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Original article

Developmental expression of stress response genes in *Theobroma cacao* leaves and their response to Nep1 treatment and a compatible infection by *Phytophthora megakarya*

Bryan A. Bailey [a,*], Hanhong Bae [a], Mary D. Strem [a], Gabriela Antúnez de Mayolo [b], Mark J. Guiltinan [b], Joseph A. Verica [b], Siela N. Maximova [b], John H. Bowers [a]

[a] *Sustainable Perennial Crops Laboratory, Plant Sciences Institute, USDA/ARS, Room 342, Building 001, Beltsville Agricultural Research Center-West, Beltsville, MD 20705, USA*
[b] *306 Wartik Laboratory, Pennsylvania State University, University Park, PA 16802, USA*

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Abstract

Developmental expression of stress response genes in *Theobroma cacao* leaves and their response to Nep1 and a compatible infection by *Phytophthora megakarya* were studied. Ten genes were selected to represent genes involved in defense (*TcCaf-1*, *TcGlu1,3*, *TcChiB*, *TcCou-1*, and *TcPer-1*), gene regulation (*TcWRKY-1* and *TcORFX-1*), cell wall development (*TcCou-1*, *TcPer-1*, and *TcGlu-1*), or energy production (*TcLhca-1* and *TcrbcS*). Leaf development was separated into unexpanded (UE), young red (YR), immature green (IG), and mature green (MG). Our data indicates that the constitutive defense mechanisms used by cacao leaves differ between different developmental stages. *TcWRKY-1* and *TcChiB* were highly expressed in MG leaves, and *TcPer-1*, *TcGlu-1*, and *TcCou-1* were highly expressed in YR leaves. *TcGlu1,3* was highly expressed in UE and YR leaves, *TcCaf-1* was highly expressed in UE leaves, and *TcLhca-1* and *TcrbcS* were highly expressed in IG and MG leaves. *NEP1* encodes the necrosis inducing protein Nep1 produced by *Fusarium oxysporum* and has orthologs in *Phytophthora* species. Nep1 caused cellular necrosis on MG leaves and young pods within 24 h of application. Necrosis was observed on YR leaves 10 days after treatment. Expression of *TcWRKY-1*, *TcORFX-1*, *TcPer-1*, and *TcGlu-1* was enhanced and *TcLhca-1* and *TcrbcS* were repressed in MG leaves after Nep1 treatment. Expression of *TcWRKY-1* and *TcORFX-1* was enhanced in YR leaves after Nep1 treatment. Infection of MG leaf disks by *P. megakarya* zoospores enhanced expression of *TcGlu-1*, *TcWRKY-1*, and *TcPer-1* and repressed expression of *TcChiB*, *TcLhca-1* and *TcrbcS*. Five of the six genes that were responsive to Nep1 were responsive to infection by *P. megakarya*. Susceptibility of *T. cacao* to *P. megakarya* includes altered plant gene expression and phytotoxic molecules like Nep1 may contribute to susceptibility.
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Keywords: Cacao; Developmental expression; Nep1; *Phytophthora megakarya*; Plant defense; Susceptibility; *Theobroma cacao*

1. Introduction

Several *Phytophthora* species, including *P. megakarya* Brasier and Griffin, *P. palmivora* (Butl.) Butler, *P. citrophthora* (R.H. Sm. and E. Sm.) Leonian, and *P. capsici* Leonian, attack the tropical tree *Theobroma cacao* L. (cacao) causing black pod disease. Symptoms include seedling blights, stem cankers, and pod rots [15,56]. *P. megakarya* is the most aggressive of the four species on cacao and poses a major threat to cacao production in western Africa [15,56]. A bioassay using leaf disks to screen for resistance to black pod in cacao reveled that increasing levels of necrosis were an indication of susceptibility to *Phytophthora* spp. [44,47]. The reaction to *Phytophthora* spp. in the leaf disk assay is highly dependent upon the leaf's stage of development. Young cacao leaves are generally highly susceptible to attack by *Phytophthora* spp. [15]. Mature leaves were used in the leaf disk assay and they could be highly resistant to specific *Phytophthora* spp. depending on the cacao genotype [43,46,49]. Selection of resistance based on the response of leaf disks from mature cacao leaves to *Phytophthora* spp. zoospore inoculation has been correlated with pod resistance [44,47,51].

Abbreviations: IG, immature green; MG, mature green; UE, unexpanded; YR, young red.

* Corresponding author. Tel.: +1 301 504 7985; fax: +1 301 504 5823.
E-mail address: baileyb@ba.ars.usda.gov (B.A. Bailey).

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The extra-cellular protein Nep1 is produced by *Fusarium oxysporum* Schlechtend:Fr. f. sp. *erythroxyli*. Nep1 causes cell death in many different dicot plant species when applied as a foliar spray [38]. Orthologues of *NEP1* (**AF036580**), the gene for Nep1 [42], have been identified in a broad range of microbes including several *Phytophthora* spp. (accession #-**AF352031.1**, **AAK25828.1**, **AF320326.1**), *Pythium aphanidermatum* (Edson) Fitzp (accession #-**AF179598**), and *Bacillus halodurans* (accession #-**BAB04114.1**). Although the importance of Nep1 in pathogenesis of *F. oxysporum* remains in question [12], Qutob et al. [46] demonstrated that *Phytophthora sojae* preferentially expresses *PsojNIP* during the necrotrophic phase of disease development on soybeans and therefore may function as a pathogenicity factor. In addition to cell death, the gene products of orthologues from the plant pathogens *F. oxysporum*, Nep1; *Phytophthora* spp., NPP1 and PsojNIP [26,46]; and *Pythium* spp., PaNie and others [54], cause similar responses in host and nonhost dicot plant species. Plant cell cultures respond to Nep1 and NPP1 by altered ion channeling and induction of active oxygen [26,34]. PaNie from *P. aphanidermatum* induces DNA laddering in carrot (*Daucus carota* L.), a primary measure for programmed cell death, in addition to production of the phytoalexin 4-hydroxybenzoic acid [54]. Foliar application of the combination of Nep1 with the plant pathogen *Pleospora papaveracea* enhances disease development on opium poppy (*Papaver somniferum* L.) [11].

Very little is known concerning the responses of cacao to biotic and abiotic stresses at the gene expression level. Recently Verica et al. [55] used subtraction library techniques to identify cacao expressed sequence tags responsive to inducers of resistance and to Nep1 treatment in mature green leaves although detailed expression data were not provided. In order to exploit genomic approaches to studying stress responses in cacao it is important to understand the influence of tissue developmental stage on gene expression. We have identified and cloned cDNA fragments showing altered expression in cacao leaves responding to pathogens and other stresses. Our primary objectives were to characterize the influence of leaf developmental stage on constitutive expression of stress response genes in *T. cacao* and to develop an understanding of the susceptible response of *T. cacao* to pathogens by characterizing the expression of nine cDNA clones in cacao leaves after treatment with Nep1, and after infection by *P. megakarya*.

2. Results

2.1. Gene expression in during leaf development

Leaf development was separated into four stages (Fig. 1): Stage 1) unexpanded leaves (UE) less than 1 cm long with limited pigmentation, Stage 2) young red leaves (YR) 5–10 cm long and pliable, Stage 3) immature green leaves (IG) 10–20 cm long and pliable, and Stage 4) mature green leaves (MG) 10–20 cm and rigid. Large differences were detected in the constitutive expression levels of the ten genes being studied depending upon the developmental stage of the leaf (Fig. 2). *TcWRKY-1* mRNA was most highly represented on Northern blots of total RNA from MG leaves where it occurred at a frequency 5.1 times greater than observed for total RNA from YR leaves and 7.7 times more frequent than observed in UE leaves. *TcORFX-1* mRNA accumulated to more than 3.5 times higher levels in YR than observed in UE and MG leaves. *TcPer-1* transcript was most highly represented on Northern blots of total RNA from YR and IG cacao leaves compared to UE and MG leaves. YR and IG leaves accumulated *TcPer-1* transcript at more than four times higher levels than MG leaves and eight times higher levels than UE leaves. Transcript of *TcGlu-1* was most highly represented on Northern blots of total RNA from developing cacao leaves compared to MG leaves. *TcGlu-1* transcript was 7.2 times higher on Northern blots of total RNA from IG leaves than observed for MG leaves. *TcLhca-1* and *TcrbcS* were more highly expressed in IG and MG leaves than observed in YR and UE leaves. *TcChiB* transcript was highly represented on Northern blots of total RNA isolated from MG cacao leaves. *TcGlu1,3* transcript was most highly represented on Northern blots of total RNA isolated from UE and YR cacao leaves. *TcCaf-1* was highly represented on Northern blots of total RNA isolated from UE leaves, consistently detectable in YR leaves, and almost undetectable in MG leaves. *TcCou-1* is most highly represented on Northern blots of total RNA isolated from YR cacao leaves. Expression of *TcCou-1* in MG leaves is near the minimal detection level for the method used.

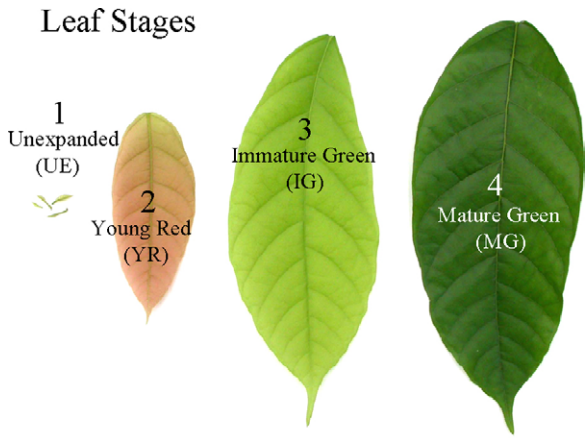

Fig. 1. Leaf development was separated into four stages starting with Stage 1) unexpanded leaves (UE), < 1 cm long with limited pigmentation; Stage 2) young red leaves (YR), 5–10 cm long, and pliable; Stage 3) immature green leaves (IG), 15–20 cm long, and pliable; and Stage 4) mature green leaves (MG) 15–25 cm, and rigid.

2.2. Nep1 induced symptoms in cacao tissues

Microscopic necrotic flecks appeared on the under side of mature green (MG) cacao leaves within 24 h of Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) treatment. Evaluation of

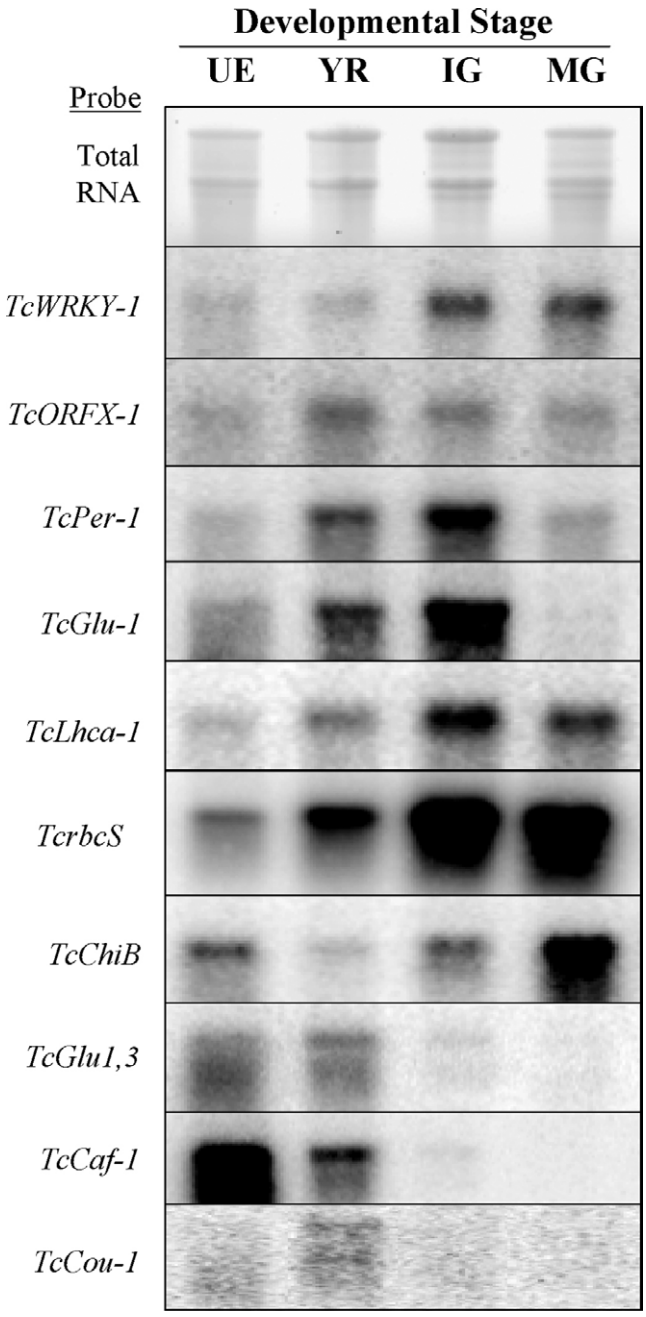

Fig. 2. Expression of 10 ESTs in *T. cacao* leaves during leaf development. Cacao leaves were harvested at unexpanded (UE), young red (YR), immature green (IG), and mature green (MG) stages of development. Representative autoradiograms are presented from four replicated samples.

the lesions on MG leaves under magnification (600 ×) revealed that Nep1 killed the majority of stomata guard cells and two or more neighboring epidermal cell around each affected stomata on the abaxial leaf surface (Fig. 3B). The overall level of visible necrosis on MG leaves in response to Nep1 treatment was less than 1% of the total leaf. Although not obvious 24 h after Nep1 treatment, elongated necrotic lesions were observed 10 d after Nep1 treatment on leaves that had been YR when the treatment was applied (Fig. 3D). The lesions on YR leaves were located on the secondary and tertiary veins of the YR leaves. The newly developed stomata between the veins were unaffected by the Nep1 treatment. YR leaves developed normally and, along with the MG leaves, persisted on the plant for weeks after Nep1 (plus Silwet-L77) treatment without showing additional symptoms. Field grown Amelonado cacao pods developed darkly pigmented necrotic lesions within 24 h of Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) treatment (Fig. 3F). It is unclear how Nep1 penetrated the pod surface but, stomata and lenticels occur on the pod surface. Cacao pods were not used in the molecular studies due to their limited availability and generally low yield of extractable RNA.

2.3. Gene expression in young red leaves treated with Nep1

Only two of the 10 genes studied were responsive to Nep1 treatment in YR cacao leaves (Fig. 4). Treatment with Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) induced accumulation of *TcWRKY-1* transcript in YR leaves (approximately 10 times control at 4 h). *TcWRKY-1* transcript decreased in both YR leaves within 24 h after treatment with Nep1 (plus Silwet-L77). Treatment with Nep1 (plus Silwet-L77) induced *TcORFX-1* transcript accumulation of four times in YR leaves when compared to the controls 4 h after treatment. The timing of *TcORFX-1* transcript accumulation in response to Nep1 was similar to that observed for *TcWRKY-1*. Treatment of cacao seedlings with Nep1 (plus Silwet-L77) did not alter expression of the remaining eight genes being studied in YR leaves (*data not shown*).

2.4. Gene expression in mature green leaves treated with Nep1

Six of the ten genes studied were responsive to Nep1 treatment in MG cacao leaves (Fig. 5). Treatment of MG leaves with Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) induced accumulation of *TcWRKY-1* transcript. The timing of induction of *TcWRKY-1* was more rapid in MG leaves (approximately two times control at 0.25 h) when compared to YR leaves (approximately 10 times control at 4 h). *TcWRKY-1* transcript decreased in MG leaves (Fig. 5) within 24 h after treatment with Nep1 (plus Silwet-L77). Treatment with Nep1 (plus Silwet-L77) induced *TcORFX-1* transcript accumulation of 2.6 times in MG leaves when compared to the respective controls 4 h after treatment. The timing of *TcORFX-1* transcript accumulation in response to Nep1 was similar to that observed for *TcWRKY-1*. Nep1 (plus Silwet-L77) treatment induced accumulation of *TcPer-1* mRNA in MG leaves by 3.3 and 5.4 times control levels 4 and 24 h after treatment, respectively. Treatment of cacao seedlings with Nep1 (plus Silwet-L77) induced *TcGlu-1* transcript accumulation in MG leaves. *TcGlu-1* mRNA was detected in MG leaves treated with Nep1 (plus Silwet-L77) at a 6.3 times higher level than controls 24 h after treatment and a 4.1 times higher level 216 h after treatment. Of the nine transcripts being studied, *TcLhca-1* and *TcrbcS* consistently showed a decrease in response to

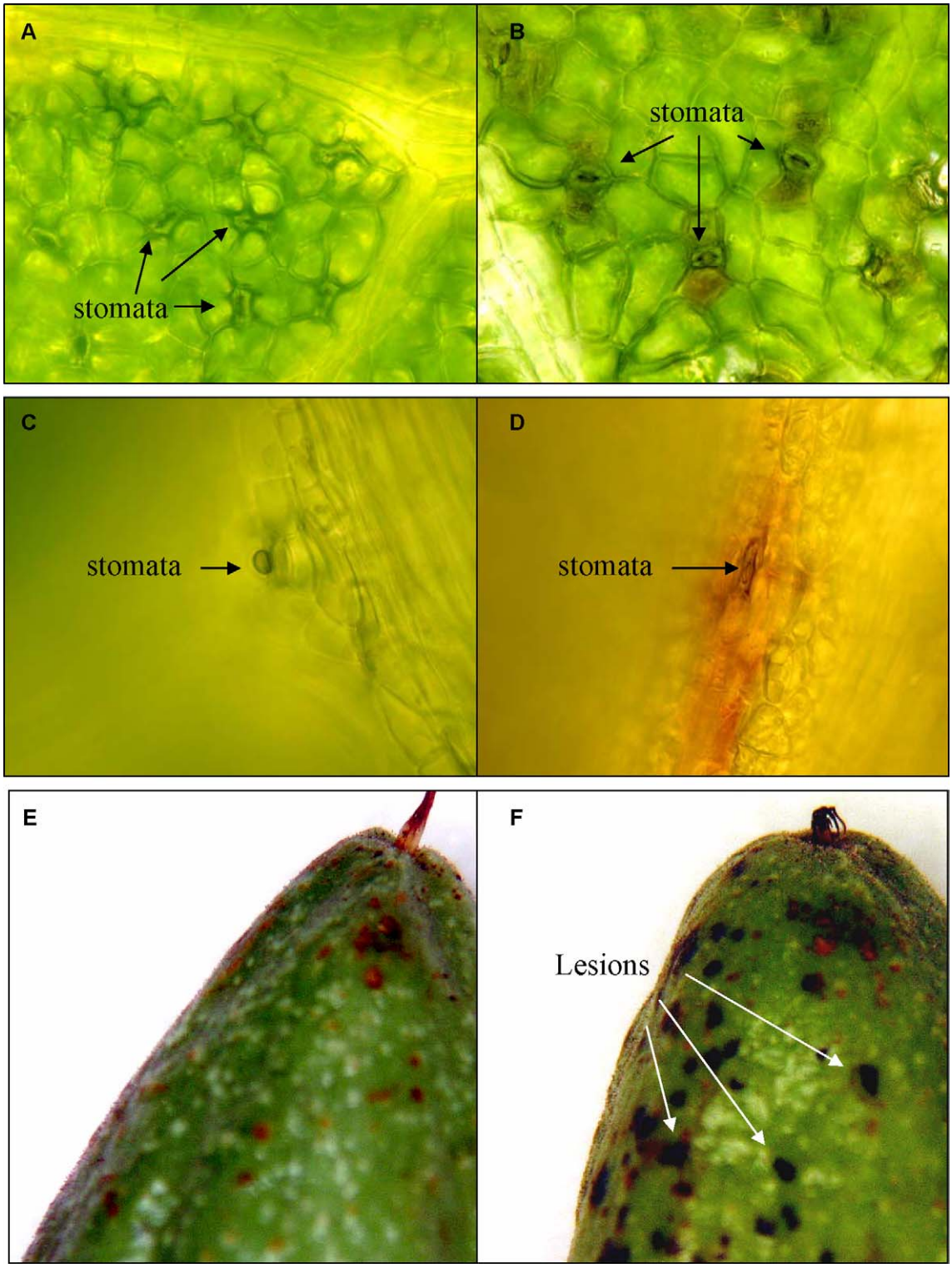

Fig. 3. Microscopic necrotic flecks on abaxial leaf surface of MG leaves (B), YR leaves (D), and young pods (F) caused by treatment of cacao seedling with Nep1. (A, C, and E) Control seedlings were treated with Silwet-L77 alone. Photos A and B (600 × magnification) and E and F (5.8 × magnification) were taken 24 h after treatment. Photos C and D (600 × magnification) were taken 10 days after treatment.

Nep1 treatment. Twenty-four hours after treatment, there was a 65% and 71% decrease of *TcLhca-1* and *TcrbcS* transcript, respectively, in Nep1 treated MG leaves when compared to controls at the same time point. Nine days after treatment, Nep1 treated MG leaves had *TcLhca-1* and *TcrbcS* transcript levels similar to controls. Nep1 treatment of MG leaves did not influence transcript accumulation for *TcChiB*, *TcGlu1,3*, *TcCaf-1*, and *TcCou-1* (*data not shown*).

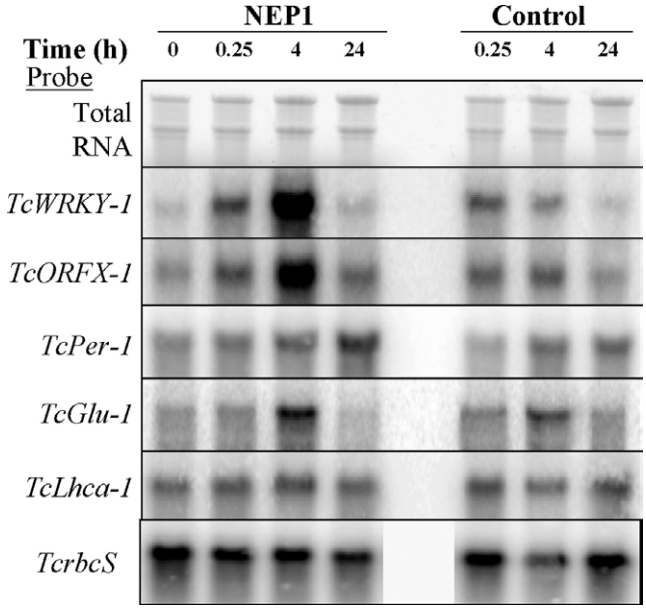

Fig. 4. Expression of 10 ESTs in young red (YR) *T. cacao* leaves after treatment with Nep1 plus Silwet-L77 or Silwet-L77. Young red leaves were harvested 0, 0.25, 4, and 24 h after treatment. Representative autoradiograms are presented from four replicated samples.

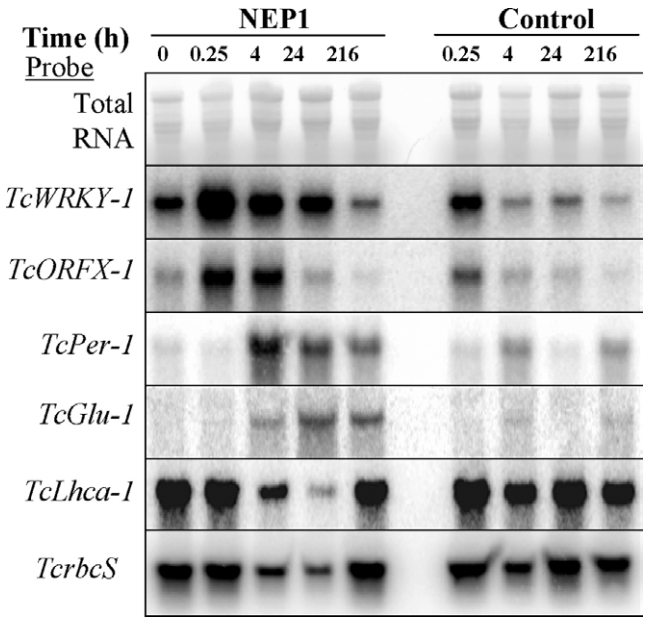

Fig. 5. Expression of 10 ESTs in mature green (MG) *T. cacao* leaves after treatment with Nep1 plus Silwet-L77 or Silwet-L77. Young red leaves were harvested 0, 0.25, 4, and 24 h after treatment. Representative autoradiograms are presented from four replicated samples.

2.5. Systemic induction

There were no differences observed in gene expression of *TcORFX-1*, *TcWRKY-1*, *TcPer-1*, and *TcGlu-1* between untreated leaves (YR and MG) from plants where the lower leaves were treated with Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) or 0.2% Silwet-L77 alone (*data not shown*). The 4 h time was selected based on the observed altered gene expression for *TcWRKY-1*, *TcORFX-1, TcPer-1*, and *TcGlu-1* at this time point in the whole plant Nep1 treatment studies.

2.6. Symptoms and gene expression in response to infection by Phytophthora megakarya

Transcript levels for seven of the 10 cacao genes being studied were severely altered in response to the cutting of 1.4 cm leaf disks and the assay conditions that followed (*data not shown*). Transcripts of the remaining three genes (*TcGlu1,3*, *TcCaf-1*, and *TcCou-1*) were almost undetectable in mature green leaves and were not induced by *P. megakarya* infection (*data not shown*). Consequently, 8.5 cm leaf disks were inoculated. Necrosis at 1–3 days after zoospore inoculation was 0%, 25–50%, and 50–85%, respectively; while controls were asymptomatic throughout the study. Transcript levels for six of the ten cacao genes being studied were altered in response to infection by *P. megakarya* (Fig. 6). *TcWRKY-1* was highly expressed at time 0, declined at 24 h, and then increased 4.5 times control levels 48 h after inoculation. Infection of MG cacao leaves by *P. megakarya* zoospores enhanced expression of *TcPer*-1 (3.1 times control) and *TcGlu-1* (16.7 times control) 2 days after inoculation. *TcChiB* (0.08 times control at 48 h), *TcLhca-1* (0.07 times control at 48 h), and *TcrbcS* (0.26 times control at 48 h) transcript levels decreased after inoculation and remained repressed when compared to controls. Transcript levels of *TcORFX-1* and the remaining three genes (*TcGlu1,3*, *TcCaf-1*, and *TcCou-1*) were unaltered by infection with *P. megakarya* (*data not shown*). *TcORFX-1* was expressed at unusually high levels for 3 days in both control and infected leaf disks.

3. Discussion

Nep1 caused necrosis in cacao leaves and pods in a time frame similar to that observed for Nep1 and related proteins in other plant species [9,26,34,38,46,54]. The necrosis on cacao leaves was centered on stomata that serve as points of entry of Nep1 into the leaf [38]. This provides clear evidence that Nep1, when combined with Silwet-L77, penetrates through stomata and causes a very localized necrosis. It was unclear what the points of entry into the pods were. The amount of necrosis observed in leaves was dependent on the stage of development. The number of stomata present may have limited the damage caused to YR leaves by Nep1. Depending on the exact age, YR leaves of cacao comun have far fewer stomata than MG leaves per unit leaf area. For example, a 7 cm YR leaf of cacao comun had 2.5 stomata per ocular view (all located near veins) while a MG leaf on the same plant had 96.3 stomata per ocular view (average of four ocular views at 600× magnification). Although apparent only after the leaves matured, Nep1 caused cell death around stomata on YR leaves, resulting in elongated lesions associated with the leaf veins. Tissue age was shown to influence the

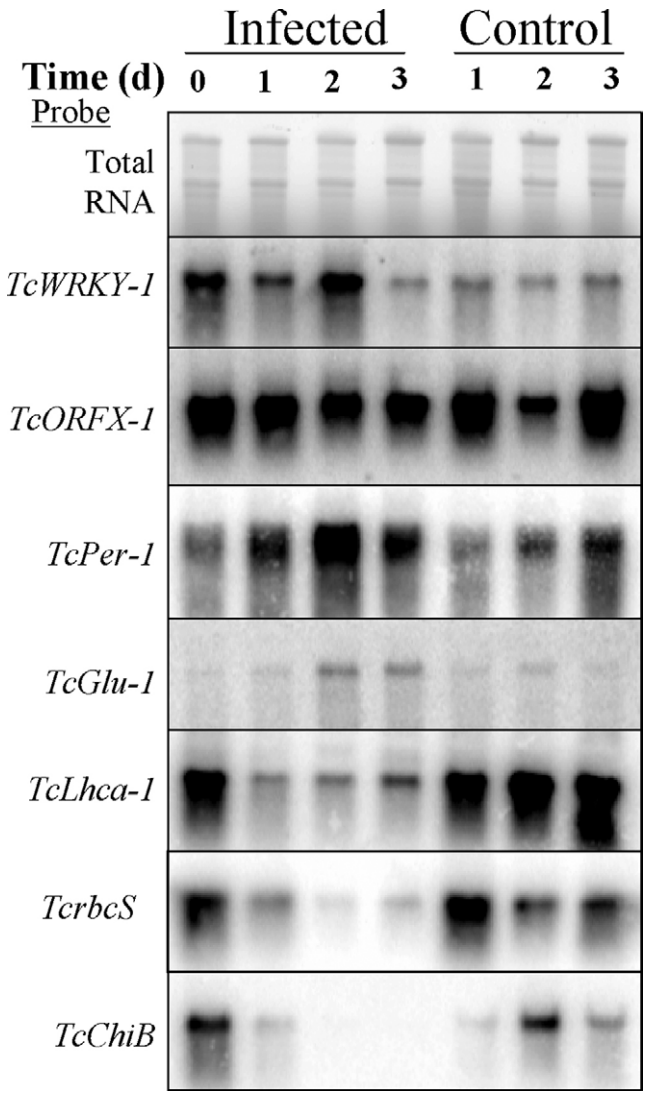

Fig. 6. Expression of seven cacao genes in cacao leaf disks inoculated with *P. megakarya* zoospores. Leaf disks were cut from green leaves and inoculated on the abaxial side with zoospores of *P. megakarya*. Controls were treated with distilled water (20 μl). Representative autoradiograms are presented from three replicated samples.

necrosis caused by a fungal xylanase in tobacco in a similar manner to that observed for Nep1 [10]. Young tobacco leaves responded with limited necrosis and ethylene induction when infiltrated with xylanase, a treatment that bypassed stomatal barriers and strongly induced both ethylene production and necrosis in older tobacco leaves [10].

Nep1 did elicit an active response in YR leaves as verified by the induction of *TcWRKY-1* and *TcORFX-1*. Of the nine cDNA clones studied, *TcWRKY-1* along with *TcORFX-1* responded most rapidly to Nep1 treatment. Both *TcWRKY-1* and *TcORFX-1* are also rapidly and transiently induced by mechanical wounding (*unpublished data*). The induction of *TcWRKY-1* and *TcORFX-1* in YR leaves was greater than observed for MG leaves although the response was delayed from 0.25 to 4 h. *TcWRKY-1* has homology to genes in the *WRKY* family (Table 1); *WRKY* genes function as transcriptional activators in many different plant response systems including the wound response, response to hormones, and plant defense responses [25,32,35,48]. *WRKY* genes cannot only act as transcriptional activators, but can also function as repressors of other *WRKY* gene family members [22]. *TcORFX*-1 has similarity to *ORFX* in tomato (*Lycopersicon* spp.) [27], a gene that functions as a negative regulator of fruit size and is hypothesized to regulate cell division. The three dimensional structure of ORFX shows relatedness to Gα subunits, and the protein shares regions of homology with RAS/RAN/RAD domains in animal systems [7]. It is possible that *TcORFX-1* functions to suppress cell division during periods of stress.

TcPer-1 is closely related to *Pod10* (AF488305), an apoplastic quiacol peroxidase from *Gossypium hirsutum* [19]. In addition, *TcPer-1* shows homology to peroxidases involved in lignification in tree species [18] and to peroxidase genes induced in an incompatible reaction in *Medicago sativa* L. to *Pseudomonas syringae* [23]. *TcPer-1* is highly expressed in YR and IG leaves, stages where cell wall expansion and lignification are expected to take place. Peroxidase activity is considered an integral part of the lignification process of cell walls in plants [3]. Enhanced lignification has been observed

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

Clone (dbEST-ID)	Size (bp)	Locus/gene	Protein/source	Identity/expected ratios
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-beta-glucanase/*A. thaliana*	92%/3e-50
TcGlu1,3	274	NM125042	Glycosyl hydrolase family 17/	94%/6e-41
		CA795103 [a]	EST/*T. cacao*	98%/1e-55
TcLhca-1 (CK144297)	290	L19651	Photosystem I 24 kDa protein/*P. sativa*	80%/1e-20
TcRBCS	555	AB006080	Rubisco small subunit/*Fagus crenata*	80%/2e-97
		CF973934 [a]	EST/*T. cacao*	98%/1e-120
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
TcCou-1 (CK144294)	221	AY250835	4-Coumarate-CoA ligase-like/*A. thaliana*	76%/4e-11

[a] Cloned by PCR amplification using primers based on accession #CA795103 or #CF973934.

in plants responding to elicitors of cell death [14]. In addition to causing cell death, Nep1 induced hydrogen peroxide production in *Nicotiana tabacum* L. cell cultures [34]. The Nep1 orthologue Npp1 induced production of superoxide anion in *Petroselinum crispum* L. cell culture [26].

TcGlu-1 shows a high degree of homology to genes encoding endo-1,4-beta-glucanases in *Arabidopsis* and other plant species (Table 1). Endo-1,4-beta-glucanases are believed to be involved in cell wall loosening involved in reorganization or degradation processes [20,28,40,45,53]. Similar to *TcPer-1*, the constitutive accumulation of *TcGlu-1* transcript while cell walls are being laid down and modified is consistent with this proposed function. The induction of *TcGlu-1* by Nep1 observed 1 and 9 days after treatment of MG leaves is relatively slow, and the induction persisted longer than for the other genes being studied; this suggests it may be a secondary response to Nep1 treatment. Nep1 is known to induce ethylene in many plant species [9], and ethylene is known to be involved in processes where endo-1,4-beta-glucanases are expressed, functioning in cell wall breakdown leading to senescence and/or fruit ripening [40].

The observed reduction in *TcLhca-1* and *TcrbcS* transcripts in response to Nep1 treatment is greater than expected considering the minimal necrosis that occurs in Nep1 treated cacao leaves. *TcLhca-1* putatively codes for a photosystem I, light-harvesting complex, pigment binding protein Lhca3 (Table 1). *TcrbcS* putatively codes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Table 1). Rubisco catalyzes the first step in photosynthetic CO_2 assimilation [50]. Nep1 affected cells that did not die following treatment. This observation is further supported by results indicating Nep1 treated leaves recover normal *TcLhca-1* and *TcrbcS* transcript levels by 9 days after treatment. Previous work in other plant species indicated chloroplast membranes were rapidly degraded in response to Nep1 treatment [38], but these results were linked to cells around stomatal openings.

Expression of *TcChiB, TcGlu1,3, TcCaf*-1, and *TcCou-1*, although developmentally regulated, was not altered by Nep1 treatment. *TcChiB* is highly expressed in untreated MG leaves. *TcChiB* (Table 1) is closely related to several chitinases including a class VII chitinase from *G. hirsutum* that is induced by salicylic acid [39], and several putative chitinases (class I/family 19) from other plant species. Chitinases, as a family, are well characterized for their function as pathogenesis-related proteins in many plant species and may function in plant defense as inducible genes or be constitutively expressed in specific tissues [13,33]. *TcGlu1,3* expression was most highly expressed in UE and YR leaves. *TcGlu1,3* is most closely related to beta-1,3-glucanases within the glycosyl hydrolase Family 17 (Table 1). Glucosyl hydrolases within Family 17 are composed of complex families with expression patterns that are both developmentally regulated and/or inducible by divergent stimuli including plant hormones and inducers of plant defense [52]. Polymers of 1,3-β glucan are components of the cell walls of *Phytophthora* spp. and other oomycetes [36]. UE and YR leaves highly expressed *TcCaf-1*, which was sometimes undetectable in maturing leaves. *TcCaf-1* shares homology with the gene for caffeine synthase (*TCS1*) isolated from *Camellia sinensis* leaves that catalyzes the final two steps in the synthesis of caffeine in *C. sinensis* (L.) Kuntze leaves [37]. As such, it also shares homology with salicylic acid *O*-methyl transferase and jasmonic acid carboxyl methyltransferase. Caffeine is typically produced in young tissues [6], an observation that is consistent with the results presented here for *TcCaf-1*. Caffeine inhibits the growth of *Crinipellis perniciosa* (Stahel) Singer in culture and it has been suggested that caffeine contributes to resistance to infection by *C. perniciosa* in cacao [2]. *TcCou-1* shows closest homology to 4-coumarate:CoA ligase (Table 1), the last enzyme of the general phenylpropanoid pathway. *TcCou-1* was difficult to detect in leaf stages other than YR and was not induced by Nep1. The enzyme 4-coumarate:CoA ligase has diverse family members showing various substrate specificities [3,24]. 4-Coumarate:CoA ligase is in the pathway leading to biosynthesis of lignins, suberin, tannins, and flavonoids [3,43].

The genes studied show significant changes in expression levels associated with leaf developmental stage. As previously discussed, the genes encoding the cDNA clones *TcWRKY-1*, *TcORFX-1*, *TcPer-1*, *TcGlu1,3*, *TcChiB*, *TcCaf-1*, and *TcCou-1* potentially function as developmentally regulated plant defense components in cacao. Arnold et al. [5] noted that concentrations of some anti-microbial compounds decrease in cacao leaves as they mature and that fungal endophytes may compensate for this loss by offering protection to cacao leaves at the mature stage. Our data indicates that the constitutive defense mechanisms used by cacao leaves differ between different developmental stages. MG leaves responded to Nep1 treatment by localized cell death associated with stomata distributed across the lower leaf surface. Nep1 treatment altered transcript levels for *TcWRKY-1*, *TcORFX-1*, *TcPer-1*, *TcGlu-1*, *TcLhca-1*, and *TcrbcS* in MG leaves. YR leaves responded to Nep1 by localized cell death associated with stomata along the veins. Only *TcWRKY-1* and *TcORFX-1* were induced by Nep1 in YR leaves disassociating the regulation of *TcWRKY-1* and *TcORFX-1*, potential transcriptional activators, from the regulation of *TcPer-1*, *TcGlu-1*, *TcLhca-1*, and *Tcrbc-1* in YR leaves.

Of the five clones of putative defense genes (*TcCaf-1*, TcGlu1, 3, *TcChiB*, *TcCou-1*, and *TcPer-1*), only *TcPer-1* was induced by Nep1. *TcWRKY-1*, *TcORFX-1*, genes putatively involved in gene regulation, were both strongly induced by Nep1 treatment. *TcWRKY-1*, *TcORFX-1*, and *TcPer-1* are also inducible by mechanical wounding (*unpublished data*) and are not specific responses to the damage caused by the Nep1 treatment. The Nep1 induced reduction in *TcLhca-1* and *TcrbcS* transcript, transcripts originating in the nucleus, and the previously described Nep1 induced damage to chloroplasts as observed in diverse plant species [38] could potentially limit energy reserves and promote susceptibility. *TcGlu-1* is related to β-1-4 glucanases that are thought to promote plant cell wall expansion [20,45,53] or breakdown [20,40] and may be associated with senescence processes.

Using PCR techniques based on areas of conserved sequence, we have identified orthologues of *NEP1* in *P. megakarya*, *P. palmivora*, *P. citrophthora*, and *P. capsici* (*unpublished data*) similar to those observed in other *Phytophthora* species [26,46]. In many cases, the timing and intensity of a plant defense response can determine the outcome of the plant–pathogen interaction [21,31]. The timing of sampling for the *P. megakarya* infection studies was delayed in comparison to the Nep1 studies. Nep1 penetrates and rapidly induces cell death within hours of treatment. In previously described cacao leaf disk assays disease development and the associated necrosis requires several days to be manifested. *The NEP1* orthologue *PsojNIP* was specifically expressed 48 h after inoculation of soybean with *P. sojae* during the necrotrophic phase of disease development [46]. The induction of active oxygen and cell death can be critical for the development of disease by necrotrophic pathogens such as *Botrytis cinerea* Pers. [29,30]. Nep1 enhanced disease development caused by *P. papaveracea* Sacc. on opium poppy when co-applied [11]. Transgenic *Colletotrichum coccodes* (Wallr.) expressing Nep1 were more virulent on *Abutilon theophrasti* Medik than wild type strains [1]. Our results are consistent with suggestions [34,46] that Nep1, PsojNIP and related proteins function to promote disease.

Cutting 1.4 cm diameter leaf disks and carrying out the leaf disk assay had profound effects on gene expression in mature green cacao leaves resulting in a change to 8.5 cm diameter leaf disk in the studies of cacao infection by *P. megakarya*. Wounding plant tissue is known to alter gene expression of many genes including plant defense genes [25,49]. It is interesting that leaf disks, likely to have highly altered gene expression due to wounding, have demonstrated resistance levels to *Phytophthora* spp. that could be correlated with resistance in the field [44,47,51]. This observation leads us to suggest that many of the defense responses critical to resistance of cacao against *P. megakarya* are not altered in leaf disks despite the altered gene expression and may be preformed or associated with the wound response itself.

Seven of the 10 genes being studied were responsive to infection of 8.5 cm diameter cacao leaf disk by *P. megakarya.* The largest changes in transcript levels for all seven genes responsive to *P. megakarya* infection occurred between 24 and 48 h after inoculation at the time of onset of tissue necrosis. It is notable that five of the six genes that were responsive to Nep1 were similarly responsive to infection by *P. megakarya* when 8.5 cm diameter leaf disks were used in the assays. The two treatments are different in many aspects: the Nep1 treatment is a single exposure to individual cacao cells while the *P. megakarya* treatments results in continually expanding areas of necrosis. Clones *TcWRKY-1*, *TcPer-1*, *TcGlu1,3*, *TcLhca-1*, and *TcrbcS* were responsive to *P. megakarya* infection and Nep1 treatment. As discussed above concerning the Nep1 response, these genes are not cacao genes specifically involved in plant defense. The changes associated with *TcPer-1*, *TcLhca-1*, and *TcrbcS* are potentially associated with negative effects including membrane damage and reduced energy and base carbohydrate production. Reductions in transcript levels of *Lhca* and/or *rbcS* have commonly been associated with plants responding to biotic and abiotic stress [8] including infection by *Phytophthora infestans*. The changes in *TcLhca-1* and *TcrbcS* reported here indicates chloroplast functions are altered by the effects of Nep1 and *P. megakarya* on the steady-state of nuclear encoded transcripts as well as by the chloroplast membrane damage previously reported [38]. In leaf disks, *TcORFX-1*, a Nep1 induced gene, was expressed at unusually high levels in controls and infected tissues for the length of the 3 days study, a possible result of the associated wounding and an indication of differential regulation between *TcWRKY-1* and *TcORFX-1* not observed with Nep1 treatment. *TcChiB* was strongly repressed during infection by *P. megakarya* although unaffected by Nep1 treatment. *TcCou-1*, *TcCaf-1*, and *TcGlu1,3* gene expression, although developmentally regulated, was not altered by either treatment.

Proteins produced from *NEP1* and its ortholoques in *Phytophthora* spp. have similar activities in a broad range of dicot species [26,46]. The response of cacao to Nep1 mimics in some ways the response of cacao to infection by *P. megakarya* in a susceptible interaction. Susceptibility of *T. cacao* to *P. megakarya* includes altered gene expression from the plant and it is postulated from these results that phytotoxic molecules like Nep1 may contribute to susceptibility. The changes in gene expression due to treatment observed for *TcWRKY-1*, *TcPer-1*, *TcGlu1,3*, *TcLhca-1*, *TcrbcS* and *TcChiB* may serve as molecular markers for the response of cacao to infection by *P. megakarya* in future studies.

4. Methods

4.1. Nep1 production

Nep1 was purified from culture filtrates of *F. oxysporum* f. sp. *erythroxyli* as previously described [9] and stored in buffer (20 mM MES, 300 mM KCl, pH 5.0) at –20 °C prior to use.

4.2. Plant production

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); 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, USA 07423-1433).

4.3. Leaf development

Leaf development was separated into four stages (Fig. 1): Stage 1) unexpanded leaves (UE) less than 1 cm long with

limited pigmentation, Stage 2) young red leaves (YR) 5–10 cm long and pliable, Stage 3) immature green leaves (IG) 10–20 cm long and pliable, and Stage 4) mature green leaves (MG) 10–20 cm and rigid. The red pigmentation of young cacao leaves is common to many cacao germplasm sources and was observed in all the germplasm used in these studies. Nep1 was applied to seedlings bearing leaves at the desired stages of development.

4.4. Spray application and sampling

Nep1 was combined with 1,1,1,3,5,5,5-heptamethyltrisiloxanyl propyl-methoxy-poly[ethylene oxide] (Silwet-L77™, Witco Corporation, 3500 South State Route 2, Friendly, WV, USA 26146) for foliar spray applications to seedlings. Seedlings were treated with Nep1 (5 μg ml^{-1}) in 0.2% v/v Silwet-L77. Seedlings treated with Silwet-L77 (0.2% v/v) were included as a control. Foliar sprays were applied with a Binks model 15 sprayer at 15 psi and at a rate of 46 ml m^{-2} (460 l ha^{-1}); sprays were applied between 10:00 and 11:00 A.M. Seedlings were maintained in greenhouse conditions until sampled. Unless otherwise indicated, leaves from Silwet-L77 (0.2% v/v) and Nep1 treated (5 μg ml^{-1}, plus 0.2% v/v Silwet-L77) seedlings were collected before treatment (0 h), 0.25, 4, 24, and 216 h after spray application, frozen in liquid nitrogen, and stored at –80 °C. Leaves not harvested for RNA extraction were observed 24 h after treatment using a Nikon Eclipse E600 at a magnification of 600X (NIKON 1300 Walt Whitman Road Melville, NY, USA 11747). Young open pollinated Amelonado cacao pods (approximately 4 cm long) were treated in the field (Raymond Schnell, Subtropical Horticulture Research Station, ARS/USDA, 13601 Old Cutler RD, Miami, FL, USA 33158) with Nep1 (20 μg ml^{-1}) in 0.2% v/v Silwet-L77 or with Silwet-L77 (0.2% v/v) using Nalgene Aerosol spray bottles (Nalge Nunc International Corporation, Naperville, IL, USA 60563). The pods were photographed 24 h after treatment using a Finepix 4700 zoom camera (Fuji Photo Film Co., Tokyo, Japan 106-8620) attached to a Nikon C-FMA dissecting microscope (Nikon Instech Co., Kanagawa, Japan 210-0005).

4.5. Studies of systemic induction of cacao genes by Nep1

Nep1 (5 μg ml^{-1} plus 0.2% v/v Silwet-L77) or 0.2% v/v Silwet-L77 alone was applied to the entire lower surfaces of the lowest four leaves of 3-month-old seedlings using a nylon paintbrush. Each treatment was applied to three plants that served as replications. Untreated YR and MG leaves from each plant were harvested 4 h after treatment, frozen in liquid nitrogen, and stored at –80 °C. The treated leaves were observed 24 h after treatment for necrosis as described above.

4.6. Inoculation of cacao leaf disks with P. megakarya zoospores

Zoospores of *P. megakarya* strain GWH 252 from the collection of the Cocoa Research Institute of Ghana (Andrews Y. Akrofi, P.O. Box 8, Akim Tafo, Ghana) were produced in axenic culture [16]. Leaf disks were cut from detached MG leaves and placed in petri dishes abaxial side up on moist sterile #2 Whatman paper moistened with sterile distilled water. Initially 25, 1.4 cm leaf disks were used per petri dish but the assay dramatically altered the constitutive expression of the genes being studied. As a result, 8.5 cm leaf disks were used (1 per petri dish). The 8.5 cm leaf disks were inoculated with 25 isolated 20 μl drops of zoospores of *P. megakarya* (3×10^4 zoospores ml^{-1}). Control leaf disks were treated with distilled water (20 μl). The petri dishes were sealed with parafilm and incubated at 25 °C for up 3 days. Three replications were harvested 0–3 days after inoculation for each treatment.

4.7. RNA isolation

The RNA isolation method was developed by adaptation of several established methods including those of Chang et al. [17], and McKenzie et al. [41], and protocols provided with the Qiagen RNeasy Mini kit, 74106 (Qiagen USA, 28159 Stanford Avenue, Valencia, CA, USA 91355). 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 [17] {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^{-1} 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 at 20 °C. 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 RLT Qiagen buffer containing 1% 2-mercaptoethanol [41]. After the addition of 250 μl 100% ethanol to the sample, the 750 μl was transferred to an RNeasy mini-column, in a 2 ml collection tube. 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.

4.8. DNase I treatment

The RNA was treated with DNase I (Roche Applied Science, Indianapolis, IN, USA 46250-0414), 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 16,000 × *g* at 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 16,000 × *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.

4.9. Confirmation of differential gene expression patterns by northern blot

Ten 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, USA 78744-1832), denatured at 50 °C for 30 min, and separated by electrophoresis at 70 V for 1.5 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, USA 94547) by upward transfer in 10× SSC buffer. After cross-linking RNA to the membrane (UV Stratalinker 8600, Stratagene, La Jolla, CA, USA 92037), membranes were air-dried and stored at –20 °C.

Nine cDNA fragments were cloned and used as probes. The nine clones were selected to represent developmentally regulated genes potentially involved in plant defense (*TcCaf-1*, TcGlu1,3, *TcChiB*, *TcCou-1*, and *TcPer-1*), gene regulation (*TcWRKY-1* and *TcORFX-1*), cell wall development (*TcCou-1*, *TcPer-1*, and *TcGlu-1*), and energy production (*TcLhca-1*). As described by Antúnez de Mayolo [4], *TcChiB*, a putative Class VII chitinase, was isolated by suppression subtractive hybridization, and *TcGlu-1*, a putative endo-1,4-beta-glucanase, was isolated by reverse transcriptase-polymerase chain reaction using primers based on consensus sequence. *TcGlu1,3*, a putative endo-1,3-beta-glucanase, was isolated by reverse transcriptase-polymerase chain reaction using primers based on the GenBank EST sequence accession #CA795103 from *T. cacao*. The primers used to amplify *TcGlu1,3* were forward 5′-GATTCTCCAAACAAT GTCTC-3′ and reverse 5′-CAGAGTCATTGATAACATGAC-3′. *TcCaf-1*, a putative caffeine synthase, was amplified by reverse transcriptase-polymerase chain reaction from total RNA isolated from YR leaves of cacao comun using primers based on consensus sequence between tea (*C. sinensis*) and coffee (*Coffea arabica* L.) caffeine synthases. The primers used to amplify *TcCaf-1* were forward 5′-ATTGAAGGAG TCTAATTTCTC-3′ and reverse 5′-TTGAACGGGAA-GATCTAC-3′. *TcrbcS*, the gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase, was isolated by reverse transcriptase-polymerase chain reaction using primers based on the GenBank EST sequence accession # CF973934 from *T. cacao*. The primers used to amplify *TcrbcS* were forward 5′-ATGGCTTCCTCAATGATCTC-3′ and reverse 5′-TTAATTTTCGTAGCCTGG-3′. Five of the cDNA clones were isolated based on differential expression in response to biotic or abiotic stresses. The methods used for differential display are described in Keates et al. [38]. The cDNA clones (putative function) and their source treatment combinations were as follows: *TcORFX-1* (*fw2.2*-like regulating cell number), Actigard (50% 1,2,3-benzothiadiazole-7-thiocarboxylic acid-*S*-methyl-ester, Syngenta Crop Protection, Greensboro, NC, USA 27419) induced; *TcWRKY-1* (WRKY family DNA binding protein), Messenger (3% Harpin, Eden Bioscience, Bothell, WA, USA 98011-8205) induced; *TcPer-1* (apoplastic quiacol peroxidase), 2,4-D (sodium salt, 1 g ai l^{-1}) induced; *TcLhca-1*(photosystem I 24 kDa protein), Nep1 (5 μg ml^{-1} plus 0.2% Silwet-L77) repressed; *TcCou-1*(4-coumarate-CoA ligase-like), Silwet-L77 (0.2%) treated.

The cDNA probes were purified using GENECLEAN *Turbo* for PCR Kit (Q-BIO gene, Irvine, CA, USA 92618-2005) following the 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 46250-0414), and gel filtered (Edge Gel Filtration Cartridges™, Edge BioSystems, Gaithersburg, MD, USA 20879-4149). Blots were prehybridized in ExpressHyb™ solution (BD Biosciences Clontech, Palo Alto, CA, USA 94303-4230) at 68 °C for 1 h.

In the studies of leaf development and Nep1 treatment effects, probes were hybridized to at least three blots with replicate samples. Probes were hybridized to two replicate blots in studies of gene expression in response to *P. megakarya* infection. 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, USA 08855-1327), and imaged at 200 μm resolution on a Typhoon 8600 Variable Mode Imager (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ, USA 08855-1327). Following imaging, the replicate blots were washed by placing in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% SDS and micro-waving on high for 10 min, scanned to verify that no probe remained, and probed a second time with radioactively-labeled 28S ribosomal cDNA probe. The figures include representative autoradiograms for probes where significant effects were observed.

4.10. Calculation of band volume

In order to estimate the relative levels of altered gene expression, 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, USA 08855-1327). To correct for variations

in loading volume on each replicate blot, the ratio of 28S ribosomal cDNA band volume for each leaf developmental stage or treatment combination (time/Nep1 rate) was determined relative to the unexpanded (UE) leaf (arbitrarily set to one) sample or 0-h (arbitrarily set to one) sample, respectively. The band volumes for blots probed with cDNAs of interest were corrected by multiplication by the appropriate 28S ribosomal cDNA ratio. The band volumes were averaged for each leaf stage or treatment combination using data from three or more replicate blots. The change in gene expression is expressed as the times increase/decrease compared to the associated control time point.

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