Molecular cloning of smut-related genes in sugarcane using suppression subtractive hybridization

Song Xiupeng¹, Chen Minghui², Tan Dandan³, Yang Litao² and Li Yangrui¹

¹Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences/Sugarcane Research Center, Chinese Academy of Agricultural Sciences, Nanning 530007, China; xiupengsong@163.com
²College of Agriculture, Guangxi University/State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Nanning 530004, China
³Biotechnology Research Institute, Guangxi Academy of Agriculture Sciences, Nanning, China

Abstract  The aim of this experiment is to reveal the molecular mechanism of sugarcane response to the smut pathogen at the beginning of the pathogen infection of sugarcane seedlings, to explore related genes, and to provide useful information for developing rational strategies to control smut at early stages of disease development. A suppression subtractive hybridization library was constructed using cDNA synthesized from RNA extracted from normal stems as driver and inoculated stems as tester. The positive clones of the libraries were sequenced randomly, analysed by BLAST and classified by GO. A total of 248 positive clones were selected for sequencing, and a total of 224 EST sequences were obtained. 188 ESTs were found to share considerable homology with known genes, while the remaining 36 ESTs had no homology with known genes. In the Gene Ontology database, the unigenes were assigned functional descriptions: 152, 129, and 139 ESTs were, respectively, involved in cell component, molecular function and biological process. Some genes related to a smut pathogen infection were obtained, while the SSH library was constructed. These genes reflected the regulation of sugarcane to smut pathogen, and can be used as candidate genes.

Key words  Sugarcane, suppression subtractive hybridization, smut, subtractive library, stress

INTRODUCTION

Sugarcane is an important sugar and biofuel crop. Sugarcane smut is caused by the basidiomycota fungus Sporisorium scitamineum (Sydow) and is a common, worldwide problem resulting in reduced sugar content and cane yield. The disease is sometimes described as culmicolous, which characterizes the outgrowth of fungus from the cane stalk. Smut is transmitted mainly by teliospores in the soil infecting planted setts, and by wind-borne teliospores infecting the standing cane (Santiago et al. 2009). The infection can take place through the open stomata in the leaves, buds or a wound in the plant tissues. It is difficult to control this disease with fungicides because the teliospores have strong stress resistance. Hence, the development and release of high-quality smut-resistant cultivars is the most economical and effective strategy for sustainable management of this disease. However, the classical sugarcane breeding and selection program takes about 14 years from the time crosses are made to the commercial release of a new cultivar.

The complexity and size of the sugarcane genome is a major limitation in genetic improvement, including the development of smut-resistant cultivars (D’Hont and Glaszmann 2001). A better knowledge of inducible molecular defense mechanisms could be useful in designing protective strategies such as over-expression of defense genes in sugarcane plants. Technologies of cDNA-AFLP, DDRT-PCR and cDNA microarray have been used to identify sugarcane genes that respond to the smut pathogen attack. Borras-Hidalgo et al. (2005) identified 62 differentially regulated genes using cDNA-AFLP. Using cDNA microarray, Que et al. (2009) successfully identified 101 differentially expressed ESTs in E. arundinaceus induced by S. scitaminea. Que et al. (2011) obtained 23 differentially expressed proteins from the smut-infected sugarcane after the smut symptoms expressed. Although there is some research about the interaction between sugarcane and smut, they all analyzed sugarcane materials with obvious smut symptoms.

If the goal is to manage plant diseases most effectively, the control measures should be introduced at an early stage of disease development (Singh et al. 2004). There are no reports on the interaction between sugarcane seedling and smut at the early stage of pathogen infection. The technology of SSH is widely used in the study of gene differential expression (Sung et al. 2004; Liu 2000; Zhu et al. 2009). This method displays specificity, sensitivity, efficiency and simple operation. We sought to identify differentially expressed genes in sugarcane seedling during the early interaction with S. scitaminea. The information obtained will give an insight on the possible mechanisms involved in sugarcane seedling response to smut.
infection and aim in the development of rational control strategies for managing smut at an early stage of disease development.

MATERIALS AND METHODS

Materials

Tissue cultured plantlets of sugarcane (Saccharum spp. hybrid) cultivar ROC22 were grown in pots (35 cm in diameter, 40 cm in depth) containing soil mix (soil/sand/organic fertilizer, 6:2:2(w/w/w)) in a greenhouse at Guangxi University, Nanning (China) that had high exposure to the sun all day. Plots were sprayed with 50% carbendazim. Robust and uniform shoots were selected for inoculation with smut teliospores when the sugarcane seedlings had 6-7 leaves. Teliospores were collected from the infected plants of sugarcane cultivar ROC22 in the field and diluted to a concentration of $5 \times 10^6$ spores/mL with ddH$_2$O for inoculation. Plantlets were inoculated at the apical portion using a syringe with 100 μL of teliospore suspension (Santiago et al. 2009). A control had only an equivalent volume of ddH$_2$O applied.

All plantlets were grown under controlled conditions (28°C, 80% relative humidity), and plants were sampled 1, 2, 3 and 4 days after inoculation by cutting the portion around the point of inoculation, and directly putting the material into liquid nitrogen. There was no difference between smut-inoculated and control plantlets in morphology when they were sampled.

Methods

RNA extraction followed the protocols of the RNAsimp Total RNA Kit (TIAN GEN). The synthesis of double stranded cDNA used the SMARTer™ PCR cDNA Synthesis Kit produced by Clontech Corporation. We constructed a Subtractive library following the protocols of the PCR-Select™ cDNA Subtraction Kit produced by Clontech Corporation.

RESULTS

Total RNA extraction and purification

We obtained high-quality RNA and the bands of 28S rRNA and 18S rRNA were clear (Fig. 1). The ratio of OD$_{260}$ and OD$_{280}$ was 1.8-2.1 (Table 1). The RNA samples were complete and could be used in downstream experiments.

![Fig. 1. Electrophoresis of the total RNA: 1-4 - samples taken 1, 2, 3 and 4 d after inoculation with smut; CK1-CK4 – check samples at 1, 2, 3 and 4 d after spraying with ddH$_2$O.](image-url)
Table 1. Concentration of total RNA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ng/μL)</th>
<th>A260/A280</th>
<th>A260/A230</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>437.3</td>
<td>2.02</td>
<td>1.69</td>
</tr>
<tr>
<td>CK1</td>
<td>355.7</td>
<td>2.05</td>
<td>2.08</td>
</tr>
<tr>
<td>2</td>
<td>450.6</td>
<td>2.02</td>
<td>1.67</td>
</tr>
<tr>
<td>CK2</td>
<td>408.3</td>
<td>1.99</td>
<td>1.70</td>
</tr>
<tr>
<td>3</td>
<td>477.2</td>
<td>1.97</td>
<td>1.51</td>
</tr>
<tr>
<td>CK3</td>
<td>372.9</td>
<td>2.04</td>
<td>1.99</td>
</tr>
<tr>
<td>4</td>
<td>463.6</td>
<td>2.07</td>
<td>1.67</td>
</tr>
<tr>
<td>CK4</td>
<td>362.5</td>
<td>1.98</td>
<td>1.81</td>
</tr>
<tr>
<td>5</td>
<td>468.6</td>
<td>2.02</td>
<td>1.62</td>
</tr>
<tr>
<td>CK5</td>
<td>376.5</td>
<td>1.99</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Synthesis of double-stranded cDNA

The double strands cDNA of the Tester (infected) and Driver (not infected) samples showed bands of PCR production dispersed between 250-2000 bp (Fig. 2).

![Fig. 2. Analysis for optimizing PCR cycle number M: DL2000 Marker; 1 and CK1: 15 cycles; 2 and CK2: 18 cycles; 3 and CK3: 21 cycles; 4 and CK4: 24 cycles; 5 and CK5: 27 cycles.](image)

Digestion of the double-stranded cDNA by restriction enzyme RsaI

After the digestion, the double-stranded cDNA was diminished and dispersed between 100-2000 bp (Fig. 3). This result suggested that the digestion was successful.

![Fig. 3. Digestion of the dscDNA M: DL 2000 Marker; 1 and 2: The tester dscDNA before and after digestion; 3 and 4: the driver dscDNA before and after digestion.](image)
**PCR products of subtraction**

The first round of PCR products dispersed between 500-2000 bp. After the second round of PCR, the production of small fragments increased significantly (Fig. 4).

![Fig. 4. Products of two rounds of PCR of subtraction cDNA and unsubtraction cDNA. M: DL 2000 Marker; 1-2: first round of PCR amplification of subtraction cDNA and unsubtraction cDNA; 3-4: second round of PCR amplification of subtraction cDNA and unsubtraction cDNA.](image)

**PCR verification of the positive clones**

The products of second round PCR were purified and ligated to the vector pMD18-T. Positive clones were selected from agar plates and verified by PCR (Fig. 5). The clones, inserted with different sizes, were selected randomly for sequencing.

![Fig. 5. PCR amplification of positive clones randomly picked from the SSH library.](image)

**Gene ontology classification of annotated unigenes**

We sequenced 224 differentially expressed genes (ESTs) that responded to smut and functional annotations were done successfully for 188 of them (Fig. 6). The results of cellular component classification at GO level 2 showed that 152 unigenes were annotated 241 times and could be divided into 8 categories (Fig. 7). Those responsible for cells, organelles and macromolecular complexes occupied the largest proportion of 36.7%, while those responsible for the symplasm, envelope and extracellular domain account for 19.3%. This indicated that smut pathogens mainly impact on the interior of cell. Molecular function classification at GO level 2 showed that 129 unigenes were annotated for 132 times and could be divided into 8 categories (Fig. 8). The largest proportion of the unigenes was found to be those for binding and catalytic activity, and they were annotated for 49 times and 61 times, respectively. The major role of these two groups of proteins is regulatory. This indicated the differential gene expression is regulated by certain factors. Biological process classification at GO level 2 showed that 139 unigenes were annotated for 273 times and could be divided into 18 categories (Fig. 9). The largest proportion of the unigenes was found to be those for metabolic process and cellular process, and they were
annotated for 85 times and 74 times, respectively. These relate to basic life parameters for plants. The unigenes for stimulus response and biological regulation were also annotated for 28 times and 17 times, respectively.

**Fig. 6.** Enrichment analysis of the differentially expressed genes.

**Fig. 7.** Cellular component ontology of the differentially expressed genes.

**Fig. 8.** Molecular function ontology of the differentially expressed genes.
DISCUSSION

Resistance genes are genes that can cause the expression of defense genes through directly or indirectly identifying the avirulence gene products of pathogens in the process of pathogenic microorganisms infecting the host. There are many kinds of plant disease resistance genes with complex relationship among each other, not only the vertical resistance of single gene function, but also the horizontal resistance of polygene functions.

Resistance-related and defense-related response genes

Eight cell wall-associated hydrolase genes (EST 13, 44, 280, 301, 310, 312, 323, 352) were obtained from this study. These kind of enzymes play an important role in the process of protecting sugarcane seedlings against smut infection, e.g. chitinase, β-1,3-glucanase, xyl glucan endonuclease. Chitin and β-1,3-glucan are major components of cell walls of plant-pathogenic fungi, but chitin has not yet been found in plants while β-1,3-glucan is only found in some plant cells. Chitinase and β-1,3-glucanase exist widely in higher plants and their roles in plant disease resistance are common (Shi et al. 2002).

These two enzymes have direct effects on pathogenic fungi. In potato, barley, rice, etc, chitinase can inhibit the growth and germination of Rhizoctonia solani and more than 20 other fungi mycelium and spores (Schlumbaum et al. 1986; Mauch et al. 1988). Chitinase can affect the extension of hypha by degrading the chitin at the end of the mycelium growth. Chitinase can also disassemble germ tubes of fungi and subsidiary cells, inhibiting the elongation of germ tubes and degrading cell walls. The resistance of plants to fungi can be improved at different levels if chitinase is expressed after genetic transformation (Nan 2006; Zuo et al. 2009). Therefore, we speculate that the cell wall-associated hydrolases are closely related to the reaction of sugarcane seedlings to smut infection.

Cytochrome P450, discovered in 1958, is a type of oxidoreductase that contains heme pigment. It is involved in many biochemical reactions, as well as having influence on aspects of defense of biological pest and disease and adverse stress (Yang et al. 2003). The expression of the gene CaCYP1 of chili cytochrome P450 increases when plants are infected by pathogens while the expression of the defense-related genes will decrease if CaCYP1 is silenced (Kim et al. 2006). Peppers are more easily infected by Xanthomonas if the gene CaCYP450A of cytochrome P450 is silenced, and overexpression of CaCYP450A in Arabidopsis thaliana increases resistance to Pseudomonas syringae (Hwang et al. 2010). Overexpression of CaCYP450A causes the accumulation of H2O2 and the death of cells, which helps prevent the infection of pathogens. EST 358 that we found is for cytochrome P450, and it may influence the reaction of sugarcane seedling to smut infection.

Zinc finger protein has a repeating amino acid structural pattern, and it can selectively bind to specific target structures, so as to control gene expression, embryo development, cell differentiation, and other important life processes. It increases significantly in expression when wheat is infected by Erysiphe graminis (Wan et al. 2004). Changes in the expression of stress response gene voz1 and voz2 of the zinc finger protein double-mutate Arabidopsis thaliana improves drought and
cold resistant ability and it significantly reduces the plant’s resistance to infection by anthrax bacteria and *Pseudomonas syringae* (Nakai et al. 2013). *BohLOL1* gene encoding proteins of green bamboo shoots contains three zinc finger protein domains and it shows obvious up-regulated expression when it is induced by auxin, cytokinin, and pathogen infection. This indicates that this gene is important to the growth and stress responses of green bamboo shoots (Yeh et al. 2011).

**Signal transduction related genes**

Signal transduction plays a vital role in the process of plants against pathogen infection. Protein kinase CDPK, which relies on Ca2+, is one of the most important signal transduction genes encoding a protein. The enzyme belongs to serine/threonine type protein kinase that exists in eukaryotic cells and that can be activation-induced by the elicitors of pathogens (Hassan et al. 2012).

Protein kinase is an important regulatory factor, more specifically, it perceives stress signals through combining with membrane receptor protein kinase, gradually enlarging the signal by phosphorylating and dephosphorylation, then causing a change of concentration of ions and molecules of Ca2+, Na+/K+, salicylic acid, abscisic acid, etc, in cells. This regulates the transcriptional expression of downstream resistance genes to eliminate the impacts of adverse stress (Lee et al. 2011; Ludwig et al. 2004). In addition, CDPK can not only mediate the signal transduction, but also regulate the expression of resistance genes (Mall et al. 2011).

Ca2+ is widely recognized as second messenger in plant growth, and it is also involved in plant responses to adverse stress (Olmos et al. 2003). When the plants are stimulated by light, pathogen infection, phytohormone or environmental stress, the Ca2+ concentration in the cytoplasm will change, and then the calcium signal can pass exogenous stimulation signals that plants experience. Calcium depends on the involvement of protein kinase and Ca2+ binding protein to further transfer the calcium signaling. The calcium messenger system participates in the process of regulating protease activity, regulating genetic expression and the formation of stimulating reactive oxygen. Hence, CDPK may be important in the signal transduction during the early stage of smut infection in sugarcane seedlings.

**Transcription factors related and elongation factors related genes**

NAC gene expression differs in different organisms, and is also induced by different stages of plant development, against pathogens, mechanical damage, low temperature and drought conditions (Hu et al. 2006; Tran et al. 2004). NAC transcription factor CaNAC1 of chili is induced by pathogens’ exogenous salicylic acid and ethylene. Elongation factors are the protein factors that promotes the extension of polypeptide chain in mRNA translation. Elongation factors in plants are encoded by multiple genes, whose expression is induced by environment and hormones. After a plant is infected by fungi, its elongation factor EF1A will be induced to express (Mahe et al. 1992).

**Other related genes**

We also obtained EST sequences that were associated with protein synthesis and degradation, and energy metabolism. Examples are ubiquitin-conjugating enzyme, hydroxyacylglutathione hydrolase, GAPDH, hexose carrier, P680 apoprotein of photosystem II, glycosyltransferase, chlide, and pyruvate dehydrogenase E1 component. These genes are involved in the formation of primary and secondary metabolites – presumably they also influence disease resistance.

**CONCLUSIONS**

Our study successfully constructed a forward SSH library of the response of sugarcane seedlings to early infections of smut, and carried out sequencing and functional analysis for some of the ESTs in the library. Our results showed that the response of sugarcane seedlings to smut infection was a complex and systematical process, and that its resistance mechanisms involved several aspects of physiological and biochemical pathways. Some of the gene sequences were obtained through sequencing and functional analysis of the gene library, but the function of a large number of gene products is still unknown. Further studies are needed to determine if these differentially expressed genes are capable for defending sugarcane plants against infection by the smut pathogen.
Clonage moléculaire de gènes liés à la maladie du charbon de la canne à sucre en utilisant l’hybridation soustractive

Résumé. Le but de cette expérience est de révéler i) le mécanisme moléculaire de la réponse de la canne à sucre à l’agent pathogène du charbon au début de l’infection de plants de canne à sucre, ii) à l’identification des gènes impliqués, et de fournir des informations utiles pour l’élaboration de stratégies pour contrôler le charbon à des stades précoces du développement de la maladie. Une bibliothèque...
d'hybridation soustractive a été construite en utilisant l'ADNc synthétisé à partir d'ARN extrait de tiges normale en tant que témoin et de tiges inoculée comme testeur. Les clones positifs des bibliothèques ont été séquencées au hasard, analysées par BLAST et classés par GO. Un total de 248 clones positifs ont été sélectionnés pour le séquençage, et un total de 224 séquences EST ont été obtenues. 188 EST ont montrés une homologie importantes avec des gènes connus, tandis que pour les 36 EST restants aucune homologie avec des gènes connus n'a été trouvé. Dans la base de données Gene Ontology, les unigènes ont des descriptions fonctionnelles; selon ces données 152, 129, et 139 EST seraient impliqués dans le composant cellulaire, la fonction moléculaire et les processus biologique, respectivement. Quelques gènes liés à l’infection par l’agent pathogène de la maladie du charbon ont été obtenus. Ces gènes reflètent la réaction de la canne à sucre en présence du pathogène de la maladie du charbon, et peuvent être utilisés comme gènes candidats.

Mots-clés: Canne à sucre, la suppression hybridation soustractive, charbon, banque soustractive, stress

Clonación molecular de genes relacionados con carbón en la caña de azúcar mediante hibridación sustractiva

Resumen. El objetivo de este experimento fue revelar el mecanismo molecular de la respuesta de la caña de azúcar al patógeno causante del carbón al comienzo de la infección por patógenos en plantas germinadas de caña de azúcar, identificar los genes relacionados y proporcionar información útil para el desarrollo de estrategias racionales para controlar el carbón en las primeras etapas de desarrollo de la enfermedad. Una genoteca de supresión de sustracción de hibridación se construyó usando ADNc sintetizado a partir de ARN extraído de tallos sin infección y tallos inoculados como testigos. Los clones positivos de las genotecas se secuenciaron al azar, analizados por BLAST y clasificados por GO. Se seleccionó un total de 248 clones positivos para la secuenciación, y se obtuvieron un total de 224 secuencias EST. Se encontraron 188 EST que comparten una considerable homología con genes conocidos, mientras que los 36 EST restantes no tenían homología con genes conocidos. En la base de datos de ontología de genes, los unigènes fueron asignados así: 152 EST participan en el componente celular, 129 EST en la función molecular y 139 EST en procesos biológicos. Se obtuvieron algunos genes relacionados con infección de patógenos como carbón mientras que la genoteca SSSH fue construido. Estos genes reflejan la regulación de la caña de azúcar para patógeno causantes del carbón y se puede utilizar como genes candidatos.

Palabras clave: Caña de azúcar, supresión de sustracción de hibridación, carbón, genoteca de sustracción, estrés