CHAPTER 3

CONSTRUCTION OF A cDNA LIBRARY WITH GENES ASSOCIATED WITH TOLERANCE TO *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* IN CAVENDISH BANANAS

ABSTRACT

Identification of banana genes involved in the defence response against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) represents an important step towards understanding disease resistance mechanisms in bananas. Suppression Subtractive Hybridisation (SSH) was used to isolate differentially expressed genes in a tolerant banana cultivar (GCTCV-218), compared to the susceptible cultivar Williams, in response to infection by *Foc*, 6 hrs post inoculation. Southern Blot analysis and a PCR showing the reduction of the housekeeping gene, actin, after the SSH, indicated that the subtraction was efficient. A cDNA library containing 736 cDNA clones was constructed. Sequencing results and BLASTX searches indicated that the cDNA library contained cDNA fragments that are associated with defence responses in other plants. The construction of a cDNA library enriched for differentially expressed transcripts is a valuble first step supporting future studies concerning resistance to *Foc* in bananas.

INTRODUCTION

Bananas (*Musa* spp.) are one of the most important sources of human nutrition, providing food and income to millions of people in the world (Jones, 2000). The crop includes dessert and cooking bananas and is planted widely throughout the tropics and subtropics. The most important dessert bananas are Cavendish (AAA) varieties, which comprise almost 90% of the export market and 40% of all bananas cultivated (Jones, 2000). Because of the clonal nature of the plant, sustainable production of bananas is threatened by diseases such as Fusarium wilt, caused by *Fusarium oxysporum* Schechtend.:Fr. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*).

No effective control strategy exists for Fusarium wilt other than replacing susceptible bananas with resistant banana varieties. For example, to continue banana production in Central America during the 1960s, Cavendish varieties had to be introduced to replace Gros Michel bananas that were highly susceptible to *Foc* race 1 (Stover, 1962). In the past three decades, however, a new race of the pathogen, *Foc* race 4, has caused devastating losses to Cavendish bananas both in the tropics and subtropics (Ploetz, 1994). Despite conventional and unconventional breeding efforts, no dessert banana variety has been developed to replace Cavendish bananas in areas that are severely affected by Fusarium wilt.

Resistance to *Foc* race 4 is found in wild banana varieties such as Calcutta 4 (AB) (Jeger *et al*., 1995). This resistance has been introduced into banana hybrids in breeding programmes such as Fundación Hondurereña de Investigacón Agrícolan (FHIA) in Honduras (Moore *et al*., 1995; Jones, 2000). The introduction of resistance into tetraploid hybrids involves the transfer of a set of chromosomes from *Musa balbisiana* Colla, a starchy banana species known for its ability to tolerate biotic and abiotic stresses (Jones, 2000). Tetraploid hybrids developed in banana breeding programmes have seldom been accepted as a replacement for sweet triploid Cavendish banana varieties, consisting exclusively of sets of chromosomes from *M. acuminata* Colla (Jones, 2000). Other efforts to introduce resistance to Fusarium wilt involve unconventional plant improvement efforts, such as induced mutations, the

production of somaclonal variants, and field selection of disease resistant plants (Hwang and Tang, 1996).

Substantial progress in the development of *Foc* race 4-resistant Cavendish bananas has been made at the Taiwan Banana Research Institute (TBRI) in Taiwan. The most promising plant from this institute is GCTCV-218, a Cavendish selection with good tolerance to *Foc* race 4 isolates belonging to vegetative compatibility groups (VCGs) 0120 (Chapter 2) and 0121 (Hwang and Ko, 2004). VCG0120 is widely distributed throughout the world, but became particularly notorious for causing disease to Cavendish bananas in the subtropics (Ploetz, 1994).

The search for genes conferring resistance to diseases and pests has become an important objective towards developing genetically improved banana plants. At least three different classes of genes play a role in the defence strategy of a plant (Glazebrook *et al*., 1997). One of these comprises genes for constitutive (passive) defence and is not directly involved in defence responses. These genes may play a role in plant resistance by inhibiting pathogen entry by, for example, forming a thick waxy cuticular layer that hinders penetration. Another class of genes are those that serve in non-specific plant defence through the production of phytoalexins, glucanases, chitinases, lignin, callose and enzymes for oxidative stress protection. In addition, antimicrobial secondary metabolites and genes coding for thionins, glutathionine S-transferases, lipoxygenases and phenylalanine ammonia-lyase (PAL) are also induced (Glazebrook *et al*., 1997). Genes in these two classes are known as minor genes for resistance and are present in all plants.

A third class of genes is required for race-specific resistance. These include major resistance (*R*) genes and result in the inhibition of pathogen growth (Jørgensen, 1994). A plant that possesses an *R*-gene has resistance to a specific pathogen containing the corresponding *avr*-gene. A *R*-gene to *Foc* in banana has recently been identified in *M. acuminata* Colla *malaccensis* Simmonds (Pereza-Escheverria *et al.*, 2004), and transgenic plants containing the *RGC*-2 gene are currently being evaluated to ascertain whether this gene confers resistance to *Foc*.

Studying genes that are expressed in plants when they are infected by pathogens is an important step towards understanding gene function and molecular mechanisms underlying plant defence responses. These genes can be isolated and identified using a highly effective and very powerful Polymerase Chain Reaction (PCR)-based technique for generating enriched complimentary (c)DNA libraries, known as Suppression Subtractive Hybridisation (SSH) (Diatchenko *et al.* 1996; Diatchenko *et al.* 1999). SSH has numerous applications, mostly in clinical medical studies (Carmeci *et al.*, 1998; Kuang *et al.*, 1998). Over the past few years, SSH has also had an impact on agriculture, where it has been successfully applied for gene discovery in plant-pathogen interactions (Birch *et al.*, 1999; Beyer *et al.*, 2001; Hein *et al.*, 2004; Lu *et al.*, 2004).

SSH involves the hybridisation of cDNA, generated from mRNA that is transcribed upon pathogen attack, from plants that contain the target genes ("tester") and plants that serve as reference material ("driver"). Gene sequences that are similar in the "tester" and "driver" material will hybridise and be removed during the SSH reaction, while those genes that are uniquely expressed in the "tester" but not in the "driver" will not be hybridised. In the process, differentially expressed genes will be selectively amplified by means of suppression PCR (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996).

Little is known regarding the molecular processes involved in resistance mechanisms, metabolic pathways and downstream signalling of the banana-*Foc* interaction. An analysis of pathogen-induced genes may lead to a better understanding of the molecular processes involved in resistance and may further contribute to the development of biotechnological strategies to fight the disease. The aim of this study, therefore, was to use SSH to isolate genes that are differentially up-regulated in GCTCV-218, but not in the susceptible Cavendish banana variety, Williams, upon challenge with *Foc* 'subtropical' race 4 VCG 0120.

MATERIALS AND METHODS

Inoculation of Banana Plants with *Foc*

Plant material and growth conditions

Micropropagated Cavendish banana plantlets tolerant (GCTCV-218) and susceptible (Williams) to *Foc* race 4 were obtained from Du Roi Laboratories in Letsitele, South Africa. All plantlets were transplanted into plastic cups containing water, fertilised regularly with a nutrient solution and maintained in a greenhouse at 18/25ºC with a 16-hrs natural sunlight/8-hrs dark photoperiod (Chapter 2). After 4 weeks the plantlets developed a strong root structure, and were transferred to an aeroponic system. The aeroponic system comprised five separated compartments that could accommodate six plants per compartment. Strips of sponge were wrapped around the pseudostems of each plant before they were stabilized in holes drilled through the Perspex lids of each compartment, with their roots hanging above a fine mist sprayer. The automated mist sprayer was set to irrigate the roots for 30 seconds every 2 min. To allow the banana roots to recover from stress related to the planting procedure, plants were kept in the aeroponic system for at least 10-14 days before inoculations were carried out. All the plants were kept under the same light and temperature conditions and were fertilised every 2 days.

Preparation of inoculum

Three highly virulent *Foc* isolates representing VCG 0120, CAV 045, 092 and 105, were used to inoculate the roots of banana plantlets. Each isolate was first grown on half strength potato dextrose agar (PDA) medium in 90-cm-diameter Petri dishes at 25^{o}C for 7-10 days. Sterile distilled water (SDW) was then poured onto the sporulating cultures, and the hyphae abraded with a sterile glass rod to loosen the spores. The spore suspensions were then removed from the Petri dishes, mixed, filtered through sterile cheesecloth, and diluted to a final concentration of 10^{5} spores.ml^{-1}.

Inoculation and sample collection

Once the banana plantlets developed into 15-cm plants, their roots were inoculated with *Foc*. For inoculation, a small wound was made by first puncturing the healthy root with a sterile needle. A single droplet of the *Foc* spore suspension was then placed onto the wound, and the inoculation points sealed with Parafilm (Pechiney Plastic Packaging, Menasha WI, USA) to ensure that the spores were not washed off during irrigation. Six plants and six roots per plant were inoculated.

Root tissue for RNA extraction was collected 0, 3 and 6 hrs after inoculation from three plants. The conidia of *Foc* germinate within in 3 hrs and by 6 hrs the plant would most likely have responded in some way. To ensure that the root tissue was fungus-free, samples were taken 1 cm away from the point of inoculation. Root tissue from three different plants was pooled for each individual time point, placed in liquid nitrogen, ground with a homogenizer (IKA A11 Basic analytical Mill, United Scientific (Pty) Ltd., San Diego, USA) and stored at -80ºC. The remaining three inoculated plants of both GCTCV-218 and Williams were maintained in the greenhouse for six weeks, after which they were evaluated for disease development. Disease rating was done using the rating scale for Fusarium wilt of banana (Carlier *et al*., 2002).

Total RNA isolation

Total RNA was extracted from 100 mg banana roots per treatment, using the RNeasy Plant Mini kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. Isolated RNA samples from the same time points were combined and stored at –80ºC. The quantity of RNA was determined with a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The RNA was analyzed for the presence of distinct ribosomal bands and the absence of degraded RNA by gel electrophoresis under non-denaturing conditions on 1% (w/v) agarose gels. Total RNA samples were treated with RNase-free DNase I (Fermentas, Life Sciences, Hanover, USA) to degrade single-stranded (ss) and double-stranded (ds) DNA (Ausubel *et al.,* 1999).

cDNA synthesis

Ds cDNA was synthesized from total RNA using a cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. First strand synthesis was carried out in a reaction volume of 21 µl, containing 20 µg RNA, 2 µl oligo dT_{15} primer (200 µM) and RNase free water. The sample was incubated at 70ºC for 10 min and immediately placed on ice. The following components were added: 8 µl 5x Reverse Transcriptase-buffer, Avian Myeloblastosis Virus (AMV), 4 µl 0.1 M dithiothreitol (DTT), 2 µl AMV RT (25 U/µl), 1 µl RNase Inhibitor (25U/µl) and 4 µl 10 mM dNTP-mix. After mixing, the sample was incubated at 42ºC for 60 min and immediately placed on ice to terminate the reaction. Second strand synthesis followed immediately by taking 40 µl of the cDNA from the first strand reaction, 30 µl 5x second strand buffer, 1.5 µl 10 mM dNTP-mix, 6.5 µl second strand enzyme blend (mixture of DNA polymerase I, *Escherichia coli* ligase and RNase H) and 72 µl redistilled water. The reaction was mixed gently and incubated at 16ºC for 2 hrs, followed by the addition of 20 µl (20 U) T4 DNA polymerase and incubation for a further 5 min. The reaction was terminated by adding 17 µl 0.2 M EDTA (pH 8.0). Ds cDNA was purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

To determine whether cDNA was contaminated with genomic DNA, a cDNA sample was screened for a small fragment of the banana actin gene by PCR-amplification, using the primers ActinF (5'ACCGAAGCCCCTCTTAACCC-3') and ActinR (5'-GTATGGCTGACACCATCACC- 3') (Fig. 1). PCR amplifications were carried out using first strand cDNA and genomic DNA as templates. The PCR was conducted in 20 µl and contained 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM dNTP's (Roche Diagnostics), 0.25 µM of each primer, 1 U Taq polymerase (Roche Diagnostics) and 10 ηg DNA. The samples were heated at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min, followed by a final elongation step at 72ºC for 7 min. The PCR was conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, Perkin Elmer, Ontario, Canada), and the PCR products were separated by electrophoresis on a 2% agarose gel.

Suppression Subtractive Hybridisation (SSH)

cDNA derived from RNA extracted 3 and 6 hrs after inoculation of GCTCV-218 with *Foc* was pooled and served as "tester", and cDNA derived from RNA extracted from Williams plants at the same time points after inoculation by *Foc* served as "driver". "Tester" and "driver" material was then subjected to SSH analysis (Diatchenko *et al.* 1996; 1999) using a PCR-Select cDNA Subtraction kit ™ (Clontech, BD Biosciences, Palo Alto, California) according to the manufacturer's instructions, but with a ratio of 300:1 "tester" to "driver" material in the primary hybridisation.

Monitoring SSH efficiency

Reduction in actin levels. cDNA enriched for differentially expressed transcripts was referred to as "subtracted tester" (ST), cDNA from infected tolerant and susceptible banana plants was referred to as "unsubtracted tester" (UT) and unsubtracted "driver" (UD), respectively. Reduction in actin levels during the SSH procedure was determined by comparing cDNA levels in the ST with that of UT and UD. Ten ηg of the ST, UT or UD was amplified with 0.33 µl of the primer pair ActinF and ActinR (15 µM stock) in a 20 µl PCR reaction. The reaction further contained 2.0 µl PCR buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl), 2.0 µl dNTP mix (2.5 mM stock) (Roche Diagnostics) and 0.2 µl Taq polymerase (5 U/µl) (Roche Diagnostics). Primers were designed to amplify a cDNA fragment of the actin gene that flanked an intron and did not contain an *Rsa*I restriction site (Fig. 1). Each sample was denatured at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min, before a final elongation step at 72ºC for 7 min. Amplification products (5 µl) were collected after 20, 25 and 30 cycles and separated on a 2% agarose gel. The amplified fragment was also sequenced to confirm that it was from the actin gene.

Presence of a banana defence-associated gene. Primers were designed from the genebank nucleotide sequence of endochitinase (AF 416677) to amplify a cDNA fragment of 149 bp that did not contain a *Rsa*I restriction site. The presence of endochitinase in the ST material was then determined by PCR amplification using 10 ηg of the ST, 2.0 µl PCR buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl), 2.0 µl dNTP mix (2.5 mM) (Roche Diagnostics), 0.33 µl of EndochitF (5'-GCCACCGTCAGAGGTTATACAG- 3') and EndochitR (5'-GACTATTAAGGGCTCCGTGGTT- 3') (15 µM stock) and 0.2 µl Taq polymerase

(5U/µl) (Roche Diagnostics). Samples were denatured at 94ºC for 2 min, then cycled 30 times at 94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 1 min, followed by a final elongation step at 72ºC for 7 min. PCR products were then visualised on a 2% agarose gel and sequenced to confirm their identity.

Southern analysis

Adaptor sequences were removed by digesting 10 µl each of ST, UT and UD cDNA with RsaI (Roche Diagnostics). PCR products were then separated through a 1.5% low melting point (LMP) agarose gel, excised and purified using a QIAquick® Gel Extraction Kit (Qiagen). The ST, UT and UD cDNA (25-50 ηg) was diluted to 47.5 µl with SDW, denatured at 95°C for 3 min, and placed on ice for 2 min. Denatured cDNA was labelled with Ready-To-Go ^{TM} labelling beads (-dCTP) (Amersham Biosciences UK Limited, England) in a reaction mixture containing buffer, dATP, dGTP, dTTP, FPLCpure^{TM} Klenow fragment and random nonamers. Twenty five µCi radioactive labelled [α-^{32}P] dCTP was added and the reaction mixture incubated at 37°C for 20 min. Unincorporated radiolabeled nucleotides were removed using MicroSpin^{TM}G-50 Columns (Amersham Biosciences) using the manufacturer's instructions. Prior to use, the labelled ds DNA was denatured at 95°C for 10 min followed by incubation on ice for 3 min. The probe was centrifuged briefly and immediately used for the hybridization

Southern-based screening was performed as described by Southern (1975). PCR products from ST, UT and UD material were separated on three replicate 2% gels, visualized under UV light and photographed, and 5 µl of each sample was transferred to a Hybond-N^{+} nylon transfer membrane (Amersham-Pharmacia). DNA transfer was carried out via alkaline capillary blotting with transfer buffer (1.5 M NaCl, 0.5 M NaOH) for 16 hrs. The membrane was rinsed in 2x Sodium Saline Citrate (SSC) (300 mM NaCl, 30 mM Na_3citrate-2$H_2$0 (pH 7.0), air-dried and cross-linked on top of an Ultraviolet Cross linker CL-508 (UVI-tec, St John's Innovation Centre, Cambridge, England) at 0.167 J (312 nm) for 2 min. Blots were wrapped in cling film and stored at –20°C. Hybridization of membranes was carried out as described by Ausubel (1999) with ^{32}P-radiolabelled PCR-amplified ST, UT and UD probes.

Subtracted library construction

To construct a cDNA library, SSH products were size fractioned on a 1.5% LMP agarose gel and separated into fragments ranging from 150-400 bp and others ranging from 400-700 bp. The fragments were then extracted from the agarose gel using a Qiaquick® Gel Extraction kit (Qiagen). Non-fractionated DNA was also purified with the Qiaquick® PCR purification kit (Qiagen) and included in the library. The cDNA clones were all ligated with pGEM-T Easy vector (Promega, Madison, USA). Ligation reactions were carried out in 10 µl reaction volumes containing 10-50 ηg insert DNA, 50 ηg pGEM-T Easy vector, 1x rapid ligation buffer and 3 Weiss units T4-DNA-Ligase (Promega). Samples were incubated for 16 hrs at 4ºC and stored at -20ºC.

Escherichia coli JM 109 competent cells (Promega) were transformed with cDNA fragments by heat-shock as recommended by the manufacturer. Following the transformation, cells were plated on Luria-Bertani (LB) agar plates (10 g l^{-1} peptone, 5 g l^{-1} yeast, 5 g l^{-1} NaCl, 15 g l^{-1} agar, 100 µg ml^{-1} ampicillin, 60 µg ml^{-1} X-gal and 60 µg ml^{-1} Isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37ºC for 16-18 hrs. Identification of the recombinant clones was done by colour screening on Xgal/IPTG indicator plates. A blue/white selection was used and 300 recombinant (white) colonies were picked for each ligation reaction (non-fractionated, and size fractionated). The library was replicated by transferring recombinant transformants to 1.5 ml tubes containing 700 µl LB amended with 50µg/ml ampicillin and grown for 20-24 hrs at 37ºC on a rotary shaker (250 rpm), and 300 µl 50% glycerol was added to each tube prior to storing at -80ºC.

Colony PCR and sequencing of selected clones

Colony PCR was conducted on 15 randomly selected clones in 25 µl reaction volumes containing 2.5 µl PCR buffer (10 mM Tris-HCl; 1.5 mM MgCl2; 50 mM KCl), 2.0 µl dNTP mix (2.5mM) (Roche Diagnostics), 0.5 µl of each of the primers T7 (5'-ATTATGCTGAGTGATATCCC-3') and SP6 (10 µM) (5'-ATTTAGGTGACACTATAGAAT-3'), 0.5 µl Taq DNA polymerase (5 U/µl) (Roche

Diagnostics), 13.5 µl SDW and 0.5 µl of the bacterial colony grown in LB. PCR conditions were as follows: Initial denaturation at 94ºC to avoid the preferential cloning of highly abundant or small DNA molecules for 2 min, 25 cycles of 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 1 min, and a final elongation step at 72ºC for 5 min. PCR products were then separated by electrophoresis on a 1.5% agarose gel to confirm successful insertion of cDNA fragments.

Cycle sequencing (Griffin and Griffin, 1993) was carried out in 10 µl reaction volumes containing 150 ηg template DNA (derived from colony PCR products), 2 µl Big Dye termination reaction mix (V3) (Applied Biosystems, Foster City, CA, USA), 2 µl primer T7 (10 µM) and 4 µl 5x dilution buffer (400 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$). Samples were cycled 25 times at 96ºC for 10 sec, 50ºC for 5 sec and 60ºC for 4 min. Sequenced products were diluted 1:20 and purified by adding 2 µl 3 M NaOAc (pH 4.6) and 50 µl cold absolute ethanol to the reactions, followed by 10 min incubation on ice and centrifugation at 10 000 rpm for 30 min at 4ºC. The supernatant was removed and the product washed to remove salts and excess dye terminators, using 250 µl 70% ethanol. This was followed by centrifugation for 10 min at 10 000 rpm at 4ºC. Samples were dried in an incubator at 37ºC for 5 min and stored at –20ºC. DNA sequences were analyzed on an ABI PRISM 377 DNA analyzer (Perkin Elmer) at the DNA Sequencing Facility of the University of Pretoria, South Africa.

Vector and SSH adaptor sequences were removed manually using Vector NTI® Suite V.6 (InforMax®, North Bethesda, USA) and database searching utilized BLAST software (Altschul *et al.,* 1990), available through the National Centre for Biotechnology Information (NCBI) (website http://www.ncbi.nih.gov/BLAST). Homologies were identified by BLASTX. The degree of sequence similarity between banana cDNA clones and known sequences was represented by E-values.

RESULTS

Inoculation of Banana Plants with *Foc*

GCTCV-218 and Williams plants point-inoculated with *Foc* developed typical internal Fusarium wilt symptoms after six weeks. The entire corm of susceptible Williams plants showed brown-purple discoloration, while the corms of tolerant GCTCV-218 plants only showed one or two brown specs. All three Williams plants had a disease severity score of 5, while the three GCTCV-218 plants had disease ratings of either 1 or 2. These results showed that the inoculation technique was effective in causing disease.

RNA isolation

Yields of between 15-30 µg total RNA for individual samples of GCTCV-218 and Williams were extracted from banana roots. However, this concentration was reduced substantially when RNA was passed through the Qiagen cleanup column, and numerous extractions had to be done to obtain sufficient material for cDNA synthesis. No degradation of RNA was visible for GCTCV-218 and Williams RNA samples at 0, 3 and 6 hrs after inoculation on 1% agarose gels, and two distinct bands were present (Fig. 2).

cDNA synthesis

Successful synthesis of cDNA for both GCTCV-218 and Williams was confirmed by PCR amplification from complementary and genomic DNA of the two banana varieties. Amplification of genomic DNA with the Actin gene primer pair resulted in a 260 bp fragment. This fragment is larger than the 170 bp fragment produced for first strand cDNA (Fig. 3). The difference in size is a result of the absence of a 90-bp intron sequence in cDNA, indicating that there was no genomic DNA present in the cDNA samples. Sequencing confirmed that the amplified product was a fragment from the actin gene.

Suppression Subtractive Hybridisation (SSH)

Reduction in actin levels. Amplification of the actin gene from ST material yielded no visible PCR product, even after 30 PCR-cycles (Fig. 4). However, specific PCR-products of 170 bp became visible after 20 cycles for the UT and UD, with intensities increasing after further PCR-cycles. Assuming that PCR amplification is 100% efficient during all amplification cycles and is, therefore, exponential, each cycle (x) difference represents a 2x times less actin template in subtracted material compared to unsubtracted material. In this subtraction procedure, ST would be expected to contain at least 210 times less actin template than UT and UD. Although these figures are theoretical, they emphasise the success of removing common sequences during the SSH.

Presence of a banana defence-associated gene. A 149 bp fragment of endochitinase was amplified in the ST material to show successful enrichment of a defence gene in the subtracted material in response to pathogen infection (Fig. 5). Sequencing results confirmed that the amplified fragment was from endochitinase.

Southern Blot analysis

Distinct bands were produced by the ST during PCR amplification compared to the product "smear" produced for the UT and UD (Fig. 6), indicating that the ST had been enriched for a number of differentially expressed products. During Southern analysis, ST probes bound primarily to the ST PCR product, with substantially less hybridization to UT and UD PCR products (Fig. 7). UT hybridised mainly to both UT and UD, representing common genes in UT. Furthermore, very little UD probe hybridized to the ST sample (Fig. 7), demonstrating that gene transcripts common to both "driver" and "tester" had been removed by the subtraction, and thus implying an enrichment for "tester" specific transcripts.

Subtracted library construction

A cDNA library of 736 banana clones expressed early after *Foc* infection was constructed, with cDNA clones ranging in size from 150 to 700 bp. The non-fractionated ST material yielded 250 clones. The fraction containing fragments between 150 and 400 bp long yielded 240 clones, and the fraction containing fragments between 400 and 700 bp long yielded 246 clones.

Colony PCR and sequencing

Colony PCR indicated that transformation of cDNA clones was successful and that the majority of vectors had only single inserts that varied in size from 150 to 600 bp (Fig. 8). Clones that had more than one insert were discarded from the library. A small subset of 15 cDNA clones was sequenced to check the quality of the cDNA library. Most cDNA clones that were sequenced (except clone 2-8) showed significant similarities to plant genes and thus were assumed to be derived from the host and not from the pathogen (Table 2).

Two of the 15 clones putatively identified by BLASTX searches showed homology to peroxidases from rice, and one each to an unspecific monooxygenase cytochrome P450, isoflavone reductase, Bowman Birk proteinase inhibitor, putative senescence associated protein, auxin protein, cytosolic monohydroascorbate reductase, UTP-glucose glucosyltransferase, reversibly glycosylated polypeptide and a putative transcription factor. Two clones had no significant homology and two showed homology to unknown proteins from barley (Table 2). Six of the 15 clones sequenced, were grouped in the functional category associated with defence/stress responses, two clones had sequences involved in metabolism and one clone had a sequence that could play a role in transcription. The level of redundancy in the 15 sequenced clones was low; only two clones for a peroxidase and an unknown protein occurred twice.

DISCUSSION

In this study, SSH was successfully utilised to isolate more than 700 cDNA clones in the tolerant Cavendish banana cultivar, GCTCV-218, in response to infection by *Foc*. A number of these clones showed significant sequence similarities to defence-associated genes. The variety of putative gene functions that were assigned to the banana clones selected for sequencing, provided confidence in the SSH library. The isolation of theses genes at an early time point reveals that the tolerant banana, GCTCV-218, recognises *Foc* and responds at the transcriptional level.

Two of the clones showed homology to a peroxidase gene from *Oryza sativa* L. This is relevant because peroxidases have been implicated in several physiological processes of importance in plant-pathogen interactions including lignification (Lagrimini et *al.,* 1987), cross-linking of cell-wall proteins (Bradley *et al.,* 1992), wound healing (Sherf *et al.,* 1993) and papillae formation (Cadena-Gomez and Nicholson, 1987). Enhanced peroxidase activity after pathogen infection has also been correlated with resistance in many different host pathogen systems.

The BLASTX search showed a clone sharing similarity with an isoflavone reductase from *Pyrus communis* L. This enzyme catalyzes the penultimate step in the synthesis of isoflavonoid phytoalexins that play a role in plant defence (Ibrahim and Varin, 1993). One clone had homology to a Bowman-Birk type protease inhibitor from kidney bean. The synthesis of theses inhibitors may be induced by pathogen infection or wounding (Qi *et al.,* 2005) and they have been linked with enhanced protection of plants against insects and microorganisms (Deshimaru *et al.,* 2004). The BLASTX search also showed a clone with homology to cytochrome P450. This compound plays a role in secondary metabolism by being involved in the phenylpropanoid biosynthesis pathway (Dowd *et al*., 2004) that leads to lignin production.

Results of this study represent the first effort to isolate genes in banana with resistance to *Foc*. Even though conventional breeding has been used by several banana improvement programmes to introduce resistance to pathogens and pests (Hwang and Tang, 1996), almost nothing is known about the genes underlying such resistance. Although several technologies are available to discover these genes (Cochran *et al.,* 1983; Duguid and Dinauer, 1990; Liang *et al.,* 1993; Lisitsyn and Wigler, 1993; Velculescu *et al.,* 1995; Schena *et al.,* 1995), SSH used in this study has numerous advantages above other gene isolation methods, and it was effective in isolating differentially expressed genes from banana. An advantage of SSH is that it allows the detection of low-abundance, differentially expressed cDNAs, such as those involved in signalling and signal transduction (Birch *et al*., 2000).

The SSH procedure appeared to be efficiently executed. Small quantities (20 µg) of RNA from banana roots were sufficient to set up the SSH experiment. The isolation of mRNA from total RNA yielded very little material (data not shown) and total RNA was thus used for the procedure. This is important in an experiment where only a limited amount of starting material can be obtained. Constitutively expressed genes, such as the actin gene were removed during subtraction hybridisation, while induced resistance genes, such as endochitinase, were found in the SSH final product. Southern blot analysis further confirmed the subtraction efficiency of the SSH technique. Southern blot data suggested that the subtracted "tester" had been enriched for differentially expressed transcripts, and that "housekeeping" gene transcripts that are common to both "driver" and "tester" had been removed by the subtraction.

From the limited number of clones sequenced in this study, it was clear that SSH was effective in enriching for pathogen-induced transcripts from as early as 3 and 6 hrs after inoculation with *Foc*. Rare transcripts, such as the putative transcription factor isolated in this study, are often not detected by other methods such as differential display (DD) (Liang and Pardee, 1992) reverse transcriptase (RT) PCR and cDNA-amplified fragment length polymorphisms (AFLP) due to competition within the PCR (Birch *et al*., 2000).

In most cases, the Clontech PCR-Select cDNA subtraction method greatly enriches for differentially expressed genes. Nevertheless, the subtracted "tester" will still contain some cDNAs that have escaped the subtraction and correspond to mRNAs that are common to both "tester" and "driver" samples. This type of background (false positives) may somewhat depend on RNA quality and purification and the performance of the specific subtraction, but it mainly arises when very few mRNA species are differentially expressed in "tester" and "driver" (Clontech PCR-Select™ cDNA Subtraction Kit User Manual, 2000). It is important and essential to subject the library to a differential screening method. Therefore, the cDNA library was screened (Chapter 4) enabling us to select clones for a more comprehensive study.

The cDNA library constructed from genes differentially expressed in Cavendish bananas after infection by *Foc* could be applied usefully in a number of ways. Firstly,

the identification of host genes involved in defence responses is one of the most critical steps leading to the understanding of disease resistance mechanisms in plants (Xiong *et al.,* 2001). Secondly, the genes can be used as markers for resistance traits in plant improvement programmes, and thirdly, the genes can be used for genetic improvement of plants via transformation. The manipulation of defence-associated genes and their signalling pathways by transgenic expression is a promising strategy to improve disease resistance in plants (Martin, 1999).

This study is the first of its kind, utilising SSH for isolating defence associated genes in banana against *Foc*. It could contribute significantly to the understanding of gene expression profiles associated with defence mechanisms in monocotyledonous plants.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G. Smith, J.A. Struhl, K., Albright, L.M. Coen, D.M. and Varki, A. 1999. Current Protocols in Molecular Biology. 4 vols. 1. Molecular Biology-Technique. 2. Molecular Biology-Laboratory manuals, John Wiley and Sons, Inc., England.

Beyer, K., Bincer, A., Boller, T. and Collinge, M. 2001. Identification of potato genes induced during colonization by *Phytophthora infestans*. *Molecular Plant Pathology* 2: 125-134.

Birch, P.R.J., Avrova, A., Lyon, G.D., Duncan, J.M. and Toth, R.L. 1999. Isolation of potato genes that are induced during an early stage of the hypersensitive response to *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* 12: 356-361.

Birch, P.R.J., Toth, I.K., Avrova, A.O., Dellagi, A., Lyon, G.D. and Pillay, D. 2000. Recent advances in the identification of differentially expressed genes. *South African Journal of Science* 96: 83-85.

Bradley, D.J., Kjellblom, P. and Lamb, C.J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline rich plant cell protein: a novel, rapid defence response. *Cell* 70: 21-30.

Cadena-Gomez, G. and Nicholson, R.L. 1987. Papillae formation and associated peroxidase activity: a non-specific response to attempted fungal penetration of maize. *Physiological Molecular Plant Pathology* 31: 51-67.

Carlier, J., De Waele, D. and Escalant, J.V. 2002. Global evaluation of *Musa* germplasm for resistance to Fusarium wilt, *Mycosphaerella* leaf spot diseases and nematodes. *INIBAP Technical Guidelines* 6, Montpellier, France.

Carmeci, C., Thompson, D.A., Kuang, W.W., Lightdale, N., Furthmayer, H. and Weigel, R.J. 1998. Moesin expression is associated with the estrogen receptor-negative breast cancer phenotype. *Surgery* 124: 211-217.

Cochran, B.H., Reffel, A.C. and Stiles, C.D. 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell:* 33: 939-947.

Clontech PCR-Select™. cDNA Subtraction Kit User Manual. 2000. Catalog #: K1804-1. BD Biosciences, Clontech.

Deshimaru, M., Yoshimi, S., Shioi, S. and Terada, S. 2004. Multigene family for Bowman-Birk type proteinase inhibitors of wild soja and soybean: The presence of two BBI-A genes and pseudogenes. *Bioscience, Biotechnology and Biochemistry* 68: 1279-1286.

Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. and Siebert, P.D. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the USA* 93: 6025-6030.

Diatchenko, L., Lukyanov, S., Lau, Y.F. and Siebert, P.D. 1999. Suppression subtractive hybridization: A versatile method for identifying differentially expressed genes. *Methods in Enzymology* 303: 349-380.

Dowd, C., Wilson, I.W. and McFadden, H. 2004. Gene expression profile changes in cotton root and hypocotyls tissues in response to infection with *Fusarium oxysporum* f. sp. *vasinfectum. Molecular Plant Microbe Interactions* 17: 654-667.

Duguid, J.R. and Dinauer, M.C. 1990. Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acids Research* 18: 2789-2792.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. 1997. Use of *Arabidopsis* for genetic dissection of plant defence responses. *Annual Review of Genetics* 31: 547-569.

Griffin, H.G. and Griffin, A.M. 1993. DNA sequencing. Recent innovations and future trends. *Applied Biochemistry and Biotechnology* 38: 147-159.

Gurskaya, N.G., Diatchenko, L., Chenchik, A., Siebert, P.D., Khaspekov, G.L., Lukyanov, K.A., Vagner, L.L., Ermolaeva, O.D., Lukyanov, S.A. and Sverdlov, E.D. 1996. Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. *Analytical Biochemistry* 240: 90-97.

Hein, I., Campbell, E.I., Woodhead, M., Hedley, P.E., Young, V., Morris, W.L., Ramsay, L., Stockhaus, J., Lyon, G.D., Newton, A.C. and Birch, P.R.J. 2004. Characterisation of early transcriptional changes involving multiple signalling pathways in the *Mla13* barley interaction with powdery mildew (*Blumeria graminis* f. sp. *hordei*). *Planta* 218: 803-813.

Hwang, S. and Ko, W. 2004. Cavendish banana cultivars resistant to Fusarium wilt acquired through somaclonal variation in Taiwan. *Plant Dis*ease 88: 580-588.

Hwang, S. and Tang, C. 1996. Somaclonal variation and its use for improving Cavendish (AAA dessert) bananas in Taiwan. In: New frontiers in resistance breeding for nematode, fusarium and sigatoka. (Eds. E. Frison, J.P. Horry and D. De Waele) INIBAP, Montpellier, France.

Ibrahim, R.K. and Varin, L. 1993. Flavanoid enzymology. In: Methods in Plant Biochemistry Vol. 9. (Ed. P.J. Lea) Academic Press, London.

Jeger, M.J., Eden-Green, S., Tresh, J.M., Johanson, A., Waller, J.M. and Brown, A.E. 1995. Banana diseases. In: Bananas and Plantains. (Ed. S. Gowen). Chapman and Hall, London.

Jones, D.R. 2000. History of banana breeding. In: Diseases of banana, abacá and enset. (Ed. D.R. Jones). CAB International, Wallingford, UK.

Jørgensen, J.H. 1994. Genetics of powdery mildew resistance in barley. *Critical Review in Plant Sciences* 13: 97-119.

Kuang, W.W., Thompson, D.A., Moch, R.V. and Weigel, R.J. 1998. Differential screening and suppression subtractive hybridisation identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Research* 26: 111-116.

Lagrimini, M.L., Burkhart, W., Moyer, M. and Rothstein, S. 1987. Molecular cloning of complementary DNA encoding the lignin forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proceedings of the National Academy of Sciences of the USA* 84: 7542-7546.

Liang, P. and Pardee, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 967-971.

Liang, P., Averboukh, L. and Pardee, A.B. 1993. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Research* 21: 3269-3275.

Lisitsyn, N. and Wigler, M. 1993. Cloning the differences between two complete genomes. *Science* 259: 946-951.

Lu, G., Jantasuryarat, C., Zhou, B. and Wang, G.L. 2004. Isolation and characterization of novel defence response genes involved in compatible and incompatible interaction between rice and *Magnaporthe grisea*. *Theoretical and Applied Genetics* 108: 525-534.

Martin, G.B. 1999. Functional analysis of plant disease resistance genes and their downstream effectors. *Current Opinion in Plant Biology* 2: 273-279.

Moore, N.Y., Bentley, S., Pegg, K.G. and Jones, D.R. 1995. Fusarium Wilt of Bananas. *Musa Disease Fact Sheet No. 5*. INIBAP.

Pereza-Escheverria, S., Dale, J., Khanna, H., Smith, M. and Collet, C. 2004. Potential resistance gene against Fusarium wilt race 4. 1st International Congress on *Musa*. Harnessing research to improve livelihoods. Penang, Malaysia, 6-9 July 2004.

Ploetz, R.C. 1994. Panama disease: Return of the first banana menace. *InternationalJournal of Pest Management* 40: 326-336.

Qi, R.-F., Song, Z.-W. and Chi, C.-W. 2005. Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application. *Acta Biochimica et Biophysica Sinica* 37: 283-292.

Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. 1995 Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-470.

Sherf, B.A., Bajar, A.M. and Kolattukudy, P.E. 1993. Abolition of an inducible highly anionic peroxidase activity in transgenic tomato. *Plant Physiol*ogy 101: 201-208.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98:503-517.

Stover, R.H. 1962. Fusarial Wilt (Panama Disease) of Bananas and other *Musa* species. *Phytopathol*ogy Paper 4. Commonwealth Mycological Institute, Kew, Surrey, England.

Velculescu, V.E., Zhang, L., Vogelstein, B. and Kinzler, K.W. 1995. Serial analysis of gene expression. *Science* 270: 484-487.

Xiong, L., Lee, M.-W., Qi, M. and Yang, Y. 2001. Identification of defence-related rice genes by suppression subtractive hybridization and differential screening. *Molecular Plant-Microbe Interactions* 14: 685-692.

```
GCAACTGTTTATGATANGGTTGAAGATCTGGCACCATACCTTCAACAATG     50

                                                5'
AGCTCCGTGTTGCTCCTGAGGAGCATCCTATTCTGCTCACCGAAGCCCCT    100
                                              specific primer
          3'
CTTAACCCAAAGACAAACAGGGAAAAGATGACCCAGATAATGTCTGAAAC    150
ACTINF

TTTCATTGTACCAGCTATGTATGTTGCTATTCAGGGCGTCCTTTCGCTCT    200

ATGYWAGTGSCSGTACTAGTGGTTAGTATCTTGTGATCGCTTACCTGATG    250

TGRAGTTGGTCATTCTTTTTATTTTCAACTRAGATGCCCAAGYTAAATGC    300

                                          3'          5'
TATTTTGACAGGCATTGTACTTGATTCTGGTGATGGTGTCAGCCATACCG    350
                                        specific primer ACTINR

TTCCTATCTATGAAGGATATGCTCTACCTCATGCCATTCTTCGTTTGGAT    400

CTTGCTGGTCGTGATCTCACGGATTGCCTGATGAANATCCTGACAGAGAG    450

GGGTTATTCATTCACTACTACTGCAGAACGGGAAATCGTAAGGGACATCA    500

AGGAGAARCTTGCCTACGTTGCTGKTGACTATGAACAGGAGCTGGATACT    550

GSCGAGACTAGCTCTGCTGTGGAGAAAASYWWKRAMCTTCCCGTTGGGCA    600

RGKTATCACGAWTGGGGCTNRARRWTYANGAGCCCCGGANGTGCTNTYCC    650

AGCCATCATTGATTGGCATGGAARCCNNTGGAGTTCATGAGACAACATAC    700

AACTCTATTATGAAGTGTGATGTGGATATCAGGAAAGATCTGTATGGCAA    750

TATTGTGCTTAGCGGTGGATCAGCAATGTTCCCTGGTATTGCCGATCGCA    800

TGAGCAAGGAGATCACAGCGCTTCCACCAAACAGCATGAAGATAAAGGTG    850

GTTGCCCCACCCGAAAGGAAATACAGCGTCTGGAT                   885
```

Figure 1. Sequence of the cloned actin gene used for Actin gene-based PCRs.

Yellow: Actin Forward primer, Blue: Actin Reverse primer and Red: Intron (90 bp).

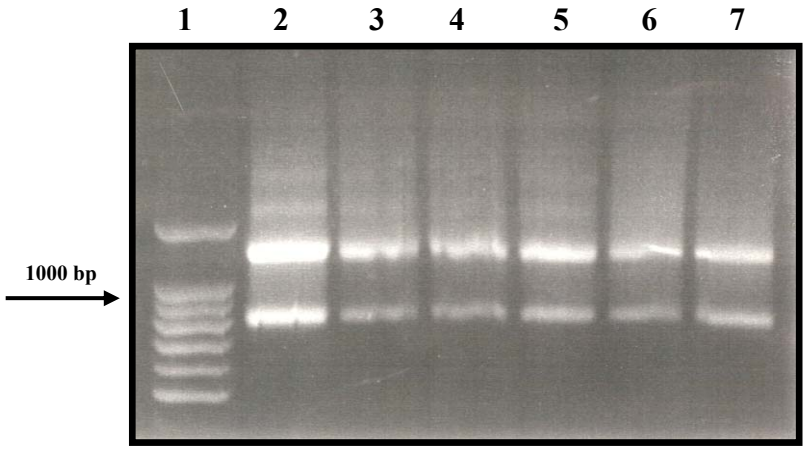

Figure 2. RNA samples from banana root material under non-denaturing conditions. Lane 1 contains the 100 bp marker (Roche Diagnostics), lanes 2-4 GCTCV-218 RNA and lanes 5-7 Williams RNA.

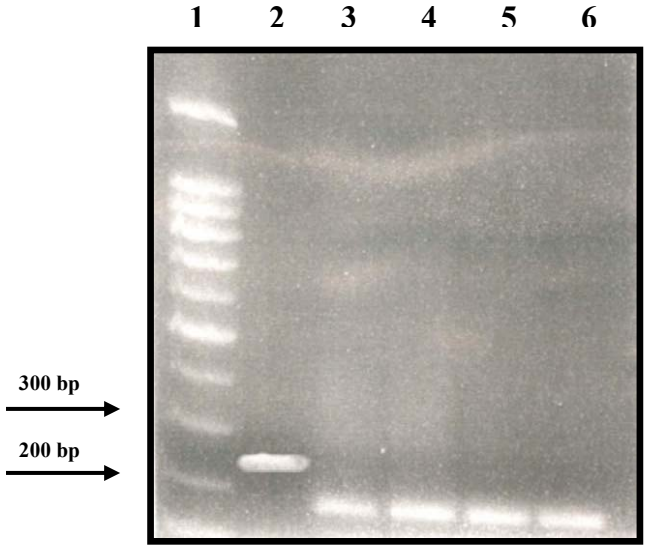

Figure 3. Actin-based control for monitoring contamination of cDNA with genomic DNA. PCR products from amplification (30 cycles) of genomic DNA (260 bp) (Lane 2), PCR products from second strand GCTCV-218 cDNA (Lanes 3 and 4) and Williams cDNA (Lanes 5 and 6) (170 bp) using primer ActinF and ActinR, separated by electrophoresis through a 2% (w/v) agarose gel. 100 bp molecular marker (Roche Diagnostics) (Lane 1) was used to estimate the size of DNA fragments.

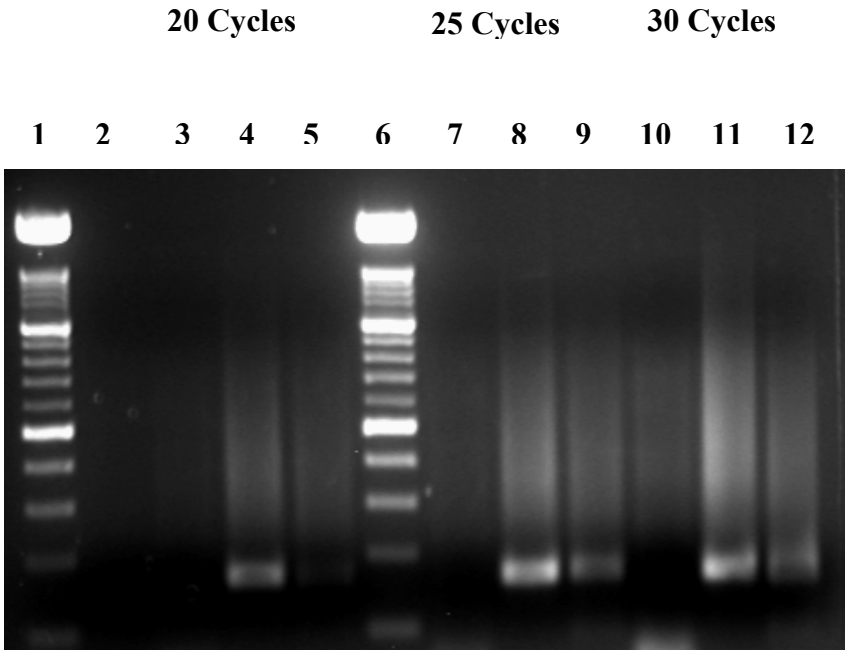

Figure 4. PCR-based assessment of the decrease in actin cDNA levels following Suppression Subtractive Hybridisation. Amplification of the banana actin gene from subtracted "tester" (ST), unsubtracted "tester" (UT) and unsubtracted "driver" (UD) cDNA samples. Lanes 1 and 6, 100 bp marker (Roche Diagnostics); lane 2, water control; lanes 3-5, ST, UT and UD after 20 amplification cycles; lanes 7-9, ST, UT and UD after 25 amplification cycles; lanes 10-12, ST, UT and UD after 30 amplification cycles.

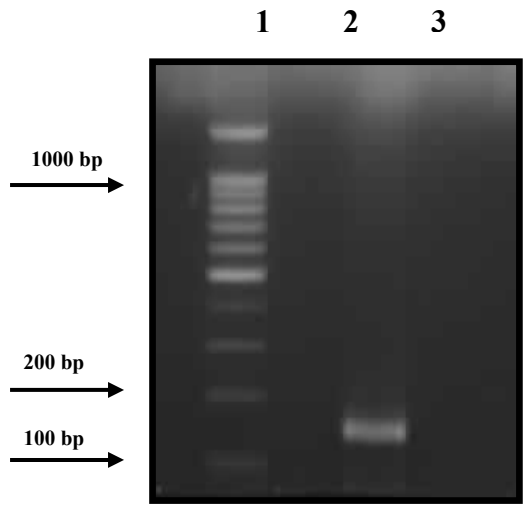

Figure 5. PCR-based assessment of the successful amplification of endochitinase in subtracted "tester" cDNA following Suppression Subtractive Hybridisation.

Lane 1, 100 bp marker (Roche Diagnostics); lane 2, water control; lane 3 endochitinase fragment (149 bp).

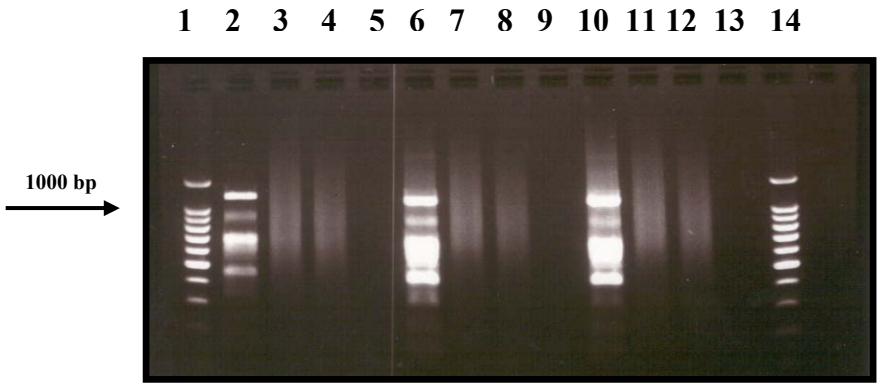

Figure 6. Agarose gel electrophoresis of Suppression Subtractive Hybridisation amplification products. Lane 1 and 14, 100 bp marker (Roche Diagnostics); lane 2, 6 and 10, subtracted "tester" PCR products; lane 3, 7 and 11, unsubtracted "tester" PCR products; lane 4, 8 and 12, unsubtracted "driver" PCR products. Lanes 5, 9 and 13, water controls.

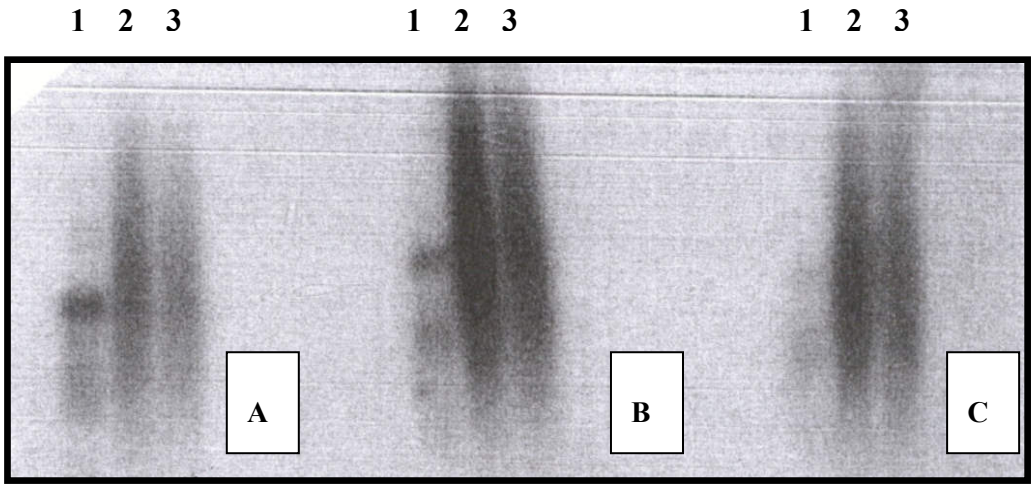

Figure 7. Southern analysis of subtracted "tester" (ST), unsubtracted "tester" (UT) and unsubtracted "driver" (UD) cDNA amplification products.

Amplification products in Fig. 6 were transferred to a positively charged nylon membrane and identical filters were independently hybridized with complex probes derived from forward ST cDNA (Filter A), UT cDNA (Filter B) and UD cDNA (Filter C). Lane 1, ST PCR products; lane 2, UT PCR products; lane 3, UD PCR products.

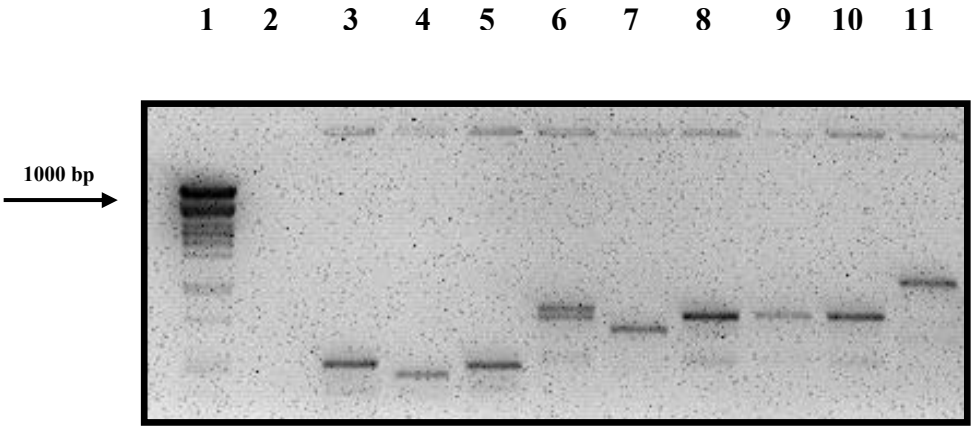

Figure 8. Colony PCR of nine selected Suppression Subtractive Hybridisation cDNA clones indicating single inserts (except lane 6) of variable sizes between 150 and 600 bp. Lane 1 contains the 100 bp molecular marker (Roche Diagnostics) used for size estimations.

Table 1. Primer and adaptor sequences used in the Suppression Subtractive Hybridisation procedure.

Primer or Adaptor name	Nucleotide sequence
PCR primer 1	5'-CTAATACGACTCACTATAGGGC-3'
Nested PCR primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested PCR2	5'-AGCGTGGTCGCGGCCGAGGT-3'
Adaptor 1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GATTATGCTGAGTGATATCCCGAGCTCGCCGGCGGGCCCGTCCA-5'
Adaptor 2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' 3'-GATTATGCTGAGTGATATCCCGTCGCACCAGCGCCGGCTCCA-5'

Table 2. Selected banana cDNA clones expressed in a tolerant banana, GCTCV-218, after *Fusarium oxysporum* f. sp. *cubense* infection, following Suppression Subtractive Hybridisation.

Clone number	Protein similarity	Origin of similar sequence	BLASTX Acc. No. of similar sequence	E-value	Putative Function
2-25	Unspecific monooxygenase, cytochrome P450	*Nicotiana tabacum* L. (tobacco)	pir|T02995	$7e^{-42}$	Defence
2-18	Isoflavone reductase related protein	*Pyrus communis* L. (pear)	gb|AA24001.1	$2e^{-70}$	Defence
1-1	Putative peroxidase	*Oryza sativa* L. (rice)	dbj|BAB19339.1	$6e^{-86}$	Defence
1-5	Putative peroxidase	*O. sativa*	emb|CAE05415.1	$2e^{-05}$	Defence/Stress
2-39	Bowman Birk type proteinase inhibitor	*Phaseolus vulgaris* L. (kidney bean)	sp|P01060|IBB2_PHAVU	$2e^{-05}$	Defence/stress
3-146	Putative senescence associated protein	*Pisum sativa* L. (pea)	dbj|BAB33421.1	$4e^{-43}$	Defence/Stress
3-130	Aux/IAA protein	*Solanum tuberosum* L. (potato)	gb|AAM29182.1	$5e^{-21}$	Auxin-related
3-91	Cytosolic monohydroascorbate reductase	*O. sativa*	dbj|BAA77214.1	$3e^{-04}$	Metabolism
3-26	UTP-Glucose Glucosyltransferase	*Arabidopsis thaliana* (L.) (thale cress)	pir|T01732	$3e^{-04}$	Metabolism (primary)
2-2	Unknown protein	*Hordeum vulgare* (L.) (barley)	gb|AAL77110.1	$5e^{-55}$	Other
2-147	Reversibly glycosylated polypeptide	*Gossypium hirsutum* L. (cotton)	emb|CAC83750.1	$1e^{-14}$	Other
1-97	No significant homology	-	-	-	Other
3-31	No significant homology	-	-		Other
1-95	Unknown protein	*H. vulgare*	AF 474373_7	$1e^{-49}$	Other
2-8	Putative transcription factor	*Periplaneta americana* L. (cockroach)	emb|CAB51041.1 (AJ243883)	$2e^{-14}$	Transcription