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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*

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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.

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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.

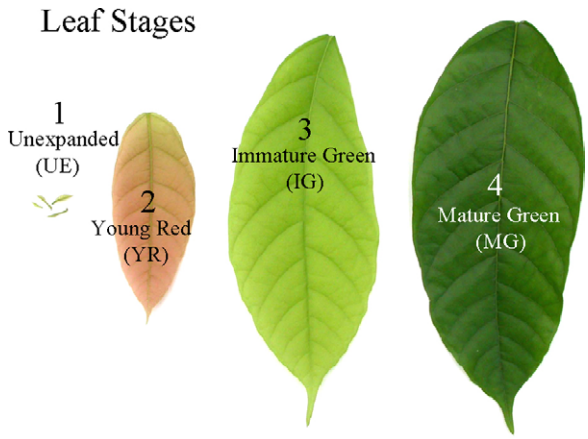

Fig. 1. Leaf development was separated into four stages starting with Stage 1) unexpanded leaves (UE), < 1 cm long with limited pigmentation; Stage 2) young red leaves (YR), 5–10 cm long, and pliable; Stage 3) immature green leaves (IG), 15–20 cm long, and pliable; and Stage 4) mature green leaves (MG)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