SUMMARY

Fusarium wilt of banana has a long and devastating history in many of the world's banana producing countries. The most pronounced damage caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), the Fusarium wilt pathogen, occurred during the 20th century in Central America, where tens of thousands of virgin forests were lost to further banana production. No control strategy is effective against Fusarium wilt other than replacement of susceptible by resistant varieties. It is, therefore, important to develop or identify resistant replacements that would not only be able to resist the pathogen, but also be acceptable to consumers.

Resistance in wild banana varieties has been identified, and hybrids have been developed by breeding programmes with good resistance to Fusarium wilt. These varieties, unfortunately, appear not to be acceptable replacements for Cavendish bananas, the sweet desert banana variety that serves as the primary export banana and constitutes almost 40% of all bananas planted in the world today. A field selection, GCTCV-218, now proved to be the Cavendish plant with the most resistance to *Foc* 'tropical' race 4 (VCG 0121) has saved the Cavendish-based banana industry in Taiwan from devastation. In this thesis, GCTCV-218 has been evaluated against *Foc* 'subtropical' race 4 (VCG 0120), the primary variant of the pathogen in subtropical banana-producing countries such as South Africa, Australia and the Canary Islands. Defence-associated genes that are differentially expressed and that were up-regulated early in the defence response against the pathogen were isolated and identified.

Greenhouse and field trials conducted at the research facilities of the Forestry and Agricultural Biotechnology Institute, University of Pretoria and in Kiepersol, South Africa, respectively, showed that GCTCV-218 had a significantly higher level of disease tolerance against *Foc* 'subtropical' race 4 (VCG 0120) when compared to the commercially grown Williams cultivar. Phenolic assays revealed that total phenolics and cell-wall bound phenolics were expressed at higher levels in GCTCV-218 after pathogen attack and seemed to play an important role in the tolerance of GCTCV-218. It was, therefore, proposed that GCTCV-218 could be considered a replacement for other Cavendish banana varieties planted in South Africa.

The genetic basis of defence mechanisms in banana to *Foc* is unknown. In this investigation, Suppression Subtractive Hybridisation (SSH) was used to construct a cDNA library, containing banana genes that were up-regulated early (3 & 6 hours after infection), in the GCTCV-218/*Foc* interaction. The efficiency of the procedure was confirmed by PCR amplification of a known defence gene (endochitinase) present in the subtracted tester material, as well as analysing the reduction of a known housekeeping gene, actin, in the subtracted material compared to unsubtracted material. Southern blot data further provided confidence in the subtraction process. A cDNA library containing 736 gene fragments was constructed and then subjected to a screening procedure to remove false positives that escaped the subtraction process.

The screening of a banana cDNA library for defence-related genes involved the development of a high-throughput cDNA microarray technique. This novel technique removed all false positives, such as housekeeping genes that escaped the subtraction as well as clones representing rDNAs. Seventy-nine genes differentially expressed in GCTCV-218 and not in Williams were selected, sequenced and subjected to BLASTX, BLASTN and DBest searches. Of these, several gene fragments showed homology to defence-associated genes, and 20 unique genes fragments were identified. These include two different peroxidases, response regulator 6, catalase 2, metallothionein, pectin acetylesterase (PAE), two different unknown proteins, salt stress, trypsin inhibitor, unspecific monooxygenase cytochrome P450, Bowman Birk proteinase inhibitor, root control, xylanase inhibitor, inhibitor CII, hypothetical protein, putative senescence-associated protein, pathogenesis-related protein 1 (*PR1*) and ribosomal protein S3a.

The significance of the defence reaction to Fusarium wilt diseases in agricultural crops depends on the tempo of plant response. When a host plant is able to respond early to pathogen invasion the pathogen is successfully contained, preventing further spread throughout the plant. The expression of genes with antimicrobial activity, such as endochitinase, suggests an induced biochemical defence response against *Foc*. The expression of PAE and *PR1* results in the deposition of lignin and callose production for cell wall strengthening. Four defence associated genes (catalase 2, pectin acetyl esterase (PAE), *PR-1* and endochitinase) were selected for expression profile analysis using Real-time reverse transcriptase PCR, with TaqMan® and Light Cycler

technology. All four genes were shown to be differentially expressed in GCTCV-218 at 3 and 6 hrs after infection, confirming SSH results. *PR-1* and PAE were induced very early (3 hrs after infection) in the GCTCV-218, while *PR3* and catalase 2 followed with a significant induction at 6 hrs after infection. This study concludes that GCTCV-218 is able to respond rapidly in response to *Foc* infection by activating both a biochemical and structural defence mechanism.

High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis

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Efficient construction of cDNA libraries enriched for differentially expressed transcripts is an important first step in many biological investigations. We present a quantitative procedure for screening cDNA libraries constructed by suppression subtractive hybridization (SSH). The methodology was applied to two independent SSHs from pearl millet and banana. Following two-color cyanin dye labeling and hybridization of subtracted tester with either unsubtracted driver or unsubtracted tester cDNAs to the SSH libraries arrayed on glass slides, two values were calculated for each clone, an enrichment ratio 1 (ER1) and an enrichment ratio 2 (ER2). Graphical representation of ER1 and ER2 enabled the identification of clones that were likely to represent up-regulated transcripts. Normalization of each clone by the SSH process was determined from the ER2 values, thereby indicating whether clones represented rare or abundant transcripts. Differential expression of pearl millet and banana clones identified from both libraries by this quantitative approach was verified by inverse Northern blot analysis.

INTRODUCTION

The identification of differentially expressed genes and examination of their patterns of expression are important to gain information about the functions relevant to processes such as cell differentiation, morphological or metabolic changes, and disease development. Various molecular techniques exist for studying differential gene expression, including representational difference analysis, suppression subtractive hybridization (SSH), differential display, differential hybridization, subtractive library construction, serial analysis of gene expression, and cDNA microarrays (1–6). In previous studies, SSH libraries were screened to identify cloned differentially expressed genes by colony blot hybridization or cDNA amplified fragment length polymorphism (AFLP) (7,8). However, these methods are time-consuming and do not allow the level of enrichment of a transcript to be quantified. SSH has also been used as a method to generate a cDNA library to use in subsequent cDNA microarray expression profiling (9). We used cDNA microarrays to screen PCR-amplified clones from SSH libraries to identify genes from pearl millet [*Pennisetum glaucum* (L.) R.Br] and banana (*Musa acuminata*) that are up-regulated during defense responses. This quantitative approach of determining the extent to which transcripts were enriched by the SSH process allowed us to identify and exclude clones that were not derived from up-regulated transcripts and to determine whether transcripts were rare or abundant.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pearl millet breeding lines ICML12=P7 and 842B were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) India and ICRISAT Zimbabwe, respectively. The seed was sterilized and germinated on half-strength Murashige and Skoog (MS) medium (10) at 25°C with a 16 h light/8 h dark photoperiod. Banana plants of a *Fusarium oxysporum* f.sp. *cubense* (*Foc*)-tolerant Cavendish selection and a *Foc*-susceptible Williams cultivar were transferred into plastic cups containing water and maintained in a greenhouse at 18°–25°C with a 16 h light/8 h dark photoperiod. Prior to inoculation, the plants were transferred to an aeroponic system for root inoculation.

Plant Treatments

Leaves of 10-day-old ICML12=P7 and 842B pearl millet seedlings were wounded by pricking leaves at 1 cm intervals with a sterile needle. The undersurfaces of the leaves were inoculated with a total of 100 µL of either 100 mg/mL chitin or a crude boiled extract of flagellin from *Bacillus* sp. Control pearl millet 842B seedlings

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were treated with deionized water. Plates containing pearl millet seedlings were sealed with Micropore™ tape (3M, Isando, South Africa) and incubated at 25°C with a 16 h light/8 h dark photoperiod.

Roots of 15 cm banana plants were inoculated with a 10^5 spores/mL *Foc* spore suspension by injecting the roots with a sterile needle. The inoculation point was sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA) to ensure that spores were not washed off by the aeroponic irrigation system. The plants were maintained under the same conditions as described above until sampling.

RNA Isolation and cDNA Synthesis

RNA was isolated from leaf and root tissue at different time intervals after the treatment and pooled prior to cDNA synthesis and SSH. Pearl millet leaves were harvested 5, 14, and 24 h post-elicitor treatment (hpe). Banana root material was sampled at 3 and 6.5 h post-inoculation (hpi) and immediately placed in liquid nitrogen.

Total RNA was prepared as previously described (11). Poly(A)$^+$ RNA was purified from total RNA using an Oligotex® mRNA Mini Kit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized from mRNA (pearl millet) and total RNA (banana) using a cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany).

Suppression Subtractive Hybridization

SSH was performed as previously described (11). Pearl millet cDNA prepared from the elicitor-treated samples was used as the "tester" and from the control sample as the "driver." Similarly, banana cDNA from *Foc*-infected Cavendish variety plants (tolerant) was used as the tester and from infected Williams (susceptible) plants as the driver. The cDNA enriched for differentially expressed transcripts was termed subtracted tester (ST), whereas unsubtracted tester (UT) cDNA was prepared from treated pearl millet plants and infected tolerant banana plants, and unsubtracted driver (UD) cDNA was prepared from control pearl millet plants and infected susceptible banana plants. The ratio of tester to driver in both experiments was 300:1. ST PCR amplification products ranging from 0.2–1.2 kb were purified and cloned as previously described (11). The banana and pearl millet ST libraries were constructed independently by two researchers.

PCR-Based Control with Actin Primers

ST, UT, and UD were used as templates in PCR with actin primers, actinF (5′-ACCGAAGCCCCTCTTAACCC-3′) and actinR (5′-GTATGGCTGACACCATCACC-3′). 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. PCR amplification was performed using *Taq* DNA polymerase (Roche Diagnostics) at 55°C. Aliquots (5 µL) were removed after 20, 25, and 30 PCR cycles and electrophoresed on a 2% agarose gel.

Southern Blot Analysis

Ten microliters of each ST, UT, or UD cDNAs were digested with *Rsa*I (Roche Diagnostics) to remove adaptors. PCR products minus adaptors were excised from a 1.5% low melting point agarose gel and purified using a QIAquick® Gel Extraction Kit (Qiagen). Products were labeled with digoxigenin (DIG)-dUTP using a DIG DNA Labeling and Detection Kit (Roche Diagnostics), according to the manufacturer's instructions. Hybridizations were performed as described previously (12), using either 5 ng/µL DIG-labeled ST, UT, or UD cDNAs. Hybridization signals were detected using CDP-Star® as outlined by the manufacturer (Roche Diagnostics).

cDNA Microarray Analysis

Libraries containing 960 pearl millet and 736 banana SSH clones were arrayed onto silanized microarray slides (Amersham Biosciences, Little Chalfont, UK) using an Array Spotter Generation III (Molecular Dynamics, Sunnyvale, CA, USA). Cloned inserts were PCR-amplified using SP6 and T7 primers, purified using Multiscreen® PCR Purification Plates (Millipore, Molsheim, France), and visualized on a 1% agarose Electro-Fast® Stretch gel (ABgene, Epsom, UK) prior to being robotically printed onto glass slides. On average, 200 and 300 pg each of pearl millet and banana SSH fragments, respectively, were spotted. The *uidA*, *luc*, and *bar* genes and a fungal rDNA internal transcribed spacer (ITS) fragment were also printed to serve as controls for global normalization. Two hundred nanograms of ST, UT, or UD cDNA probes, following the removal of adaptor sequences, were labeled by the incorporation of Cy™5 or Cy3 dUTP (Amersham Biosciences) using Klenow enzyme (USB, Cleveland, OH, USA) as previously described (13). Each hybridization was performed in duplicate with the reverse cyanin dye labeling of the probes. To ensure that high background hybridization does not occur due to incomplete removal of adaptor sequences, we recommend prehybridization with unlabeled adaptor sequences. Reactions were spiked with cyanin-labeled *uidA* (0.3 ng), *luc* (0.03 ng), and *bar* (3 ng) genes and a fungal ITS fragment (3 ng). After incubation at 37°C for 20 h, the probes were purified using a Multiscreen® PCR Purification Plate (Millipore, Molsheim, France) and eluted in 45 µL sterile distilled water. The probe was dried in a SPD111V vacuum centrifuge (Savant, Holbrook, NY, USA) and resuspended in DIG Easy Hyb Buffer (Amersham Biosciences). The glass slide was initially incubated in pretreatment solution [3.5× standard saline citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA)] without probe at 60°C for 20 min. The glass slide was placed in a HybUP hybridization chamber (NB Engineering, Pretoria, South Africa) with the probe at 42°C for 16 h. After hybridization, the slides were washed for 4 min at 42°C with 1× SSC/0.2%

Table 1. Primary Microarray Data

http://fabinet.up.ac.za/microarray/SSH/Banana_raw_data.xls
http://fabinet.up.ac.za/microarray/SSH/Banana_modified_data.xls
http://fabinet.up.ac.za/microarray/SSH/Banana_microarray_image.jpg
http://fabinet.up.ac.za/microarray/SSH/PearlMillet_raw_and_modified_data.xls
http://fabinet.up.ac.za/microarray/SSH/PearlMillet_microarray_image.jpg

SDS, 0.1× SSC/0.2% SDS (twice), followed by three washes in 0.1× SSC for 1 min at room temperature. The slides were rinsed with distilled water, dried with high-pressure nitrogen, and scanned with a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA). The computer program ArrayVision™ (Molecular Dynamics) was used to localize and integrate every spot on the array (Table 1).

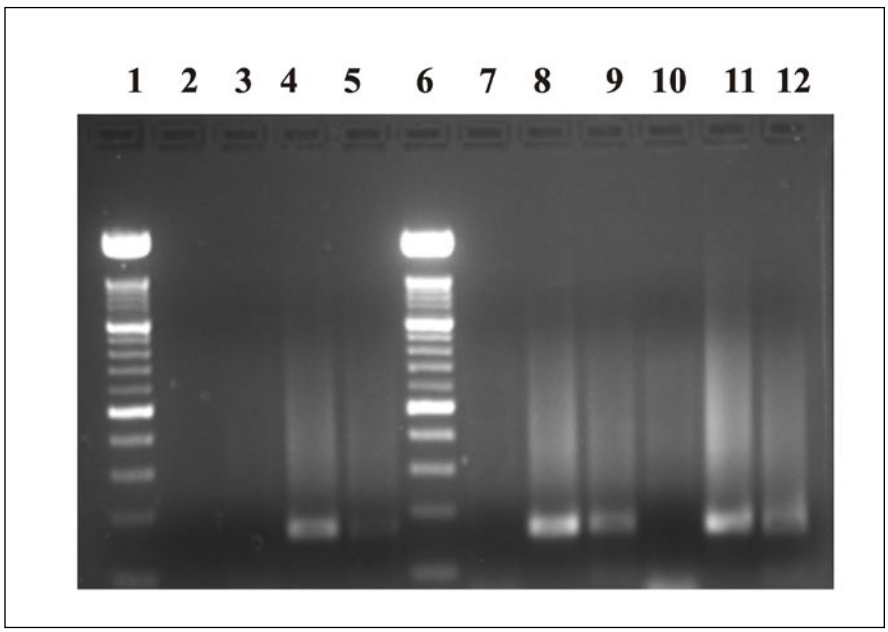

Figure 1. Amplification of the banana actin gene from subtracted tester, unsubtracted tester, and unsubtracted driver cDNA samples. Lanes 1 and 6, DNA Molecular Weight Marker XIV, 100 bp ladder (Roche Diagnostics); lane 2, water control; lanes 3–5, subtracted tester (ST), unsubtracted tester (UT), and unsubtracted driver (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.

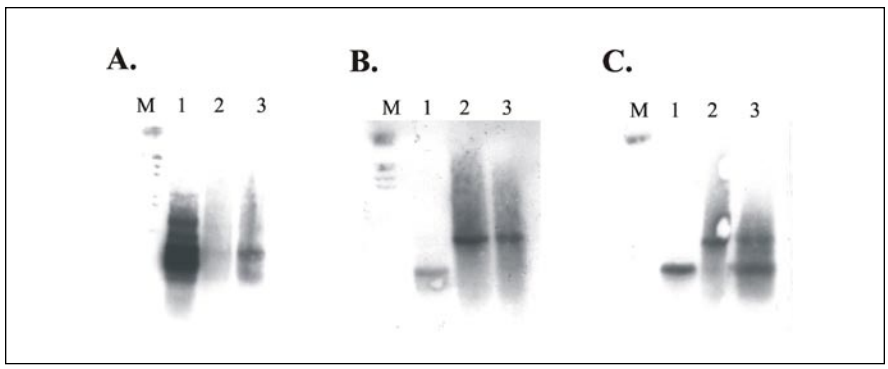

Figure 2. Southern hybridizations to pearl millet subtracted tester (lane 1), unsubtracted tester (lane 2), and unsubtracted driver (lane 3) to analyze library-specific enrichment following SSH. Identical filters were independently hybridized with complex probes derived from (A) subtracted tester (ST), (B) unsubtracted tester (UT), and (C) unsubtracted driver (UD). M represents a digoxigenin (DIG)-labeled Marker III (Roche Diagnostics). SSH, suppression subtractive hybridization.

Enrichment ratios of ST:UD (ER1) and ST:UT (ER2) were calculated from experiments performed in duplicate. For each clone, background signal intensities were subtracted, signal intensities of duplicate spots on glass slides were averaged, and spots with a signal-to-noise ratio of less than 2 (banana) or 3 (pearl millet) were rejected. Global normalization of data for the cyanin dye effect was performed using a control gene set to calculate normalization functions c and c′ for each pair of dye swap slides (14). To determine ER1, slides were hybridized with ST and UD. ER1 was calculated using the following formula (14):

$$\tfrac{1}{2}[\log_2 \text{Cy3 ST/Cy5 UD} - c - (\log_2 \text{Cy3 UD/Cy5 ST} - c')]$$

ER2 was calculated in the same way, following hybridizations with ST and UT. UT/UD values (Table 2) were calculated from the ER1 and ER2 values as follows. Because ER1 - ER2 ~ $\log_2$ ST/UD - $\log_2$ ST/UT = $\log_2$ UT/UD, therefore UT/UD = antilog of (ER1 - ER2) in the base 2. The primary data sets, along with a figure showing hybridization to the microarrays, are shown in Table 1.

rRNA redundancy for pearl millet and banana libraries was determined by hybridizing glass microarray slides with pearl millet and banana rDNA probes, respectively. A clone was considered to have hybridized to an rDNA probe if its fluorescence was more than two standard deviations above local background fluorescence (15).

Inverse Northern Dot Blots

Amplified, denatured inserts of selected clones from banana and pearl millet SSH libraries were applied to a positively charged Hybond™ nylon membrane (Amersham Biosciences) as previously described (11). Poly(A)$^+$ mRNA, used for pearl millet probe generation, was isolated from 50 µg freshly prepared total RNA (tester and driver). Banana tester and driver probes were prepared from 30

µg total RNA. cDNA was labeled with DIG-dUTP using the DIG DNA Labeling and Detection Kit. Hybridizations were performed as described previously (12), using 20 ng/µL cDNA. Hybridization signals were detected using CDP-Star.

ArrayVision was used to calculate signal density, following normalization by comparing values of rDNA dots. Inverse Northern expression ratios were calculated by dividing normalized density measurements for each clone hybridized with the tester

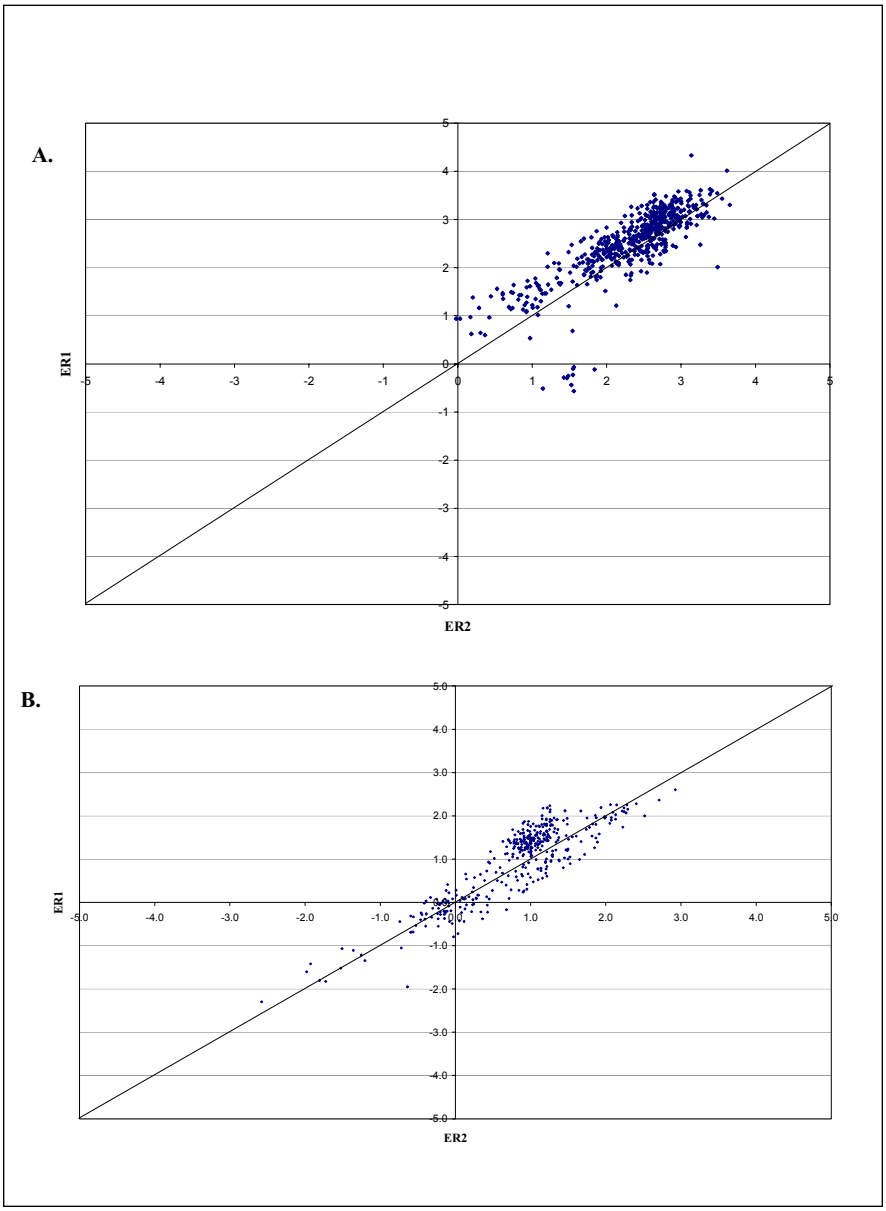

Figure 3. Screening pearl millet (A) and banana (B) SSH libraries on glass microarray slides. Suppression subtractive hybridization (SSH) enrichment ratio 1 (ER1) is plotted against SSH enrichment ratio 2 (ER2). ER1 was calculated for each clone by $\log_2$ transforming the value of the subtractive tester (ST) fluorescence divided by the unsubtracted driver (UD) fluorescence. ER2 for each clone was calculated by $\log_2$ transforming the value of the ST fluorescence divided by the unsubtracted tester (UT) fluorescence. The diagonal line indicates clones derived from transcripts of equal abundance in UD and UT (i.e., ER1 = ER2). Clones that lie above the diagonal line represent transcripts that are induced upon treatment (ER1 > ER2), while those below the line indicate transcripts that have escaped the subtraction (ER1 < ER2). Clones above the line with a positive ER2 value represent rare transcripts, whereas clones above the line with a negative ER2 value are regarded as abundant and have been reduced in relative concentration during normalization.

probes, with values for the same clones hybridized with the driver probe.

RESULTS AND DISCUSSION

SSH cDNA Library Construction

To identify differentially expressed pearl millet defense response genes, SSH was performed between cDNAs from the downy mildew-resistant line ICML12=P7 that had been wounded and treated with elicitors and cDNAs from an untreated downy mildew susceptible line, 842B. SSH experiments for banana were designed to isolate unique defense response genes from a tolerant variety in response to *Foc*. SSH was therefore performed using two near-isogenic banana cultivars, a Cavendish selection (tolerant) and Williams (susceptible) from the Cavendish group of banana varieties.

Two tests were performed to investigate the efficiency of the SSH. First, PCR was used to amplify a representative housekeeping gene (actin) to show the successful removal (reduction in the levels) of this gene in the ST material (result for the banana SSH shown in Figure 1). Specific PCR products were visible after 20 cycles from the UT and UD material, whereas no PCR product was detected in the ST material even after 30 PCR cycles. In addition, the efficiency of subtraction was evaluated by Southern blot analysis. Complex ST probes hybridized mainly to the ST PCR products, with far less hybridization to UT and UD PCR products (Figure 2A). Furthermore, very little UT and UD probe hybridized to the ST material (Figure 2, B and C), demonstrating gene transcripts common to both UD and UT had been removed by the subtraction and thus implying enrichment for tester-specific transcripts. Similar results were obtained with the banana SSH (data not shown). The SSH procedure yielded cDNA libraries of 960 pearl millet clones and 736 banana clones.

Screening of the SSH Libraries Using Glass Slide Microarrays

Table 2. Validation of Microarray Screening of Selected Pearl Millet and Banana SSH Clones by Inverse Northern Data

Clone No.	ER1[a]	ER2[b]	UT/UD Ratio[c]	Inverse Northern Expression Ratio[d]
Pearl Millet				
3-D5	1.4	0.2	2.3	34.3
4-H11	1.2	0.3	1.9	21.1
10-B7	1.0	0.2	1.7	11.1
4-H9	0.6	0.3	1.2	9.3
5-C3	1.5	0.7	1.7	7.9
1-B7	1.6	0.5	2.1	7.2
6-A2	1.2	0.7	1.4	6.3
8-D7	1.0	0.4	1.5	6.2
4-A2	0.9	0.03	1.8	5.8
4-E12	0.9	-0.02	1.9	5.4
7-D7	1.6	0.8	1.7	5.3
2-A12	3.6	3.4	1.1	3.6
6-F1	2.6	2.0	1.5	3.3
2-A8	3.6	3.3	1.2	2.6
6-H1	2.2	1.9	1.2	2.5
6-C2	3.0	2.7	1.2	2.3
6-D1	2.6	2.5	1.1	1.5
3-H3	4.3	3.1	2.3	1.3
6-G2	2.0	1.7	1.2	1.1
10-C6	-0.3	1.5	0.3	0.9
Banana				
2-35	0.3	-0.1	1.3	6.9
2-21	1.8	0.9	1.9	1.5
2-18	2.1	1.5	1.6	1.4
2-45	1.4	1.0	1.3	1.0
1-1	0.8	0.9	0.9	0.98
2-14	0.7	0.9	0.9	0.97
3-94	-0.1	0.2	0.8	0.94
2-134	1.5	1.6	0.9	0.1
3-156	0.6	0.8	0.9	0.1

SSH, suppression subtractive hybridization; ST, subtracted tester; UD, unsubtracted driver; UT, unsubtracted tester.
[a,b]Enrichment ratio 1 and 2 (ER1 and ER2) were calculated from the microarray screening as $\log_2$(ST/UD) and $\log_2$(ST/UT), respectively.
[c]UT/UD = antilog of (ER1 - ER2) in the base 2.
[d]The inverse Northern expression ratio was calculated as follows: density of tester/density of driver samples after normalization of the data using an rDNA clone.

cDNA microarray technology was used to perform a high-throughput screen of the pearl millet and banana SSH cDNA libraries to identify genes expressed in response to pathogen elicitor treatment and *Foc* infection, respectively, and to obtain information about the relative abundance of these gene transcripts upon induction of plant defense responses. The aim was to identify and discard housekeeping and rRNA genes that had escaped subtraction and to select defense response-associated genes for sequencing.

The SSH libraries arrayed on the glass slides were screened with rDNA clones from pearl millet or banana. The rDNA probes hybridized to 5% of the pearl millet clones and 28% of the banana clones (data not shown). The banana library had been made from total RNA, whereas the pearl millet library had been made from mRNA, which may explain why more banana rDNA clones escaped subtraction.

After hybridization with combinations of the SSH cDNAs, enrichment ratios were calculated for ST:UD (ER1) and ST:UT (ER2). A positive ER1 or ER2 value indicates transcripts that have been enriched during subtraction relative to their levels in UD or UT, respectively. Conversely, negative ER1 or ER2 values indicate transcripts that have been reduced in abundance during SSH relative to their levels in UD or UT, respectively, due to normalization. Normalization equalizes the concentration of individual transcripts, which may be present at very different concentrations prior to normalization (2). This has the advantage of enriching for rare transcripts in the subtracted tester samples and reducing the levels of abundant transcripts.

Relative abundance of cDNAs in UD and UT was visualized by plotting ER1 versus ER2 for individual pearl millet and banana clones (Figure 3). The diagonal line on each graph (ER1 = ER2) in Figure 3 represents similar levels of enrichment/normalization during SSH relative to UD and UT. Clones lying on this line are derived from transcripts of equal abundance in UD and UT. The majority of clones (77% for the pearl millet SSH and 63% for the banana SSH) lie above this line (ER1 > ER2), indicating a greater abundance of these transcripts in UT than in UD and confirming that each SSH has enriched for transcripts that are up-regulated in the tester. Clones above the diagonal line with positive ER2 values are likely to be derived from low abundance transcripts; expression of some of these may be difficult to detect in Northern blots, and accurate comparisons of gene expression between tester and driver may require real-time reverse transcription PCR (RT-PCR). Several clones above the diagonal line showed negative ER2 values, representing transcripts enriched relative to levels in UD and reduced in abundance during normalization relative to levels in UT. This was more evident in the banana SSH (Figure 3B). Such clones represent abundant transcripts specifically up-regulated in the tester that should be readily detected in Northern blot analyses.

Inverse Northern Blot Analysis of Selected Transcripts

To validate conclusions drawn from comparisons of ER1 and ER2 and to confirm that clones are derived from differentially expressed transcripts, clones from each library were selected for inverse Northern blot analyses. These clones were arrayed in duplicate on dot blots and hybridized to freshly prepared nonamplified tester and driver cDNAs.

ER1 and ER2 ratios from the microarray screening (Figure 3), together with the inverse Northern expression ratios of the selected pearl millet and banana clones, are shown in Table 2. Clones with ER1 > ER2 had inverse Northern expression ratios greater than one, confirming that these clones represented transcripts that were up-regulated in the tester compared to the driver (data for 19 pearl millet and 4 banana clones are shown in Table 2). Clones where ER1 < ER2 showed inverse Northern expression ratios of less than one (10-C6, 1-1, 2-14, 3-94, 2-134, 3-156) confirmed that they represented transcripts that were more abundant in the driver than the tester and that these clones had escaped the subtraction process. In order to relate the inverse Northern and microarray data, a ratio of UT/UD was calculated from the ER1 and ER2 ratios (Table 2). As expected, inverse Northern ratios correlated with UT/UD ratios (i.e., clones with inverse Northern ratios >1 had UT/UD values >1), whereas clones with inverse Northern ratios <1 gave UT/UD <1 (Table 2).

Although the majority of clones in the two SSHs showed ER1 > ER2, there were differences between the two SSHs (Figure 3). The pearl millet SSH, which used mRNA for the subtraction and was based on treated tester and untreated driver material, yielded a majority of clones that were enriched in ST relative to UT. In contrast, the banana SSH, based on total RNA and involving tester and driver samples treated in the same way (i.e., both challenged with *Foc*), but in which different defense responses were anticipated, yielded more clones that were reduced in abundance in ST relative to UT. Many of the clones

enriched in this latter SSH were of equal or greater abundance in UD than UT. Therefore, whereas the vast majority of clones in the pearl millet SSH could be confidently included in downstream analyses as likely derived from up-regulated genes, many of the clones in the banana SSH would be eliminated from further analyses. This emphasizes the value of the approach for quantitative characterization of the SSH and in selecting clones for further study.

We have effectively demonstrated the use of cDNA microarrays to screen two independently constructed pearl millet and banana SSH libraries in a rapid, high-throughput manner. The major advantages of our screening method are that it provides an objective and quantitative way to identify differentially expressed genes as well as to determine the relative abundance of transcripts in the original UT samples.

Previous studies have used inverse Northern blot analysis to screen SSH libraries in which PCR products or colonies are dotted onto nylon membranes, and the driver and tester cDNAs are labeled with radioactivity (2,7). This method has disadvantages; namely, that comparisons are made between two separate membrane hybridizations, which introduces error, and the interpretation is qualitative (7) unless a laboratory has access to a phosphorimager. In contrast to membrane-based methods, hybridization to glass slide cDNA microarrays can be performed with different fluorescent tags, which allows a direct comparison of the relative abundance of transcripts in ST, UT, and UD. Furthermore, hybridizations are performed on a small surface area, which reduces the amount of labeled probe needed. Finally, the computerized scanning of the array provides a high-throughput quantitative method to choose which genes to sequence and study further using Northern blot analysis, real-time RT-PCR, or a custom microarray.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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