

## Large-scale identification of differentially expressed genes in maize inbreds susceptible and resistant to *Fusarium* ear rot

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### Abstract

*Fusarium* ear rot is a destructive disease in maize mostly caused by the fungus *Fusarium verticillioides* (FV), which results in reduction of grain yield. To understand the host response to FV infection in maize, we examined gene expression changes in bract tissue of resistant inbred line Bt-1, as well as susceptible inbred line Ye478, at the fourth day after inoculation with FV, based on the genechip experiment. Results showed that seven expressed genes were specifically up-regulated (>1.5-fold) in response to FV in Ye478, while 482 genes were significantly up-regulated in Bt-1, compared to their controls. Overall, the identity of the up-regulated genes indicates that the response of maize bract tissue to FV infection involves a complicated host-pathogen interaction. To the best of our knowledge, this study represents the first large-scale identification of genes differentially expressed in maize ear rot after FV infection, providing new insight into the host processes potentially involved in maize defense against this pathogen.

**Keywords:** Ear rot; Genechip; *Fusarium verticillioides*; up-regulated genes; *Zea mays*.

**Abbreviations:** PR-Pathogenesis-related protein, P450-cytochrome P450, UDP-UDP-glucosyltransferase, SAM-S-adenosylmethionine synthase, WRKY-WRKY transcription factor, MYB-MYB transcription factor, MAPK-MAP kinase, CDPK-CDPK-related protein kinase, POD-peroxidase, GL-germin-like.

### Introduction

*Fusarium* ear rot, predominantly caused by *Fusarium verticillioides*, *F. proliferatum*, and *F. subglutinans*, is among the most destructive diseases for its decrease of grain yield in maize (Robertson-Hoyt et al., 2006; Reid et al., 1999). Especially, a high incidence of ear rot occurs in the moist and humid regions of Southwest China, as well as other regions with similar longitude in other countries (Ali et al., 2005). The symptom for *Fusarium* ear rot usually consists of a white or light pink mold on bracts or kernels (Munkvold, 2003). Biochemical treatments and planting resistant maize inbred lines are the most common methods for controlling this disease. However, current resistant inbred lines are only partially resistant and severe outbreaks of ear rot can occur when climatic conditions are favorable for the pathogen (Chungu, et al., 1996). It has been proven that genetic manipulation of genes involving in pathogen defense responses has been frequently demonstrated to improve disease resistance in a range of plants, sometimes more effectively and with more stable and longer-lasting effects (Gao et al., 2007; Grison et al., 1996; Datta et al., 2002; Maruthasalam et al., 2007). While nevertheless, few genes associated with maize ear rot resistance against FV have been identified and their inheritance remains uncertain. Within the past several years, considerable progress has been made in the understanding of the resistance mechanisms involved in maize ear rot caused by FV infection, including isolation of disease resistance genes, characterization of defense responses, and elucidation of signal transduction leading to activation of defense responses (Casacuberta et al., 1991; Cordero et al., 1992; Multani et al., 1998; Poppenberger et al., 2003). In response to FV infection, the disease resistance genes Hm1 and ZmGC1 (guanylyl cyclase-like protein) were

isolated in maize and found to be highly involved in resistance to ear rot (Johal et al., 1992; Yuan et al., 2008). It is widely accepted that plant resistance to diseases such as maize ear rot is a multigenic trait linked to quantitative trait loci (QTL) (Young, 1996). In our previous studies, 10 resistant QTLs on chromosomes 1, 2, 3, 4, 6, 7 and 9 for FV ear rot resistance using maize inbred lines R15 (resistant parent) and Ye478 (susceptible parent) have been mapped on the chromosomes (Zhang et al., 2006). Although QTLs mapping has advanced our knowledge regarding the genetic mechanisms of disease resistance, the molecular processes and gene regulation of the maize defense system for ear rot remain poorly understood. In this study, one objective was to characterize host gene expression changes in two maize inbred lines, which could improve our knowledge of defense mechanism in maize ear rot.

### Results

#### *Time course of pathogen invasion between Bt-1 and Ye478*

FV invasion of maize bract tissue in resistant inbred line Bt-1 and susceptible inbred line Ye478 was observed through SEM at 24, 48, 72, 96, 120, and 144 h after inoculation (hpi). Microscopic images of infected tissue 48, 72, and 120 hpi are presented in Fig. 1. Once FV was able to invade the bract tissue, more hyphae assembled into the stoma by 120 hpi in both inbred lines, although invasion was delayed in resistant line Bt-1, as shown in Fig. 1 (left lower panel). This phenomenon indicates that more possible defense mechanisms were activated in inbred line Bt-1 than Ye478.

### FV-induced gene expression changes in maize

To identify specifically expressed genes in response to FV inoculation at the fourth day, genechip hybridization were performed using RNAs from the independent FV-infected bract tissues and their controls (Table 1). In total, 482 unique genes were found to be up-regulated more than 1.5-fold in resistant line Bt-1 (ANOVA,  $p < 0.05$ ) when compared to controls, using the GeneChip Maize Genome Array platform. However, only seven genes were up-regulated in susceptible line Ye478 (these same genes were also found among the 482 genes in Bt-1), indicating that gene expression in the resistant genotype responded strongly to FV. No gene showed greater than a 1.5-fold decrease in either inbred line. A set of genes associated with defense activities, such as PR-1 (4.5-fold), PR-2 (15.3-fold), PR-4 (chitinase) (1.6-fold), P450 (29.9-fold), UDP (11.4-fold), SAMS (16.5-fold), MAPK (2.4-fold), CDPK (2.8-fold), MYB (4.9-fold), WRKY (4.7-fold), POD (5.0-fold), GL (9.8-fold) were specially induced based on genechip data, respectively. Further analysis of the 482 FV-induced genes identified in Bt-1 indicated that 372 are already annotated, since the remaining 110 are unknown, based on the UniGene assignment.

### Sequence analysis and functional categorization

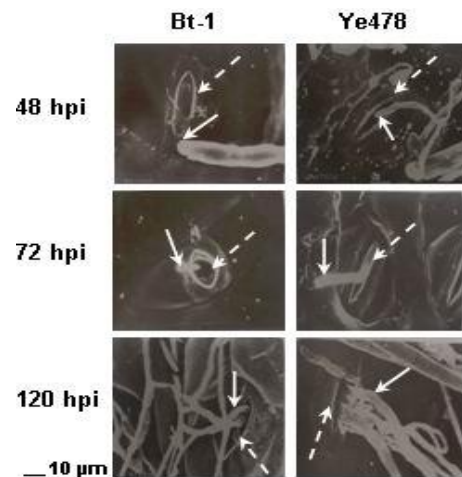
Based on genechip analysis, we were able to identify and assign putative function of the FV-induced genes into eight functional categories in resistant inbred line (Fig. 2) and all the differential genes are shown in supplementary Table 2. The largest category “defense anti-microbial proteins” was the most abundant and accounted for 15% of the identified genes. Other categories included “metabolism and energy” (14%), “signal transduction” (12%), “transcriptional regulators” (11%), “reactive oxygen scavengers” (11%), “protein destination” (7%), “transporters” (5%) and “stress proteins” (3%). A total of 22% of the genes were “unclassified or with unknown function proteins”. Further classification at detailed levels were significantly enriched in seven cellular component categories, five molecular function categories, and nine biological process categories by GO annotation (Fig. 3).

### Confirmation of differentially expressed genes by semi-quantitative RT-PCR

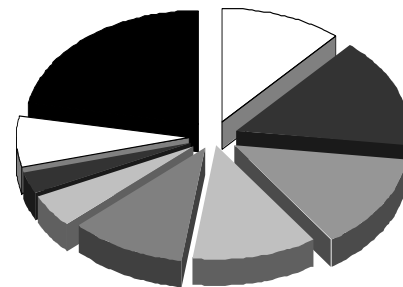
According to the Affymetrix maize GeneChip analysis, expression patterns of 12 representative marker genes were investigated using RT-PCR throughout each of six stages in resistant inbred line Bt-1 after FV infection. Results confirmed that the expression levels for all selected genes were differentially expressed during FV invasion. Further, most of them were distinctly induced comparing to the  $\beta$ -actin control levels, which did not differ between samples (Fig. 4). Expression patterns of these representative genes in RT-PCR were compatible with the results obtained from genechip data.

### Discussion

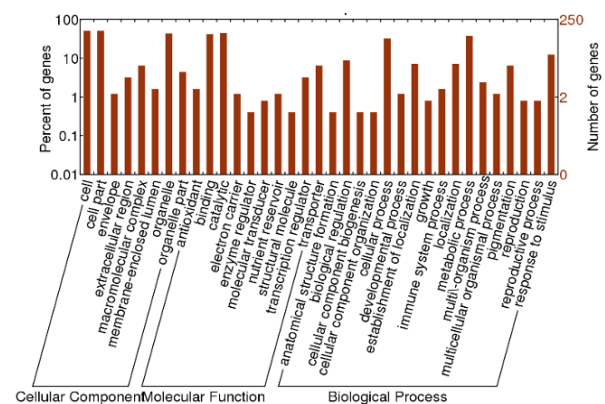
Ear rot disease in maize has a direct effect on maize kernels and bract tissues; thus, investigation of the defense responses that occur in bract tissues following inoculation with FV will improve our understanding of the host-pathogen interaction. Genechip analysis performed in this study indicates that the interaction between maize and FV results in a range of induced genes encoding important proteins in plant defense. By comparing the responses of two distinct genotypes (resistant and susceptible) upon FV infection, the lower number of



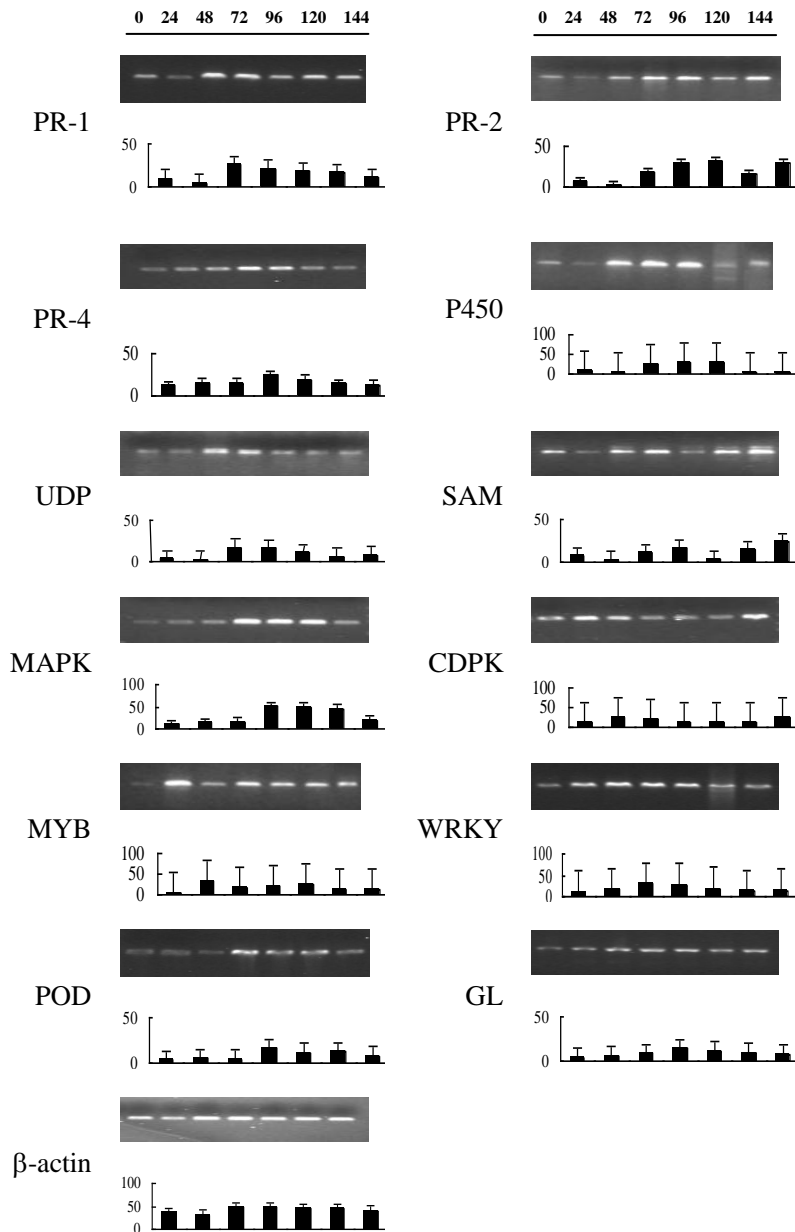
**Fig 1.** Electronic microscope images of the hyphea invasion procedure in stomas of both Bt-1 and Ye478 inbred lines at 48, 72, and 120 hpi ( $\times 1300$ ). The hyphae of FV are marked with solid arrows and the stomas of host tissues are marked with dashed arrows.



**Fig 2.** Functional categorization and percentage of FV-inoculated genes based on their putative functions. Genes were classified into 9 categories. Percentages represent the proportions of genes belonging to a particular functional group of the total number of genes, including genes with known functions, unclassified and unknown functions. A total of 489 genes from genechip analysis were submitted to GeneBank to predict the putative functional classification of individual proteins, including genes with known and unknown functions.



**Fig 3.** Molecular annotation on the differential genes from genechip data.



**Fig 4.** Expression profiles of 12 differentially expressed genes selected from genechip analysis. Maize bract tissues collected at 0, 24, 48, 72, 96, 120, and 144 h after FV-inoculation were analyzed by RT-PCR in resistant inbred line Bt-1.  $\beta$ -actin genes were amplified as RT-PCR controls.

up-regulated genes in Ye478 may be due to the constitutive expression of many defense genes at a higher or lower level than in Bt-1 prior to FV infection and expected to be only weakly modulated in response to FV attack in Ye478. However, the situation is different in resistant inbred line Bt-1 for the strong modulating in the response. This different transcriptional change in pathogen-affected tissues is also consistent with previous findings in other plant-pathogen interactions (Fung et al., 2008; Schenk et al., 2003; Tao et al., 2003). Moreover, it is possible that there were no down-regulated genes (>1.5-fold) in maize bract tissue after FV infection because both inbred lines maintain up-regulation of defense genes in response to FV infection. Other possible explanations are that the abundance of those down-regulated genes is too low and hard to screen in both inbred lines, or that the maize GeneChip does not represent the entire maize genome. Future work through

multiple approaches is needed for further understanding of the mechanisms of altered gene expression between resistant inbred line Bt-1 and susceptible inbred line Ye478 after FV infection. FV-inducible genes classified as different functional categories, such as defense anti-microbial proteins, metabolism and energy, signal transduction, transcriptional regulation, reactive oxygen scavengers or genes involved in other functions. Many genes identified by genechip were associated with roles in disease defense for its direct or indirect anti-fungal activities in response to FV infection. It is possible that the constitutively elevated expression of these genes in maize play important roles in modulating the response to FV infection or enhancing plant protection system. In summary, this study is the first step toward better understanding of the molecular responses triggered in maize after FV infection resulting in ear rot disease.

## Materials and methods

### Plant materials and inoculation procedures

Two maize inbred lines, resistant Bt-1 and susceptible Ye478 with different genetic background, were identified for this study in preliminary evaluation through many years of field trial. The line Bt-1 is derived from the tropical germplasm with high resistance to *Fusarium* ear rot and the line Ye 478 is an elite inbred in China with highly susceptible to *Fusarium* ear rot in southwest China. The spores of FV were prepared by washing conidia from the cultures and diluting to a final concentration of approximately  $1.0 \times 10^6$  spores/mL in water. Milky stage maize plants were treated with 3 mL on each bract by injection. The infected bract tissues were collected six times at a 24-h interval after inoculation.

### Microscopic observation

The invasion procedure of fungus on bract tissue was investigated by SEM at 24-h intervals from the inoculated bract tissues immediately after injection and controls. Samples were initially fixed for 3 h with 2.5% (v/v) glutaraldehyde in 0.024  $\mu\text{mol.L}^{-1}$  phosphate buffer (pH 6.8). After several washes in buffer, the specimens were post-fixed with 1% (v/v) osmium tetroxide for 5 h at 4 °C. Tissue dehydration was carried out in a series of ethanol dilutions and replaced by isoamyl acetate, followed by critical point drying and carbon coating. Observations were made on a JEM-100CX scanning electron microscope operating at 25 kv.

### Sampling and Affymetrix chip hybridization

The fourth day-post-inoculation and mock-inoculation bract tissues were sampled by collecting two independent biological replicates, each consisting of independent maize bract tissue. Inoculated bracts and controls were frozen immediately in liquid nitrogen and sent to the Bioassay Laboratory of CapitalBio Corporation (Beijing, China) for cDNA synthesis, labeling, hybridization with the maize Affymetrix GeneChip Maize Genome Array (Affymetrix, Santa Clara, CA, USA), which contains 17,555 probe sets representing 14,850 maize gene transcripts. Resulting Affymetrix data files are publically available from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number **GSE19501**. Data analysis was performed using Affymetrix GeneChip Operating Software Version 1.4 (GCOS). Statistical analysis was performed to identify genes that were differentially expressed in FV-infected samples compared to controls using analysis of variance (ANOVA,  $p < 0.05$ ) across all replicates. Genes were then described as 'up-regulated' or 'down-regulated' if their change in expression was  $> 1.5$ -fold.

### Annotation and sequence alignment

Annotation of gene sequences was performed by searching the NCBI database (<http://www.ncbi.nlm.nih.gov/>) for homology sequences using the Basic Local Alignment Search Tool X (BLASTx) algorithm. Genes were classified as unknown if no reasonable alignments. The putative physiological functions of sequences were classified according to the GeneOntology annotation of component function, biological process, and cellular component ontologies.

### Confirmation of differentially expressed genes by performing semi-quantitative RT-PCR

The PCR reactions were performed in 30 cycles of 94 °C for 30 s, 30 s at the primer-specific annealing temperature, and 72 °C

for 30 s. Specific primers used in sets for PCR were designed for target genes using Primer Premier 5.0 (Table 3). The intensity of each band (area) was estimated with Image J software (USA) and normalized with the intensity of the internal control  $\beta$ -actin gene. Each PCR reaction was repeated at least three times.

## Conclusion

This study represents the first large-scale identification of genes differentially expressed in maize ear rot after FV infection in two different genetic background inbred lines. Genes identified here that warrant further investigation into their potential to improve disease resistance include those encoding FV-induced defense genes. Future studies may reveal how specific manipulation of the components of host defense affect the outcome of interaction between maize and FM. Manipulation of defense genes has been demonstrated to improve resistance to pathogen infection for a range of diseases, and a similar approach of over-expressing one or more genes encoding defense compounds may improve resistance of maize to ear rot disease.

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