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A cDNA-AFLP protocol with reciprocally arranged 2-enzyme sequential digestion and silver staining detection

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Abstract: This study provides a modified protocol to the standard cDNA-amplified fragment length polymorphism (AFLP) procedure that has been used to detect differentially expressed transcripts in various systems. Modifications aimed to improve the overall coverage of the technique and the isolation of single fragments from each of the cDNA species. The protocol utilizes oligo-d(T) coupled magnetic beads to isolate and synthesize, and 2-enzyme sequential digestions of ds-cDNA molecules with flip-flop strategy on the beads to release transcript tags representing individual mRNA type and quantity. The protocol has been applied to detect *Puccinia sorghi*-induced expressional changes in maize leaf material sampled at 8 time points following inoculation. cDNA-AFLP analysis of this material pooled into 2 time intervals (6–24 h and 36–96 h), along with their controls, revealed banding patterns in which 10–20 differentially expressed message tags were present among the 40–80 bands detected per primer combination. Three hundred and ten differentially expressed message tags were sequenced, and the majority with known functions were found to be associated with plant–microbe interactions. Although it requires a few additional steps, this protocol appears to be effective in revealing differentially expressed messages, and the study demonstrates that pooling the material in the preparation of templates and silver staining detection provides economical alternatives for surveying and identifying expressional modulations occurring over relatively longer time periods.

Key words: Maize, *P. sorghi*, plant–microbe interaction, cDNA-AFLP, differential gene expression

1. Introduction

Genome-wide expression analysis methods provide valuable tools for displaying gene expression modulations induced by certain conditions, developmental stages, and various other biological phenomena. Studies of such expressional changes can give clues about what mechanisms, physiological processes, and biochemical pathways are activated or inactivated in response to applied conditions. Such displays may also provide information in relating the large number of cDNA sequences compiled in GenBank databases with unknown functions to certain events and phenomena. Relatively straightforward differential expression analysis methods such as mRNA differential display (DDRT-PCR) and cDNA-amplified fragment length polymorphism (AFLP) are 2 alternatives that do not require sequence/cDNA information and allow detection of spatial and temporal gene expression changes occurring in response to various internal or external factors in many genes simultaneously (Liang and Pardee, 1992; Bachem et al., 1996). These methods, however, have certain drawbacks, such as high false-positive rates and transcriptome coverage concerns. The latter is especially true for cDNA-AFLP, to which several improvements have been made (Breyne et al., 2003; Fukumura et al., 2003; Vuylsteke et al., 2007; Weiberg et al., 2008; Korpelainen et al., 2010; Liu et al., 2011).

A protocol with reciprocally arranged 2-enzyme sequential digestion for improving cDNA-AFLP coverage is presented and applied to detect differentially expressed genes in the maize–maize common rust pathosystem.

2. Materials and methods

2.1. Biological materials, growth conditions, and experimental setups

A near-isogenic (NIL) *Rp1* line, *Rp1-G*, one of the *Rp1* NILs developed by Hooker and his colleagues in the 1960s (Hooker, 1969), along with the A188 inbred and a *Puccinia sorghi* isolate (T09) prepared from a locally collected rust sample, were used as biological materials in the study. Rust isolate was prepared via inoculation of susceptible seedlings in a rust-free room with spores obtained from a fresh single pustule of the rust sample. The isolate was found to be avirulent on a set of *Rp1* differentials including *Rp1-G* and *Rp1-D* (Hooker, 1969), and virulent on the inbred, A188,

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which was presumed to contain no resistance genes and thus to be susceptible to all known rust races. The isolate grew on A188 without any resistance reaction, while no rust growth was observed on *Rp1-G*, which displayed typical water-soaking signs for 24–48 h in and around infection sites with the subsequent disappearance of all infection symptoms. Unlike *Rp1-G*, *Rp1-D* and several other lines developed typical hypersensitive responses of varying degrees and phenotypes; in some, small pustules surrounded necrotic areas.

Before inoculation, both A188 and *Rp1-G* seedlings were grown and maintained in rust-free chambers. Seven-day-old seedlings were divided into control and inoculation (treatment) groups. Control-group plants of both resistant and susceptible genotypes were rubbed with ddH_2O, while treatment groups of both genotypes were inoculated with freshly collected urediniospores of T09 by rubbing with ddH_2O-wetted fingertips as in the control groups. Following inoculation, both control and treatment plants were incubated overnight (in dark) at 18 °C in a chamber with >95% humidity and subsequently transferred to a room with a 16/8-h day/night photoperiod at 24–27 °C. As 3 parallel experiments, control and treatment group tissue samples were collected at 6, 12, 18, 24, 36, 48, 72, and 96 postinoculation hours (pih). In each sampling event, an approximately 7-cm segment (extending from tip to base) of the second leaf (which was about 0.1 g) was taken, placed into sterile 1.5-mL Eppendorf tubes within a few seconds, frozen in liquid nitrogen, and stored until grinding and addition of extraction buffer. The sampling intervals were selected according to the observations of Rijkenberg et al. (1992).

2.2. Total RNA isolation

For total RNA isolation, approximately 100 mg of leaf tissue frozen in liquid nitrogen was ground into a fine powder in 1.5-mL Eppendorf tubes using a pointed-tip screwdriver cooled in liquid nitrogen. Without thawing, 1 mL of RNAzol (Molecular Research Center) extraction buffer was added to the powder and mixed to obtain a homogenate, which was either stored at –20 °C for later use or processed for RNA extraction according to the manufacturer's instructions. Briefly, the extraction procedure was as follows: homogenates were kept at room temperature (RT) for 10–15 min with occasional mixing. Following the addition of either 400 µL ddH_2O or DEPC-treated water, homogenates were mixed vigorously for 15 to 20 s and kept at RT for 10–15 min, and then DNA, proteins, and carbohydrates were selectively precipitated via centrifugation at 12,000 × *g* for 15 min. Subsequently, 1 mL of supernatants was transferred into nuclease-free Eppendorf tubes, and RNA was precipitated via centrifugation at 12,000 × *g* for 7–8 min after the addition of 400 µL 75% ethanol (prepared with DEPC-treated water) to each supernatant tube and storing at RT for 5–10 min. RNA pellets were washed twice, each time with 400 µL of 75% ethanol prepared with DEPC-treated water and were briefly dried to evaporate the ethanol. RNA was dissolved in 50–100 µL of DEPC-treated water. Nucleic acid concentrations were measured spectrophotometrically at 260 nm, and the average yield was found to be approximately 60 µg/100 mg leaf tissue with an A_{260}/A_{280} ratio of 1.7–1.8.

cDNA-AFLP template preparations were carried out using RNA extracted from homogenate samples pooled for specific time intervals; for example, in the A188 treatment, 1 pool (AT1) was constituted by combining 250 µL of homogenate samples from each of the 6, 12, 18, and 24 h time-points using sterile P1000 bore tips. In the same way, 6 homogenate pools including control pools of susceptible (A188) and resistant (*Rp1-G*) genotypes—A188 control, AT1 (6–24 h), AT2 (36–96 h), *Rp1-G* control, GT1 (6–24 h), and GT2 (36–96 h)—were constituted from individual time-point samples, and RNA from pooled homogenates was extracted as described above. RNA was also extracted from rust spores germinated overnight on sterile dH_2O. A total of 7 RNA samples were used in cDNA-AFLP template preparations.

2.3. mRNA purification and cDNA and ds-cDNA syntheses

From total RNA, mRNA isolations were carried out using the NEB (New England Biolabs) magnetic mRNA separation system according to the manufacturer's instructions. Using 0.5 mg of oligo-$d(T)_{25}$ coupled magnetic beads and approximately 50 µg of total RNA per reaction, mRNA isolation and washing steps were performed. At the last step, without mRNA elution, cDNA syntheses were carried out using bound oligo-$d(T)_{25}$ as the primer with 200 U of reverse transcriptase H^- enzyme (Fermentas) in 30-µL reaction volumes containing other constituents as follows: 1X Fermentas RT H^- buffer (50 mM Tris-HCl, pH 8.3 at 25 °C, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT), 1 mM of each of the dNTPs, and 20 U of RiboLock (Fermentas). Subsequently, the reaction mixtures were combined individually with the 120-µL ds-cDNA synthesis cocktail, which contained 1X *E. coli* DNA polymerase I buffer (50 mM Tris-HCl pH 7.5 at 25 °C, 10 mM $MgCl_2$, 1 mM DTT), 1 µL of 10 mM dNTP mixture, 1.5 U of RNase H (Fermentas), and 3.5 U of *E. coli* DNA polymerase I; synthesis was carried out with incubation at 15 °C for 2 h.

2.4. Restriction enzyme combination selection via in silico cDNA-AFLP simulations

The sequences of 8878 cDNA clones obtained from a 2-week-old B73 seedling shoot cDNA library (Barkan Lab) were used to choose an appropriate enzyme combination in the web-based AFLPinSilico program (Rombauts et al., 2003), which gives distribution, sizes, and number

of transcript tags (cDNA-AFLP fragments) for the given AFLP primer pairs. With the specified enzyme pair and 3' extension nucleotide combinations, this program retrieves sequences from the data file and simulates a transcriptome-wide cDNA-AFLP experiment to determine the sizes and numbers of transcript tags. A number of enzyme combinations with the majority of possible +2 extension nucleotide combinations (16 × 16) at 3' ends of both AFLP primers were tried; based on the results, the *Taq* I (T↓CGA) and *Csp6* I (G↓TAC) enzyme combination was found to be optimal for our cDNA-AFLP experiments, covering about 65%–70% of the transcripts.

2.5. Reciprocally arranged sequential Csp6 I and Taq I digestions of ds-cDNAs

Following ds-cDNAs synthesis, reaction tubes were mixed to obtain homogeneous mixtures; each was divided into 3 parts of 50 µL each, and ds-cDNAs in each tube were separated by pelleting magnetic beads on a magnetic stand and removing the reaction mixture by pipetting. One 50-µL part was saved for future use; the remaining 2 ds-cDNA pellets were washed twice with 40 µL of either 1X *Csp6* I or 1X *Taq* I restriction enzyme buffers. In each wash, supernatants were removed by pipetting after pelleting ds-cDNAs on the magnetic stand. After the final wash, pellets were dissolved again in 40 µL of 1X respective restriction enzyme buffer containing 1 µL (10 U) of either *Csp6* I or *Taq* I per reaction. Digestions were performed at the appropriate enzyme optimum temperatures (which are 65 °C for *Taq* I and 37 °C for *Csp6* I) for 2 h. Following the first digestion, the remaining ds-cDNAs coupled to beads were pelleted, the supernatants were dumped, and the pellets were washed with the second restriction enzyme buffers by suspending and pelleting ds-cDNAs in 40 µL of 1X respective buffer. Subsequently, 40 µL of 1X respective restriction enzyme buffer containing 1 µL (10 U) of either *Csp6* I or *Taq* I per reaction was added to the pellet tubes, and the reaction tubes were incubated at the respective enzyme optimum temperatures as in the first digestion step. These sequential digestions with their reciprocal arrangements in 2 parallel digestion experiments (called flip-flop) liberated mRNA tags (cDNA restriction fragments) from the magnetic-bead–coupled ds-cDNAs. Following the second digestion, all reaction tubes were heated to 65 °C for 10 min to release restriction fragments from their cutting sites. After that, the remaining magnetic-bead–coupled ds-cDNAs were again pelleted on the magnetic stand, and supernatants were transferred into new tubes. mRNA tags released via sequential digestions of the same group, e.g., AT1 ds-cDNAs cut with *Csp6* I–*Taq* I and *Taq* I–*Csp6* I sequential combinations (flip-flops), were combined into the same tube and used as one preparation in adapter ligation. As in homogenate pooling, 7 groups of restriction fragments derived from 2 reciprocally arranged sequential digestions of ds-cDNAs were obtained with these manipulations.

2.6. cDNA-AFLP analysis

Csp6 I and *Taq* I (ds) adapters were prepared as 50-µM solutions from individually synthesized oligos (Invitrogen, Life Technologies) having sequences (underlined positions are cohesive ends) *Csp6* I top strand, 5'-CTCGTAGACTGCCTACC-3'; *Csp 6* I bottom strand, 5'-TAGGTAGGCAGTC-3'; *Taq* I top strand, 5'-GATGAGTCCTGACCGAAC-3'; and *Taq* I bottom strand, 5'-CGGTCAGGACTCAT-3' by heating for 2 min at 99 °C, then keeping at 65 °C for 10 min and cooling to room temperature in 10 mM Tris buffer (pH 7.6) containing 100 mM NaCl. Individual adapter ligations were performed in 50-µL reaction volumes, each containing 50 pmol (1 µL) of each of these ds-adapters, 40 µL of *Csp6* I↔*Taq* I double-digestion solution, 1X T4 DNA ligase buffer, and 1 U of T4 DNA ligase, with incubation at 22 °C for 2 h.

Preselective amplification-PCR experiments were carried out with 5 pmol each of the *Csp6* I + 0 (5'-TCGTAGACTGCCTACCTAC-3') and *Taq* I + 0 (5'-GACGATGAGTCCTGACCGA-3') primers and 5 µL of 5-fold diluted adapter ligation solution in 30-µL PCR reactions, which additionally contained 1X PCR buffer (10 mM Tris-HCl pH 8.8 at 25 °C, 50 mM KCl, 0.08% Nonidet P40), 1.5 mM $MgCl_2$, 200 µM dNTP mix, and 1.5 U of Taq DNA polymerase (Fermentas). PCR conditions were as follows: 94 °C for 30 s denaturation, 56 °C for 30 s annealing, and 72 °C for 1 min extension, which was repeated for 23 cycles. Amplification product size range and quantity were assessed via electrophoresis of 5 µL of reaction mixture in a 2% 0.5X TBE agarose gel, which was run at 10 V/cm for 1 h. An amplification product smear ranging between 50 and 700 bp was observed in all 7 of these reactions.

Selective amplification-PCRs were carried out with 5 pmol from each of the *Csp6* I (5'-TAGACTGCCTACCTACNN-3') and *Taq* I (5'-GATGAGTCCTGACCGANN-3') primers having 2 selective nucleotide extensions at their 3' ends in 15-µL reaction mixtures, each additionally containing 3 µL of 50-fold diluted preamplification products, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mixture, and 0.5 U of Taq DNA polymerase (Fermentas). PCRs were carried out in the following conditions: 1 cycle with the denaturation segments at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 60 s; during the subsequent 12 cycles, denaturation and extension time/temperature values were kept the same, but the annealing temperature was lowered by 0.7 °C per cycle. This touchdown period was followed by an additional 24 cycles with the time/temperature profiles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C, with a final extension step at 72 °C for 5 min.

PCR products were mixed (1:1) with 2X sequencing loading dye (containing 98% deionized formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol) and denatured for 3 min at 90 °C, and 4 µL was loaded onto preheated 6% denaturing polyacrylamide gels containing 7 M urea. Samples were electrophoresed in 1X TBE for 2 h at 50 W until the xylene cyanol dye (comigrates with about 100 bp) positioned at about 15 cm of the gel front. cDNA-AFLP bands were visualized via silver staining as described by Bassam et al. (1991). Dried gels were photographed using a digital gel documentation system (Eastman Kodak Company). A total of 74 primer combinations were used to detect differentially expressed messages in the interaction of susceptible and resistant genotypes with *P. sorghi*.

2.7. Isolation and sequence characterization of differentially expressed mRNA tags

Differentially expressed bands were recovered from the glass-backed dried gels by rehydrating the gel at the band position with a 4-µL ddH_2O drop for 1 min, peeling it with the same 10-µL tip, and transferring into 75 µL of ddH_2O in 0.5-mL tubes. These solutions were then boiled for 15 min at 100 °C and incubated at 4 °C overnight; 3 µL of the solution was then reamplified in 25-µL reaction mixtures using the same primer combinations and conditions as in the selective amplification PCR. Products were gel-purified by running in 2% 0.5X TBE agarose gels and excising and eluting fragments from the gel slices. A large number (238 of 310) of these products were directly sequenced using either of the cDNA-AFLP primers, while 72 fragments were ligated into T-vectors (pTZ57 or pUC18), transformed into *E. coli* JM107 cells, and sequenced with either of the plasmid-anchored M13 primers. Both types of sequencing experiments were conducted by commercial service providers (either Refgen Biotechnology or Bioer), and the results were provided as sequencing files. Homology screens of the sequences were carried out in the National Center for Biotechnology Information nucleotide and protein sequence databases using BLAST routines (http://www.ncbi.nlm.nih.gov/BLAST). Sequence homologs with the lowest E-values, along with their related literature information, were collected in Word files, and homologs with the lowest E-values were considered to be the sequence homologs of the transcript-derived fragments (TDFs).

2.8. sqRT-PCR confirmations of interaction-induced expressional modulations

TDFs showing clear expressional modulations with presumed plant–microbe interaction associated functions were selected for sqRT-PCR confirmations. sqRT-PCR primer pairs were designed using a web-based program, Primer3Plus (http://sourceforge.net/projects/primer3), with its modified parameters for amplifying products of between 90 and 260 bp in length, which mostly covers the BLAST homology segments. The first 5 primer pairs developed by the program were further examined visually for their cross-dimer, self-dimer, hairpin, etc. forming characteristics in a PrimerPremier6.1 program demo (www.premierbiosoft.com). One primer pair with better characteristics than the others for each TDF was ordered to be synthesized. Adapter and primer oligos used throughout this study were synthesized by Invitrogen, Life Technologies, and all enzymes and related reagents were purchased from Fermentas (Lithuania) unless otherwise specified. All PCR reactions were performed in an Eppendorf (Hamburg, Germany) thermal cycler.

RNA samples were prepared essentially as in initial cDNA-AFLP analysis from leaf tissue samples collected at the same time points. Without pooling, RNA was extracted as 2 replicates, and quality and quantity measurements were made using an ACTGene nanodrop. A one-tube qRT-PCR kit with HotStart Taq DNA polymerase (Bioron GmbH, Ludwigshafen, Germany) was used to carry out reverse transcriptions and PCR amplifications of individual samples in 25-µL reaction volumes, each containing 9.5 µL of qPCR reaction mixture, 5 pmol of each of the forward and reverse gene specific primers, about 50 ng of total RNA, and 0.125 µL of RT/HotStart Taq DNA polymerases. Time and temperature profiles of the synthesis reactions were as follows: cDNA synthesis at 45 °C for 30 min, HotStart *Taq* activation at 95 °C for 10 min, followed by a 35-cycle PCR, each cycle consisting of a denaturation step at 92 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 50 s; amplifications were finalized with a 5-min extension step at 72 °C. Entire reaction mixtures were then mixed individually with 5 µL of 6X loading dye (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) and run for 2 h in 2% 0.5X TBE agarose gels containing 0.4 µg/mL ethidium bromide to visualize the (expected) size of PCR products with changes in quantity at each sampling point. Maize actin 1 and 18S rRNA were used as housekeeping genes to compare the quantity changes in the amplification profiles of sqRT-PCR experiments (Table 1). For each of these genes, 2 pairs of primers, 1 pair amplifying the relatively longer target segment (in the case of actin 1 gene, a 245-bp product surrounding the intron 1 region was targeted to detect genomic DNA contamination in RNA preparations), and 1 pair amplifying shorter products (~120 bp), were designed and tested to assess the RNA template quantity. Housekeeping gene primers always amplified expected product sizes, and adjusted template concentrations appeared to be relatively the same throughout the time points.

Table 1. Primer sequences for the TDFs, expressional modulations of which were tested via sqRT-PCR.

Primer ID*	Primer sequences (F/R, 5'→3')	Homologous gene
GG/GA-A1 185	GTATGCCGCCCTGACCATTGAG	Full-length cDNA clone ZM_BFb0229H10 mRNA
	GGTGGTGGACGAAGGCTACAAC	
GG/TA-A3 178	CCTTCGTCTGTGCCTTCT	ZM clone 289536 60S ribosomal protein L24 mRNA
	TCCATTGTGGGTGCTTCC	
GG/TA-G4 195	GCCTCTGTTCTGAGCCGCATAC	TBC domain containing protein
	ACCACTGCCGACATGAGAATCC	
HKG1-2 124	TTTGACTCAACACGGGGAAA	ZM 18S rRNA
	CAGACAAATCGCTCCACCAA	

*The primer IDs designated to indicate *Taq* I and *Csp6* I primer extension combinations (separated by the slash) after dash designations are A for A188 inbred and G for *Rp1-G* NIL; the number accompanying the letter indicates the identification orders of the TDF, and the following number is the product size in bp.

3. Results and discussion

DNA chips are considered as the state-of-the-art method of high-throughput gene expression analysis whenever the genome sequence or expressed sequence tag libraries for a species are available. Several alternatives exist that do not require such an extensive genomic characterization, and they can be carried out in moderately equipped laboratories with relatively low costs. cDNA-AFLP, one such technique, is a universally applicable, robust, and straightforward method of choice for global gene expression profiling studies. However, the cDNA-AFLP procedure is not perfect in every aspect: it has limited coverage due the absence of restriction sites for the enzyme combinations used in analysis in some cDNA types of the transcriptome. Coverage of cDNA-AFLP analyses may be optimized by using different numbers and combinations of restriction enzymes. A number of modified protocols and theoretical projections have been developed to address this problem to some degree (Vuylsteke et al., 2007; Weiberg et al., 2008; Liu et al., 2011).

A modified cDNA-AFLP protocol with the features of 2 reciprocally ordered 4-cutter enzyme digestions has been developed to improve the coverage of the technique. This procedure is based on mRNA isolation and first and second strand cDNA synthesis on oligo-d(T) coupled magnetic beads. Subsequent restriction enzyme digestion manipulations are also performed on magnetic beads as illustrated in Figure 1. cDNA manipulations on magnetic beads allow both sequential and flip-flop digestions to be performed, which are also presumed to facilitate the purification of single mRNA tags from each of the cDNA species at the end, which can be used to quantify expressional changes, as well (Breyne et al., 2003; Vuylsteke et al., 2007). Choosing appropriate enzyme combinations along with flip-flop and sequential digestions in the preparation of templates have been proposed to increase the overall coverage of cDNA-AFLP analyses (Weiberg et al., 2008).

This protocol was successfully applied to detect gene expression modulations in the maize and common maize rust pathosystem using a rust race that is virulent on the A188 inbred and avirulent on the *Rp1-G* differential line. In both susceptible and resistant genotypes, 74 primer combinations were used to survey messages expressed differentially in response to rust inoculation during the time period of 6 to 96 pih in cDNA-AFLP templates prepared from pooled leaf material (T1 pool of 6, 12, 18, 24 h and T2 pool of 36, 48, 72, 96 h sampling points; see Section 2) along with their pooled controls. A total of 930 differentially regulated TDFs were isolated from band profiles of 40–80 bands per primer combination (Figure 2). Nearly a quarter of the combinations did not produce usable banding patterns, from which no TDFs were characterized. Sequencing 310 TDFs revealed that the majority of the tags were either known to have a role in or were associated with plant–microbe interactions (Figure 3; Table 2). The majority of the messages showed parallel expressional modulations in both resistant and susceptible genotypes, while a small number of TDFs differed in their expressional changes, as in one example seen in Figure 3. Using sequence information of the tags and their presumed homologs, gene-specific primers were designed for 10 of these tags, and sqRT-PCR confirmations of their expressional modulations were carried out. The majority (about two-thirds) of the expressional changes were confirmed as in cDNA-AFLP profiles (Figure 4). Among the differentially regulated genes that are known to be associated with plant–microbe interactions, PR2, chitinase, a sequence homologous to WRKY47 of rice, serine/threonine kinases, potassium channel/transporter

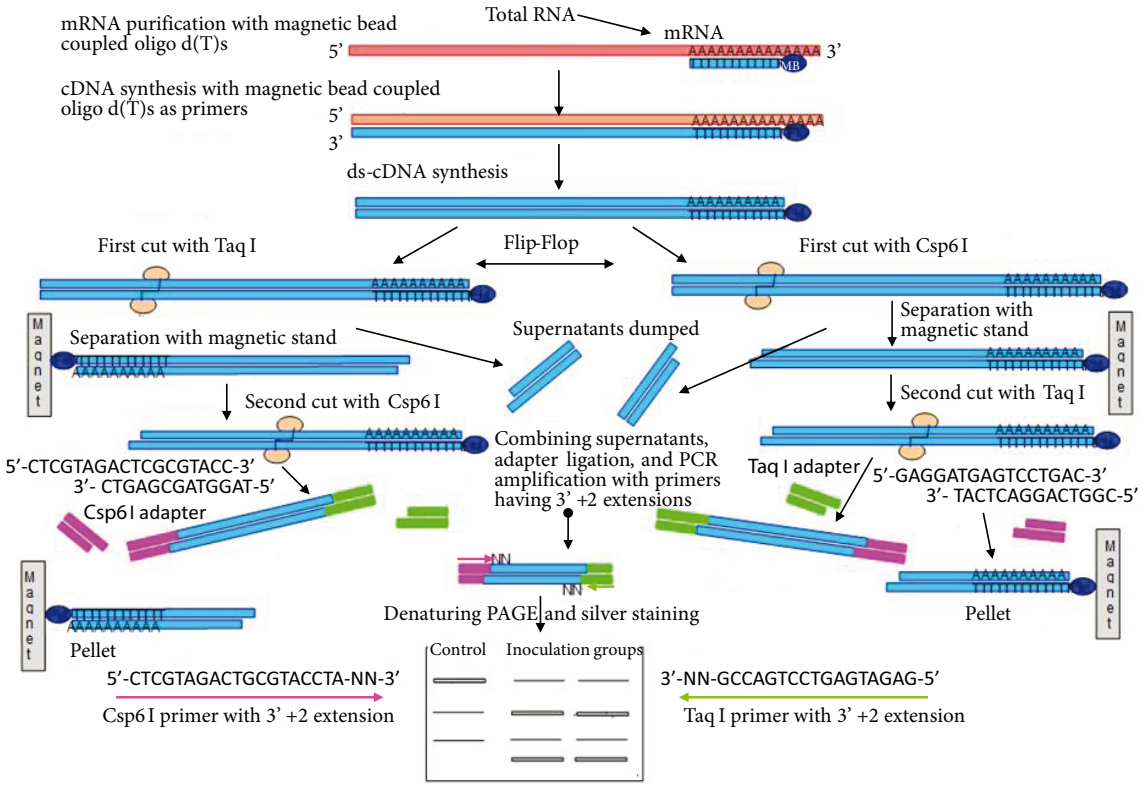

Figure 1. cDNA-AFLP procedure with the flip-flop sequential digestion.

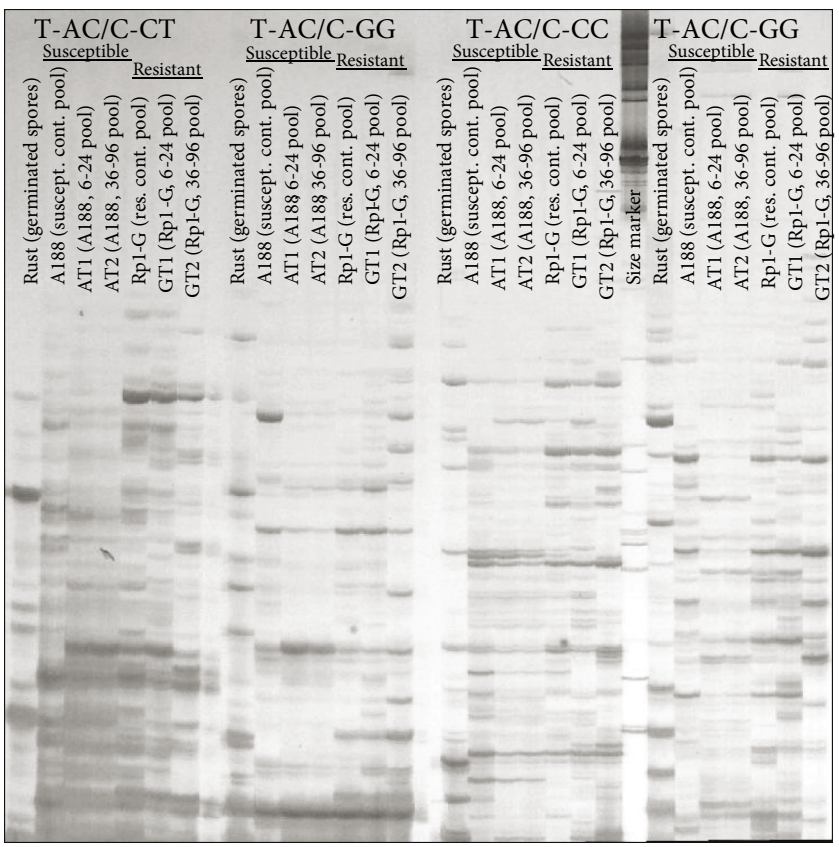

Figure 2. A sample cDNA-AFLP display. Primer combinations are given on top of each set in the picture.

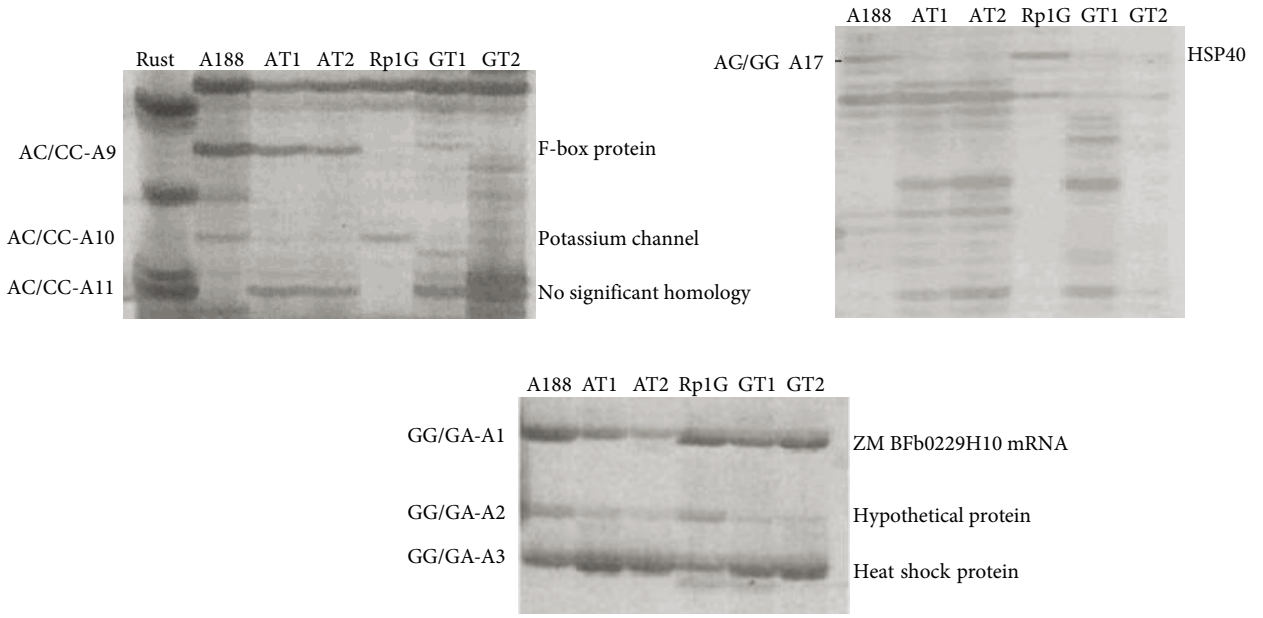

Figure 3. Samples of cDNA-AFLP gel sections showing (annotated) differentially expressed message bands in control (AC: A188 control; GC: *Rp1G* control) and treatment group pools (AT1 and GT1 for 6–24 h; AT2 and GT2 for 36–96 h time periods). Primer combinations of the band profiles are given in figures. No significant homology was found for AC/CC-A11 TDF.

Table 2. List and functional groupings of the sequence characterized 100 example TDFs having homologies to known sequences and presumed to play a role in plant–microbe interactions (full list and their features will be published elsewhere).

TDF ID*	Accession #	GenBank sequence showing the highest homology	E-val.
Disease resistance, signal transduction, redox state, and transcriptional regulation			
GG/CG-G4	CA452584.1	ZM cDNA clone, Kr1N-2_A10, mRNA sequence	$5e^{-141}$
AC/CC-A1	AY574035.1	ZM rust resistance protein rp3-1 (rp3-1) gene	$1e^{-102}$
GG/GC-A3	DQ417752.1	ZM B73 pathogenesis-related protein 2	$6e^{-111}$
TC/GG-A14	EU724506.1	ZM subsp. *parviglumis* isolate chiI_Z10 chitinase	$7e^{-31}$
GG/GG-A16	GQ369449.1	*S. bicolor* lipoxygenase encoding LOX7 cds	$1e^{-5}$
GG/CA-A2	EU955207.1	ZM PAP fibrillin protein	$1e^{-123}$
GG/CG-G26	EU956267.1	ZM LRR containing protein 40 mRNA	0.71
GG/GG-A1	EU974247.1	ZM Ptr ToxA-binding protein 1 mRNA	0.0
GG/CA-G1	EU966070.1	ZM APx1 - cytosolic ascorbate peroxidase	0.0
TG/GT-G6	BT069892.1	ZM secretory peroxidase full-length cDNA	$4e^{-33}$
GG/TA-G7	EU959346.1	ZM peroxiredoxin-5 mRNA	$6e^{-100}$
GG/CA-G3	EU969009	ZM thioredoxin H-type mRNA	$3e^{-135}$
AC/TG-A4	EU957399.1	ZM thioredoxin H-type 5 mRNA	$3e^{-99}$
TC/TG-G2	EU956301.1	ZM phytosulfokines 2 precursor, mRNA	$1e^{-11}$

Table 2. (Continued).

TDF ID*	Accession #	GenBank sequence showing the highest homology	E-val.
AC/CC-A8	EU963527.1	ZM ferredoxin-NADP reductase, leaf isozyme mRNA	$3e^{-50}$
TC/TA-G4	EU962667.1	ZM MPK14 - putative MAPK mRNA	$1e^{-91}$
TC/TA-G11	XM_002463351.1	*S. bicolor* hypothetical protein mRNA similar to WRKY TF 47	$4e^{-15}$
TC/TA-G1	EU967389.1	ZM auxin-repressed 12.5 kDa protein mRNA	$8e^{-140}$
TC/TA-A12	EU963078.1	ZM vacuolar ATP synthase subunit G mRNA	$3e^{-25}$
TC/TG-A8	HM004525.1	ZM auxin response factor 10 (ARF10) gene	0.81
GG/CG-G3	DQ417753.1	ZM serine/threonine kinase protein	$1e^{-76}$
TC/AT-G1	BT067381.1	ZM protein phosphatase 2C mRNA	$9e^{-62}$
TC/AT-A6	JF951920.1	*T. aestivum* R1R2R3-MYB protein mRNA	3.4
GG/CT-G12	HQ858666.1	ZM C2C2-CO-like transcription factor mRNA	$3e^{-50}$
AC/TG-A12	EU975275.1	ZM transcription factor Dp-1 mRNA	$2e^{-33}$
AC/TG-G1	XM_002460225	*S. bicolor* hypothetic protein mRNA, similar to LZIP protein	$5e^{-21}$
TG/GA-G6	NM_001111880.2	ZM general regulatory factor1 (grf1), mRNA	$1e^{-50}$
TC/TG-A14	AJ850298.1	ZM putative MADS-domain transcription factor	$4e^{-04}$
TC/TG-A16	EU968313.1	ZM bHLH transcription factor mRNA	$2e^{-05}$
AC/AG-G15	NM_001152803.1	ZM putative transcription factor (x1), mRNA	$4e^{-39}$
AC/CT-A1	EU952992.1	ZM transcription factor BTF3 mRNA	$8e^{-120}$
GG/GG-A15	EU965748.1	ZM mitotic checkpoint protein BUB3 mRNA	$2e^{-17}$
AC/CC-A19	EU966238.1	ZM glycine-rich protein A3 mRNA	$2e^{-09}$
AC/TG-A10	AJ131535.1	ZM hydroxyproline-rich glycoprotein gene	$2e^{-15}$
TC/AT-A1	S57628.1	ZM metallothionein-like protein gene	$2e^{-44}$
GG/GG-G1	ACG27569.1	ZM S-adenosylmethionine-dependent methyltransferase	$4e^{-47}$
TC/TA-A8	EU972158.1	ZM TCP-domain protein mRNA	$6e^{-58}$
GG/CA-G2	BT063931.1	ZM CBL-interacting protein kinase 09	0.0
GG/TA-G4	NM_001155160.1	ZM TBC domain containing protein mRNA sequence	$2e^{-18}$
GG/TA-G5	EU946841.1	ZM TBC domain containing protein mRNA sequence	$6e^{-149}$
GG/GC-A9	AF548024.1	ZM translationally controlled tumor protein-like protein mRNA	$1e^{-13}$
Protein synthesis, folding, and degradation			
GG/TA-A4	EU965866.1	ZM 60S ribosomal protein L24 mRNA	$2e^{-120}$
GG/TA-A5	EU958222.1	ZM 60S ribosomal protein L7-2 mRNA	$1e^{-106}$
GG/CG-G10	EU967200.1	ZM 50S ribosomal protein L40 mRNA	$2e^{-100}$
AC/CT-G11	EU965008.1	ZM 40S ribosomal protein S7 mRNA	$5e^{-168}$
TG/GA-G5	EU959849.1	ZM eukaryotic translation initiation factor 5A mRNA	$8e^{-78}$
AC/AG-G16	EU955647.1	ZM peptide chain release factor 2 mRNA	$4e^{-33}$

Table 2. (Continued).

TDF ID*	Accession #	GenBank sequence showing the highest homology	E-val.
TC/TA-A6	EU975854.1	ZM chaperone protein DnaJ mRNA	$3e^{-09}$
AC/GG-A17	EU963627.1	ZM heat shock protein DnaJ, N-terminal mRNA	$7e^{-17}$
GG/GA-A3	EU962980.1	ZM 17.4 kDa class I heat shock protein 3 mRNA	$1e^{-106}$
AC/CC-A9	EU974403.1	ZM F-box protein mRNA	$1e^{-39}$
TC/TA-A13	EU963701.1	ZM DNA repair protein RAD23 mRNA	$6e^{-31}$
TC/TA-A10	EU959683.1	ZM ubiquitin-like protein 5 mRNA	$3e^{-51}$
GG/CA-A5	EU960598.1	ZM ubiquitin-activating enzyme E1 domain-containing protein 1	$1\ e^{-119}$
TC/CG-A7	EU968199.1	ZM ubiquitinating enzyme mRNA	$1e^{-65}$
GG/TA-G8	EU953077.1	ZM ubiquitin ligase SINAT4 mRNA	$3e^{-114}$
GG/GC-A2	U29159.1	ZM MubG1 ubiquitin gene	$8e^{-79}$
Photosynthesis, cellular metabolism, and transport			
GG/TA-G9	EU963847.1	ZM chlorophyll a-b binding protein 4 mRNA	$4e^{-47}$
AC/CC-G11	EU963404.1	ZM photosystem I reaction center subunit IV A mRNA	$3e^{-22}$
AC/GG-A7	EU956354.1	ZM photosystem I reaction center subunit XI mRNA	$9e^{-83}$
TC/AT-G4	EU958093.1	ZM oxygen evolving enhancer protein 3 mRNA	$9e^{-24}$
GG/CA-A21	NM_001111915.1	ZM cytosolic glyceraldehyde-3-phosphate dehydrogenase	$3e^{-23}$
TC/GG-A11	J04502.1	ZM chloroplast cytochrome b559 alpha- and beta-subunit	$4e^{-25}$
TC/GG-A6	EU968076.1	ZM shikimate dehydrogenase mRNA	$3e^{-113}$
GG/CG-A4	EU962919.1	ZM ATP-citrate synthase mRNA	$2e^{-146}$
GG/CG-A12	EU953656.1	ZM adenylate kinase mRNA	$6e^{-64}$
GG/GA-A8	HQ697603.1	ZM pyruvate orthophosphate dikinase 1 (PPDK1) mRNA	$1e^{-42}$
AC/TG-A1	EU963441.1	ZM acyl carrier protein mRNA	$2e^{-136}$
TG/GA-G4	EU963336.1	ZM sorbitol dehydrogenase mRNA	$2e^{-07}$
GG/GC-A5	EU964884.1	ZM 3-beta-hydroxysteroid-delta-isomerase mRNA	$2e^{-46}$
TG/GT-G7	ZMU08403	ZM carbonic anhydrase (LOC542302), mRNA	$1e^{-04}$
AC/GG-A11	EU975063.1	ZM 4-nitrophenylphosphatase mRNA	$8e^{-52}$
AC/GG-A5	BT067700.1	ZM, nitrilase 1 mRNA	$2e^{-95}$
AC/AG-A9	EU963619.1	ZM chorismate mutase mRNA	$3e^{-50}$
AC/CC-G4	EU966588.1	ZM adenosine 5'-phosphosulfate reductase-like mRNA	$1e^{-49}$
AC/CG-G3	EU962752.1	ZM threonine endopeptidase mRNA	$2e^{-54}$
AC/CC-A6	U44087.1	ZM beta-D-glucosidase precursor (glu2) mRNA	$6e^{-58}$
GG/GG-A3	NM_001111826.1	ZM glutamine synthetase4 (gln4), mRNA	$2e^{-130}$
GG/CG-A17	EU963258.1	ZM glutamine synthetase mRNA	$3e^{-50}$
GG/GG-A5	X65928.1	ZM glutamine synthetase mRNA for gs1-3	$2e^{-99}$
AC/AG-A10	AF348367.1	ZM beta-keto acyl reductase gene	$1e^{-43}$
AC/CC-A10	Y07632.1	ZM potassium channel mRNA	$7e^{-47}$

Table 2. (Continued).

TDF ID*	Accession #	GenBank sequence showing the highest homology	E-val.
GG/CA-G6	EEE56594.1	*O. sativa* prenyltransferase domain containing protein	$1e^{-08}$
TC/GG-A16	Z26595.1	ZM zmcpt mRNA triose phosphate/phosphate translocator	$2e^{-28}$
Other stress-related messages, structural proteins, and ungrouped ones			
AC/TG-A2	EU956477.1	ZM wound responsive protein mRNA	$3e^{-134}$
TC/GG-G9	EU957315.1	ZM ultraviolet-B-repressible protein mRNA	$8e^{-04}$
AC/CG-G1	DQ078764.1	ZM cold-inducible unknown mRNA	$6e^{-100}$
GG/CG-G5	BG320377.1	ZM mRNA sequence from cold-stressed seedling	$1e^{-31}$
TC/TG-A5	NM_114122.4	*A. thaliana* aspartyl protease family protein mRNA	0.14
AC/TG-A6	EU970858.1	ZM GRAM domain containing protein mRNA	$1e^{-91}$
AC/CT-G6	EU964858.1	ZM PREG-like protein mRNA	$9e^{-67}$
TC/GG-A21	AY744462.1	*Pithecia pithecia* growth hormone-like protein 2 (ghlp) gene	0.15
TC/GG-G5	EU957475.1	ZM protein binding protein mRNA	$2e^{-22}$
AC/CC-G2	EU974377.1	ZM zinc-binding protein mRNA	$3e^{-93}$
TG/GT-G2	BT036488.1	ZM_BFb0118L12 mRNA (penta pep. rep)	$4e^{-71}$
TG/GT-G8	EU960226.1	ZM light-induced protein 1-like mRNA	$9e^{-12}$
AC/CC-A3	NM_001156148.1	ZM nuclear gene encoding mitochondrial protein	$2e^{-78}$
AC/CC-G3	EU963985.1	ZM mitochondrial-processing peptidase beta subunit mRNA	$1e^{-80}$
AC/CC-A18	NM_001153830.1	ZM regulatory subunit mRNA	$5e^{-04}$
TC/TA-G7	EU935003.1	ZM kanadi1 (kan1) mRNA	$4e^{-40}$
GG/GC-A1	BT042794.1	ZM BFc0041M16 mRNA	0.0

*TDF IDs designated to indicate *Taq* I and *Csp6* I primer extension combinations (separated by the slash) after dash labels are A for A188 inbred and G for *Rp1-G* NIL; the number accompanying the letter indicates the identification orders of the TDF in the respective line.

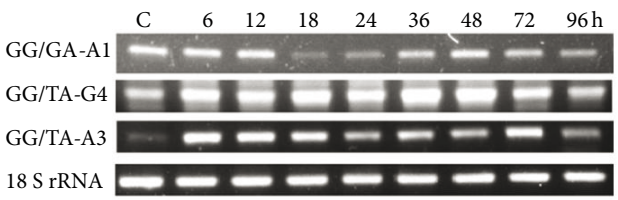

Figure 4. sqRT-PCR confirmations of 3 TDFs. Expressional modulations were tested at 8 time points covering the period of 96 pih. Housekeeping gene is 18 S rRNA.

proteins, and MAP kinases are the most important. These and other interesting messages will be studied further to determine their roles and expressional modulations in plant–microbe interactions.

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