynch 2000). The expression of *TcAIL1*, an AP2-like ethylene responsive transcription factor, was 6.1-fold repressed in parthenocarpic pods (Table 6). An AP2 transcription factor was repressed in transgenic-induced parthenocarpic tomato fruits (Martinelli et al. 2009), suggesting that repression of this TcEST could potentially be a factor in parthenocarpy. The repressed expression of GA-20 oxidase (see above) suggests reduced GA synthesis in parthenocarpic pods. GA-20 oxidase is induced by pollination (Lange et al. 1994) and limits development of parthenocarpic fruit in tomatoes (Serrani et al. 2007).

Synthetic cytokinins are known to induce parthenocarpy in kiwifruit (Iwahori et al. 1988), pears (Zhang et al. 2008), and watermelon (Maroto et al. 2005). The highest induction of TcESTs by *M. perniciosa* occurred with putative cytokinin oxidase/dehydrogenase (CKX). *TcCKX5* was 9.9-fold induced in parthenocarpic pods, and *TcCKX3* was 26.2-fold induced (Table 6). CKXs regulate cytokinin levels during seed development (Emery 2006). CKXs irreversibly inactivate cytokinins to reduce overall levels (Schmülling et al. 2003) and delay cell differentiation in reproductive meristems (Bartrina et al. 2011). The induction of *TcCKX3* and *TcCKX5* during parthenocarpy may be associated with the absence of seed (Emery 2006) or from elevated cytokinin levels that induce genes targeting reduction of cytokinins. Expression of *TccrtXa*, putatively related to zeatin *O*-glucosyltransferase (Martin et al. 1999), was repressed in parthenocarpic fruit (Table 6). Zeatin *O*-glucosyltransferase converts zeatin to *O*-glucosylzeatin and is considered important for protection against cytokinin oxidases (Martin et al. 1999).

Auxins are known to induce parthenocarpy in tomatoes and cucumbers (Kim et al. 1992). Parthenocarpic pods had increased expression of *TcGH3.17a* and *TcGH3.1*, putatively related to indole-3-acetic acid (IAA)–amido synthetase (Table 6 and 7). IAA–amido synthetase conjugates excess IAA to amino acids to help maintain auxin levels (Staswick et al. 2005). Expression of putative auxin response factor 18 (*TcARF18*) was induced in parthenocarpic pods (Table 6). *AtARF18* is a transcriptional repressor of auxin genes (Guilfoyle and Hagen 2007). Infection of tomatoes with a GUS fusion of an auxin-responsive promoter–reporter gene with the solanaceous biotype (S-biotype) of *M. perniciosa* suggests increased auxin levels in the primary xylem of *M. perniciosa* brooms (Marelli et al. 2009). *M. perniciosa*-infected cacao leaves had 2.5× more auxins than healthy leaves (Kilaru et al. 2007). There is an increase in IAA concentrations in compatibly pollinated cacao flowers, while IAA concentration remained stable in incompatibly pollinated flowers (Baker et al. 1997), suggesting an importance of IAA in flower and fruit development. The induced expression of TcESTs putatively associated with reducing auxin levels suggests that an elevated auxin environment exists within flower cushions/parthenocarpic pods.

Altered expression patterns of cytokinin-, auxin-, and gibberellin-related TcESTs suggest attempts to reduce hormone levels in parthenocarpic pods. It is possible that elevated hormone levels in the flower cushions are being translocated to pods. Others species have the ability to transport IAAs, cytokinins, and GAs from stems and leaves to the fruit (Davies 2010), indicating that phytohormones

affecting fruit do not need to be produced in the fruits. *M. perniciosa* may induce hormone production in flower cushions or produce hormones itself during the early development of parthenocarpic pods.

This molecular analysis is the first look at the interaction of *M. perniciosa* in the development of parthenocarpic cacao pods. *M. perniciosa* appears to be utilizing detoxification of plant metabolites, cell wall degradation, and adjusted metabolism for nutrient acquisition in a nutrient depleted environment during the biotrophic phase of witches' broom disease. With the exception of increased expression of *MpDOPA*, all the phytohormone-related MpESTs were strongly repressed compared to expression in *M. perniciosa* liquid culture. Since *M. perniciosa*-produced hormones would be from an irregular source and quantity compared to those produced by cacao, induction of MpESTs related to phytohormones may not be necessary for *M. perniciosa* to alter cacao hormone levels. The altered expression observed for TcESTs related to hormone biosynthesis and regulation suggests some attempt by cacao to control the hormones produced during *M. perniciosa*-induced parthenocarpy. *M. perniciosa* employs elevated expression *MpSOD2*, *MpCTA1*, *MpATG8*, *MpAs*, *MpAK*, *MpPLY*, and *MpPME* during the biotrophic phase of parthenocarpy, targeting nutrient acquisition and survival.

Knowledge of the genes involved in the interaction of cacao with *M. perniciosa* could aid cacao breeders by identifying candidate genes for manipulation through breeding. Identifying new source trees with elevated constitutive expression or inducible expression levels of defense genes could reduce the time needed to breed resistant trees by providing more direct selection methods (i.e., marker assisted selection). The difference in EST expression between branch brooms and parthenocarpic pods suggests a difference in resistance between branch and cushion infections. This research has partially identified the molecular signature of the reaction between cacao and *M. perniciosa* and has identified several highly inducible cacao defense genes for further study and has partially described the regulation of many other potentially important cacao genes during induction of parthenocarpy by *M. perniciosa*. Considering other true fungi (*Moniliophthora roreri*, *Ceratocystis cacaofunesta*, etc.) cause severe diseases in cacao, altering expression of some of the identified defense genes could contribute to broad-spectrum disease resistance.

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