

ANALYSIS OF PANTOEA AGGLOMERANS X FUSARIUM GRAMINEARUM DIFFERENTIAL GENE EXPRESSION

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Introduction

Loss of yield and grain quality caused by *Fusarium graminearum*, the main causing agent of scab in important cultures such as wheat, maize, and barley, has been a reality in Brazilian agriculture, causing substantial economic losses. An efficient and promising strategy to be adopted in order to protect cultivated plants against such diseases is the selection of antagonist microorganisms. The diversity of such microorganisms which efficiently control *F.graminearum* is represented by different species, amongst them, *Pantoea agglomerans*. These organisms might have an important impact in scab control, isolated or in an integrated management program with chemical treatment. The present work aimed at identifying differentially expressed sequences by macroarray in pathogenic fungi-antagonistic microorganisms interactions, considering the *F. graminearum* – *P. agglomerans* model. This technology has been efficiently used to generate information concerning the expression of mRNA from cells or tissues upon various stress conditions.

Materials and Methods

Culture conditions and cDNA library construction: After 7 days of growth on PDA medium (at 28°C, 16 h photoperiod) the fungal mycelia from *F.graminearum* were immediately ground in liquid nitrogen. Total RNA extraction was done according to the protocol of Sokolovsky et al., (1990). The mRNA was purified with the Oligotex mRNA isolation kit (Qiagen), following manufacturer’s directions. The cDNA library was constructed using the SuperScript Plasmid System for cDNA Synthesis and Cloning kit (Invitrogen), according to manufacturer’s instructions. Plasmid DNAs were purified using a modified alkaline lysis method (Sambrook *et al.*, 1989). Sequencing reactions were conducted using the DYEnamic ET Dye Terminator sequencing kit (GE Healthcare). cDNA inserts were sequenced from the 5’ end with T7 primer and the products separated and analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems). Base calling, quality control and clustering of the ESTs were performed using softwares Phred (Ewing et al., 1998), Cap3 (Huang and Madan,1999) and Consed (Gordon et al., 1998). Sequence similarity search of the unique sequences were conducted using BLAST programs (Altschul et al., 1990) against the GenBank non-redundant (nr) database.

cDNA Macroarray analysis: The membranes were constructed by Brazilian Clone Collection Center (BBC)-Unesp, Jaboticabal, SP (<http://www.bcccenter.fcav.unesp.br>). In each membrane 1.014 individual clones (in triplicate on 4 x 4 array) were spotted as bacterial colonies. These membranes were first hybridized with a plasmidial probe using the *overgo* method (McPherson, <http://www.tree.caltech.edu/protocols/overgo.htm>). Total RNA of the *F. graminearum* in the presence and in the absence of the *P. agglomerans* were used to synthesize labeled probes for hybridization to macroarrays. Total RNA were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen), oligo-dT₁₈ primers and labeled with $\alpha^{33}\text{P}$ -dCTP (Amersham Biosciences). After hybridization (described by Schummer, 1999 modified) and washing, the membranes were exposed to a Image Plate (IP, Kodak) and the images were obtained by scanning

using the STORM[®] PhosphorImager (GE Healthcare). The images were quantified using the *ArrayVision* 8.0 software (Imag. Res. Inc. 2003, <http://www.imagingresearch.com>). The plasmidial probes data were used to quantify the DNA spotted onto the arrays. The “acetyl-CoA synthetase” and “Glyceraldehyde 3-phosphate dehydrogenase” genes were used as controls for normalization. The model proposed by Wolfinger et al., 2001 was used to detect the differentially expressed genes.

Results and Discussion

A total of 1,903 valid ESTs were obtained by our group, resulting in 1,148 unique sequences after the assembly exercise. Of these, 1,014 clones were used for macroarray analysis. The statistical analysis has suggested 28 differentially expressed *F. graminearum* genes ($P < 0.05$), being 19 up-regulated and nine down-regulated in the fungi-bacteria interaction. Among the up-regulated genes, we were able to identify: a) a Glyoxylate pathway regulator - GPR1, an integral membrane protein involved in the molecular process of adaptation to acetic acid in ascomycetous fungus; b) a sterigmatocystin 7-O-methyltransferase, involved in the biosynthesis of aflatoxins in fungi, associated with defense and virulence mechanisms; c) a Cu-oxidase, that oxidize different types of inorganic and organic substances to establish an infection; d) a glutathione S-transferases –GSTs, involved in resistance to oxidative stress in fungi; e) transporter of oligopeptides (OPT), amino acids (NAAP1) and ammonium (MEP); f) fungal transcription factors, mainly acting on genes encoding for enzymes involved in degradation of the biopolymers and g) flavin-containing monooxygenase (FMO), a family of microsomal enzymes important for the oxidative metabolism of a wide variety of natural and synthetic compounds. Among the down-regulated genes, a) a mitochondrial hydrolase (H⁺-transporting ATP synthase); b) the proteases dipeptidylpeptidase III and endopeptidase K, involved in cytoplasmatic and nuclear protein degradation; c) the basic-leucine zipper (bZIP), a eukaryotic transcription factor; d) a component of the respiratory chain complex (COX6A); e) a protein (DNAJ-like protein homolog) that participates actively in the hyperosmotic and heat shock responses; f) a conserved hypothetical protein with a glycosyl hydrolases motif, that participates of cell wall organization and biogenesis; g) a probable binding protein component of ABC transporter, responsible for translocation of a variety of compounds across cellular membranes and h) a zinc-finger protein ZPR1 involved in mitotic cell cycle and cell cycle control, essential for cell viability. The interaction with eEF-1alpha has been shown to be essential for normal cellular proliferation. The last one was identified as a conserved hypothetical protein and no domain was identified until this moment. A genomic library of the antagonistic bacterium *P.agglomerans* has been constructed. The clustering and assembly resulted in 887 unique sequences with 194 contigs and 693 singletons. This database will also be used to construct a macroarray to identify differentially expressed genes of the *P.agglomerans* in the presence and absence of the *F. graminearum* as probes. The data provided by this work suggest starting points for a number of experimental investigations to the understanding of gene expression profile and biology associated to pathogenic fungi-antagonist microorganism interactions.

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