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Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*

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Abstract *Theobroma cacao* L. plants over-expressing a cacao class I chitinase gene (*TcChi1*) under the control of a modified CaMV-35S promoter were obtained by *Agrobacterium*-mediated transformation of somatic embryo cotyledons. Southern blot analysis confirmed insertion of the transgene in eight independent lines. High levels of *TcChi1* transgene expression in the transgenic lines were confirmed by northern blot analysis. Chitinase activity levels were measured using an in vitro fluorometric assay. The transgene was expressed at varying levels in the different transgenic lines with up to a sixfold increase of endochitinase activity compared to non-transgenic and transgenic control plants. The in vivo antifungal activity of the transgene against the foliar pathogen *Colletotrichum gloeosporioides* was evaluated using a cacao leaf disk bioassay. The assay demonstrated that the *TcChi1* transgenic cacao leaves significantly inhibited the growth of the fungus and the development of leaf necrosis compared to controls when leaves were wound inoculated with 5,000 spores. These results demonstrate for the first time the utility of the cacao transformation system as a tool for gene functional analysis and the potential utility of the cacao chitinase gene for increasing fungal pathogen resistance in cacao.

Keywords Fungal pathogen resistance · *Theobroma cacao* · Chitinase · Transgenic · *Colletotrichum gloeosporioides*

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Introduction

Theobroma cacao L. (cacao) is a small understory tree endemic to the lowland rainforests of the Amazon basin (Wood and Lass 1987). Cacao was domesticated in pre-Columbian times by the Olmec and/or Mayan civilizations. Its seeds (cocoa beans) were used to produce beverages for the royalty and in religious ceremonies (Coe and Coe 1996; Motamayor et al. 2002; Emch 2003), and also as currency. Today, cacao is grown throughout the world in the humid tropics, often in agroforestry ecosystems with other fruit and commodity crops. Cacao is the main raw ingredient for the world chocolate industry, which is estimated to be approximately $58 billion per year. Additionally, cacao-growing regions are largely centered in important biodiversity hotspots, impacting 13 of the world's most biologically diverse regions (Piasentin and Klare-Repnik 2004).

For a number of reasons, long-term efforts toward breeding for disease resistance in cacao has yielded only limited success. First, the long generation time and large size of cacao plants make it difficult to conduct the necessary long-term and multi-located field trials. Additionally, because many of the cacao pathogens are opportunistic, sources of resistance have not co-evolved for some of the most devastating of the major cacao diseases. More recently, researchers have begun to apply the tools of molecular genetics to develop genomic resources and molecular markers to help speed up cacao breeding programs (Bennett 2003). As a tool to study the functional genomics of cacao candidate resistance genes and as a possible means to integrate resistance genes into breeding programs, we have recently developed a genetic transformation system for cacao (Maximova et al. 2003). The system employs *Agrobacterium*-mediated transformation of cotyledon pieces from somatic embryos and a visible marker gene encoding the green fluorescent protein (GFP) for rapid screening of primary regenerants. As a first test of this system, we chose to

examine the function of a cacao chitinase gene, by over-expressing it in transgenic cacao plants and testing the effect on fungal pathogen resistance.

Chitinases are members of the pathogenesis-related protein family (PR-proteins), some of which have been shown to play a role in plant defense by degrading the chitin of fungal cell walls (Collinge et al. 1993; Neuhaus 1999). In particular, the class I chitinases, which accumulate to high levels in vacuoles in response to wounding and pathogen infection, have been reported to be important in these regards. Using transgenic approaches, chitinase genes from plants and microorganisms have been introduced into different plant species in order to enhance resistance against a broad range of fungal pathogens (Kramer and Muthukrishnan 1997). Tobacco has been transformed with chitinases from various other plants and microorganisms with varied results (Linthorst et al. 1990; Jach et al. 1995; Vierheilig et al. 1995; Kellmann et al. 1996; Carstens et al. 2003; Patil and Widholm 1997). Broglie et al. (1991) reported enhanced resistance to *Rhizoctonia solani* in tobacco plants expressing a bean chitinase. Other crops transformed with plant chitinases that resulted in enhanced fungal pathogen resistance include tomato (Tabaeizadeh 1997), carrot, cucumber, pickling cucumber (Punja and Raharjo 1996; Raharjo et al. 1996), rose (Marchant et al. 1998), rice (Liu et al. 2004), and grape (Yamamoto et al. 2000). Additionally, tobacco and potato (Lorito et al. 1998), broccoli (Mora and Earl 2001), and apple (Bolar et al. 2001) were transformed with an endochitinase derived from the parasitic fungus *Trichoderma harzianum* and these plants exhibited wide-spectrum resistance to the foliar pathogens *Alternaria alternata, A. brassicae, A. solani*, *Botrytis cinerea, R. solani*, and *Venturia inaequalis*.

Previously we have reported the regeneration of multiple transgenic lines carrying a class I endochitinase gene isolated from cacao (Maximova et al. 2003). The cacao *TcChi1* gene (NCBI accession U30324) was isolated by PCR based on tobacco chitinase sequences and shown to be expressed in cacao fruit in response to fungal elicitor treatment (Snyder-Leiby and Furtek 1995). Here, we report on the evaluation of these plants and demonstrate the enhanced fungal pathogen resistance to *Colletotrichum gloeosporioides* conferred by the chitinase gene. For the purpose of this study we have developed an in vivo bioassay using detached young leaves of *T. cacao* infected with *C. gloeosporioides* spores. The symptoms observed in laboratory conditions were similar to those observed on infected cacao plants in field conditions, where *C. gloeosporioides* attacks primarily young and soft cacao leaves causing brown lesions surrounded by a characteristic clear yellow halo (Mohanan et al. 1989).

Our results indicated that the cacao *TcChi1* gene product is active against this fungal pathogen, and that it is possible to increase the resistance of cacao against pathogens by over-expression of this gene. Furthermore, our results demonstrate that the transgenic cacao system can be used as a tool to study the functions of cacao genes and perhaps, in the future, for crop improvement.

Materials and methods

Genetic transformation

Plant transformations were performed as previously described (Maximova et al. 2002, 2003) via *Agrobacterium tumefaciens* co-cultivation with cacao somatic embryo explants. All transformations were performed with the cacao genotype PSU-Scavina 6 (S6). S6 plants were established in a greenhouse at Penn State in 1993 from clonal material introduced from Mayaguez, PR, USA. DNA fingerprinting has shown that this clone is not identical to the bona fide Scavina 6 clone at the International Cacao Germplasm collection in Trinidad, so we have appended the designation PSU to distinguish it from authentic Scavina 6 (M. Guiltinan, unpublished). For brevity, we also refer to PSU-Scavina 6 as S6 in this text. This clone was used as source for explants during our prior research on somatic embryogenesis and transformation protocol development (Maximova et al. 2002, 2003). The genotype demonstrated the highest somatic embryogenesis and genetic transformation potential compared to a number of other genotypes. In brief, staminodes dissected from immature cacao flowers of S6 were cultured to produce somatic embryos by methods previously described (Li et al. 1998; Maximova et al. 2002). Cotyledons from primary somatic embryos were co-cultivated with *A. tumefaciens* (AGL1) containing binary plasmids (described below) and further cultured on 50 mg/l geneticin selection for 2 weeks under conditions conducive for secondary embryogenesis (Maximova et al. 2002). Transgenic secondary somatic embryos were selected based on green fluorescence resulting from the expression of the enhanced green fluorescence protein (*EGFP)* marker gene (Clontech, Palo Alto, CA, USA).

Transformations were performed with pGAM00.0511 (Maximova et al. 2003) (Fig. 1), which contains the cacao *TcChi1* chitinase gene (Snyder 1994; Snyder-Leiby and Furtek 1995), *EGFP*, and the neomycin phosphotransferase II (*NPTII)* (De-Block et al. 1984) marker genes, all under the control of high-level constitutive E12-Ω CaMV-35S promoter (Mitsuhara et al. 1996). One control transgenic line was regenerated after transformation with pGH00.0126 containing the *EGFP* and *NPTII* marker genes only (Maximova et al. 2003). Additionally non-transformed S6 plants were regenerated by somatic embryogenesis (Maximova et al. 2002) and used as non-transformed controls.

Clonal propagation of transgenic and control plant lines

The individual lines described here originate from independent transformation events. Multiple individuals of each line were obtained either through repetitive somatic embryogenesis (Maximova et al. 2002) or through rooted cuttings (Maximova et al. 2005). All control and

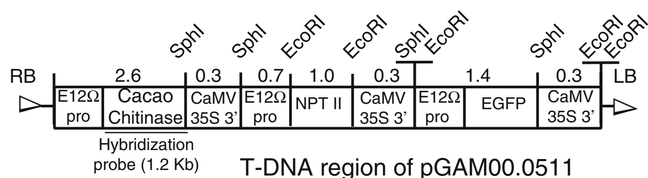

Fig. 1 Illustration of the T-DNA region of pGAM00.0511 binary plasmid containing the *TcChi1* transgene (Maximova et al. 2003). Components of this vector include: E12-Ω 35S promoter (Mitsuhara et al. 1996), the cacao genomic DNA sequence encoding a basic chitinase class I (TcChi1) (Snyder 1994; Snyder-Leiby and Furtek 1995), the CaMV-35S terminator sequence, the NPTII kanamycin selectable marker gene (De-Block et al. 1984), and the EGFP gene encoding green fluorescent protein (Clontech). The modified CaMV-35S derivative, E12-Ω promoter, drives all transgenes. Only restriction enzyme recognition sites important for the Southern Blot analysis (Fig. 1) are shown at the *top* of the map and are abbreviated as follows: *E, Eco*RI; *Sh, Sph*I. The 1.2 kb restriction fragment containing the *TcChi1* coding region of the cacao endochitinase (*underlined*) was used as probe for the Southern and northern analyses. *RB* and *LB* indicate the right and left borders of the Ti plasmid T-DNA region, respectively. Number of base pairs between restriction sites is indicated *above* the map in kb. The drawing is not to scale to allow labeling

transgenic plantlets were acclimated to greenhouse conditions and multiplied by rooted cuttings as previously described (Maximova et al. 2005). Briefly, single leaf, semi-hardwood cuttings, with stems approximately 4 cm long, and with leaves pruned to one-third of the initial length were treated with rooting solution [1:1 mixture of α-naphthalene acetic acid (NAA) and α-indole-3-butyric acid (IBA)]. After hormone treatment, the cuttings were inserted in wet sand and placed under intermittent mist (10 s every 10 or 15 min) for 4–6 weeks at a light intensity of approximately 100 μmol/m^2/s PAR (85% shade). During the misting period the cuttings were fertilized every 3–4 days with Hoagland's nutrient solution (160 ppm N). Rooted cutting with two or more roots and a growing axillary bud were transplanted to pots with soil. After transplanting, the plants were removed from the mist and maintained under 50% shade and a relative humidity of 60–65% at approximately 28°C. Water and fertilizer were applied five or more times daily as needed by drip irrigation. Plants were grown in the greenhouse for 7–9 months before analysis. To collect tissue samples for the different assays, 2–6 trees per individual line were used.

Fluorescent imaging of GFP

Fluorescent GFP images of transgenic leaves were obtained as previously described (Maximova et al. 2003). Images were captured using a Nikon SMZ-4 stereodissecting microscope equipped with an epi-fluorescence attachment, a 100 W mercury light source, and a 3-CCD video camera system (Optronics Engineering, Goleta, CA, USA). The fluorescence imaging filters used were a 520–560 nm emission filter and a 450–490 nm excitation filter. Fluorescence intensity of each image was measured as the mean integrated pixel value (mIPV) using NIH Image 1.6 image processing and analysis software. Mean IPV (ranging from 0 to 255 each) was calculated by dividing the sum of the intensity values of all pixels in a given image by the total number of pixels in that image. To ensure equal imaging conditions of all measurements, all images were acquired at 30× magnification with a 30 s exposure time with no changes in lighting setup. Using a set of representative samples ranging from the lowest to the brightest fluorescence, exposure time was determined to result in a minimum of 99% of all pixels below the saturation point (255) in the brightest image. Three individual leaves from three trees per line were measured for a total of nine measurements per line. The means of replicate mIPV ± SE measurements were calculated and significant differences were established by Fisher Protected LSD test at the $P < 0.05$ level of significance.

Southern genomic blot analysis

Genomic DNA was isolated from mature cacao leaves as previously described using a modified CTAB extraction protocol for latex-containing plants (Michiels et al. 2003) and additionally purified by CsCl gradient ultracentrifugation (Ausubel et al. 2001). Per tissue sample, 5 μg of genomic DNA was incubated overnight with 60 units of *Eco*RI or 50 units of *Sph*I (Promega, Madison, WI, USA). DNA was separated on 1% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham Bioscience, Piscataway, NJ, USA) in 10× sodium chloride/sodium citrate (SSC). Following transfer, DNA was fixed to the membranes via UV crosslinking (120 mJ). Membranes were prehybridized in ExpressHyb solution (Clontech) supplemented with 100 μg salmon sperm DNA for 3 h at 60°C. The blots were hybridized with 1.2 kb *TcChi1* gene probe (Fig. 1) generated by PCR from pGAM00.0511 as described by Maximova et al. (2003). Using Megaprime DNA labeling kit (Amersham Bioscience), 50 ng of the probe was labeled with α-32P-dCTP, denatured by boiling, quenched on ice, and added directly to the prehybridization mix. Hybridizations were carried out at 60°C for 20–24 h. Membranes were washed twice at 55°C in 2× SSC, 0.5% sodium dodecyl sulfate (SDS) for 20 min, and twice at 55°C in 1× SSC, 0.5% SDS for 20 min. Radiographic imaging was performed via storage phosphorimaging (Molecular Dynamics, Sunnyvale, CA, USA).

Northern blot analysis

Total RNA was extracted from mature cacao leaves using a modified protocol for highly viscous samples rich in polyphenols and polysaccharides (Zeng and Yang 2002). Additionally, RNA was purified following the Qiagen RNeasy clean up protocol (QIAGEN, Valencia,

CA, USA). Twenty micrograms of total RNA was denatured for 30 min at 55°C with 1:1 (v/v) sample and glyoxal/DMSO loading dye (Amersham Bioscience). RNA samples were separated on 1.5% agarose gels, transferred to nylon membranes (Hybond-N +, Amersham Bioscience) in 10× SSC, and fixed to the membranes via UV crosslinking (120 mJ). The probe preparation for the northern blot was identical to that for the Southern blots described above. Probe labeling, hybridization, washing, and radiographic imaging were also performed as described above. Sizes of hybridizing fragments were determined by comparison with mobility of known RNA markers (RNA ladder, Ambion, Inc., Austin, TX, USA). Hybridizing band intensity was quantified by phosphorimaging (Molecular Dynamics, Amersham Bioscience) using ImageQuant software (Molecular Dynamics, Amersham Bioscience). All measurements were within the linear range of the phosphorimager.

In vitro endochitinase activity assay

Total protein extracts from two leaves from two different trees per line (total of four extracts/line) were extracted as follows. Frozen leaves were placed in liquid nitrogen with polyvinyl pyrrolidone (PVP) (0.07 gm of PVP per gm of leaf tissue) and ground to a fine powder. The powder was then added to ice-cold extraction buffer [50 mM sodium acetate, pH 5.0, 2 mM EDTA, 3 mM sodium metabisulfite, 5 mM dithiothreitol (DTT), and 250 mM sodium chloride, 7 ml per gm of leaf tissue] in sterile 30 ml Oakridge tubes, then swirled briefly to suspend the powder. Samples were centrifuged at 10,000*g* at 4°C for 15 min and the supernatants transferred to fresh tubes. Total protein content was quantified using Bio-Rad standard protein microassay (Bio-Rad Laboratories, Richmond CA, USA). Endochitinase activity was quantified using a fluorometric assay (Mora and Earl 2001). For each individual sample, the assay contained 20 μg of total protein in a final volume of 100 μl. To the extracts, 50 μl of substrate solution [0.2mM methylumbelliferyl B-D-*N*,*N*′,*N*″-triacetylchitotrioside (Sigma M-5369) in 100 mM sodium acetate, pH 5.0] was added and reactions were incubated on an orbital shaker (200 rpm), at 37°C for 40 min after which 50 μl of STOP buffer (0.2 M sodium carbonate) was added. Each sample was assayed in triplicate. Background fluorescence of each sample was determined by assays identically prepared except that the STOP buffer was added prior to the 37°C incubation. Standard curves were determined using a series of dilutions of 1 μM 4-methylumbelliferone (4-MU) (Sigma M-1508) in 0.2 M sodium carbonate (STOP buffer) prepared by the manufacturer's instructions. All treatment and control samples, including the standard curve dilution set, were incubated together in a black, 96-well microtiter plate (#3650, Corning, Inc., Corning, NY, USA). Fluorescence generated by reaction byproduct (4-MU) was recorded at 360/485 ηm ex/em with a fluorescence plate reader (FluoroCount Packard BioScience, Meriden, CT, USA) using the FluoroCount software package. To normalize the fluorescence intensity value of each sample, the respective background fluorescence values were subtracted. Based on the standard curves, endochitinase activity was calculated as nmols of 4-MU generated per min per μg of total protein. Means of all technical and biological replicates were calculated and variation was established by paired comparisons among all possible pairs using Fisher Protected LSD test at the $P < 0.05$ level of significance.

In vivo evaluation of antifungal activity of transgenic cacao plants against *C. gloeosporioides*

Colletotrichum gloeosporioides was isolated from infected roots of a 2-month-old S6 cacao plant. The identity of the pathogen was determined by culture morphology on PDA media and sequence homology of the ITS region to reference sequences of isolates of *C. gloeosporioides* available from NCBI database (J.-P. Marelli, data not shown). *C. gloeosporioides* single spore cultures were grown on 2% water agar for 2–4 weeks at 30°C under a 12:12 h day:night cycle. When acervuli were formed on the entire surface of the plate, 3 ml of sterile water was poured over the culture and the conidia were harvested by scraping the surface of the culture with a glass rod. The resulting conidia suspension was filtered through sterile miracloth (Calbiochem, La Jolla, CA, USA) and diluted to 500,000 conidia/ml with sterile distilled water.

For each bioassay, two large fully expanded light green and soft cacao leaves were collected from control and transgenic lines established in the greenhouse from rooted cuttings (corresponding to stage C leaves as defined in the Guiltinan lab, http://guiltinanlab.cas.psu.edu/Research/Cocoa/leaves/stages.jpg). All leaves collected were 7–10 days old and appeared to be at the same developmental stage. Leaf discs were cut to fit into 100×15 mm^2 Petri dishes (VWR, West Chester, USA) containing two layers of sterile filter paper soaked in 2.5 ml sterile distilled water. Ten areas (1.5 mm^2) were sampled on each leaf (five on each side of the main vein). Immediately before inoculation, the selected areas were wounded with eight insect pin needles bunched and taped together. Single drops of 10 μl of inoculum (5,000 conidia) were placed on eight of the ten wounded areas and the last two areas were left as water-only controls without infection. Wounding and inoculation steps were performed under a magnifying lens to improve visualization. Plates were incubated at 30°C in a 12:12 h light:dark cycle under fluorescent light for 4 days. Images of the control and infected areas after 4 days of incubation were captured using Nikon SMZ-4 dissecting microscope and a 3-CCD video camera system (Optronics Engineering). The area of necrosis was measured using Scion Image for Windows (version beta 4.0.2,

Scion Corporation). The average area was calculated and the data were statistically analyzed using the SAS software (version 9.1, SAS Institute, Inc., Cary, NC, USA). The mean areas of necrosis per genotype were separated using a *t* test LSD.

Results

The objective of this study was to evaluate the potential function of the *TcChi1* gene product in pathogen defense and specifically to determine if it plays a role as an antifungal defense protein. This gene was chosen for the study because it was previously described to be expressed in fruit pericarp in response to wounding and fungal cell wall elicitors (Snyder 1994). Hence it is likely to be an important pathogenesis response protein in cacao, although a direct functional analysis was not demonstrated. We studied the function of *TcChi1* gene by constitutively over-expressing it at levels much higher than the endogenous gene, using a very highly active, modified viral promoter. Furthermore, we reasoned that in the future, acceptance of transgenic cacao by the public could be facilitated if cacao genes were reintroduced into cacao, rather than using genes isolated from a different species.

Generation of transgenic plant lines

Co-cultivation was carried out with cacao somatic embryo cotyledons and *A. tumefaciens* strain AGL1 containing binary vectors with the *EGFP* and *NPTII* genes with and without the cacao *TcChi1* gene (Fig. 1). Transgenic somatic embryos from nine independent transformation events were cultured to maturity, then converted to plants, and acclimated to greenhouse conditions. Rooted cuttings of the T0 plants were used to produce plants (clonal lines) for replicated experiments. Consistent with our previous report (Maximova et al. 2003), no visible phenotypic differences were observed between the *TcChi1* transgenic plants and control transgenic plants (without the *TcChi1* gene) or non-transgenic S6 control plants.

GFP fluorescence intensity variation in transgenic cacao

As an early screen to identify high-level *EGFP*-expressing plants, the intensity of green fluorescence in mature leaves from different transgenic lines was measured and compared to that of control leaves (Fig. 2). All plants evaluated were propagated via somatic embryogenesis and grown for 9–10 months after acclimation to greenhouse conditions. Consistent with our previous observations, the fluorescence intensity of individual leaf samples varied significantly among the transgenic lines (Maximova et al. 2003). The intensities, expressed as mean integrated pixel values, ranged from 25.1 ± SE 2.4 mIPV for line 61.8 which was only slightly above the background (14.8 ± SE 0.045) seen in non-transgenic plants, to 215 ± SE 15.8 mIPV for line 47.1. One *EGFP*-expressing line containing the *EGFP* and *NPTII* genes only (Fig. 2, GFP lacking the *TcChi1* gene) was selected as a control and it showed *EGFP* expression at levels similar to the highest *TcChi1*-containing line.

Transgene integration and expression

To investigate T-DNA integration and copy number in the GFP-expressing plants, genomic DNA from one control non-transformed S6 and all transgenic lines were evaluated by Southern blot hybridization (Fig. 3a, b). As a control, plasmid pGAM00.0511 DNA containing the *TcChi1* gene was diluted to equimolar equivalents and included in the analysis (Fig. 3a, b). DNA was digested with *Eco*RI (Fig. 3a) and *Sph*I (Fig. 3b), blotted, then hybridized to the 1.2 kb cacao *TcChi1* probe (Fig. 1). In all plant genomic DNA samples, the endogenous *TcChi1* gene was detected as a large fragment above 12.2 kb (Fig. 3a, b). Only this single band was detected in the control S6 (Fig. 3a) and GPF lines (Fig. 3a, b), consistent with the previous report by Snyder (1994), concluding that the *TcChi1* gene was single copy. All but one of the transgenic plant lines (56.2) transformed with the *TcChi1* gene vector exhibited additional hybridization bands indicating the presence of the transgene (Fig. 3a, b). Line 44.1 digested with *Eco*RI showed no hybridizing bands (Fig. 3a), but the digest with *Sph*I resulted in a 3 kb hybridizing band

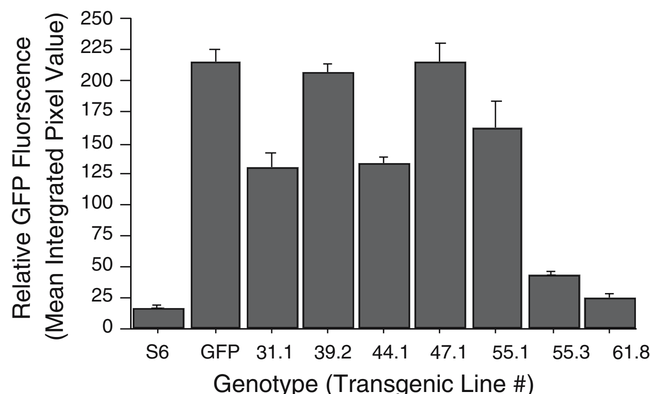

Fig. 2 Evaluation of *EGFP* fluorescence of control and transgenic cacao leaves. Three young fully expanded light green leaves from each of three trees (total of nine leaves per line) were observed under a fluorescence stereomicroscope equipped with a digital CCD camera. The fluorescence intensities of the digital images were measured and expressed as the mean integrated pixel values (mIPV). Mean IPVs ± SE of control and transgenic leaves were calculated. The genotypes measured were: PSU-Scavina 6 (S6), control untransformed plants of identical genotype used for all in transformations, *EGFP*, control plants transformed with the *EGFP* and *NPTII* genes only (pGH00.0126, Maximova et al. 2003), and a series of transgenic plant lines, indicated with various numbers, which contained the *EGFP, NPTII*, and the *TcChi1* genes

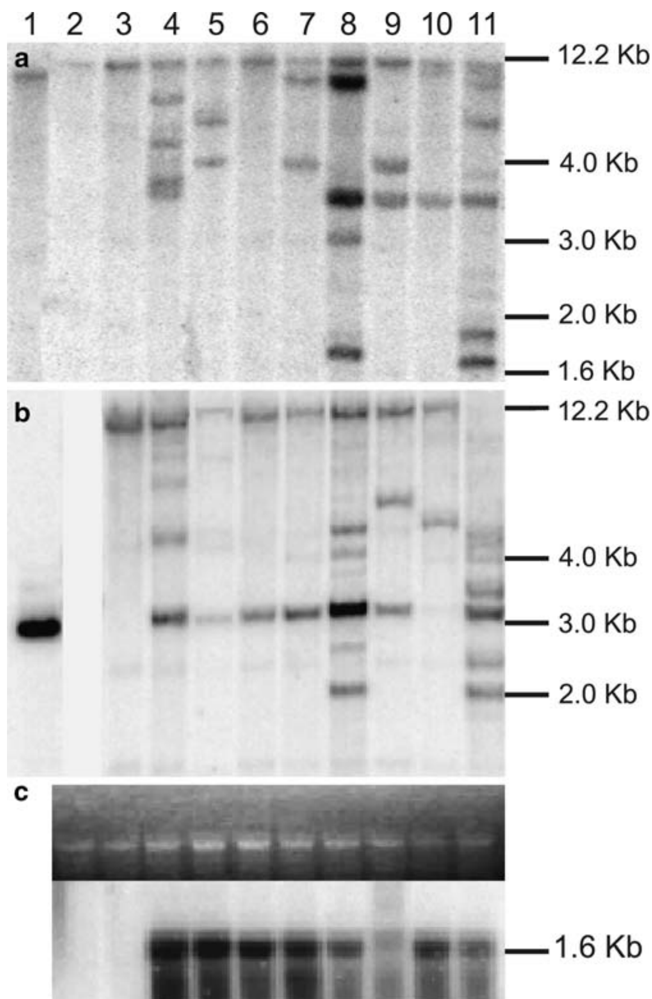

Fig. 3 Southern (**a** and **b**) and northern (**c**) analysis of DNA and RNA isolated from leaf tissue of control and transgenic cacao plants. Nucleic acids were isolated from leaves of plants as follows: *lane 2* non-transgenic PSU-Scavina 6 (S6), *lane 3* transgenic plants transformed with pGH00.0126 control vector without chitinase (GFP) (Maximova et al. 2003), *lanes 4–11* eight transgenic lines transformed with pGAM00.0511 (Maximova et al. 2003; Fig. 1) (lines 31.1, 39.2, 44.1, 47.1, 55.1, 55.3, 56.2, and 61.7 in *lanes 4–11*,respectively). For the Southern analysis, DNA from pGAM00.0511 (*lane 1*) and genomic DNA from transgenic and non-transgenic cacao leaves were digested with *Eco*RI and *Sph*I in different reactions (**a** and **b**, respectively). The blots were hybridized to the *TcChi1* genomic restriction fragment (see Fig. 1). In **b**, the S6 control was not included. Sizes and positions of molecular weight markers are indicated on the *right side* of the gels in kb. **c** Northern blot analysis. Total RNA (20 μg) isolated from all plant samples was separated by electrophoresis and blotted onto nylon membranes, followed by hybridization with the same *TcChi1* probe as the Southern blots (see Fig. 1). The gel was stained with ethidium bromide prior to transfer (*top panel*) and autoradiogram was captured by phosphorimager analysis after hybridization (*bottom panel*). Size of *TcChi1* transcript is indicated (1.6 kb)

(Fig. 3b). From the map of *TcChi1* (Fig. 1), *Eco*RI was expected to cut in the middle of the T-DNA, 2.9 kb away from the right border, and *Sph*I was predicted to cut 1.9 kb away from the right border. The analysis of the *Sph*I blot resulted in the discovery of additional *Sph*I site on pGAM00.0511 DNA outside of the T-DNA borders that generated a 3 kb hybridizing fragment. This fragment was detected in seven of the eight chitinase transgenic lines analyzed, suggesting transfer and insertion of a portion of the plasmid backbone DNA into the plant genome. Similar results have been reported by a number of authors who also observed transfer of DNA outside of the T-DNA borders (Kononov et al. 1997; Matzke and Matzke 1998). Together, these results indicate that each of the transgenic lines contain the *TcChi1* transgene, although in most cases it appears that the integration sites may include multiple and, possibly, longer than expected insertions.

In order to assess the expression of the *TcChi1* gene in the transgenic plants, northern blot analysis was performed with total RNA isolated from leaves (Fig. 3c). No *TcChi1* transcript was detected in the control S6 and GFP plants, suggesting that the *TcChi1* gene is not expressed in cacao leaves at levels detectable by this method. However, high levels of the transgene mRNA were observed in transgenic *TcChi1* plants, with the exception of line 55.3.

Increased levels of chitinase activity in *TcChi1* transgenic plants

In order to evaluate if *TcChi1* over-expression contributed to an increase of chitinase activity in the leaves of the transgenic plants, an in vitro endochitinase activity assay was used (Fig. 4). Light green, opaque, fully expanded (longer than 4.5 in.) leaves were harvested from all plants and total protein was extracted. S6 and GFP lines were used as controls. The chitinase activity levels of the two controls were detectable, but were very low and not significantly different from each other, indicating the endogenous chitinase activity in cacao leaves. Protein extracts isolated from the *TcChi1* transgenic leaves exhibited a three to seven fold increase in total chitinase activity compared to the controls. All of the *TcChi1*-containing plants showed statistically significant increases in chitinase activity compared to control plants ($P < 0.001$) (Fig. 4).

In vivo evaluation of antifungal activity of transgenic cacao plants against *C. gloeosporioides*

To assess the in vivo functionality of the *TcChi1* protein in the transgenic lines, we developed an excised leaf challenge assay against the pathogenic fungus *C. gloeosporioides*. This species has been reported as a pathogen of economic importance for cacao in India and Venezuela (Wood and Lass 1987). The strain of *C. gloeosporioides* used in this study was isolated from infected cacao plants at The Pennsylvania State University, University Park, PA, USA. When young cacao leaves were wound inoculated with this strain, they exhibited pathogenicity, severe lesions and necrosis in 4 days

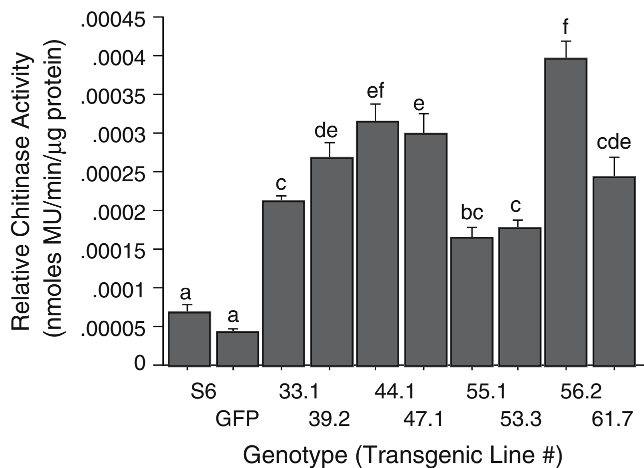

Fig. 4 Endochitinase protein activity assay of control and transgenic cacao plants. Total protein was extracted from control non-transgenic PSU-Scavina 6 (S6), control pGH00.0126 (GFP) (Maximova et al. 2003) and eight transgenic pGAM00.0511 (*TcChi1* chitinase) lines (Maximova et al. 2003). Four leaves from each line were collected (two from each of two clonal replicate plants). Total protein was extracted from each sample, and these were assayed in triplicate (total of 12 measurements per line). The total relative chitinase activity was analyzed by fluorometric assay using methylumbelliferyl as a substrate. The fluorescence of the 4-methylumbelliferone (MU) reaction product was recorded at 360/485 ηm ex/em. Means of all 12 replicate measurements from each line ± SE are presented. Mean separation was performed with Fishers Protected LSD test at $P < 0.05$ level of significance. Different letters (*a, b, c, d, e, f*) indicated significant difference

(Fig. 5d, e). When leaves from S6 and GFP control plants were inoculated, we observed rapid formation of lesions (Fig. 5d, e) compared to mock inoculated leaves (Fig. 5a, b). On the contrary, plants over-expressing the *TcChi1* gene showed reduced necrosis after inoculation (Fig. 5f) compared to the inoculated control plants (Fig. 5d, e). Lesion formation on the transgenic plants was similar to mock inoculated control and transgenic plants. To measure lesion sizes, digital images were taken and image analysis was performed. The mean lesion sizes of multiple replicates are presented in Fig. 6. The analysis established that the transgenic plants reproducibly showed enhanced resistance, as measured by the significantly smaller lesion formation compared to either of the control plant lines (Fig. 6). The reduction of the lesion size correlated with the increase of in vitro protein activity (Fig. 6). Two of the lines with the least chitinase activity showed the least reductions in lesion sizes, while two lines with much higher levels of chitinase activity showed the smallest lesion sizes. Taken together, our results indicate that the high expression of the *TcChi1* gene in the transgenic plants contributes to enhancing resistance to infection in our in vitro assay.

Discussion

Fungal diseases constitute a major challenge to the millions of cacao farmers throughout the tropical regions where *T. cacao* is grown. Despite efforts at breeding for resistance, a large proportion of the potential cacao crop is lost yearly to several major pathogens. Understanding mechanisms by which cacao responds to pathogen infection could lead to the development of molecular markers of key defense genes, which could be applied to accelerate breeding programs. Analysis of transgenic plants provides a powerful tool for functional studies of defense genes in cacao. We investigated transgenic expression of a known plant defense response gene encoding a class I chitinase and have demonstrated the effectiveness of its encoded protein in inhibiting the growth of a cacao leaf pathogen. While similar studies have been reported for other plant species, this is the first report of such an analysis in *T. cacao* and, to our knowledge, for any tropical tree species.

Our results demonstrate that the transgene was integrated into the cacao genome in varying copy numbers and expressed to varying degrees, in each of the independent lines studied. Measurement of *EGFP* expression levels and protein activity in the transgenic plants revealed that there was little correlation between the *EGFP* fluorescence and the expression of the linked chitinase transgene. However, as expected, we observed a good correlation between chitinase activity and fungal pathogen resistance. When evaluated for fungal pathogen resistance, the two lines with lower protein activity (55.1 and 55.3) also developed significantly larger lesions than the two lines with higher protein activity (56.6 and 61.7). This supports our hypothesis that the *TcChi1* gene product acts as an antifungal defense protein in cacao. Similarly, the two control lines (S6 and GFP) that contained undetectable levels of *TcChi1* mRNA in leaves also displayed lower chitinase activity than any of the chitinase expressing transgenic lines. Consistent with the hypothesis that the *TcChi1* gene product functions in defense, the two control lines developed the largest lesion sizes of all lines tested. These results are consistent with an earlier report where *TcChi1* expression was detected in the fruit pericarp in response to wounding and ethylene, which indirectly implicated a role for this gene in defense (Snyder 1994). From our data we can conclude that the *TcChi1* transgene product contributes significantly to defense against *C. gloeosporioides* in laboratory conditions.

Colletotrichum spp. are a hemibiotrophic fungi (Mendgen and Hahn 2002) that cause anthracnose in a variety of plant species including *Solanaceae* plants (tomato, pepper) and many tropical cultivated crops such as mango, papaya, avocado, coffee, and coconut. After penetrating the host, the fungus initially grows intracellularly and establishes as a biotroph for one or a few days (O'Connell et al. 2000). In a later phase, secondary narrower hyphae are formed that kill the host cells and proliferate necrotrophically. Cacao anthracnose, caused by *C. gloeosporioides*, is one of the major cacao problems in India and has been reported to cause significant damage to trees in Brazil (Wood and Lass 1987) and in Venezuela, where the variety 'Porcelana' is very suscep-

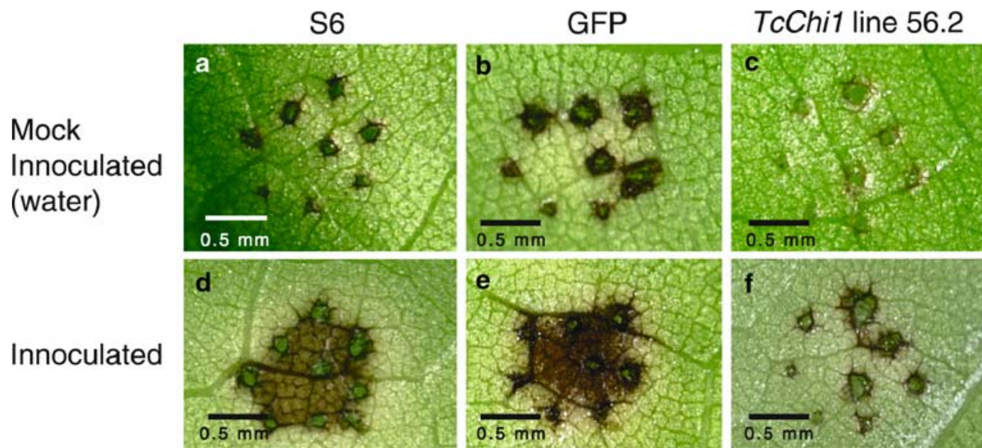

Fig. 5 Representative images of in vivo leaf infection assay of control and transgenic lines. Two young, fully expanded cacao leaves from each line were collected and wounded at ten different areas per leaf (five on each side of the leaf main vein, total of 20 areas per line). Sixteen areas per line were inoculated with *Colletotrichum gloeosporioides* (5,000 conidia per inoculated area). The necrosis of the control and infected areas was evaluated 4 days after inoculation. *Top row* non-inoculated plants, *bottom row* inoculated plants. Images include non-inoculated: control non-transgenic Scavina 6 (S6) (**a**), control GFP (**b**), chitinase 56.2 line (**c**), and *C. gloeosporioides* inoculated: control non-transgenic S6 (**d**), control transgenic GFP (**e**), and transgenic chitinase 56.2 line (**f**)

tible to this disease (Cedeño and Carrero 2003). *C. gloeosporioides* attacks primarily young and soft cacao leaves causing brown lesions surrounded by a characteristic clear yellow halo (Mohanan et al. 1989). With the development of the disease, the lesions expand and gradually coalesce to form large blighted areas that can lead to defoliation. The disease also affects mature and young fruits. The symptoms on mature fruit appear as dark sunken lesions that expand and agglomerate but rarely damage the beans. Infection of the cherelles (young fruits) causes wilt and death, but the mummified fruits remain attached to the truck (Mohanan et al. 1989).

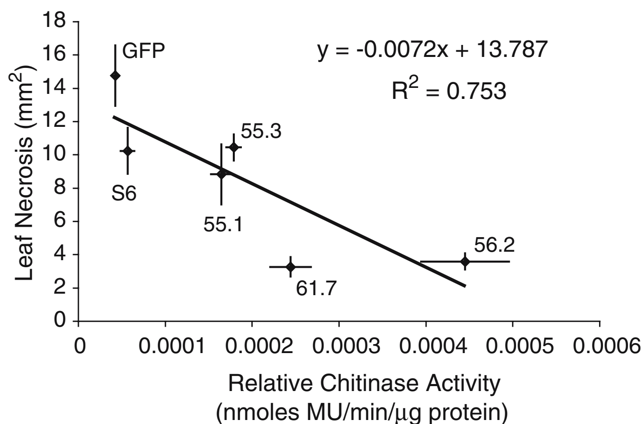

Fig. 6 Correlation of *Colletotrichum* resistance with *TcChi1* chitinase activity. Young, fully expanded cacao leaves from control non-transgenic PSU-Scavina 6 (S6), control transgenic GFP, and four transgenic pGAM00.0511 (chitinase) lines were wounded prior to inoculation with *Colletotrichum gloeosporioides* as described in Fig. 5. Disease necrosis was evaluated 4 days after inoculation. The area of necrosis was measured using Scion Image for Windows (version beta 4.0.2, Scion Corporation). The average areas of necrosis were calculated. Correlations were established between the average necrotic areas and total chitinase activities as presented in Fig. 4

Interestingly, *Colletotrichum* sp. M1 has also been described as the most frequently encountered endophyte of *T. cacao* mature and old leaf samples collected from five lowland sites with mixed forest cover across Panama (Arnold et al. 2003). The same authors also reported that endophyte-free *T. cacao* seedlings inoculated with mixture of endophytes representing three common endophyte genera of *T. cacao* including *Colletotrichum*, *Xylaria*, and *Fusarium*/*Nectria* demonstrated increased resistance to black pod disease (*Phytophthora* sp.). Hence the possibility that *Colletotrichum* spp. in *T. cacao* could behave as beneficial endophytes as well as fungal pathogens is not surprising given that different species, or even different genotypes of the same species, could have alternatively pathogenic or symbiotic/mutualistic effects. Furthermore, it is important to acknowledge that because of their typical behavior as a hemibiotrophic fungi and also due to their very broad host range, *Colletotrichum* spp. are commonly employed as experimental organisms for the studies of fungal biotrophy (Perfect et al. 1999; Mendgen and Hahn 2002), the switch between biotrophy and necrotrophy (Dufresne et al. 2000), and also endophytic versus pathogenic behavior (Freeman and Rodriguez 1993). The recent description of the interactions between *C. destrictivum* and *Arabidopsis thaliana* was a milestone in the investigations of plant–fungal interactions (O'Connell et al. 2004). The protocols developed for genetic transformation of *Colletotrichum* spp. with GFP via protoplast transformation (Dumas et al. 1999) and *Agrobacterium*-mediated transformation (O'Connell et al. 2004) have also made it possible to conduct cytological studies of the penetration and colonization processes at the cellular level. Accordingly, we consider that the fast and easy *Colletotrichum* leaf disk infection assay developed for this study not only provided the necessary evidence for the antifungal activity of the *TcChi1* but

could also potentially be utilized as a tool for future research of the interactions between cacao and hemibiotrophic fungi, representative of which is the important *Crinipellis perniciosa* fungus.

In conclusion, although functional genomic analysis is a difficult and time-consuming process in cacao trees, this experimental approach allows us to make specific and strong inferences about gene function that would be impossible in any other way. Information gained by this method is very useful in developing markers for breeding programs and for screening of germplasm collections. For example, with this knowledge in hand, it would be possible to screen cacao germplasm collections for genotypes with elevated levels of *TcChi1* gene or for expression of the gene in unique tissue-specific manners. The selected genotypes could further be utilized in breeding programs. Furthermore, based on its known expression pattern, it is likely that the *TcChi1* gene contributes to fungal pathogen resistance in pods (cacao fruit) and would map to the location of QTL markers associated with resistance. If so, we could develop molecular markers for the *TcChi1* gene to be used in marker-assisted selection breeding programs.

More directly, our results suggest that the *TcChi1* gene can confer enhanced resistance in transgenic plants when over-expressed. In the specific example presented in this manuscript, the DNA constructions were not designed for commercial deployment due to the incorporation of the *EGFP* marker gene. To ensure these genotypes were not released, none of the cacao plants developed in this research have been moved outside of our laboratories and greenhouses, excluding the possibility of field-testing. Although public acceptance has limited the applications of transgenics to cacao improvement, in the future it is possible that this gene could be used to enhance fungal pathogen resistance in commercial varieties of cacao. Furthermore, the utility of the *TcChi1* gene in other plant species has yet to be demonstrated and this is currently being tested in our laboratory.

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References

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100:15649–15654

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (2001) Current protocols in molecular biology. Wiley, New York

Bennett AB (2003) Out of the Amazon: *Theobroma cacao* enters the genomic era. Trends Plant Sci 8:561–563

Bolar J, Norelli J, Harman G, Brown S, Aldwinckle H (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. Transgenic Res 10:533–543

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Carstens M, Vivier MA, Pretorius IS (2003) The *Saccharomyces cerevisiae* chitinase, encoded by the CTS1–2 gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. Transgenic Res 12:497–508

Cedeño L, Carrero C (2003) Antracnosis del cacao. Universidad de los Andes http://www.ulauniversidad.com.ve/vnews/display.v/ART/2003/06/30/3f009da56c4c4?in_archive=1

Coe SD, Coe MD (1996) The true history of chocolate. Thames and Hudson, New York

Collinge D, Kragh K, Mikkelsen J, Nielsen K, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3:31–40

De-Block M, Herrerra-Estrella L, Van Montagu M, Shell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and their progeny. EMBO J 3:1681–1689

Dufresne M, Perfect S, Pellier AL, Bailey JA, Langin T (2000) A GAL4-like protein is involved in the switch between biotrophic and necrotrophic phases of the infection process of *Colletotrichum lindemuthianum* on common bean. Plant Cell 12:1579–1589

Dumas B, Centis S, Sarrazin N, Esquerré-Tugayé M-T (1999) Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. Appl Environ Microbiol 65:1769–1771

Emch M (2003) The human ecology of Mayan cacao farming in Belize. Hum Ecol 31:111–132

Freeman S, Rodriguez RJ (1993) Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260:75–78

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 8:97–109

Kellmann JW, Kleinow T, Engelhardt K, Philipp C, Wegener D, Schell J, Schreier PH (1996) Characterization of two class II chitinase genes from peanut and expression studies in transgenic tobacco plants. Plant Mol Biol 30:351–358

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J 11:945–957

Kramer KJ, Muthukrishnan S (1997) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27:887–900

Li Z, Traore A, Maximova S, Guiltinan MJ (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao L.*) using thidiazuron. In Vitro Cell Dev Biol Plant 34:293–299

Linthorst HJ, van Loon LC, van Rossum CM, Mayer A, Bol JF, van Roekel JS, Meulenhoff EJ, Cornelissen BJ (1990) Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol Plant Microbe Interact 3:252–258

Liu M, Sun ZX, Zhu J, Xu T, Harman GE, Lorito M (2004) Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from *Trichoderma atroviride*. J Zhejiang Univ Sci 5:133–136

Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filipone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95:7860–7865

Marchant R, Davey MR, Lucas JA, Lamb CJ, Dixon RA, Power JB (1998) Expression of a chitinase transgene in rose (*Rosa hybrida* L) reduces development of blackspot disease (*Diplocarpon rosae* Wolf). Mol Breed 4:187–194

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1:142–148

Maximova SN, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan M (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In Vitro Cell Dev Biol Plant 38:252–259

Maximova S, Miller C, Antunez de Mayolo G, Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. Plant Cell Rep 21:872–883

Maximova SN, Young A, Pishak S, Miller C, Traore A, Guiltinan MJ (2005) Integrated system for propagation of *Theobroma cacao* L. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. Springer, Berlin Heidelberg New York, pp 209–229

Mendgen K, Hahn M (2002) Plant infection and the establishment of fungal biotrophy. Trends Plant Sci 7:352–356

Michiels A, Van den Ende W, Tucker M, Van Riet L, Van Laere A (2003) Extraction of high-quality genomic DNA from latex-containing plants. Anal Biochem 315:85–89

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol 37:49–59

Mohanan RC, Kaveriappa KM, Nambiar KKN (1989) Epidemiological studies of *Colletotrichum gloeosporioides* disease of cocoa. Ann Appl Biol 114:15–22

Mora A, Earl E (2001) Resistance to *Alternaria brassicola* in transgenic broccoli expressing a *Trichoderma harzianum* endochitinase gene. Mol Breed 8:1–9

Motamayor JC, Risterucci AM, Lopez PA, Ortiz CF, Moreno A, Lanaud C (2002) Cacao domestication I: the origin of the cacao cultivated by the Mayas. Heredity 89:380–386

Neuhaus J-M (1999) Plant chitinases (PR-3, PR-4, PR-8, PR-11). In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press, New York, pp 77–107

O'Connell RJ, Perfect SE, Hughes HB, Carzaniga R, Bailey JA, Green JR (2000) Dissecting the cell biology of *Colletotrichum* infection processes. In: Prusky D, Freeman S, Dickman M (eds) Host specificity, pathology, and host–pathogen interaction of *Colletotrichum*. American Phytopathology Society Press, St Paul, pp 57–77

O'Connell R, Herbert C, Sreenivasaprasad S, Khatib M, Esquerre-Tugaye M-T, Dumas B (2004) A novel *Arabidopsis–Colletotrichum* pathosystem for the molecular dissection of plant–fungal interactions. Mol Plant Microbe Interact 17(3):272–282

Patil VR, Widholm JM (1997) Possible correlation between increased vigor and chitinase activity expression in tobacco. J Exp Bot 48:1943–1950

Perfect SE, Hugues HB, O'Connel RJ, Green JR (1999) *Colletotrichum*: a model genus for studies on pathology and fungal–plant interactions. Fungal Genet Biol 27:186–198

Piasentin F, Klare-Repnik L (2004) Biodiversity conservation and cocoa agroforests. Gro Cocoa 5:7–8. http://www.cabicommodities.org/Acc/ACCrc/

Punja ZK, Raharjo SHT (1996) Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. Plant Dis 80:999–1005

Raharjo SHT, Hernandez MO, Zhang YY, Punja ZK (1996) Transformation of pickling cucumber with chitinase-encoding genes using *Agrobacterium tumefaciens*. Plant Cell Rep 15:591–596

Snyder T (1994) Isolation and characterization of a genomic chitinase clone form *Theobroma cacao* L. PhD thesis, Intercollege Program in Plant Physiology, The Pennsylvania State University

Snyder-Leiby TE, Furtek DB (1995) A genomic clone (accession no. U30324) from *Theobroma cacao* L. with high similarity to plant class I endochitinase sequences. Plant Physiol 109:338

Tabaeizadeh Z (1997) Transgenic tomato plants expressing *L. chilense* chitinase gene demonstrate resistance to *Verticillium dahliae*. Plant Physiol 114:299

Vierheilig H, Alt M, Lange J, Gut-Rella M, Wiemken A, Boller T (1995) Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Appl Environ Microbiol 61:3031–3034

Wood GAR, Lass RA (1987) Cocoa. Longman Scientific & Technical, copublished by Wiley, New York

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000) Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. Plant Cell Rep 19:639–646

Zeng Y, Yang T (2002) RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant Mol Biol Rep 20:417a–417e