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Gene expression in leaves of *Theobroma cacao* in response to mechanical wounding, ethylene, and/or methyl jasmonate

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Abstract

Yield of *Theobroma cacao* (cacao), the source of chocolate, is limited by disease and insect pests. Developmental stage influences the resistance of cacao leaves to disease and insect pests. Mechanical wounding, ethylene, and methyl jasmonate induce resistance to pests in many plant species. The effects of mechanical wounding, ethylene, and methyl jasmonate on gene expression was studied in cacao leaves at two developmental stages, young red (YR) and mature green (MG). Differential expression was observed for genes putatively encoding a DNA binding protein (*TcWRKY-1*), a protein regulating cell division (*TcORFX-1*), a Type III peroxidase (*TcPer-1*), an endo-1,4-β-glucanase (*TcGlu-1*), a class VII chitinase (*TcChiB*), a caffeine synthase (*TcCaf-1*), and a light-harvesting complex protein (*TcLhca-1*). Wounding induced *TcWRKY-1* and *TcORFX-1* in YR and MG leaves. Elevated *TcPer-1* mRNA levels were detected in YR and MG leaves after wounding. Wounding induced *TcChiB* in YR leaves and repressed *TcLhca-1* in MG leaves. Ethylene (12 μL L^{-1}) induced *TcPer-1* in YR and MG leaves but induced *TcGlu-1* in MG leaves only. Ethylene repressed *TcLhca-1* and *TcCaf-1* in YR leaves. Ethylene repressed *TcLhca-1* and *TcChiB* in MG leaves. Methyl jasmonate (0.2 mM) induced *TcCaf-1* and *TcChiB* in YR leaves and *TcPer-1* and *TcChiB* in MG leaves. Ethylene/methyl jasmonate combined induced *TcChiB* in YR leaves and *TcGlu-1* in MG leaves. 1-Methylcyclopropene, an inhibitor of ethylene action, blocked ethylene induced responses but did not block responses to wounding or methyl jasmonate. The cacao response to wounding, ethylene, and/or methyl jasmonate was influenced by developmental stage. Cross-talk between ethylene and methyl jasmonate action on cacao gene expression resulted in synergistic and antagonistic responses. It is critical to account for tissue developmental stage when studying the molecular responses of cacao to mechanical wounding, ethylene, and methyl jasmonate. The constituative and inducible defense strategies used by cacao are dependent on the developmental stage of the tissues involved.

Keywords: *Theobroma cacao*; Methyl jasmonate; Ethylene; Wounding; Induced resistance

1. Introduction

Theobroma cacao (cacao) is a tropical tree native to the rain forest understory of South and Central America [1]. The source of chocolate, cacao is now grown around the world in tropical climates with sufficient rainfall [1]. There are several major diseases of cacao that threaten production [2]. The diseases include Black Pod, caused by several *Phytophthora* spp., Frosty Pod, caused by *Crinipellis roreri*, and Witches' Broom, caused by *Crinipellis perniciosa* [1,2]. Cacao production in South and Central America has been greatly reduced due to a disease complex involving these pathogens [1,2]. In addition, cacao has several major insect pests that impact its production [1]. In recent years new efforts have been made on an international scale by cacao producers, government agencies, and associated industries to develop control measures for insect pests and diseases of cacao. Control measures include development of improved cultural methods, integrated pest management strategies,

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Table 1
Cacao cDNA clones and their tentative identification based on sequence comparisons (blastx)

dbEST-ID	Size (bp)	Locus/gene	Protein/source	Identity (%)/expected ratios
Clone				
TcWRKY-1 (CK144293)	304	NM106732	WRKY family transcription factor/*A. thaliana*	56/2E−10
		CA798062	EST/*T. cacao*	99/1E−66
TcORFX-1 (CK144295)	440	AY097189	fw2.2 gene (evolution of fruit size)/*L. pennellii*	72/6E−20
TcPer-1 (CK144296)	639	AF488305	Apoplastic quiacol peroxidase/*G. hirsutum*	87/2E−76
TcGlu-1 (AY487173)	258	NM105114	Endo-1,4-β-glucanase/*A. thaliana*	92/3E−50
TcLhca-1 (CK144297)	290	L19651	Photosystem I 24 kDa protein/*P. sativa*	80/1E−20
TcChiB (CF973685)	440	AF527943	Class VII chitinase precursor/*G. hirsutum*	80/1E−74
		CA794516	EST/*T. cacao*	98/1E−90
TcCaf-1 (CK144298)	470	AB031280	Caffeine synthase/*C. sinensis*	60/4E−50
		CA796721	EST/*T. cacao*	97/3E−99

and identification of resistant germplasm. Associated with this work, we are characterizing the responses of cacao to biotic and abiotic treatments known to induce resistance to pests in other plant species. The wound response and the plant hormones ethylene and methyl jasmonate are the subjects of this study.

Wounding of plant tissues has been shown to induce complex molecular responses many of which are considered plant defense responses [3–5]. The form of wounding, whether strictly mechanical or combined with other components such as insect saliva [6,7], is critical to determining the plant's response. Wounding can induce formation of jasmonates and ethylene, each of which are capable of independently inducing signal transduction pathways leading to resistance against plant pests. Jasmonatic acid and the related compound methyl jasmonate are part of the octadecanoid pathway in plants [8,9], and in some incidences contribute to resistance to insects [10,11] and pathogens [12,13]. Jasmonates have been shown to function as regulators of many different plant defense genes [10,13]. Ethylene is a gaseous plant hormone extensively studied due to its involvement in senescence and stress responses, in addition to plant defense [14]. Ethylene is known to induce specific subsets of pathogenesis-related proteins in many plant species [15,16]. The effects of ethylene have been associated with resistance to many different plant pathogens [13,14,16] and insects [14,17–19]. Ethylene has also been shown to contribute to susceptibility to disease [14,16] and insect pests [20].

In order to optimize pest management strategies it is important to understand the genetic capabilities of cacao germplasm to defend itself against disease. Although diverse biotic and abiotic treatments are known to induce defense responses in many plant species, the outcome of any particular plant/pest/inducer interaction is typically dependent on each individual component of the interaction plus the environment the interaction takes place in. Our objective in this study was to characterize the responses of cacao leaves at two stages of maturity, young red leaves (YR, 5–10 cm long, very pliable) and mature green leaves (MG, 10–20 cm, very rigid), to mechanical wounding and to treatment with ethylene, methyl jasmonate, and the combination of ethylene/methyl jasmonate. To do this we studied the effects of the above treatments on expression of developmentally regulated cacao genes associated with responses to stress including resistance to plant disease (Table 1).

2. Materials and methods

2.1. Plant production and leaf development

Open pollinated seeds of *T. cacao* variety comun (Lower Amazon Amelonado type) were collected by Alan Pomella from established plantings at the Almirante Cacau Inc. farm (Itabuna, Bahia, Brazil). Seeds were planted in 15.2 cm pots filled with a soilless mix (2:2:1, sand:perlite:promix) and seedlings were grown in ambient light and temperature conditions in a greenhouse for up to 9 months. Heat was provided when temperatures were below 20 °C and cooling was provided by water-cooled air when temperatures exceeded 29 °C. In addition, humidity was maintained at or above 75% during daylight hours using a misting system (Atomizing Systems Inc., Ho-Ho-Kus, NJ). Leaf development was separated into two stages—Stage 1: young red leaves (YR), 5–10 cm long, very pliable and Stage 2: mature green leaves (MG) 10–20 cm, very rigid.

2.2. Sources of cDNA clones

Seven cDNA fragments were cloned and used as probes. The probes were chosen for their diversity as related to developmental and metabolic processes within the plant and/or their potential involvement in plant defense. As described by Antúnez de Mayolo [21], *TcChiB* was isolated by suppression subtractive hybridization, and *TcGlu-1* was isolated by reverse transcriptase-polymerase chain reaction using primers based on consensus sequence. *TcCaf-1* was amplified by reverse transcriptase-polymerase chain reaction from total RNA isolated from YR leaves of cacao comun using primers based on a consensus sequence between tea (*Camellia sinensis*) and coffee (*Coffea arabica* L.) caffeine synthases. The primers used to amplify *TcCaf-1* were forward 5′-ATTGAAGGAGTCTAATTTCTC-3′ and

reverse 5′-TTGAACGGGAAGATCTAC-3′. Four of the cDNA clones were isolated based on differential expression in cacao leaves responding to biotic or abiotic stresses. The methods used for differential display are described in [22]. The cDNA clones and their source treatment combinations were as follows: *TcORFX-1*, induced by Bion, an inducer of plant defense (19 h); *TcWRKY-1*, induced by messenger, an inducer of plant defense (2 h); *TcPer-1*, induced by 2,4-D (48 h); *TcLhca-1*, repressed by Nep1, a necrosis inducing peptide.

2.3. Mechanical wounding and ethylene/methyl jasmonate treatments

The mechanical wound treatment consisted of rolling a Groomax™ hard slicker brush (Pacific Coast Distributing Inc., Phoenix, AZ) over selected leaves backed with Styrofoam. The wound treatment resulted in 16 uniformly spaced perforations of the leaf per square inch. Controls were unwounded leaves on separate seedlings. Seedlings used in the wound studies were maintained on a greenhouse bench where misting was applied at 0.25 h intervals. Leaves were harvested before wounding (time 0), 0.25, 4, and 20 h after wounding. Methyl jasmonate (0.2 mM) was mixed with distilled water and applied as a foliar spray to cacao seedlings using a Binks model 15 sprayer at 15 psi and at a rate of 46 mL m^{-2} (460 L ha^{-1}). Controls were sprayed with distilled water. Sprays were applied between 10:00 a.m. and 11:00 a.m, a time point when stomata were consistently open. After treatment, seedlings were immediately placed in glass cylinders and the glass cylinders were sealed with plastic wrap. Ethylene was injected into the cylinders, where required, at a concentration of 12 μL L^{-1}. Seedlings in the ethylene/methyl jasmonate studies were maintained in the cylinders, in the greenhouse, out of direct sunlight for 0.25, 4 or 20 h prior to harvesting YR and MG leaves. Harvested leaves were frozen in liquid nitrogen, and stored at −80 °C.

2.4. Treatment with 1-methylcyclopropene (1-MCP)

The compound 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action was used in an effort to confirm the direct action ethylene in the observed induced responses to ethylene, methyl jasmonate, and mechanical wounding. Plants were treated with 1-MCP at 500 nL L^{-1} for 4 h prior to treatment with wounding, ethylene, or methyl jasmonate. The 1-MCP powder was placed in a beaker in the glass cylinders containing plants and 1% potassium hydroxide was added to the beaker. The cylinders were immediately sealed. After the 4 h treatment with 1-MCP, either ethylene was injected into the sealed cylinders or the plants were removed from the cylinders and treated by wounding or with methyl jasmonate as described above. For the 20 h treatments with wounding, ethylene, or methyl jasmonate, the plants were replaced in the cylinders and the 1-MCP treatment was repeated and the cylinders resealed. For the 0.25 h treatments, plants treated with methyl jasmonate and ethylene were maintained in the sealed cylinders but, the plants treated by wounding were maintained on the greenhouse bench for the 0.25 h treatment period.

2.5. RNA isolation

The RNA isolation method was developed by adaptation of several established methods including those of Chang et al. [23], and McKenzie et al. [24] and protocols provided with the Qiagen RNeasy Mini kit, 74106 (Qiagen, USA, Valencia, CA). All solutions used were either treated overnight at 37 °C with 0.1% DEPC then autoclaved or were mixed with DEPC-treated and autoclaved water. Leaf material was ground in liquid nitrogen and transferred to labeled 50 mL tubes containing hot (65 °C) extraction buffer [31], {2% CTAB (hexadecyltrimethyl-ammonium bromide), 2% PVP (polyvinylpyrrolidone), *K*-value 28–32, 100 mM Tris base, pH 8.0, 25 mM diNaEDTA, 2 M NaCl, 0.5 g/L spermidine (*N*-[3-aminopropyl]-1,4-butane-diamine)} with 2% 2-mercaptoethanol added just before use. The ground leaf material and buffer were mixed and incubated at 65 °C for 15–20 min. An equal volume of chloroform was added to each tube, mixed, and centrifuged at 8000 × *g* for 30 min. The chloroform extraction was repeated, and the RNA precipitated overnight at 4 °C with 1/3 volume of 8 M lithium chloride. The precipitated RNA was centrifuged for 1 h at 8000 × *g* at 4 °C in a pre-chilled rotor. The supernatant was carefully decanted and the pellet was re-suspended in 500 μL Qiagen buffer RLT containing 1% 2-mercaptoethanol [32]. After the addition of 250 μL 100% ethanol to the sample, the 750 μL was transferred to an RNeasy mini-column. The sample was centrifuged for 1 min at 16,000 × *g* and washed with 700 μL RW1 buffer. After centrifugation for 15 s at 16,000 × *g*, the column was transferred to a new collection tube and washed twice with 500 μL RPE buffer, centrifuged at 16,000 × *g* for 15 s the first time and 2 min the second time. The RNA was eluted with two 50 μL washes with water, yielding a total eluant of 100 μL.

2.6. DNase I treatment

The RNA was treated with DNase I, by the addition of 100 μL DEPC water, 20 μL 10× DNase I buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin) and 4 μL DNase I to each tube. After a 30 min incubation at 37 °C, the samples were extracted with an equal volume of 3:1 phenol:$CHCl_3$ and centrifuged at 14,000 × *g* and 4 °C for 10 min. The aqueous layer was precipitated overnight with 20 μL 3 M NaOAc, pH 5.5 and 600 μL 100% ethanol at −80 °C. The samples were centrifuged 10 min at 14,000 × *g* at 4 °C. The pellets were washed in 80% ethanol and air-dried in a 50 °C heat block

for 3–5 min. The pellets were suspended in 50 μL water, and quantified using a spectrophotometer and integrity verified with a BTPE gel as described below.

2.7. Confirmation of differential gene expression patterns by Northern blot

The 10 μg (1 μg μL^{-1}) of total RNA was mixed with an equal volume of glyoxal/DMSO load dye (NorthernMax-Gly™ load dye, Ambion Inc., Austin, TX), denatured at 50 °C for 30 min, and separated by electrophoresis at 70 V for 2 h on a 1.3% agarose gel containing 300 mM Bis–Tris (bis[2-hydroxyethyl]iminotris [hydroxymethyl] methane), 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and 10 mM EDTA, at pH 8.0 (1× BPTE). RNA was attached to Zeta-Probe®GT membrane (Bio-Rad Laboratories, Hercules, CA) by upward transfer in 10× SSC buffer. After cross-linking RNA to the membrane (UV Stratalinker 8600, Stratagene, La Jolla, CA), membranes were air-dried and stored at −20 °C.

The cDNA probes were purified using the GENECLEAN Turbo for PCR Kit (Q-BIO gene, Carlsbad, CA) following manufacturer's directions. For each probe, 35 ng of cDNA was labeled with [α-^{32}P] dCTP using a Random Primed DNA Labeling Kit™ (Roche Diagnostics Inc., Indianapolis, IN), and gel filtered (Edge Gel Filtration Cartridges™, Edge BioSystems, Gaithersburg, MD). Blots were prehybridized in ExpressHyb™ solution (BD Biosciences Clontech, Palo Alto, CA) at 68 °C for 1 h.

For all studies, probes were hybridized to at least four blots with replicate samples. Radioactively labeled cDNA probes were denatured at 95–100 °C for 5 min. The probe was added to 15 mL of fresh ExpressHyb™ solution, and blots were incubated with continuous shaking at 68 °C for 2 h. Blots were washed according to the ExpressHyb™ protocol, covered in plastic wrap, and exposed to a storage phosphor screen (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ) and imaged at 200 μm resolution on a Typhoon 8600 Variable Mode Imager. Following imaging, the replicate blots were washed, scanned to verify that no probe remained, and probed a second time with radioactively labeled 28S ribosomal cDNA probe.

2.8. Calculation of band volume ratios

Blots probed with 28S ribosomal cDNA were imaged and band volumes were calculated using ImageQuant Version 5.2^{c} (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ). To correct for variations in loading volume on each replicate blot, the ratio of 28S ribosomal cDNA band volume of each wound treatment/time point combination or ethylene/methyl jasmonate treatment/leaf stage combination was determined relative to the unwounded 0 h YR leaf (arbitrarily set to 1) sample or the YR leaf air-treated control at 0.25, 4 or 20 h (arbitrarily set to 1), respectively. The band volumes for blots probed with cDNAs of interest were corrected by multiplication by the appropriate 28S ribosomal cDNA ratio. For ease of presentation, corrected band volumes for each wound treatment/time point combination or ethylene/methyl jasmonate treatment/leaf stage combination were converted to ratios relative to the unwounded 0 h YR leaf sample (arbitrarily set to one) or the YR leaf air-treated control at 0.25, 4 or 20 h (arbitrarily set to 1), respectively. The ratios were averaged for the wound study and ethylene/methyl jasmonate studies (0.25, 4 and 20 h time points analyzed separately) using data from three or more replicate blots. The figures include representative northerns and the average band volume ratios (plus or minus one standard error) for each treatment combination. Data for 0.25 and 4 h treatments with ethylene, methyl jasmonate, of ethylene/methyl jasmonate combined were either non-significant or showed trends similar to the 20 h time points and are not presented.

3. Results

3.1. Gene expression in response to mechanical wounding, ethylene, and methyl jasmonate treatment

Mechanical wounding rapidly induced accumulation of WRKY transcript (Fig. 1A). The wound induced expression of *TcWRKY-1* was observed after 0.25 h, but declined to near control levels by 4 h after wounding. The level of induction exceeded 7 times control levels in both YR and MG leaves. Variability in WRKY transcript in response to ethylene, methyl jasmonate, and the ethylene, methyl jasmonate combination was limited and did not exceed two times the control at 0.25, 4, or 20 h after treatment (data not shown). ORFX mRNA accumulated 2.75–5.14 times higher levels in wounded leaves than observed in unwounded leaves in YR and MG leaves, respectively (Fig. 1B). The timing of the response was similar to that observed with WRKY with transient expression 0.25 h after wounding. As was also observed for WRKY, variability in ORFX transcript in response to ethylene, methyl jasmonate, and the ethylene, methyl jasmonate combination was limited and did not exceed two times the control for any treatment time point (data not shown).

Peroxidase transcript was most highly represented on Northern blots of total RNA from YR leaves compared to MG leaves (Fig. 2). Induction of peroxidase in YR leaves by wounding was two times the control 4 h after wounding (Fig. 2A). In MG leaves wounding induced accumulation of peroxidase transcript to 2.75 and 5.78 times control levels after 4 and 20 h, respectively (Fig. 2A). Methyl jasmonate induced peroxidase transcript in MG leaves to 6.06 times the air-treated peroxidase transcript level 20 h after treatment (Fig. 2B). Ethylene and the combination of ethylene/methyl jasmonate induced peroxidase transcript accumulation of more than four times that observed in air-treated seedlings in YR leaves after 20 h of treatment (Fig. 2B). These same

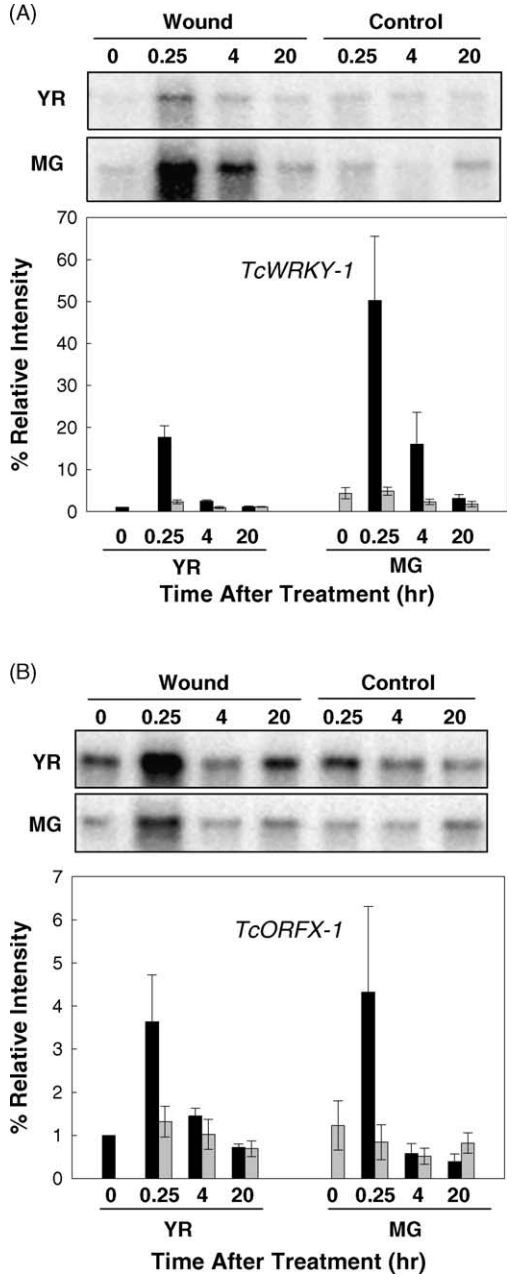

Fig. 1. Expression of: (A) *TcWRKY*-1 and (B) *TcORFX-1* in young red (YR) and mature green (MG) *T. cacao* leaves after mechanical wounding. YR and MG cacao leaves were harvested 0, 0.25, 4, and 20 h after mechanical wounding (black bars). Controls (gray bars) were unwounded leaves on separate seedlings. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the YR time leaf samples at time 0 on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

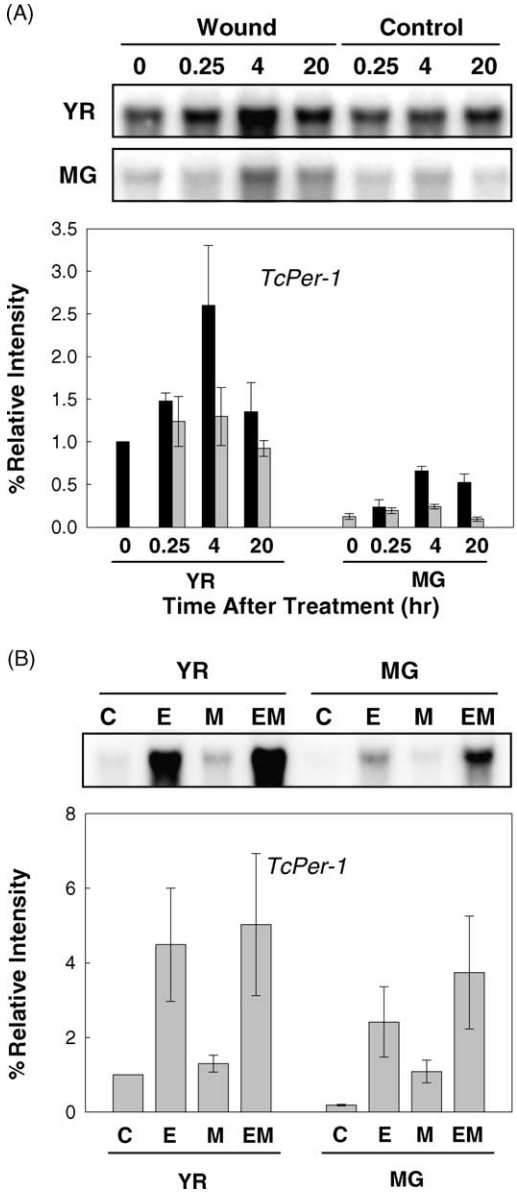

Fig. 2. Expression of *TcPer-1* in young red (YR) and mature green (MG) *T. cacao* leaves after mechanical wounding or treatment with ethylene (E), methyl jasmonate (M), or ethylene/methyl jasmonate combined (EM). (A) YR and MG cacao leaves were harvested 0, 0.25, 4, and 20 h after mechanical wounding (black bars). Controls (gray bars) were unwounded leaves on separate seedlings. (B) MG and YR leaves were harvested from cacao seedlings treated with ethylene (E, 12 μL L^{-1}), methyl jasmonate (M, 0.2 mM), or ethylene/methyl jasmonate (EM) combined for 20 h in sealed chambers. Controls (C) were maintained in sealed chambers with air only. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the (A) YR time leaf samples at time 0 or (B) air-treated YR leaf sample on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

treatments induced peroxidase transcript accumulations of more than 20 times the air-treated seedlings in MG leaves (Fig. 2B).

Transcript of endo-1,4-β-glucanase was most highly represented on Northern blots of total RNA from air-treated YR leaves compared to air-treated MG leaves (Fig. 3). Endo-1,4-β-glucanase transcript levels were near the minimal level of detection in MG leaves. Four hours after wounding endo-1,4-β-glucanase transcript was detected at two times higher levels than observed in unwounded YR leaves

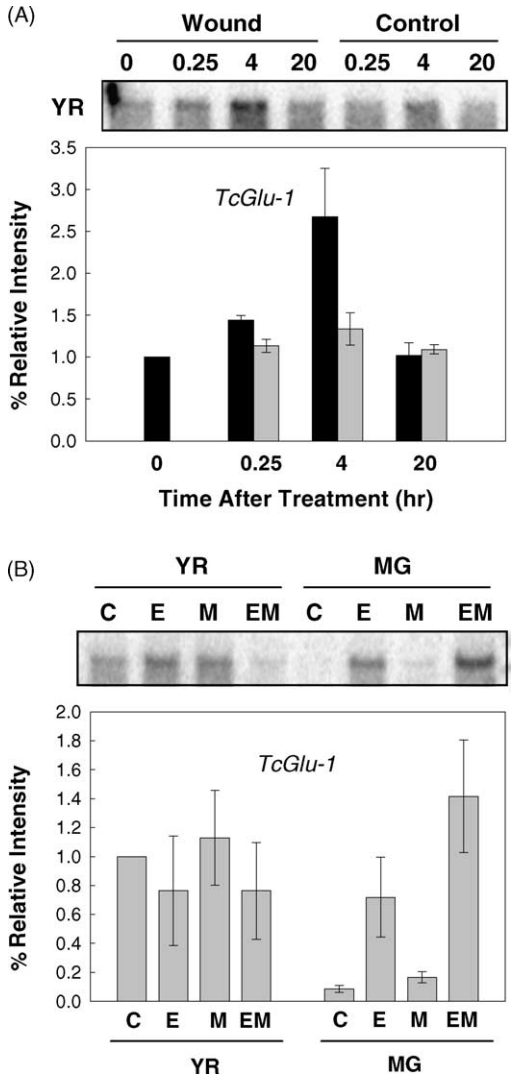

Fig. 3. Expression of *TcGlu-1* in young red (YR) and mature green (MG) *T. cacao* leaves after mechanical wounding or treatment with ethylene (E), methyl jasmonate (M), or ethylene/methyl jasmonate combined (EM). (A) YR and MG cacao leaves were harvested 0, 0.25, 4, and 20 h after mechanical wounding (black bars). Controls (gray bars) were unwounded leaves on separate seedlings. (B) MG and YR leaves were harvested from cacao seedlings treated with ethylene (E, 12 μL L^{-1}), methyl jasmonate (M, 0.2 mM), or ethylene/methyl jasmonate (EM) combined for 20 h in sealed chambers. Controls (C) were maintained in sealed chambers with air only. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the (A) YR time leaf samples at time 0 or (B) air-treated YR leaf sample on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

(Fig. 3A). Wounding did not alter expression of endo-1,4-β-glucanase in MG leaves (data not shown). Ethylene, methyl jasmonate, and the ethylene/methyl jasmonate combination had minimal effect on endo-1,4-β-glucanase transcript accumulation in YR leaves after 20 h (Fig. 3B). Endo-1,4-β-glucanase transcript was strongly induced in MG leaves after 20 h of treatment with ethylene (eight times air-treated control) and ethylene/methyl jasmonate (15.78 times air-treated control) (Fig. 3B).

As expected, the gene encoding photosystem I, light-harvesting complex, pigment binding protein Lhca was more highly expressed in MG leaves than observed in YR (Fig. 4B). There was a 52% decrease in Lhca 20 h after wounding in MG leaves (Fig. 4A). Lhca transcript levels were not affected by wounding in YR leaves (data not shown). Ethylene alone induced a 68% reduction in Lhca

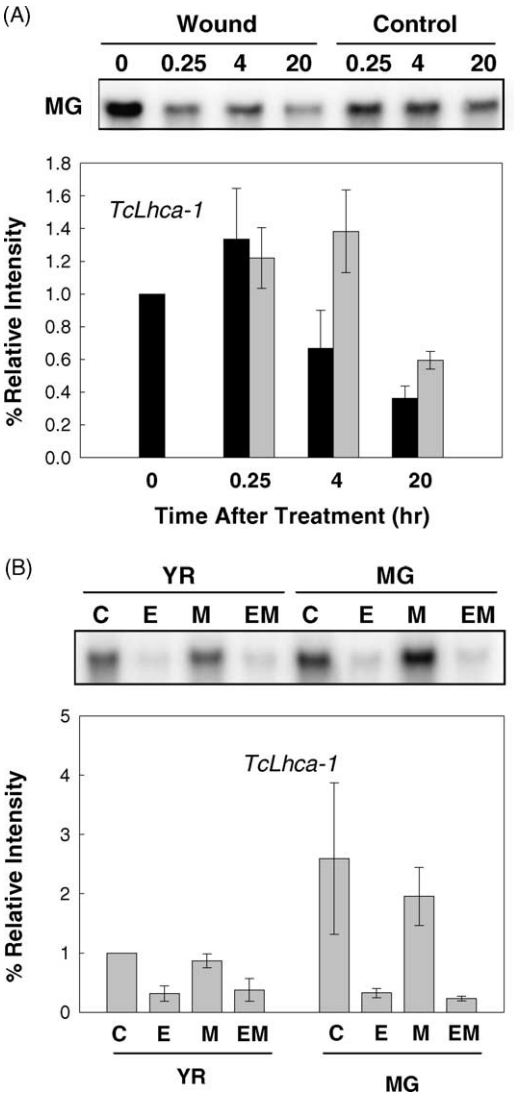

Fig. 4. Expression of *TcLhca-1* in young red (YR) and mature green (MG) *T. cacao* leaves after mechanical wounding or treatment with ethylene (E), methyl jasmonate (M), or ethylene/methyl jasmonate combined (EM). (A) YR and MG cacao leaves were harvested 0, 0.25, 4, and 20 h after mechanical wounding (black bars). Controls (gray bars) were unwounded leaves on separate seedlings. (B) MG and YR leaves were harvested from cacao seedlings treated with ethylene (E, 12 μL L^{-1}), methyl jasmonate (M, 0.2 mM), or ethylene/methyl jasmonate (EM) combined for 20 h in sealed chambers. Controls (C) were maintained in sealed chambers with air only. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the (A) YR time leaf samples at time 0 or (B) air-treated YR leaf sample on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

transcript in YR leaves and an 88% reduction in Lhca transcript in MG leaves after 20 h of treatment (Fig. 4B). The ethylene/methyl jasmonate combination induced a 63% reduction in Lhca transcript in YR leaves and a 91% reduction in Lhca3 transcript in MG leaves after 20 h of treatment (Fig. 4B). Methyl jasmonate alone did not significantly affect Lhca transcript accumulation.

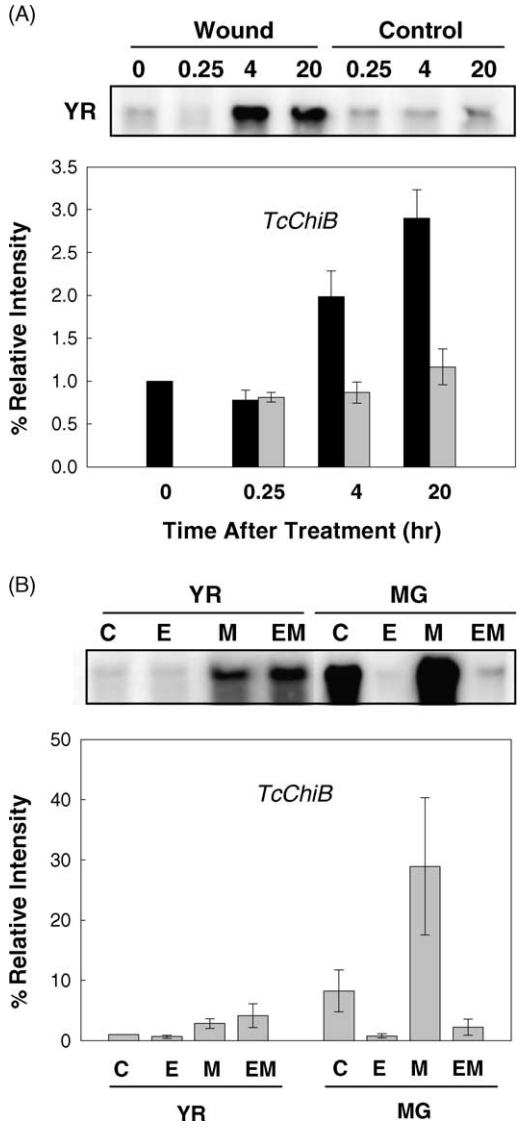

Fig. 5. Expression of *TcChiB* in young red (YR) and mature green (MG) *T. cacao* leaves after mechanical wounding or treatment with ethylene (E), methyl jasmonate (M), or ethylene/methyl jasmonate combined (EM). (A) YR and MG cacao leaves were harvested 0, 0.25, 4, and 20 h after mechanical wounding (black bars). Controls (gray bars) were unwounded leaves on separate seedlings. (B) MG and YR leaves were harvested from cacao seedlings treated with ethylene (E, 12 μL L^{-1}), methyl jasmonate (M, 0.2 mM), or ethylene/methyl jasmonate (EM) combined for 20 h in sealed chambers. Controls (C) were maintained in sealed chambers with air only. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the (A) YR time leaf samples at time 0 or (B) air-treated YR leaf sample on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

Twenty hours after wounding, chitinase transcript was detected at levels 2.48 times that observed for controls in YR leaves (Fig. 5A). Wounding did not affect chitinase transcript accumulation in MG leaves (data not shown). Methyl jasmonate alone, and ethylene/methyl jasmonate in combination, induced accumulation of chitinase transcript in YR leaves to levels 2.85–4.12 times transcript levels observed in YR leaves from air-treated plants after 20 h of treatment (Fig. 5B). Ethylene and ethylene/methyl jasmonate in combination reduced chitinase transcript levels by at least 73% in MG leaves compared to MG leaves from air-treated seedlings (Fig. 5B). Methyl jasmonate induced accumulation of chitinase transcript in MG leaves to levels 3.51 times that observed in MG leaves from air-treated seedlings (Fig. 5B). Chitinase transcript was detected at more than 8.25 times higher levels on Northern blots of total RNA from MG leaves than observed on Northern blots of total RNA from YR leaves (Fig. 5B).

Caffeine synthase transcript was easily detectable on Northern blots of total RNA from YR leaves (Fig. 6) but almost undetectable on Northern blots of total RNA from MG leaves (data not shown). Mechanical wounding did not significantly alter expression of caffeine synthase in YR or MG leaves (data not shown). Ethylene and ethylene/methyl jasmonate treatment for 20 h reduced caffeine synthase transcript levels in YR leaves by at least 74% that observed for YR leaves from air-treated seedlings (Fig. 6B). Methyl jasmonate induced accumulation of caffeine synthase transcript in YR leaves to levels 2.56 times higher than that observed for YR leaves from air-treated seedlings (Fig. 6).

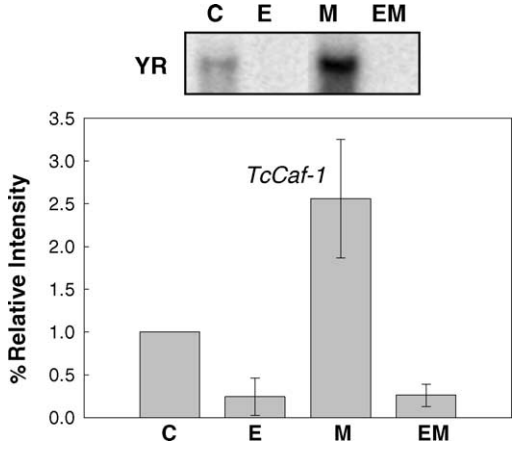

Fig. 6. Expression of *TcCaf-1* in young red (YR) *T. cacao* leaves after treatment with ethylene (E), methyl jasmonate (M), or ethylene/methyl jasmonate combined (EM). YR leaves were harvested from cacao seedlings treated with ethylene (12 μL L^{-1}), methyl jasmonate (0.2 mM), or ethylene/methyl jasmonate combined for 20 h in sealed chambers. Controls (C) for were maintained in sealed chambers with air only. Corrected band volumes for each leaf stage/treatment by time combination were converted to ratios relative to the air-treated YR leaf sample on individual blots. The ratios were averaged for each leaf stage/treatment by time combination using data from blots of four or more replicate samples.

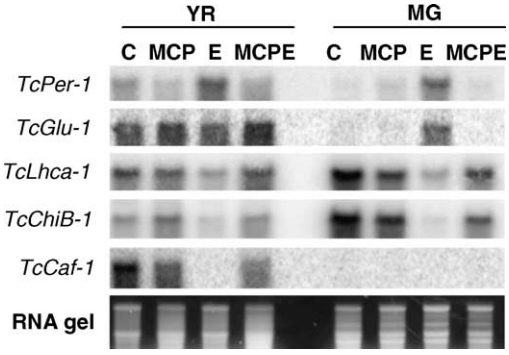

Fig. 7. Inhibition of ethylene (E) action in cacao leaves by 1-methylcyclopropene (MCP), a competitive inhibitor of ethylene receptors. YR and MG leaves were harvested from cacao seedlings treated with 1-MCP (MCP, 500 nL L^{-1}), ethylene (12 μL L^{-1}), or 1-MCP (500 nL L^{-1}) and ethylene (12 μL L^{-1}), combined for 20 h in sealed chambers. Controls (C) for were maintained in sealed chambers with air only. Three replicate Northern blots were probed with *TcPer-1*, *TcGlu-1*, *TcLhca3-1*, *TcChiB-1*, and *TcCaf-1*.

3.2. 1-Methylcyclopropene blocks ethylene action but not wound response or methyl jasmonate responses

The 1-methylcyclopropene (1-MCP) treatment consistently blocked the action of ethylene in cacao leaves whether ethylene enhanced transcript levels or reduced transcript levels (Fig. 7). The 1-MCP treatment alone did not consistently alter transcript levels for any of the genes studied. The 1-MCP failed to alter the wound and methyl jasmonate responses for any of the genes studied at both 0.25 and 20 h after treatment (data not shown). Similar to previously presented data, the influence of tissue ontogeny on gene expression was apparent. Constitutive expression of peroxidase, endo-1,4-β-glucanase, and caffeine synthase was higher in YR leaves while constitutive expression of Lhca3 and chitinase was higher in MG leaves (Fig. 7).

4. Discussion

4.1. Gene expression and related gene function

Mechanical wounding rapidly and transiently induced *TcWRKY-1* and *TcORFX-1*. Changes in transcript levels of *TcWRKY-1* and *TcORFX-1* in cacao leaves in response to ethylene or methyl jasmonate were inconsistent at the time points used and always less than two times the levels observed in the appropriate controls. *TcWRKY-1* has homology to genes in the WRKY gene family (Table 1). WRKY genes function as transcriptional activators in many different plant response systems including mechanical wounding [25–27]. WRKY gene induction by wounding is often rapid and transient, similar to that observed for *TcWRKY-1*. *TcORFX-1* has similarity to *ORFX* in *Lycopersicon* species [28,29], a gene that functions as a negative regulator of fruit size and is hypothesized to regulate cell division. Mechanical stresses have been shown to alter plant growth [30]. This is the first report of a gene related to *ORFX* being induced by mechanical wounding.

TcPer-1 transcript accumulation in cacao leaves was enhanced by mechanical wounding, treatment with ethylene, and treatment with methyl jasmonate. Mechanical wounding induced *TcPer-1* in both YR and MG leaves. Ethylene strongly induced *TcPer-1* in both YR and MG leaves, but methyl jasmonate induced *TcPer-1* in MG leaves only. *TcPer-1* is related to *Pod10* (AF488305), an apoplastic quiacol peroxidase from *G. hirsutum* [31] in addition to many peroxidases involved in the lignification processes [32–34]. Increases in peroxidase transcript and enzyme activities have been noted in plants responding to mechanical wounding [33]. *TcPer-1* is highly expressed in YR leaves, a stage where cell wall expansion and lignification is expected to take place. Ethylene [33,35] and methyl jasmonate [33,36,37] have been shown to enhance transcript levels and enzyme activities for peroxidases in leaves as a component of induced resistance.

TcGlu-1 was transiently induced by mechanical wounding (observed after 4 h only). *TcGlu-1* transcript accumulation was induced in MG cacao leaves by ethylene treatment for 20 h. Expression of *TcGlu-1* in cacao leaves was not influenced by treatment with methyl jasmonate. *TcGlu-1* shows homology to genes encoding endo-1,4-β-glucanases in *Arabidopsis thaliana* and other plant species (Table 1). Endo-1,4-β-glucanases are believed to be involved in cell wall loosening involved in reorganization or degradation processes [38,39]. The constitutive accumulation of *TcGlu-1* transcript while cell walls are rapidly expanding (YR) is consistent with this proposed function. Similar observations were made by Brummell et al. [40] for the endo-1,4-β-glucanase gene *Cel4* that was expressed at high levels in rapidly expanding tissues of tomato (*Lycopersicon esculentum*) and was also inducible by ethylene.

TcLhca-1 transcript was more resistant to change in YR leaves as compared to MG leaves. Both mechanical wounding and ethylene treatment had a greater effect in reducing *TcLhca-1* transcript levels in MG leaves than observed in YR leaves. *TcLhca-1* transcript levels in YR and MG cacao leaves was repressed by exposure to ethylene but was not affected by exposure to methyl jasmonate. *TcLhca-1* putatively codes for a photosystem I, light-harvesting complex, pigment binding protein Lhca3 (Table 1). Exposing leaves to ethylene has been shown to induce breakdown of chloroplasts as a component of the senescence process [41,42]. The effect is considered a secondary response to ethylene related to senescence processes and could involve interactions with many other plant hormones [14]. The strong repression (85% reduction in MG leaves) of *Lhca-1*, a nuclear encoded gene, by ethylene suggests ethylene also influences chloroplast function by action in the nucleus.

TcChiB transcript accumulation was induced by wounding in YR leaves where constitutive expression was low, was inhibited by ethylene in MG leaves where constitutive expression was high, and was induced by methyl jasmonate

in YR and MG leaves. The constituative expression of *TcChiB* in MG cacao leaves was more than eight times higher than observed in YR leaves in the wounding studies and was approximately two times higher than the *TcChiB* transcript levels observed in wounded YR leaves. Based on sequence homology, *TcChiB* is related to several chitinases including a class VII chitinase from *Gossypium hirsutum* that is induced by salicylic acid [43] (Table 1). Chitinases, as a family, are well characterized for their function as pathogenesis-related proteins in many plant species [44,45]. Chitinases may function in plant defense as inducible and/or constitutively expressed genes in many cases [46] and have been shown in several cases to be inducible by wounding, ethylene, and methyl jasmonate [47,48]. It is unusual to find a putative chitinase gene repressed by ethylene, as the opposite is often observed [48,49].

TcCaf-1 transcript accumulation was not affected by wounding, was enhanced in YR leaves by treatment with methyl jasmonate for 20 h, and was repressed in YR leaves by treatment with ethylene for 20 h. Cacao *TcCaf-1* has homology (Table 1) with the caffeine synthase (TCS1) isolated from *Camellia sinensis* leaves [50]. TCS1 catalyzes the final two steps in the synthesis of caffeine in *C. sinensis* leaves [50]. It was suggested by Aneja and Gianfagna [3] that caffeine contributes to resistance to infection by *C. perniciosa* in cacao. Caffeine is typically produced in young tissues [51] an observation that is consistent with the results presented here for *TcCaf-1*. Aneja and Gianfagna [3] provided evidence that caffeine biosynthesis is enhanced in response to wounding. *TcCaf-1*, a putative caffeine synthase clone, was induced by methyl jasmonate but not by mechanical wounding. There are other EST clones from cacao in GenBank with homology to genes encoding caffeine synthase in other plants species. Coffee has several genes encoding caffeine synthases, one that catalyzes the last two steps in the biosynthetic pathway [52], and others that catalyze only the final step converting theobromine to caffeine [53]. It is possible the induction of caffeine production in response to wounding observed by Aneja and Gianfagna [3] was a result of activation of other caffeine synthase genes or due to other processes altered in response to wounding. Mechanical wounding can induce other stress responses [5]. Winz and Baldwin [54] found that treating *Nicotiana attenuata* roots with ethephon, a compound converted to ethylene by plants, repressed nicotine production and putrescine methyltransferase transcript production, thought to be the rate limiting step in nicotine biosynthesis, resulting from both wounding and *Manduca sexta* herbivory, an effect that could be reversed by treatment with 1-MCP pretreatment, a inhibitor of ethylene action. Aneja and Gianfagna [3] found that AVG, inhibitor of ethylene biosynthesis, also enhanced caffeine production in wounded cacao leaf tissues which when combined with our results for *TcCaf-1*, lead us to suggest a regulatory role for ethylene as a repressor of caffeine biosynthesis in cacao leaves.

4.2. Mechanical wounding and interactions between ethylene and methyl jasmonate

The results discussed above hint at the complexity of the interaction between ethylene and methyl jasmonate when applied to cacao leaves. Both synergistic and antagonistic interactions were observed between the effects of ethylene and methyl jasmonate on gene expression in cacao leaves. Ethylene sometimes blocked the effect of methyl jasmonate at the concentrations used (*TcChiB* and *TcCaf-1*). Methyl jasmonate never blocked the effect of ethylene. In several instances the combination of ethylene and methyl jasmonate augmented the response to treatment (*TcPer-1* and *TcGlu-1*). This type of genetic cross-talk, both synergistic [55,56] and antagonistic [54,56], between ethylene and methyl jasmonate have been reported in the literature and the specific nature of the interaction is dependent on the gene and tissue type being studied.

1-Methylcyclopropene (1-MCP) consistently blocked ethylene action under the conditions used consistent with its function as an inhibitor of ethylene action [57]. Based on the results of the 1-MCP studies, the responses to methyl jasmonate were independent of ethylene even when synergistic (that is wounding or methyl jasmonate did not induce ethylene which induced the observed changes in gene expression). Although jasmonates and ethylene production are commonly considered as components of the response to mechanical wounding, mechanical wounding does have effects independent of the octadecanoid pathway [4,5]. The responses of *TcPer-1* and *TcChiB* to mechanical wounding were not dependent upon ethylene but the involvement of the octadecanoid pathway cannot be ruled out since they were both induced by methyl jasmonate. The response to *TcLhca-1* to mechanical wounding was independent of ethylene based on the MCP studies while methyl jasmonate treatment alone had no effect on *TcLhca-1* expression.

TcWRKY-1 and *TcORFX-1* are responsive to mechanical wounding and do not respond to ethylene or methyl jasmonate (Table 2). When combined with enhanced expression of *TcPer-1* and *TcChiB* in YR leaves or enhanced expression of *TcPer-1* and repressed expression to *TcLhca-1* in MG leaves, they serve as indicators of the wound response in cacao leaves (Table 2). Enhanced expression of *TcPer-1* and *TcChiB* in MG leaves or enhanced expression of *TcChiB* and *TcCaf-1* in YR leaves are indicative of cacao leaves responding to methyl jasmonate (Table 2). Similar comparisons can be made for the response of MG and YR leaves to ethylene and YR leaves to the ethylene/methyl jasmonate combination (Table 2). The response of MG leaves to ethylene/methyl jasmonate combined is distinguishable from the response to ethylene only based on relative expression levels (Table 2).

Six of the seven genes studied show consistent differences in expression levels between YR and MG leaves. *TcChiB* is expressed at more than eight times higher levels in MG leaves than observed in YR leaves. *TcCaf-1*,

Table 2
T. cacao transcript levels in young red (YR)/mature green (MG) leaf stage or after treatment

Gene[a]	Leaf stage[b]	Treatment							
		Wound[c]		Ethylene		MJ		ET/MJ	
		YR	MG	YR	MG	YR	MG	YR	MG
TcWRKY-1	MG (4.33)	7.70 (0.25)	10.38 (0.25)	–	–	–	–	–	–
TcORFX-1	–	2.75 (0.25)	5.14 (0.25)	–	–	–	–	–	–
TcPer-1	YR (8.33)	2.00 (4)	2.75 (4), 5.78 (20)	4.49	13.39	–	6.06	5.03	20.78
TcGlu-1	YR (14.29)	2.00 (4)	–	–	8.00	–	–	–	15.78
TcLhca-1	MG (2.59)	–	0.48 (4)	0.32	0.12	–	–	0.37	0.09
TcChiB	MG (8.25)	2.28 (4), 2.48 (20)	–	–	0.09	2.85	3.51	4.12	0.27
TcCaf-1	YR (>100)	–	–	0.24	–	2.56	–	0.26	–

Ratios (treatment/control) were based on the average of three or four replicated experiments. Ratios were included if greater than 2.0 or less than 0.5.
[a] The detailed description about the genes is in Table 1.
[b] The numbers in parenthesis are the relative transcript level compared to different leaf stages.
[c] The numbers in parenthesis are the treatment time (hour). For other treatments, data from 20 h sampling are presented.

for example is almost undetectable in MG leaves. This is consistent with the findings of Aneja and Gianfagna [3] that caffeine levels are elevated in young red leaves of cacao. Aneja and Gianfagna [3] also provided evidence that caffeine contributes to resistance of cacao to infection by *C. perniciosa*, a pathogen that attacks young actively growing tissues (1). Tissue ontogeny can affect sensitivity of plants to ethylene [58,59], methyl jasmonate [60], and wounding [60,61]. The response of cacao leaves to wounding, ethylene, and methyl jasmonate was strongly influenced by the stage of leaf development. For example, *TcGlu-1* was induced by ethylene in MG leaves but not in YR leaves where constitutive expression was high. Ethylene blocked the induction of *TcChiB* by methyl jasmonate in MG leaves but not in YR leaves. As described here, accounting for tissue ontogeny is critical when studying the molecular responses of cacao to mechanical wounding, ethylene, and methyl jasmonate. Young cacao tissues are generally highly susceptible to attack by *Phytophthora* spp. (2), the causal agents of black pod. Mature leaves have been used to screen for resistance to *Phytophthora* spp. and have shown high levels of resistance to specific *Phytophthora* spp. [62,63]. In addition, the young shoots and associated young leaves are preferred feeding sites for several cacao insect pests [1]. The constitutive and inducible defense strategies used by cacao are dependent on the developmental stage of the tissues involved. With this understanding, differences in constituative and inducible expression of cacao defense genes in divergent cacao germplasm source can be more accurately characterized.

We have identified genes useful in the further characterization of the responses of cacao to wounding, ethylene, and methyl jasmonate. The methods developed will be useful for identifying other genes responsive to these treatments. The knowledge gained concerning these genes, such as *TcChiB*, and *TcCaf-1* as methyl jasmonate induced plant defense genes, can be exploited in studying the responses of cacao to diverse plant pathogens and insect pests. In addition, our newly gained understanding of the responses of cacao to wounding, ethylene, and methyl jasmonate will be used to identify beneficial organisms and abiotic treatments capable of inducing resistance to diseases and/or insects in cacao through pathways involving mechanical wounding, ethylene, and or methyl jasmonate.

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