Fingerprinting of *Ustilago Scitaminea* (Sydow) in Egypt Using Differential Display Technique: Chitinase Gene the Main Marker

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Abstract: The sugarcane smut disease caused by *Ustilago scitaminea* causes the greatest yield losses and is the most difficult to control through producing host genetic resistance, in part because the pathogen occasionally produces a new race that overcomes prior host resistance. In the smut fungi, few features are available for use as taxonomic criteria (spore size, shape, morphology, germination type, and host range). DNA-based molecular techniques are useful in expanding the traits considered in determining relationships among these fungi. Approximately 17 smut infected samples from five different sugarcane cultivars were collected from different governorates in Upper Egypt. Only 12 fungal isolates were succeeded to grow on Potato Dextrose Agar (PDA), the mycelium of each isolate was subjected to RNA extraction, followed by differential display technique to classify these isolates. Determination of the identity of sugarcane smut races and maintenance of resistant commercial varieties has proven to be difficult. So, it is essential to know the fungus race to find the suitable tools for controlling. For that reasons this study focusing on the scanning of the fungal mRNA, especially the one of the functional genes like chitinase gene. This study concluded that chitinase genes are the most suitable for genotyping study between fungal isolates. Differential display had the capability to demonstrate the changes carried out in fungal genome and could be a good substitute to the DNA sequence for fingerprinting of *Ustilago* sp., because of it is so easy, achieved in no time, cheap and impressive.

Key words: Smut disease, Sugarcane, chitinase gene, fungi, differential display

INTRODUCTION

Smut disease of sugarcane, caused by the fungus *Ustilago scitaminea* (H and P Sydow), can cause considerable yield losses and reduction in sugarcane quality[1]. Sugarcane smut was first reported in South Africa in 1877, and many observations were made in Africa and Asia in the following decades[2, 3]. Sugarcane smut was observed for the first time in the Ord River Irrigation Area of Western Australia in July 1998. Australian sugarcane cultivars are currently being screened in Indonesia to obtain smut resistance ratings, and results show that 70% of Australian cultivars are susceptible[4]. As the use of resistant cultivars is the best option for long term control of smut a high priority of Australian sugarcane breeding programs is to increase the level of smut resistance in commercial cultivars.

*Ustilago scitaminea* as well as other *Ustilago* species readily hybridize between races and even between species[5]. Determination of the identity of sugarcane smut races and maintenance of resistant commercial varieties has proven to be difficult. Sugarcane resistance to *U. scitaminea* appears to be the result of several characteristics and is probably determined by a number of genes[6, 7]. In addition, commercial sugarcane varieties are polyploid hybrids of several *Saccharum* species. Genetic resistance in this case does not follow the strict gene-for gene pattern as seen in some pathogen-host interactions, although differences in variety susceptibility to different smut isolates have been reported[8, 9, 10].

Successful disease management strategies and development of resistance requires an understanding of variation in the pathogen population. While neutral genetic markers such as AFLPs may not detect changes in pathogenicity genes such markers can track changes in pathogen populations across geographic areas and over time, determine whether resistance screening trials are being done in the appropriate location, and reveal
evidence of pathogen genotype by host genotype interactions\cite{11}.

Mycoparasitic *Trichoderma* species secrete chitinases and glucanases enzymes that attack cell wall polymers in other fungi and have been exploited in the development of bio-control strategies\cite{12}. In other studies, differential display (RNA fingerprinting) has been used to identify transcripts that are aberrantly regulated in human tumors\cite{13, 14}, differentially expressed during mouse brain development\cite{15}, or differentially expressed during peroxide stress in *Salmonella*\cite{16}.

The aim of this study is genotyping screening of the races of *Ustilago* species in Egypt using the chitinase gene primers.

**MATERIALS AND METHODS**

**Samples Collection:** A total of 17 smut infected samples from five different sugarcane cultivars were collected from Giza, El-Mania, Souhag, Qena and Aswan Governorates in Upper Egypt (Table 1).

**Fungal Isolation and Identification:** Teliospores were obtained by shaking the whip on a sterilized plastic sheet. Fungal spores were suspended for 24 hours in 1.5% copper sulphate solution for sterilization\cite{17}. A loop of fungal suspension was streaked on Petri plates containing (PDA) and then incubated at 30°C for 10 days. Tips of fungal mycelium were taken and transferred to tubes containing PDA medium, incubated at 30°C for 10 days after that kept in a refrigerator at 4°C as stock cultures for further studies. To identify the fungal isolates, teliospores were microscopically examined and measured according to their morphological characteristics using Carl zeiss eyepiece micrometer as described by\cite{18}(1952).

**Molecular Characterization of *Ustilago Scitaminae:***

**RNA Extraction and cDNA Synthesis:** Total RNAs of the fungal spores were prepared using RNA-easy kit according to manufacturer’s instructions (QIAGEN). The RNA was dissolved in DEPC-treated water, and total RNA analyzed on 1.2% agarose gel. For cDNA synthesis 2.5μl from RNA was combined with 5μl of a 2x reverse transcription mixture containing (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl$_2$, 20 mM dithiothreitol), 2.5μl dNTPs (4 mM), 1μl oligd T primer (Promega), 13μl of RNAs free water and 1μl (50unit/μl) of Murine Leukemia Virus (MLV) reverse transcriptase and incubated at 37°C for 1 hr, followed by one cycle for killing the enzyme at 75°C for 10 minutes\cite{19}.

**Differential Display for the Collected Fungal Isolates:** The cDNA was subjected to second PCR using arbitrary primers, chi 25 (5- GAY TTR GAT TGG GAA TAY CC -3), chi 15 (5- GGY GGY TGG AAT GAR GG-3), A2 (5- GAA ACG GGT GGT GAT CGC-3) and A4 (5-GGA CTG GAG TGT GAT CGC-3). The PCR reaction was performed as following; a 25 μl reaction mixture containing; 2.5μl 10x Taq DNA polymerase buffer (10 mM Tris HCl (pH 8.3), 25 mM KCl), 2.5 μl 50mM MgCl$_2$, 2μl primer (40 Pmol/μl), and 0.25μl of Taq polymerase (AmpliTaq, Perkin-Elmer, 5u/ µl), 2.5 μl from the cDNA, 2.5 μl dNTPase 4mM and 12.75 μl of dH$_2$O. The PCR reaction was performed in 9700 thermal cycler (Perkin-Elmer) and the PCR conditions was performed as following: initial denaturation 95°C for 5 min, followed by 40-cycles (94°C for 1 min; 53°C for 1 min. and 72°C for 2 min; final extension, 72°C for 10 min). Percentage of similarity between the 12 smut fungal isolates based on the differential display band pattern was carried out using 3 random primers according to\cite{20}. The results were analyzed according to\cite{21, 21}.

**RESULTS AND DISCUSSIONS**

**Results:** A total of 17 smut infected samples from five different sugarcane cultivars were collected from Giza, El-Mania, Souhag, Qena and Aswan Governorates in Upper Egypt. A total of 12 *Ustilago scitaminae* fungal cultures were isolated from these different smut infected sugarcane cultivars. Three, one, two, five and one isolates were collected from Giza, El-Menia, Souhag, Qena and Aswan Governorates, respectively as shown in Table 1.

DNA-based molecular techniques are useful in expanding the traits considered in determining relationships among these isolates. This work was conducted to determine the variability and distribution of sugarcane smut races in Egypt by differential display technique using chitinase gene as specific primers and another 20 *mers* arbitrary primer as were used in this study for more confirmation.

The results presented in figure 1 showed differential display for 12 fungal isolates using chitinase 25 and 15 as arbitrary primers. Primer chi 25 grouped the isolates into 9 groups, group one contains G1 and C1 whenever group two contains B1 & B2 and group 3 contains B8 and B9 (Fig.1). In addition, the rest of the isolate each one formed a separate group. Whenever, the primer chi 15 was able to differentiate between the all examined isolates except the isolates B4 and B8 which they were much closed to each other (Fig.2). Whenever, the results presented in figure (3) revealed that primer A2 succeeded to distinguish between the 12 isolates and there was no similarities observed among them. In case of the primer A4 as shown in figure (4) the same result was obtained in compared with the primer A2.
Table 1: Source of Ustilago scitaminea Syd. isolates used in this study

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Sugar cane variety</th>
<th>Locality</th>
<th>Governorate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (G1)</td>
<td>Gt54-9</td>
<td>*SCRI</td>
<td>Giza</td>
</tr>
<tr>
<td>2 (C1)</td>
<td>NC03310</td>
<td>SCRI</td>
<td>Giza</td>
</tr>
<tr>
<td>3 (C3)</td>
<td>Gt54-9</td>
<td>Hawamdih</td>
<td>Giza</td>
</tr>
<tr>
<td>4 (C6)</td>
<td>Gt54-9</td>
<td>Abo-Korkas</td>
<td>El-Menia</td>
</tr>
<tr>
<td>5 (B1)</td>
<td>Gt54-9</td>
<td>Gerga</td>
<td>Souhag</td>
</tr>
<tr>
<td>6 (B2)</td>
<td>Gt54-9</td>
<td>Dar-Elsalam</td>
<td>Souhag</td>
</tr>
<tr>
<td>7 (B3)</td>
<td>Gt54-9</td>
<td>Nag-Hammadi</td>
<td>Qena</td>
</tr>
<tr>
<td>8 (B4)</td>
<td>G85-37</td>
<td>Nag-Hammadi</td>
<td>Qena</td>
</tr>
<tr>
<td>9 (B6)</td>
<td>G99-165</td>
<td>El-Mattana</td>
<td>Qena</td>
</tr>
<tr>
<td>10 (8)</td>
<td>G54-9</td>
<td>El-Mattana</td>
<td>Qena</td>
</tr>
<tr>
<td>11 (9)</td>
<td>G99-103</td>
<td>El-Mattana</td>
<td>Qena</td>
</tr>
<tr>
<td>12 (11)</td>
<td>Gt54-9</td>
<td>Kom-Ombo</td>
<td>Aswan</td>
</tr>
</tbody>
</table>

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Fig. 1: Differential display for the smut fungal isolates using chitinase 25 primer.

Fig. 2: Differential display for the smut fungal isolates using chitinase 15 primer.

Fig. 3: Differential display for the smut fungal isolates using chitinase A2 primer.
Fig. 4: Percentage of similarity between the 12 smut fungal isolates based on the Differential display band pattern.

Data presented in figure 4 showed that the differential display technique classified the twelve isolates into 10 different groups. Group1 contains G1 and C3, group 2 include B9 and B11, whenever, the other isolates each one made one group. In addition, the similarity between the isolates was demonstrated based on the band pattern obtained by the AP-PCR. The similarity percentage between G1 and C3 was 100% and the same percent was obtained in case of B9 and B11. The lowest similarity 30% was observed between B8, B9 and B11.

**Discussion:** RNA fingerprinting using arbitrarily primer PCR (RAP) allows the semi-quantitative simultaneous comparison of the abundances of several hundred randomly sampled RNAs. In RAP first-strand cDNA synthesis by reverse transcriptase is initiated from an arbitrarily chosen primer at sites in the RNA that best match the primer. Second-strand synthesis is initiated by extension of the same arbitrary primer at sites of adequate match on the first-strand cDNA product by using Taq polymerase. The products of cDNA synthesis are then amplified by PCR and displayed on a gel as a fingerprint representing between 10 and 50 RNAs, depending on the choice of arbitrary primer. Any differences in the pattern produced by a primer in different RNA populations reflect abundance differences in individual RNAs or between the individuals.

Sugarcane smut disease caused by *Ustilago scitamineae* (Sydow) causes the greatest yield losses and is the most difficult to control through producing host genetic resistance, in part because the pathogen occasionally produces a new race that overcomes prior host resistance. However, it is become necessary to identify how many fungal isolates distributed in Egypt. As well as, studying which cultivars are susceptible for infections with such pathogen and which genotype. Many question needs to answerers to protect our economy in Egypt from such disease. We tried in this study to evaluate most of sugarcane cultivars cultivated in five governorates and to what extent these cultivars are resistance and or susceptible to smut disease. Whenever, selecting the most resistance cultivars for all *Ustilago* genotypes will solve the problem.

Due to the increasing areas infected with smut in the sugarcane fields in Egypt, we decided to fingerprint the races presented in Egyptian fields in this study. The spores were collected from infected sugarcane plants from different five different governorates in Upper Egypt. The results obtained by differential display techniques showed that there are at least 10 different races from the *Ustilago* sp. in Egyptian field; these results come in agree with who used specific PCR primers for amplification of 459 bp fragment, followed by band elution, RFLP and finally sequencing to distinguish among microbial isolates. make a fingerprinting for the sugarcane cultivars resistance and susceptible for the smut disease. The primers chitinase 25 gave 20-24 different band patterns with different molecular weights (50bp-3000kbp), whenever, 10-15 band patterns were obtained with primer chitinase 15 and the molecular weights of the obtained bands was ranged from 100bp to 10kbp. The differences in the band patterns revealed how many RNAs had been scanned when we used these primers to differentiate between the fungal isolates. Chitinase gene was considered as a good guide to tell us how the fungal react with the environment, i.e the fungal able to generate a new mutation for the new environment. Through the reaction between the chitinase gene and the environmental conditions we can obviously demonstrated the differences between the obtained isolates. In conclusion the Chitinase gene is a functional gene but at the same time it is considered as a one of the conditional genes.
As well as another primer A2 also was used as arbitrary primers for fungal isolates screening it succeeded to scan more than 10 RNAs in the examined isolates. The differential display classified the 12 fungal isolates into to 10 groups; group one contains the isolate G1 and C3, but groups two contains the isolates B9 and B11. Whenever, the other 8 isolates; each isolate considered as one group. In conclusion we recommended the usage of the differential display as tool for fingerprinting the very close individuals in the same species.

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REFERENCES
