cDNA-AFLP DIFFERENTIAL DISPLAY OF SUGARCANE (*SACCHARUM* SPP. HYBRIDS) GENES INDUCED BY CHALLENGE WITH THE FUNGAL PATHOGEN *USTILAGO SCITAMINEA* (SUGARCANE SMUT)

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Abstract

Sugarcane smut, caused by the fungal pathogen *Ustilago scitaminea,* has been severe in almost all sugarcane growing areas at one time or another. To date, the most satisfactory method of control is the use of resistant varieties. The aim of this study is to gain insight into resistance mechanisms with the possibility of isolating specific gene fragments to be used as RFLP probes in marker and mapping efforts. The cDNA-AFLP technique was used to detect sugarcane differential gene expression in response to challenge by *U. scitaminea* in resistant N52/219 and susceptible Co301 genotypes. Sequence homology searches of isolated genic fragments have identified a putative chitin receptor kinase, a Pto ser/thr protein kinase interactor, and an active gypsy type LTR retro-transposon expressed in the resistant variety in response to challenge.

Introduction

Smut disease of sugarcane is caused by a fungus, *Ustilago scitaminea* (H&P Sydow). Initially discovered in India, smut was first seen in Natal, South Africa, in 1877 and has since been severe in nearly all sugar-growing areas of the world (Antoine, 1961). To date, the most satisfactory method of controlling smut is the use of resistant varieties. Hence, to improve breeding for such varieties, the need exists for knowledge of resistance mechanisms.

Teliospores of *U. scitaminea* germinate on the inner scales of young buds and form apressoria within 24 h (Waller, 1970). Germinating teliospores form a short filament which buds off to form haploid sporidia. Fusion of sporidial germ tubes results in dikaryotic infectious hyphae which enter the bud meristem within 6 to 36 h (Alexander and Ramakrishnan, 1980). Inability of infectious hyphae to penetrate buds tightly enclosed within scale leaves has led to the hypothesis that bud morphology plays a major role in sugarcane smut resistance (Waller, 1970). Lloyd and Naidoo (1983) have suggested that increased concentrations of bud glycosidic substances correlate with higher resistance ratings. These two mechanisms form part of preformed resistance to the pathogen. Pertinent to this study, however, is the cascade of defense mechanisms induced in sugarcane by pathogen challenge.

Several methods have been developed for the detection of differentially expressed genes, and among these, methods for the fingerprinting of messenger (m)RNA have been shown to be efficient and convenient (Liang and Pardee, 1992). Amplified Fragment Length Polymorphism (AFLP) is a Polymerase Chain Reaction (PCR)-based fingerprinting method that was first developed by Vos *et al*. (1995). It is robust and reliable and has been successfully used for fingerprinting the genome of sugarcane varieties (Thokoane *et al*., 1999). The aim of this study was to investigate induced resistance mechanisms in sugarcane in response to challenge by the pathogen *U. scitaminea* using the cDNA-AFLP differential display technique.

Materials and methods

Preparation of material, RNA isolation and cDNA synthesis

Single budded setts of resistant (N52/219) and susceptible (Co301) varieties were spray-inoculated with 10^8.ml^{-1} teliospores. in 0.01% (v/v) Tween-20 while controls were mock inoculated with water and 0.01% (v/v) Tween-20. Setts were placed in a tray ¾-filled with moist sand with the buds facing upwards, covered with Saran wrap, and incubated at 26 °C in the glasshouse. Each inoculation sample consisted of 36 single budded setts. After seven days, total RNA isolated from pooled breaking buds using TRIzol reagent (Life Technologies), was quantified spectrophotometrically, and the integrity checked on a 1% (w/v) denaturing agarose gel. Poly A^+ RNA was then isolated using oligo dT-coated paramagnetic beads (Dyanabeads, Dyanal) and cDNA subsequently synthesised using SMART(TM) methodology (Clonetech). All procedures were according to manufacturers' instructions.

cDNA-AFLP and reamplification of fragments

Differential display was performed on cDNA using the AFLP (Gibco, Life Technologies) method outlined in the manufacturer's manual. This was followed by separation of fragments on a 5% polyacrylamide gel which was then dried on to filter paper and exposed to X-ray film (Kodak BioMax MR-2) for 3-6 days. Using a tooth pick, a mixture of ^{33}P isotope and black India ink (1:10) was dotted on the filter paper: three dots on the top edge and two at the bottom for the purpose of orientation. Differential bands were marked on an autoradiography film, whereafter, the X-ray was superimposed on the dried gel with the aid of the ink dots. Areas with bands of interest were punched through the X-ray film on to the filter paper, then excised using a sharp scalpel. Each piece of filter paper was moistened with 10µl TE buffer (10µM TrisCl, 1µM EDTA; pH 8.0) to scrape off the gel containing the fragment. The gel was then soaked in 100µl TE and boiled at 95 °C for 5 min, cooled on ice for 2 min, then

centrifuged for 15 min (12 000rpm). A pre-incubation step of 37 °C overnight prior to boiling was also tested. Eluted DNA (in approximately 80µl TE) was transferred into a fresh Eppendorf tube. To determine template concentration optimal for PCR, various template dilutions (0; 1:10; 1:20; 1:50 and 1:100) were used in a PCR mixture with the final concentrations as follows: 1.3ng.µl^{-1}BSA, 2µM $MgCl_2$ 0.1µM dNTPs, 0.2µM primers and 0.01U Taq polymerase (Promega) in a total volume of 30 µl. Primers and PCR conditions were the same as those used initially to generate each band of interest. Re-amplified fragments were separated on a 1% (w/v) agarose gel, extracted and purified through a column (Qiagen).

Cloning and sequencing

Re-amplified fragments were cloned into pGEM®-T Easy vector (Promega) and transformed into *Escherichia coli* competent cells (strain JM109). Clones were identified using blue/white selection on LB plates [0.5% (w/v) NaCl, 0.5% (w/v) Bacto®-yeast extract, 1% (w/v) Bacto®-Tryptone and 1.5 % (w/v) agar] containing 0.08 mg.ml^{-1} X-Gal, 0.1 mg.ml^{-1} IPTG and 0.1 mg.ml^{-1} ampicillin]. Recombinant (white) cells were screened by PCR for the presence of an insert using T7 and SP6 primers. Amplified inserts were subjected to single pass sequencing via dye terminator sequencing followed by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Sequences were edited manually to remove vector sequences, thereafter comparative sequence analysis was conducted with BLAST algorithm against non-redundant protein databases (BLASTX) (Altschul *et al*., 1990) at htpp://www.ncbi.nlm.nih.gov/blast/blast.cgi

Results and Discussion

In this study, a concentration of 10^8.ml^{-1} teliospores was used for the inoculation of single budded setts. A preliminary study on smut teliospore germination indicated that germination at that concentration was greater than 90%. This concentration also fell within the range most commonly reported in the literature, between $2.5x10^6$ and $5x10^8$ spores.ml^{-1} (Comstock *et al*., 1983). Sections obtained from an infected susceptible variety (Co301) were stained with 0.5% (w/v) aniline blue in a lactophenol solution (10 ml phenol, 10 ml glycerol and 10 ml lactic acid) and viewed under a light microscope. Results revealed advanced inter-cellular hyphal development seven days after inoculation (results not shown). Although hyphae have been reported to begin entry into the bud meristem within 36 hours (Alexander and Ramakrishnan, 1980), those authors also reported that hyphae were observed inter-cellularly after three days (72 h). In this preliminary study, a longer period (seven days) of hyphal development was chosen to allow complete interaction and response elicitation in sugarcane.

A wide variation in total RNA yields was obtained (280 mg.g^{-1} to 640 mg.g^{-1} fresh mass tissue). Poly A^+ RNA was isolated and full length cDNA approximately 0.3 to 15 kb in size was synthesised (Figure 1A). Using the cDNA as template, a total of 56 primer combinations was used to generate cDNA-AFLPs. An average of 70 bands were generated per primer combination and about 15% of these were polymorphic. Polymorphisms of interest were scored and each polymorphic band was assigned an identity (Figure 1B). Using TDF 47A as an example, the first three letters reflect the source of polymorphism (Transcript Derived Fragment), the subsequent numbers reflect the AFLP primer number (47) and the last letter indicates the number of the particular polymorphism (A for the first polymorphic band generated with primer 47). Interesting polymorphisms were classified into two categories. Type 1 polymorphisms were bands present only in the resistant variety that had been challenged (Figure 1B TDF 21A). This indicated possible activation or up-regulation of genes in the resistant variety in response to challenge. Type 2 were of bands present in the resistant challenged and unchallenged as well as susceptible challenged as shown in Figure 1B (TDF 21B and C). This could be an indication of pre-formed resistance mechanisms in N52/219 (resistant) or, as described by Lyon (2000), it could be a result of late induction of a resistance response in Co301, a susceptible variety.

Bands of interest had to be PCR amplified for subsequent cloning and sequencing. However, PCR-inhibiting contaminants from polyacrylamide gels have been reported to co-elute with DNA (Koo and Jaykus, 2000). To minimise this, two methods of eluting DNA were considered, 1) boiling at 95 °C for 5 minutes or 2) pre-incubation at 37 °C overnight followed by boiling. Eluted DNA from the two methods was diluted in water for PCR as follows: 0, 1:10, 1:20, 1:50 and 1:100. Diluting the template could also minimise any traces of contaminants that might have co-eluted with the DNA. Results indicated no amplification when DNA was eluted at 95 °C (results not shown) even after a series of dilutions. Overnight pre-incubation at 37 °C resulted

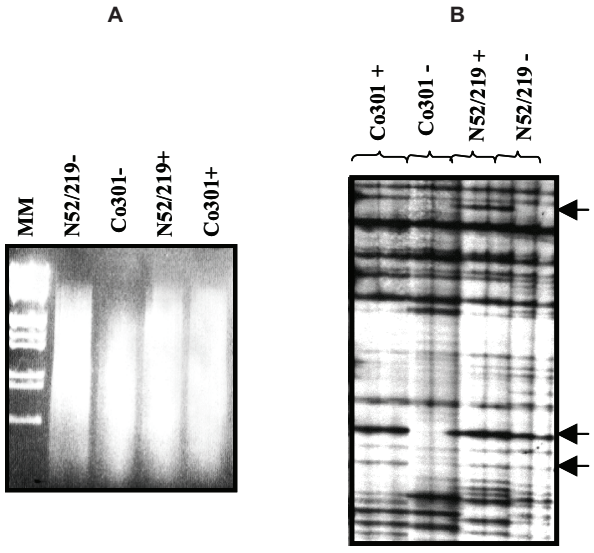

Figure 1. cDNA (A) and cDNA-AFLPs (B) generated from smut challenged and unchallenged sugarcane buds. (A) Full length cDNA fragments generated using SMART technology. cDNA fragments (0.3-15 kb) were synthesised from RNA of resistant variety (N52/219) and susceptible variety (Co301) both challenged (+) and unchallenged (-). Lane MM: Standard molecular marker (M3) (λ DNA-Eco RI/Hind III), (B) A cDNA-AFLP differential display. TDF 21A shows type 1 polymorphism where a band is present in the resistant (N52/219) variety challenged (+) only. TDF 21 B and C show type 2 polymorphism with bands present in the susceptible (Co301) challenged (+) and unchallenged (-) and N52/219 challenged.

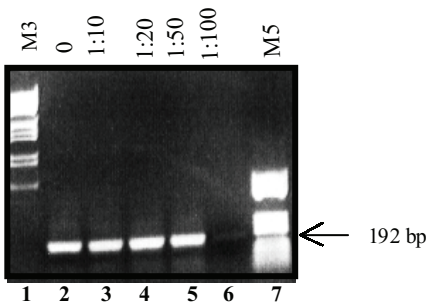

Figure 2. Yields of a re-amplified cDNA-AFLP fragment after a series of dilutions. Lanes 1 and 7: standard molecular markers M3 (λ DNA-Eco RI/Hind III) and M5 (pBR322 DNA-Hae III) respectively. Re-amplified fragment is approximately 180 bp.

in high amplification yields in undiluted samples as well as diluted samples of up to a 50-fold dilution (Figure 2). A very faint band was observed with the 1:100 dilution which was ascribed to limited DNA template. These findings suggest that contaminants are more likely to co-elute at elevated temperatures than lower temperatures. In support of our findings, Koo and Jaykus (2000) reported better re-amplification yields with DNA eluted at ambient than at elevated temperatures. Re-amplified fragments were cloned and subsequently sequenced from plasmid vector.

Currently, only 13 TDFs of type 1 polymorphism have been sequenced and their putative identities of some of these are shown in Table 1. As protein kinases are strongly involved in the early stages of signal transduction in tomato (Hammond-Kosack and Jones, 1996), their identification in this study is of particular significance. Two of the TDFs, 47H and O (Table 1), encode a putative Pto-like kinase and an S-receptor-like kinase (RLK), respectively. It is interesting to note that Pto kinase is an R-gene product involved in resistance against *Xanthomonas campesteris pv oryzae*. On the other hand, the S-RLK is a chitin binding type, and chitin elicitors have been found to induce defense mechanisms in suspension-cultured cells of crops such as rice, tobacco and tomato (Hammond-Kosack and Jones, 1996). A Typ3/gypsy type long terminal repeat (LTR)–retrotransposon, also induced when the resistant variety is challenged (TDF 47E), was used as probe across 78 well characterised sugarcane genotypes (DNA digested with Dra I) to determine association with resistance. Four polymorphic bands were observed; however, none of these was significantly associated with smut resistance (results not shown).

The cDNA-AFLP differential display method has been successful in producing a large number of differential transcripts. Preliminary sequence analyses of these transcripts has led to interesting putative identities of TDFs, some of which may be involved in signalling. Further sequencing of TDFs is in progress and TDFs will also be used as probes across varieties phenotypically characterised with respect to disease resistance, or a population segregating for resistance, to assess the association of a polymorphism with smut resistance ratings.

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Table 1. Homologies to some of the Transcript Derived Fragments (TDFs) sequenced to date. Sequence homologies are of TDFs present in N52/219 challenged only (Type 1). E-value is an expectation value: the lower the E-value, the more significant the homology.

Fragment	size (bp)	Accession #	Homology indicated by Blast X search	E-values
TDF 47A	426	gb/AF200533.1	Maize Cellulose synthase (Ces A)	10^{-111}
TDF 47E	475	gb/AAG12877.1	Ty3/Gypsy LTR - retrotransposon	8×10^{-31}
TDF 47H	404	pir//T01711 gb/AAG29223.1	- serine/threonine protein kinase - Pto-like kinase	8×10^{-25} 10^{-07}
TDF 47J	168	dbj/BAB11562.1	hypothetical protein	4×10^{-32}
TDF 47M	170	gb/AAA57051.1	dihydropteridine reductase	0.002
TDF 47O	118	gb/AAD52097.1	S-receptor-like kinase (chitin binding)	3×10^{-08}
TDF 47X	111	sp/P08735	Gpc1 Maize glyceraldehyde 3-phosphate dehydrogenase	7×10^{-48}

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