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GENETICS AND GENOMICS

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Isolation of ESTs from cacao (*Theobroma cacao* L.) leaves treated with inducers of the defense response

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Abstract Pathogenic diseases represent a major constraint to the growth and yield of cacao (*Theobroma cacao* L.). Ongoing research on model plant systems has revealed that defense responses are activated via signaling pathways mediated by endogenous signaling molecules such as salicylic acid, jasmonic acid and ethylene. Activation of plant defenses is associated with changes in the expression of large numbers of genes. To gain a better understanding of defense responses in cacao, we have employed suppressive subtractive hybridization (SSH) cDNA libraries, macroarray hybridization analysis, high throughput DNA sequencing and bioinformatics to identify cacao genes induced by these signaling molecules. Additionally, we investigated gene activation by a phytotoxic elicitor-like protein, Nep1. We have identified a unigene set of 1,256 members, including 330 members representing genes induced during the defense response.

Keywords *Theobroma cacao* · Defense · Salicylic acid · Methyl jasmonate · Ethylene · Nep1

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Introduction

Cacao (*Theobroma cacao* L.) is endemic to the Amazon basin and Central America (Wood and Lass 1985); however, it is presently grown in over 5 million hectares of tropical lowlands around the world. Pathogenic diseases are a major problem for cocoa production, causing annual crop losses of 30–40%. In its endemic region, cacao is susceptible to a number of coevolved pathogens, such as *Crinipellis perniciosa*, the causal agent of witches broom disease, and *Moniliophthora roreri*, the causal agent of frosty pod rot (Wood and Lass 1985). Outside its center of origin, cacao is susceptible to a variety of opportunistic pathogens from indigenous hosts.

Gaining a better understanding of how cacao plants respond to pathogens is central to the task of reducing the devastating affects that pathogenic diseases cause to cocoa production. Toward this end, a variety of genetic and genomic resources have recently been developed in cacao to assist in this process, including a high-density linkage map, and the identification of disease-associated quantitative trait loci (QTL) (Bennett 2003). Recently, a gene discovery and array analysis program was initiated in cacao (Jones et al. 2002). In that study, 4,455 expressed sequence tags (ESTs) from untreated leaves and seeds were sequenced and assembled into a unigene set consisting of 1,380 members. Analysis of this gene set identified 46 sequences similar to either defense genes or other genes with known defense-associated functions.

In the present study, we undertook a gene discovery program to identify genes expressed in cacao leaves treated with inducers of the defense response. We identified a unigene set of 1,256 members representing genes expressed in defense-elicited leaves, including 330 representing genes induced by these signaling molecules.

Materials and methods

Plant growth and elicitor treatment

Cacao pods (Forastero, Comum variety) were provided by A. Pomella from the Almirante Center for Cacao Studies (Bahia, Brazil). Seeds were germinated in potting soil and grown under greenhouse conditions (daylength, 11–13 h) at 24°C. Benzothiadiazole (BTH; Syngenta, http://www.syngenta.com) was dissolved in water (1,000 mg l^{-1}) and applied as a foliar spray. Control plants were sprayed with water. Nep1 (5 μg ml^{-1}) was combined with 0.2% (v/v) Silwet-L77 (Witco; http://www.cromptoncorp.com/) for foliar spray applications. Seedlings treated with Silwet-L77 (0.2%, v/v) were included as a control. Methyl jasmonate (0.12 m*M*) was mixed with distilled water and applied as a foliar spray. Controls were treated with water. Foliar sprays were applied to 4-month-old seedlings (3–4 true leaves) with a Binks model 15 sprayer (ITW Industries; http://www.binks.com) at approximately 1 kgf cm^{-2} (=9.81 N) and at a rate of 46 ml m^{-2}. After treatment with methyl jasmonate or distilled water (methyl jasmonate control), seedlings were placed in glass cylinders, and the cylinders were sealed with plastic wrap. Ethylene was injected into the cylinders containing methyl jasmonate-treated seedlings at a concentration of 12 μl l^{-1}. For each treatment, mature green leaves were collected at the time points indicated (BTH, 30 min and 2 h; Nep1, 30 min; methyl jasmonate/ethylene, 2 h), frozen in liquid nitrogen, and stored at −80°C.

cDNA library construction

The RNA isolation method was developed by adaptation of several established methods (Chang et al. 1993; McKenzie et al. 1997), and protocols provided with the Qiagen RNeasy Mini kit (Qiagen; http://www1.qiagen.com/). Leaf material was ground in liquid nitrogen, mixed with extraction buffer (Chang et al. 1993) and incubated at 65°C for 20 min. Samples were extracted with chloroform and centrifuged at 8,000 *g* for 30 min. RNA was precipitated overnight at 4°C with one-third volume of 8 *M* LiCl, centrifuged for 1 h at 8,000 *g* at 4°C, resuspended in 500 μl RLT Qiagen buffer, and purified with the Qiagen RNeasy mini-kit according to the manufacturer's instructions. The RNA was treated with DNase I (Roche; http://www.roche-applied-science.com) for 30 min at 37°C, extracted with phenol/chloroform (3:1) and precipitated with 3 *M* Na acetate, pH 5.5 and ethanol using standard procedures. Poly A+ RNA was purified using a Micro-FastTrack 2.0 kit (Invitrogen; http://www.invitrogen.com/) according to the manufacturer's instructions.

Elicitor-induced cDNAs were generated by suppression subtractive hybridization (SSH) (Diatchenko et al. 1996) using the PCR-select cDNA subtraction kit (BD Biosciences Clontech; http://www.bdbiosciences.com/clontech/) according to the manufacturer's instructions. Briefly, 2 μg poly A+ RNA from control and defense-elicited leaves were used for synthesis of the driver and tester cDNA pools, respectively; 2 μg skeletal muscle poly A+ RNA were used for synthesis of a control driver cDNA pool to assess subtraction efficiency (see below). Driver and tester cDNAs were digested with *Rsa*I, phenol/chloroform extracted, ethanol precipitated and resuspended in water. Tester cDNA was then split into two pools, each of which was ligated to a different adapter (supplied in the cDNA subtraction kit). An unsubtracted tester control was prepared as described by the manufacturer to serve both as a positive control for ligation and a negative control for subtraction. Subtractive hybridization and PCR amplification were carried out according to the manufacturer's protocol with the following changes. Primary PCR was performed for 30 cycles (94°C, 10 s; 63°C, 30 s; 71°C, 90 s). Secondary PCR was performed for 14 cycles (94°C, 10 s; 66°C, 30 s; 72°C, 90 s). PCR products from both the primary and secondary PCR reactions were analyzed according to the manufacturer's instructions to ensure the success of the subtraction. A control subtraction was performed using skeletal muscle cDNA as a driver and skeletal muscle cDNA spiked with *Hae*III-digested $\phi\chi$174 DNA as a tester (see kit for details). Subtracted cDNAs were cloned into the pCR4-TOPO vector and transformed into One Shot chemical competent *Escherichia coli* (Invitrogen), plated on LB, 1.5% Difco agar (Becton Dickinson; http://www.bd.com/clinical/products) supplemented with 75 mg l^{-1} ampicillin and grown overnight at 37°C.

Differential screening

Colonies were picked and transferred to 384-well microtiter plates containing LB/8% glycerol supplemented with 75 mg l^{-1} ampicillin using a QPix2 robot (Genetix; http://www.genetix.com/productpages/genomics.htm) and grown overnight at 37°C. Bacterial clones were arrayed in six replicates onto 22×22 cm^2 nylon membranes using a QPix2 robot. The membranes were placed on LB agar plates supplemented with 75 mg l^{-1} ampicillin and incubated at 37°C. The next day, colonies were lysed in situ and the resulting cDNAs were fixed to membranes according to the methods of Green et al. (2002).

Membranes were prehybridized in 10 ml ExpressHyb solution (Clontech) supplemented with 100 μg ml^{-1} salmon sperm DNA and 100 μl Differential Screening Blocking Solution (Clontech) for 3 h at 72°C. Forward and reverse subtracted cDNAs were generated as above for use as hybridization probes. Probes (100 ng) were labeled with α-^{32}P-dCTP using the Megaprime DNA labeling kit (Amersham; http://www.apbiotech.com/) and added directly to the prehybridization solution. Membranes were first hybridized with the reverse subtracted (control) probe. Hybridization was carried out at 72°C for 20–24 h. Membranes were washed twice at 68°C in 2× sodium chloride/sodium citrate (SSC), 0.5% sodium dodecyl sulfate (SDS) for 20 min, and twice at 68°C in 0.2× SSC, 0.5% SDS. Radiographic imaging was performed via storage phosphor imaging (Molecular Dynamics; http://www.mdyn.com/). Membranes were stripped by boiling in 0.5% SDS for 20 min, washed in 0.4 *N* NaOH at 42°C for 20 min, followed by two washes in 0.5 *M* Tris-HCl (pH 7.5) at 25°C. Hybridization with the forward subtracted probe and subsequent washes were carried out as above.

Identification of up-regulated genes

Upregulated genes were identified using the ArrayVision software package (Imaging Research; http://www.imagingresearch.com/). Each cDNA was represented six times on each membrane. Signal intensity for each spot was measured as the normalized density (nDens). Briefly, the density value for each spot (sDens) was determined to be the measured density minus the background in the area surrounding each spot. The average sDens for all spots was considered the reference density (sRef). The nDens is expressed as a multiple of the reference density value, (nDens = sDens/sRef). Differential density analysis was performed by dividing the experimental nDens by the control nDens for each spot [nDens (exp)/nDens (cont)]. Those clones that showed a ratio of 2.0 or greater in at least four of the six replicates were designated as up-regulated clones.

cDNA sequencing and analysis

Single-pass DNA sequencing was carried out with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems; http://www.appliedbiosystems.com/), using an ABI Prism 3700 DNA analyzer (Applied Biosystems). Vector trimming and contig assembly were performed using ContigExpress application in the VectorNTI software package (Informax; http://www.informaxinc.com/). Sequences were considered members of a contig if they were at least 85% identical in a 20 bp overlap. The resulting consensus sequence for each contig and the sequence for each EST not included in any contig were considered the unigene set. The predicted unigene sequences were used to query the predicted protein set of *Arabidopsis thaliana* ecotype Columbia, *Oryza sativa* cv.

japonica, and the Plant Gene Indices from the Institute for Genomic Research (TIGR, http://www.tigr.org/) using BLASTX (Altschul et al. 1990). Unigenes with no significant match in these databases were then used to query the non-redundant nucleotide database at GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN and the non-redundant protein database maintained at GenBank using BLASTX. Matches to sequences in the database were reported if $E \leq 10^{-6}$.

Sequences were assigned to functional classes using a combination of methodologies. Cacao sequences were used to identify the most closely related *Arabidopsis* sequences using the following resources: BLAST (Altschul et al. 1990), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg), and the gene families available at The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org). Functional classes were assigned to each cacao sequence based on that of its most closely related *Arabidopsis* sequence, according to the Munich Information Center for Protein Sequences *A. thaliana* database (MAtDB) [(http://mips.gsf.de/proj/thal/db/)].

Results and discussion

We have undertaken a gene discovery project aimed at identifying ESTs representing cacao genes expressed early in the defense response. In a recent cacao gene discovery program, Jones et al. (2002) identified 1,380 unigenes from a data set containing 4,455 ESTs (69% redundancy) from untreated leaves and beans. Slightly more that 3% of their unigene sequences were predicted to encode defense-associated genes. In order to maximize our efforts to identify defense genes in cacao, we generated subtracted, normalized cDNA libraries from leaves treated with the following defense signaling molecules: the salicylic acid analog BTH, methyl jasmonate and ethylene (MJE), and the elicitor-like protein, Nep1 (Bailey 1995). Subtraction and normalization was carried out to enrich the libraries for cDNAs representing low-abundance genes or genes expressed in response to the above treatments. Analysis of the libraries shows that the cDNA inserts ranged from 100 bp to 1.1 kb, with an average size of 486 bp (N=100; SD=175).

Identification of upregulated clones

cDNA clones representing genes upregulated by defense inducers were identified by differential screening. Each library was separately screened with cDNA probes from both control and treated leaves. For example, the BTH library was screened with cDNA probes from both control and BTH-treated leaves. Similarly, Nep1 and MJE libraries were screened with probes from control and Nep1- or MJE-treated leaves. In all, 8,600 cDNA clones (2,688 BTH, 3,224 MJE, 2,688 Nep1) were screened, and 475 upregulated clones were identified (139 BTH, 211 MJE, 125 Nep1). Overall, 5.5% of the cDNAs in our libraries were identified as upregulated, slightly lower than the percentages (7–16%) reported by other recent studies (Wang et al. 2002; Cramer and Lawrence 2004). One possible explanation for this lies in our experimental design. We specifically constructed our libraries from poly A+ RNA isolated from leaves collected 30 min to 2 h after each treatment in order to identify early defense-induced genes. As such, transcripts for some defense genes may not have had sufficient time to accumulate.

Contig assembly

All 475 upregulated cDNA clones and 1,639 randomly chosen cDNA clones across all libraries (996 BTH, 265 MJE, 378 Nep1) were isolated and sequenced. Contig assembly analysis was performed to determine the number of different sequences present. The 2,114 ESTs in our data set were assembled into 1,256 unigenes (41% redundancy). A full listing of all unigenes, the ESTs comprising each, and the corresponding GenBank accession numbers can be viewed at the following website: http://guiltinanlab.cas.psu.edu/Research/Cocoa/genomics.htm.

A total of 330 upregulated genes are represented in the unigene set. Among these, 68 were unique to the BTH library, 102 to the Nep1 library, and 154 to the MJE library. One sequence (21 kDa seed protein/trypsin inhibitor) was found in all three libraries. Three were found in both BTH and Nep1 libraries (60S ribosomal protein, carbonic anhydrase, and a sequence with no significant match). Two were found in both the BTH and MJE libraries (heat shock protein, and a sequence with no significant match).

Sequence annotation

Putative functions were assigned to the predicted protein for each sequence in the unigene set. Overall, 865 of the unigenes (68.9%) matched annotated plant genes (including hypothetical or unknown proteins). The remaining 391 unigenes (31.1%) did not match other sequences in the database. Table 1 shows BLAST scores and annotations for a subset of the unigenes representing genes whose expression was upregulated following treatment with inducers of defense responses (see below). A complete listing of the BLAST scores and annotations can be viewed at the following web site: http://guiltinanlab.cas.psu.edu/Research/Cocoa/genomics.htm.

Functional classification

The 865 unigene sequences with matches to previously identified plant genes were assigned to functional classes according to MIPS (http://mips.gsf.de/proj/thal/db/) (Fig. 1a). The following functional classes were used: growth (cell growth, cell division and DNA synthesis), defense (cell rescue, defense, cell death and ageing), signaling (cellular communication/signal transduction), cellular organization, transport (cellular transport and transport mechanisms, and transport facilitation), energy, metabolism, transcription, protein synthesis, and protein destination. Twenty-nine percent of the 865 annotated

Table 1 Annotations and BLAST scores for cacao leaf unigene sequences representing genes whose expression is up-regulated by inducers of the defense response. Sequences are grouped by functional class. Accession numbers beginning with *At* correspond to sequences from *Arabidopsis* and can be accessed at TAIR (http://www.arabidopsis.org). TIGR accession numbers are preceded by a three-letter code that indicates the plant gene index from which the sequence was retrieved. *ath* Arabidopsis, *gar* cotton, *gma* soybean, *han* sunflower, *les* tomato, *lja* lotus, *lsa* lettuce, *mcr* ice plant, *mtr* Medicago, *osa* rice, *stu* potato, *tae* wheat, *zma* maize. The TIGR plant gene indicies can be accessed at http://www.tigr.org/tdb/tgi/plant.shtml. Elicitors used in libraries: *BTH* Benzothiadiazole, *MJE* methyl jasmonate and ethylene, *Nep1* elicitor-like protein Nep1

Annotation	BLAST score	Accession number	Library
Cell growth, cell division and DNA synthesis			
Pasticcino 1 protein	5.00×10^{-29}	gma_TC137684	BTH
Alpha-tubulin	2.00×10^{-35}	gar_TC8581	Nep1
Cell rescue, defense, cell death and ageing			
Chitinase-like protein	1.00×10^{-65}	mcr_TC3160	BTH
Heat-shock protein 80	1.00×10^{-84}	gma_TC131723	BTH/MJE
Beta-cyanoalanine synthase	2.00×10^{-31}	gma_TC134055	MJE
Class I chitinase	2.00×10^{-77}	gar_TC11178	MJE
clpB heat shock protein-like	1.00×10^{-94}	les_TC99670	MJE
DnaJ protein homolog	1.00×10^{-91}	gar_TC9182	MJE
dnaK-type molecular chaperone	1.00×10^{-41}	gma_TC134160	MJE
Heat shock protein 70 cognate	10^{-131}	gar_TC9297	MJE
Heat-shock protein 90	6.00×10^{-33}	gar_TC9481	MJE
Manganese superoxide dismutase 1	2.00×10^{-87}	gar_TC9056	MJE
Regulatory protein NPR1	1.00×10^{-16}	At3g57130	MJE
RSH-like protein	2.00×10^{-64}	les_TC104278	MJE
SRG1 protein-related protein	4.00×10^{-52}	mtr_TC54568	MJE
UDP-glucose glucosyltransferase-like protein	4.00×10^{-53}	gma_TC128457	MJE
Blue copper protein	6.00×10^{-16}	gma_TC124801	Nep1
Class IV chitinase	4.00×10^{-72}	ath_TC155743	Nep1
Cu/Zn-superoxide dismutase	2.00×10^{-16}	ath_TC172573	Nep1
Monodehydroascorbate reductase	3.00×10^{-27}	gar_TC8977	Nep1
Zeaxanthin epoxidase	9.00×10^{-09}	lsa_TC3266	Nep1
Cellular communication/signal transduction			
Gamma response I protein	1.00×10^{-55}	At3g52120	BTH
Protein kinase PK4	8.00×10^{-25}	At1g29230	BTH
Putative AtBgamma protein	4.00×10^{-73}	gma_TC124086	BTH
ATMPK9	1.00×10^{-32}	At1g53510	MJE
Calreticulin	3.00×10^{-34}	stu_TC49193	MJE
Casein kinase-like protein	1.00×10^{-60}	ath_TC183419	MJE
Ethylene response sensor	3.00×10^{-61}	gar_TC11967	MJE
G protein-coupled receptor	3.00×10^{-49}	At1g52920	MJE
GTP-binding protein	1.00×10^{-58}	At5g11480	MJE
Protein phosphatase type 2C	5.00×10^{-35}	At1g07430	MJE
Protein tyrosine phosphatase	2.00×10^{-54}	gar_TC11616	MJE
Putative protein kinase	1.00×10^{-26}	gma_TC122758	MJE
Rac GTPase activating protein 2	4.00×10^{-19}	lja_TC2660	MJE
Somatic embryogenesis receptor-like kinase	4.00×10^{-57}	At1g34210	MJE
Mitogen activated protein kinase kinase	5.00×10^{-49}	mtr_TC53957	Nep1
Probable protein phosphatase 2C	4.00×10^{-55}	mtr_TC45036	Nep1
Protein phosphatase type 2C	8.00×10^{-74}	ath_TC162732	Nep1
Receptor-like kinase	2.00×10^{-42}	At1g34210	Nep1
Sphingosine kinase	8.00×10^{-93}	ath_TC162229	Nep1
Cellular organization			
Ankyrin-like protein	1.00×10^{-36}	At1g04430	BTH
Caffeoyl-CoA O-methyltransferase	4.00×10^{-87}	gma_TC132746	BTH
ETAG-A3	2.00×10^{-18}	gar_TC9458	BTH
Extensin-like protein	5.00×10^{-23}	At1g49750	BTH
Extracellular dermal glycoprotein (EDGP)	3.00×10^{-15}	At1g03230	BTH
Gluthatione reductase	3.00×10^{-31}	At3g54660	BTH
Annexin	1.00×10^{-80}	gar_TC8800	MJE
Cellular transport and transport mechansims			
ADP-ribosylation	4.00×10^{-85}	osa_TC75953	MJE
Sugar transporter protein	10^{-102}	mtr_TC43602	MJE
Synaptobrevin 7B	7.00×10^{-91}	At1g04750	MJE
Vacuolar processing enzyme	3.00×10^{-82}	gar_TC9509	Nep1
Energy			
Chlorophyll a/b-binding protein	4.00×10^{-57}	stu_TC53388	BTH
Chlorophyll a/b-binding protein	1.00×10^{-81}	gma_TC132158	BTH
Chlorophyll a/b-binding protein	7.00×10^{-54}	gma_TC132158	BTH
Ferredoxin I	8.00×10^{-50}	lja_TC2218	BTH
Fructose bisphosphate aldolase	2.00×10^{-12}	At2g21330	BTH
NADH dehydrogenase	4.00×10^{-61}	gar_TC10105	BTH
NADH-plastoquinone oxidoreductase subunit J	1.00×10^{-28}	lja_NP459509	BTH
Oxygen-evolving enhancer protein 3-2	1.00×10^{-53}	gma_TC120160	BTH
Photosystem I chain II precursor	8.00×10^{-30}	mcr_TC3589	BTH
Oxygen-evolving enhancer protein 2	1.00×10^{-09}	mcr_TC3567	MJE
Photosystem II 10 kDa polypeptide	9.00×10^{-28}	gma_TC132143	MJE
Photosystem II protein X precursor	8.00×10^{-20}	ath_TC149445	MJE

Table 1 (continued)

Annotation	BLAST score	Accession number	Library
Thioredoxin-m	3.00×10^{-29}	At1g03675	MJE
Fructose bisphosphate aldolase	8.00×10^{-35}	At2g21330	Nep1
H+-transporting ATP synthase-like protein	5.00×10^{-19}	gar_TC9440	Nep1
NADH dehydrogenase	0	ath_TC193046	Nep1
NADH-ubiquinone oxidoreductase	1.00×10^{-39}	gma_TC133555	Nep1
Photosystem I protein psaH precursor	2.00×10^{-16}	les_TC99254	Nep1
Photosystem I reaction center subunit VI	9.00×10^{-11}	gma_TC119102	Nep1
Probable fructose bisphosphate aldolase	1.00×10^{-39}	gar_TC8653	Nep1
Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	1.00×10^{-40}	gar_NP385866	Nep1
Ribulose-1,5-bisphosphate carboxylase/ oxygenase activase 2	1.00×10^{-74}	gar_NP385866	Nep1
Metabolism			
Acetyl-CoA carboxylase	9.00×10^{-77}	At1g36050	BTH
Acyl-CoA oxidase	3.00×10^{-36}	gma_TC135657	BTH
Asparagine synthetase	5.00×10^{-45}	han_TC2557	BTH
Glutamyl-tRNA reductase 1	6.00×10^{-58}	les_TC98501	BTH
Lil3 protein	2.00×10^{-69}	gar_TC12144	BTH
Methionine synthase	10^{-103}	gar_TC8601	BTH
Methylenetetrahydrofolate dehydrogenase	5.00×10^{-20}	gar_TC11680	BTH
Carbonic anhydrase isoform	2.00×10^{-86}	gar_ TC11928	BTH/Nep1
12-oxophytodienoate reductase OPR2	2.00×10^{-79}	gma_TC132909	MJE
4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein	3.00×10^{-47}	At3g14990	MJE
ACS12	1.00×10^{-62}	stu_TC48077	MJE
Alpha-amylase	2.00×10^{-15}	At1g69830	MJE
Alpha-glucosidase I	2.00×10^{-22}	mtr_TC46782	MJE
Ammonium transporter	8.00×10^{-52}	stu_TC47246	MJE
Anthranilate N-benzoyltransferase-like	6.00×10^{-53}	ath_TC161680	MJE
ATP citrate lyase	6.00×10^{-14}	gar_TC17391	MJE
Carotenoid 9 10–9′ 10′ cleavage dioxygenase	1.00×10^{-95}	gma_TC120527	MJE
Cytochrome P450	4.00×10^{-07}	ath_NP034755	MJE
Cytochrome P450	4.00×10^{-94}	ath_TC174971	MJE
Galactinol synthase	7.00×10^{-46}	At1g60470	MJE
GcpE protein	2.00×10^{-92}	gma_TC120513	MJE
Glucosidase II alpha subunit	3.00×10^{-13}	ath_TC162432	MJE
Glycine dehydrogenase	1.00×10^{-73}	mtr_TC51393	MJE
Sulfolipid synthase	8.00×10^{-43}	ath_TC152680	MJE
Neutral invertase	9.00×10^{-77}	gma_TC121654	MJE
O-linked GlcNAc transferase	1.00×10^{-60}	les_TC98302	MJE
Omega-6 fatty acid desaturase	1.00×10^{-10}	han_TC526	MJE
Porphobilinogen deaminase (Hydroxymethylbilane synthase)	5.00×10^{-19}	stu_TC53966	MJE
P-Protein precursor	1.00×10^{-62}	stu_TC53677	MJE
Probable thiamin biosynthesis protein	2.00×10^{-43}	han_TC1559	MJE
Putative acyl-CoA synthetase	1.00×10^{-40}	stu_TC53563	MJE
Pyruvate dehydrogenase	10^{-111}	gar_TC9199	MJE
Shikimate kinase	7.00×10^{-52}	les_TC101392	MJE
Shikimate kinase	1.00×10^{-54}	les_TC101392	MJE
1-Aminocyclopropane-1-carboxylate deaminases	9.00×10^{-71}	At1g48420	Nep1
Magnesium chelatase subunit	10^{-115}	gma_TC120638	Nep1
Methylmalonate semi-aldehyde dehydrogenase	2.00×10^{-94}	At2g14170	Nep1
nifU-like protein	5.00×10^{-67}	At4g22220	Nep1
Nitrate reductase	3.00×10^{-22}	mcr_TC4108	Nep1
Phenylalanine-ammonia lyase	1.00×10^{-97}	gar_TC10686	Nep1
Phytoene synthase	5.00×10^{-94}	gar_TC13167	Nep1
Putative ribonuclease E	6.00×10^{-28}	At2g04270	Nep1
S-Adenosylmethionine decarboxylase	2.00×10^{-14}	lja_TC1942	Nep1
Protein destination			
ATP-dependent Clp protease	4.00×10^{-12}	mcr_TC3521	BTH
F-box containing tubby family protein	5.00×10^{-51}	At1g25280	BTH
N-Acetylglucosaminyl-phosphatidylinositol biosynthetic protein GPI1	2.00×10^{-13}	At3g57170	BTH
Polyubiquitin	2.00×10^{-81}	At4g02890	BTH
21 kDa seed protein precursor	8.00×10^{-61}	gar_TC11652	BTH/MJE/Nep1
Chloroplast FtsH protease2	10^{-109}	At1g50250	MJE
Chloroplast membrane protein (ALBINO3)	5.00×10^{-94}	At1g24490	MJE
Chloroplast membrane protein (ALBINO3)	6.00×10^{-18}	gma_TC121213	MJE

Table 1 (continued)

Annotation	BLAST score	Accession number	Library
O-Sialoglycoprotein endopeptidase	4.00×10^{-35}	At5g26110	MJE
Probable AAA-type ATPase	2.00×10^{-65}	stu_TC54884	MJE
Probable legumain	3.00×10^{-54}	gma_TC119971	MJE
Prolyl aminopeptidase-like protein	7.00×10^{-87}	ath_TC152522	MJE
Putative chaperonin	2.00×10^{-08}	les_TC100635	MJE
Ubiquitin-specific protease 16	2.00×10^{-19}	ath_TC173656	MJE
Xaa-Pro aminopeptidase 2	10^{-104}	mtr_TC44326	MJE
FK506-binding protein (peptidylprolyl isomerase)	1.00×10^{-19}	gma_TC134394	Nep1
Intracellular protease, PfpI family	3.00×10^{-65}	At3g02720	Nep1
Probable cullin protein	2.00×10^{-35}	mtr_TC52234	Nep1
Ubiquitin extension protein	1.00×10^{-39}	les_TC98489	Nep1
Protein synthesis			
30S ribosomal protein S1	10^{-108}	les_TC101014	BTH
40S ribosomal protein S2	3.00×10^{-49}	gar_TC8811	BTH
60S ribosomal protein L35	4.00×10^{-54}	gar_TC9254	BTH
Ribosomal protein-like	2.00×10^{-15}	ath_TC191904	BTH
RuBisCO subunit binding-protein beta subunit	2.00×10^{-49}	gma_TC132417	BTH
RuBisCO subunit binding-protein beta subunit	9.00×10^{-47}	gma_TC132417	BTH
60S ribosomal protein L7	2.00×10^{-69}	gar_TC9241	BTH/Nep1
30S RIBOSOMAL PROTEIN S20	4.00×10^{-34}	At3g56010	MJE
60S RIBOSOMAL PROTEIN-like	6.00×10^{-51}	gar_TC9511	MJE
RRM-containing protein	6.00×10^{-55}	At4g00830	MJE
30S ribosomal protein S10	6.00×10^{-35}	At3g13120	Nep1
40S ribosomal protein S18	3.00×10^{-17}	zma_TC130204	Nep1
Eukaryotic translation initiation factor 5 (eIF-5)	2.00×10^{-54}	mcr_TC4001	Nep1
Ribosomal protein 30S subunit	5.00×10^{-43}	At5g24490	Nep1
Ribosomal protein L1 protein	3.00×10^{-33}	gma_TC120499	Nep1
Ribosomal protein L36a.e	4.00×10^{-55}	lsa_TC3307	Nep1
Transcription			
High mobility group protein HMG-beta1	3.00×10^{-55}	gar_TC9595	BTH
Histone deacetylase	10^{-168}	gma_TC136421	BTH
Probable splicing factor	2.00×10^{-41}	ath_TC154562	BTH
RNA splicing protein	4.00×10^{-48}	hvu_TC31616	BTH
Alanyl-tRNA synthetase	2.00×10^{-26}	gma_TC137409	MJE
ARID DNA binding domain	4.00×10^{-15}	At1g76510	MJE
bZIP transcriptional activator	1.00×10^{-55}	gma_TC135846	MJE
DNA-binding protein WRKY3	1.00×10^{-17}	mtr_TC53525	MJE
DNA-directed RNA polymerase II	0	mtr_TC52887	MJE
DRH1 DEAD box protein-like	9.00×10^{-85}	ath_TC167972	MJE
EIN3-like protein	10^{-100}	lja_TC324	MJE
Histone H2B like protein	1.00×10^{-13}	ath_TC186206	MJE
PHAP2B protein	3.00×10^{-51}	gma_TC134709	MJE
Probable RNA helicase	5.00×10^{-31}	gar_TC9990	MJE
WD1521	5.00×10^{-37}	gar_TC10596	MJE
Homeodomain GLABRA2 like 1 protein	4.00×10^{-20}	gar_TC13367	Nep1
Transcriptional regulator	2.00×10^{-12}	At4g00050	Nep1
Transport facilitation			
Porin-like protein	3.00×10^{-23}	gar_TC10966	BTH
Aquaporin	6.00×10^{-45}	At2g45960	MJE
ATP carrier protein	5.00×10^{-22}	tae_TC56538	MJE
MgATP-energized glutathione S-conjugate pump	2.00×10^{-28}	gma_TC121244	MJE
Peptide transporter	5.00×10^{-33}	At1g18880	MJE
Putative ABC transporter protein	1.00×10^{-68}	At1g17840	MJE
Probable glutamate receptor	7.00×10^{-61}	ath_TC169248	Nep1
Transporter protein	3.00×10^{-30}	stu_TC43204	Nep1
Triose phosphate/phosphate translocator	2.00×10^{-10}	gma_TC120047	Nep1

sequences had no known function, as they matched neither hypothetical nor unknown sequences (Fig. 1a). The largest class of sequences with known functions was predicted to play roles in metabolism (17%). Eleven percent are predicted to function in energy. The majority of the remaining sequences are distributed among transcription (9%), defense (8%), protein synthesis (6%), protein destination (7%), and signaling (5%). Less than 5% of the sequences were assigned to cellular organization, growth or other functional classes such as ionic homeostasis or transposable elements. The functional distribution of unigene sequences within each library was similar for most classes to the data as a whole; however, there were some specific differences.

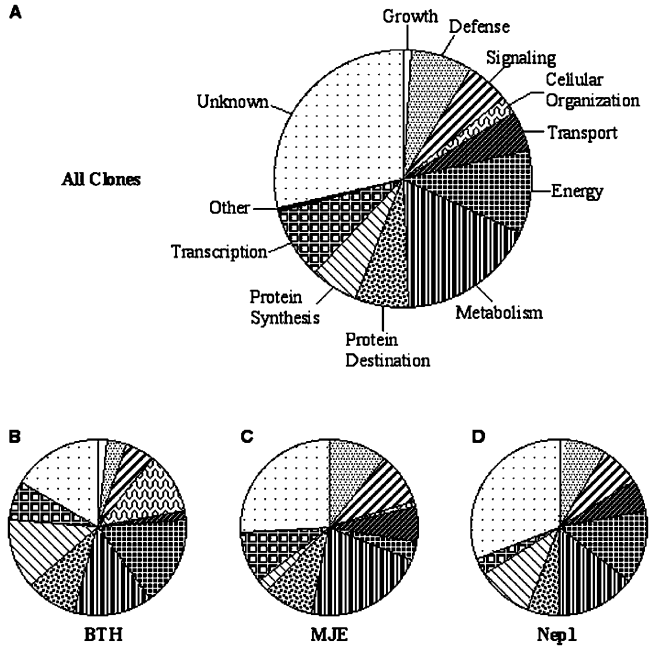

Fig. 1 Distribution of putative functional classes of **a** all annotated unigene sequences from cacao leaves treated with inducers of the defense response and the annotated unigene sequences representing candidates for genes upregulated by **b** the salicylic acid analog, benzothiadiazole (BTH), **c** methyl jasmonate/ethylene (MJE), and **d** the elicitor-like protein Nep1

BTH library

BTH is a salicylic acid analog that has been shown to trigger systemic acquired resistance (SAR) and pathogenesis-related (*PR*) gene expression via the salicylic acid signaling pathway (Gorlach et al. 1999). Seventy-four genes upregulated by BTH are represented in the unigene set. The functional distribution of BTH-induced genes shows, relative to the data as a whole, that there was an increase in the percentage of genes involved in cellular organization and protein synthesis, and a decrease in the percentage of genes involved in defense and transport (Fig. 1b).

MJE library

Ethylene and jasmonates have been shown to play a role in the regulation of wound responses and in defense against some pathogens (Ton et al. 2002). A recent microarray study in Arabidopsis showed that approximately 50% of genes induced by ethylene were also induced by jasmonate (Schenk et al. 2000). In order to maximize our efforts in identifying ethylene-regulated and jasmonate-regulated genes, cacao leaves were simultaneously treated with both inducers.

A total of 157 genes upregulated by MJE are represented in the unigene set. The functional distribution of MJE-induced genes shows that there was a slight increase in the percentage of genes involved in defense and signaling. In addition, there was a large decrease in the percentage of genes with a function in energy (Fig. 1c).

Nep1 library

Nep1 is a fungus-derived protein that induces a number of processes similar to those induced by defense elicitors, such as an increase in extracellular pH, K^+ efflux, reactive oxygen species (ROS) production, ethylene production, hypersensitive cell death and changes in gene expression (Keates et al. 2003); 106 genes upregulated by Nep1 are represented in the unigene set. Overall, the functional distribution of Nep1-induced clones was more similar to the data set as a whole than to either the BTH- or MJE-induced clones. However, there was a large decrease in the percentage of clones that function in transcription (Fig. 1d).

The main objective of this study was to identify genes expressed early in cacao defense responses. Overall, 8% of the sequences in our data set are similar to genes with bona fide defense functions. Among BTH-induced sequences, the percentage is lower (4%). There are two possible explanations for this observation. First, many of the sequences in our libraries were less than 500 bp. As such, strong homology to genes with known defense functions could not be definitively assigned. For example, some of our induced sequences share weak similarity to cytochrome P450-like sequences, protein kinase-like sequences or genes encoding WRKY-like transcription factors. Some of these sequences are likely to function in defense; however, in the absence of strong homology to specific sequences with characterized defense functions, the cacao sequences were conservatively assigned to more general functional classes (metabolism, signaling or transcription), potentially under-representing the percentage of defense-related genes in our libraries. Second, because we specifically constructed our libraries from poly A+ RNA collected 30 min to 2 h after treatment in order to identify early defense-induced genes, there may not have been sufficient time for transcripts for some defense genes to accumulate. For example, transcripts for some Arabidopsis *PR* genes do not accumulate to significant amounts until 4–8 h after treatment (Lorenzo et al. 2003). As such, the identification of some defense genes may require the construction of additional libraries with extended time periods following application of defense signaling molecules.

The assignment of genes to a given functional class does not preclude a defense-related function for that gene. For example, sequences assigned to the metabolism or transcription classes may play roles in the biosynthesis of defense-related secondary metabolites or in the transcriptional activation of other defense-related genes. As illustrated below, genes induced by the defense signaling molecules from a variety of functional classes have potentially important defense-related functions.

Defense and defense-related genes expressed in cacao leaves

Eight percent of the genes upregulated by defense inducers were similar to known defense genes in other plants. Of these, one-third were predicted to encode heat shock proteins and were isolated from the MJE library. Heat shock proteins are a large class of proteins that function as molecular chaperones by directing the refolding and activation of proteins that have become unfolded or insoluble due to environmental stresses (Queitsch et al. 2002).

A sequence similar to *NPR1* was also induced. NPR1 is a transcriptional regulator that mediates the transcription of salicylic acid- and jasmonate-responsive genes, including many *PR* genes (Pieterse et al. 1998). Several *PR* genes were represented, such as *SRG1*, a gene of unknown function that is a member of the PR-10 family (Truesdell and Dickman 1997), and chitinases, members of the PR-3 and PR-4 families (Neuhaus et al. 1999). Chitinases have been shown to enhance resistance against fungal pathogens, presumably by degrading fungal cell walls (Neuhaus et al. 1999).

There were also several induced genes predicted to encode proteins involved in antioxidant defense, such as two superoxide dismutases, dehydroascorbate reductase, zeaxanthin epoxidase, and blue copper proteins. Antioxidant defense proteins function by reducing various ROS produced in response to pathogens or other stresses, thereby preventing the potentially overwhelming effects that ROS could pose to the plant itself (Bolwell et al. 2002).

Metabolism

With the exception of genes of unknown function, genes with predicted roles in metabolism composed the largest class induced by the defense signaling molecules. For example, P-Protein is a subunit of glycine decarboxylase and is the major pathogen-induced nitric oxide (NO) synthesizing enzyme (Chandok et al. 2003). NO is a signaling molecule that plays a role in the activation of the hypersensitive response (HR), induction of defense gene expression and accumulation of antimicrobial phytoalexins (Delledonne et al. 2001).

In addition, we identified several genes involved in the biosynthesis of defense-related secondary metabolites (phenylpropanoids, carotenoids, isoprenoids, and phytoalexins). Collectively, these metabolites function in a variety of defense-related processes, including the induction of wound response, antimicrobial and antifungal defense, and antioxidant defense (Dixon 2001).

Signaling

Eight percent of the upregulated genes are predicted to play roles in signaling. These sequences encode protein kinases, protein phosphatases, B-gamma proteins, G-proteins and calcium-binding proteins. The signaling pathways mediated by these proteins could regulate a variety of defense responses, such as the formation of ROS, the induction of HR, production of a phytoalexins, and the expression of defense-related genes (Zhang and Klessig 2001).

Cellular organization

Several genes upregulated by defense signaling molecules are predicted to function in cellular organization, particularly in modification of the cell wall. For example, genes encoding extensin (hydroxyproline-rich glycoprotein) and caffeoyl-CoA O-methyltransferase (lignin biosynthesis) were induced. Lignification and cross-linking of extensins (and other cell-wall proteins) in response to pathogens are proposed to form a physical barrier to further pathogen attack (Showalter 1993).

Transcription

The defense signaling molecules also induced several genes encoding a variety of transcription factors, including an NPR1-like protein, WRKY and bZIP proteins, and EIN3. In addition, there were also several genes predicted to encode other transcriptional regulators, such RNA helicases, splicing factors, histones or other DNA binding proteins. Collectively, these proteins could serve to regulate the transcription of a variety of defense-related genes (Singh et al. 2002).

Protein synthesis and protein destination

Approximately 14% of the induced genes are predicted to function in protein synthesis or protein destination. Almost all the genes with predicted roles in protein synthesis encoded ribosomal proteins or other proteins (translation initiation factor) that likely function in translation. Among proteins predicted to function in protein destination, approximately one-half encoded proteases. Protease activities have been implicated in cell death during HR, but the specific proteases involved have not been identified (Beers et al. 2000). In addition to proteases, we identified a protease inhibitor—a 21 kDa seed protein (Kunitz trypsin inhibitor)—which was induced by all the elicitor treatments in our study. This trypsin inhibitor was first isolated from cacao seeds and is thought to function in defense against herbivorous insects (Dodo et al. 1992).

We also identified several induced genes that are predicted to function in the ubiquitin/26S proteasome pathway, including ubiquitin, cullin, F-box proteins and an AAA-type ATPases. The ubiquitin/26S proteasome pathway regulates ubiquitin-mediated breakdown of many proteins and plays a key role in the regulation of a variety of processes including regulation of defense-related genes (Devoto et al. 2003).

Energy

In addition to genes with defense-related functions, defense signaling molecules induced several genes encoding proteins predicted to function in photosynthesis, such as ferrodoxin, chlorophyll a/b binding proteins, rubisco and oxygen-evolving enhancer proteins. This is consistent with a previous report suggesting that there may be some cross-talk between defense pathways and the phytochrome A/red light mediated signaling pathway (Schenk et al. 2000). In addition to their energy roles, some of these proteins may have defense functions. For example, Yang et al. (2003) have recently shown that oxygen-evolving enhancer protein (OEE) is phosphorylated by a protein complex containing Wall-associated kinase 1, a PR protein required for survival of plants during the pathogen response (He et al. 1998). Activation of OEE by Wak1 is thought to modulate formation of ROS, which could function in defense signaling, the induction of defense-related genes, and the regulation of the HR (Yang et al. 2003).

Conclusions

In this study, we have identified 1,256 unigene sequences representing genes expressed in defense-elicited cacao leaves. Comparison of our unigene set to that of Jones et al. (2002) shows that only 10% of the sequences overlap (data not shown), giving rise to a combined unigene set of approximately 2,500 sequences. Annotation of the sequences shows that many had no significant match in the public databases. These sequences are a potentially valuable resource for identifying novel defense genes. In addition, candidate defense genes can be used to search for colocalization with QTLs associated with resistance to cacao diseases.

Little overlap was observed between the upregulated clones from each of the libraries suggesting that each defense-signaling molecule may regulate a unique set of genes. However, the number of clones sampled may have been insufficient to effectively address this issue. To this end, a synthetic oligonucleotide microarray representing sequences from the combined international data set has been constructed, and will be used to explore the regulatory interactions between defense signaling pathways. These studies will undoubtedly shed light on defense signaling pathways in cacao, and offer great potential for improvement of cacao disease resistance.

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