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Transcriptional changes in developing maize kernels in response to fumonisin-producing and nonproducing strains of *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides infects maize producing ear rot, yield loss and the accumulation of fumonisins. In the present study, a transcriptomic approach was employed to investigate the molecular aspects of the interaction of susceptible/resistant maize genotypes with fumonisin-producing/nonproducing strains of *F. verticillioides* over a time course of 4 days after inoculation. The fumonisin-nonproducing strain led transcription in susceptible maize kernels, starting from 48 h post inoculation, with a peak of differentially expressed genes at 72 h after inoculation. Pathogen attack altered the mRNA levels of approximately 1.0% of the total number of maize genes assayed, with 15% encoding proteins having potential functions in signal transduction mechanisms, and 9% in the category of transcription factors. These findings indicate that signalling and regulation pathways were prominent in the earlier phases of kernel colonization, inducing the following expression of defense genes. In the resistant maize genotype, the *fum1* mutant of *F. verticillioides*, impaired in this polyketide synthase gene (PKS), provoked a delayed and weakened activation of defense and oxidative stress-related genes, compared to the wild-type strain. The inability to infect resistant kernels may be related to the lack of PKS activity and its association with the lipoxygenase pathway. Plant and fungal 9-lipoxygenases had greater expression after *fum1* mutant inoculation, suggesting that PKS plays an indirect effect on pathogen colonization by interfering with the lipid mediated cross-talk between host and pathogen.

1. Introduction

Fusarium verticillioides is a pathogen of maize and it causes ear rot and contamination of grain with the mycotoxins fumonisins. These toxins disrupt sphingolipid metabolism through the inhibition of the enzyme ceramide synthase [1]. Fumonisins are polyketides and are synthesized by the condensation of acetyl units by a polyketide synthase (PKS), composed of seven functional domains (ketoacyl synthase, acyl transferase, acyl carrier protein, ketoacyl reductase, dehydratase, methyl transferase, and enoyl reductase) [2]. In *F. verticillioides*, fumonisin biosynthetic genes (*FUM*) are clustered into 17 co-regulated genes, designated as *FUM1*–*FUM3*, *FUM6*–*FUM8*, *FUM10*, *FUM11*, and *FUM13*–*FUM19* [3]. Another relevant *FUM* cluster gene is *FUM21*, which is adjacent to *FUM1* and encodes a Zn(II)2Cys6 binuclear DNA-binding transcription factor that regulates expression of almost all *FUM* genes [4]. Gene deletion analysis has confirmed that the disruption of *FUM1*, *FUM6* and *FUM8* blocked fumonisin production [5].

It has been reported that fumonisin production does not have a major effect on the ability of *F. verticillioides* to cause maize ear rot [6]. A rigorous test of the importance of fumonisins in ear rot and ear infection would use strains that are identical except for a gene that confers fumonisin production. Isogenic fumonisin-nonproducing mutants were generated by disrupting *FUM1*, the gene encoding PKS, that is required for fumonisin biosynthesis [7]. *Fum1* mutants are 100% reduced in fumonisin production in culture but, in field tests, they were able to cause ear rot following different inoculation methods. The results provided evidence that production of fumonisins was not required for maize ear rot by *F. verticillioides* and suggest that it is unlikely that fumonisin resistance would be

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an effective way to control this disease or fumonisin contamination in maize [8]. However, studies with fumonisin-producing and non-producing strains of *F. verticillioides* revealed that the severity of root and seedling rots was significantly correlated with the fumonisin levels and the degree of distruption of sphingolipid metabolism in maize seedlings [1]. Moreover, *F. verticillioides* produces several other toxins (e.g., fusaric acid, fusarins and moniliformin) and additional compounds still remaining unidentified that may contribute to the virulence of this fungus. It is possible that methods to control *F. verticillioides*-induced maize ear rot and fumonisin contamination will become apparent if the relationships of these toxins and virulence are critically examined [9]. Much progress has been done on *F. verticillioides* genomic resources, which has provided researchers with powerful tools to speed the process of identifying new fungal genes and understanding their role in pathogenesis events and mycotoxin biosynthesis [10,11].

The molecular interaction between the fungus and the plant is not well known and only recently comprehensive approaches of transcriptome and proteome analysis have become available. Proteomic studies were performed in germinating maize embryos to identify the response to fungus infection at protein level [12,13]. Different kinds of enzymes were detected such as antioxidant enzymes, Cu/Zn-superoxide dismutase, glutathione-S-transferase and catalase, and pathogenesis-related (PR) proteins β-1,3-glucanases and chitinases [14]. The up-regulation of the enzyme aldolase and the down-regulation of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were observed, that suggested a sugar metabolism alteration in infected plants. More recently, differential expression of messenger RNAs provided intriguing results [15,16]. A high level of variability was detected for the response to *F. verticillioides* infection between susceptible and resistant maize lines. Similar functional categories of genes were involved in the response to infection in resistant and susceptible maize genotypes, but, in the susceptible line, the genes were induced after pathogen infection, while in the resistant line, they were transcribed at high level before infection and provided basic defense to the fungus. This result suggested that plant basal defense genes in seed tissues may be involved in reducing colonization of *F. verticillioides* and fumonisin synthesis [17].

Specific genes putatively providing resistance to *Fusarium* pathogens have also been identified. The disruption of the maize 9-lipoxygenase gene (9-*LOX*) determined increased resistance to fungal pathogens and reduced fumonisin contamination [18,19]. Plant LOXs catalyse the incorporation of molecular oxygen into PUFA, primarily linoleic (C18:2) and linolenic (C18:3) acids, at either position 9 or 13, in a stereospecific manner and, therefore, are referred as linoleate 9-LOXs or 13-LOXs [20]. In plants, oxylipins are involved in different events: they stimulate signals implicated in the onset of plant defenses against pathogens and pests, have antimicrobial effects, provide building units of physical barriers against pathogen invasion, regulate plant cell death and have a major role in the formation of phytohormones and in senescence [21]. Also in fungi, by-products of LOX enzymatic activities play a relevant role in different events, that is by modulating morphogenesis and secondary metabolism (e.g., mycotoxins biosynthesis) [22]. Therefore the oxyilipins and the PUFA produced by plants can control, by a cross-kingdom cross-talk, the synthesis of toxins by pathogenic fungi and, vice versa, fungi may alter plant-defense responses by releasing plant-like oxylipins (e.g., jasmonic acid) into the host cell [20]. In fact, hydroperoxides formed from linoleic and linolenic acid can play different physiological roles, as reported for *Aspergillus*, where 9-LOX stimulates the expression of aflatoxin-related genes, while 13-LOX inhibits their induction.

In previous studies [15,16], we found significant differences between resistant and susceptible genotypes of maize in response to infection with fumonisin producing strains of *F. verticillioides*. Our objective in this study was to investigate at the transcriptional level the host response to *fum1* mutant strain of *F. verticillioides* over a time course of 4 days after inoculation. The data obtained provide essential tools to investigate the molecular aspects of the interaction of fumonisin-producing/nonproducing strains of *F. verticillioides* with susceptible/resistant maize genotypes.

2. Materials and methods

2.1. Maize inbreds, growth conditions, fungal strains and inoculation assays

The maize inbred lines CO441 and CO354 were used as resistant and susceptible genotypes, as previously reported [15,23,24]. Both the lines were developed by the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada (AAFC), Ottawa, Canada, and were maintained by sibling at the Institute of Agronomy in Piacenza.

For microarray experiments, seeds of the lines were planted in pots (40 cm diameter, 35 cm height) and 10 plants of each line were grown up. Before inoculation, pots were transferred to an environmentally controlled greenhouse with day-time and night-time conditions of 28 °C and 20 °C temperature, respectively, and a light regime of 16 h using lamps at intensity of 500 μmol m^{-2} s^{-1} (Master TLD 58 W/830, Royal Philips Electronics, Eindhoven, The Netherlands).

We used the wild-type *F. verticillioides* strain ITEM 10514, a fumonisin-producing strain, and its corresponding *fum1* mutant ITEM 10515, a fumonisin-nonproducing strain, generated from ITEM 10514 by partial deletion of the *FUM1* gene [7]. Cultures were maintained on Petri plates (9 cm diameter) in Potato Dextrose Agar (PDA; infusion from potatoes 200 g; dextrose 15 g; agar 20 g; H_2O to 1 L) and incubated at 25 °C with a 12 h photoperiod for 14 days. Conidia were collected by rinsing plates with sterile water, scraping the agar surface with a scalpel and filtering the conidia suspension through sterile cloth. Spore suspension was obtained by adding 200 ml of sterile water to a final concentration of 3.5×10^6 conidia/ml based on microscopic counts using a Bürker chamber.

Maize ears for microarray analysis were inoculated at 15 days after pollination (DAP) using a pin-bar inoculator. The inoculating device consists of two 100 mm-long rows of 10 needles mounted on a wooden bar. Pins were dipped in the conidial suspensions and the bar was pressed through the husks sideways and into the centre of the ear, penetrating the kernels to a depth of 5–10 mm.

Seeds were collected at 12, 24, 48, 72 and 96 hours after inoculation (hai). Control seeds were sampled at the same inoculation times listed above and considered as uninoculated. Kernels were sampled only in the area around the point of inoculation, to evaluate fungal growth and colonization and to avoid mechanical damage due to needle-prick, as previously reported (see Fig. 1 in [16]). Three biological replicates were taken for each time point.

2.2. RNA extraction and microarray analysis

The collected samples were ground in liquid nitrogen with a pestle and mortar and total RNA was extracted from 2.5 g of seeds using Trizol protocol (Invitrogen, Carlsbad, CA, USA). RNA was then purified with the RNA Clean up protocol (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The amount and the quality of the total RNA were estimated by fluorimetric assay (Qubit, Invitrogen) as well as by agarose gel electrophoresis.

We used the SAM 1.2 Chip, a 15,680 element cDNA microarray chip generated at Iowa State University's Center for Plant Genomics, USA. Total RNA was reverse transcribed following the protocol of the Superscript Direct Labelling Kit (Invitrogen). The