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Infection with toxigenic and atoxigenic strains of *Aspergillus flavus* induces different transcriptional signatures in maize kernels

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**ABSTRACT**

The application of atoxigenic *Aspergillus flavus* strains in maize fields has been shown to be an effective strategy for controlling contamination of aflatoxins, potent carcinogens produced by the fungus. This study monitored the expression levels of 18 defense genes against toxigenic and atoxigenic *A. flavus* strains in developing maize kernels over a time course of 96 h after inoculation. A stronger upregulation of genes encoding pathogenesis-related proteins, oxidative stress-related proteins, transcriptional factors and lipoxygenases were observed in response to the atoxigenic strain. On the other side, this strain showed a significant enhanced growth in the later stages of infection, measured as copy number of the constitutive calmodulin gene. These results suggest that overexpression of maize-defense-associated genes observed in response to the atoxigenic strain could contribute to an aflatoxin reduction. The identification of genes significantly affecting the resistance to *A. flavus* or aflatoxin accumulation would accelerate the development of resistant cultivars.

**1. Introduction**

Aflatoxins are polyketide-derived secondary metabolites primarily produced by *Aspergillus flavus* in several host crops, highly toxigenic and carcinogenic to humans and animals consuming contaminated food and feed (Bhatnagar et al. 2006). Aflatoxin contamination of maize is a serious safety issue worldwide but, until few years ago, it has not been signaled as a matter of concern in Europe (Logrieco & Moretti 2008). However, the alarming contaminations reported in 2003, 2012 and 2015 for Italy and south Europe, respectively, must be mentioned (Giorni et al. 2007; Levic et al. 2013).

Aflatoxin mitigation in maize through proper cropping systems, such as tillage, irrigation, early sowing and harvesting, is commonly not sufficient for the production of grain compliant with legal limits. Several attempts to develop varieties resistant to fungal growth and/or aflatoxin contamination have been carried out using breeding strategies (conventional and based on genetic modifications). As a result, many new sources of resistance to *Aspergillus* infection and the subsequent accumulation of aflatoxins in corn have been identified and released (Chen et al. 2015). In the long term, the creation of resistant germplasm with desirable agronomic characteristics could be the most economically efficient control measure for all markets (Williams et al. 2015).

The biocontrol strategy, based on the competitive exclusion of toxigenic by applying atoxigenic *A. flavus* strains in field, has been shown to significantly reduce aflatoxin contamination in the USA and African countries and may reduce the problem in the short term as well (Ehrlich 2014). Atoxigenic strains of *A. flavus* were reported to reduce the contamination of aflatoxin up to 90% in maize in in-vitro studies carried out with Italian strains (Mauro et al. 2015). Comparable results are reported in field trials managed in the USA and Africa (Abbas et al. 2006; Atehnkeng et al. 2016). Use of biocontrol products with atoxigenic *A. flavus* active ingredients is a proven method for reducing the aflatoxin content and will be crucial in the future; in fact, *A. flavus* and other aflatoxin-producing fungi thrive under the hot and dry environmental conditions recently experienced and predicted due to the ongoing climate change (Battilani et al. 2016). However, for this biocompetition strategy to achieve its full potential in mitigating the issue of aflatoxin contamination, management programs that optimize both biocontrol’s long-term and area-wide benefits are still needed.

Plants respond to fungal pathogens through various defense mechanisms that are host–pathogen dependent (Kelley et al. 2012). The fungal pathogens are basically classified into groups of biotrophs, hemibiotrophs and necrotrophs (Oliver & Ipcho 2004). The plant resistance to necrotrophic fungi is known to be quantitative and controlled by multiple genes (St Clair 2010). Toxigenic strains of *A. flavus* are characterized with the features of necrotrophic fungal pathogens (Kelley et al. 2012) and jasmonate- and ethylene-dependent signaling pathways are likely involved in such resistance systems, triggered by fungal toxins or other fungal effectors (Oliver & Ipcho 2004). The identification of specific genes playing a precise role in host resistance to *A. flavus* or aflatoxin accumulation would enable more rapid selection of resistant maize inbred lines and hybrids. Previous gene mapping studies led to the identification of several *A. flavus* resistance-related quantitative trait loci (QTLs) in maize (Brown et al. 2013). Interestingly, QTLs for resistance to aflatoxin accumulation appeared to be derived by both resistant and susceptible parental lines (Willcox et al. 2013). Nevertheless, the molecular mechanisms underlying the significant aflatoxin reduction in resistant maize lines or the significant
aflatoxin accumulation in susceptible maize lines are yet to be determined.

The aim of this study was to understand the molecular response of maize after infection with toxigenic and atoxigenic \textit{A. flavus} strains. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed by assaying a set of selected defense-related candidate genes on developing kernel samples of a susceptible inbred line over a time course of 96 h. The results report interesting differences between toxigenic and atoxigenic strains in the expression level of the assayed genes and will help in understanding the complex genetics involved in plant-pathogen interaction.

2. Materials and methods

2.1. Plant and fungal materials

The maize inbred line CO354 was used as a susceptible genotype to \textit{A. flavus} infection (Lanubile et al. 2015). The line was developed by the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada (Reid et al. 2009), and was maintained by pollinating sister plants at the Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy. The study was carried out in open air and a randomized block design with three treatments (inoculation with water, toxigenic and atoxigenic strains of \textit{A. flavus}) and three replicates were adopted. Six plants distributed in three plastic pots were used for each replication. Each pot (40 cm diameter and 35 cm height) contained 25 kg of dry loam soil with the following properties: pH 7.5, organic matter 1.80%, available P 15.5 mg kg$^{-1}$ and exchangeable K 130 mg kg$^{-1}$. Maize seeds were sown in late April 2013 and plants were grown 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 suspension through sterile cloth. The suspension was adjusted to a final concentration of 3.5 × 10$^6$ conidia/ml using a Bürker chamber.

2.2. Fungal inoculation and sample collection

Maize ears were inoculated 15 days after hand-pollination using a side-needle inoculator, as reported by Lanubile et al. (2015). The inoculated and immediately adjacent kernels were collected at 24, 48, 72 and 96 h post-inoculation (hpi). Control ears were mock-inoculated (inoculation with water) at the same inoculation time listed above. Three biological replicates were prepared for each time point, collecting kernels from six different maize ears. They were ground in liquid nitrogen with a pestle and mortar and used both for gene expression analyses and aflatoxin quantification.

2.3. RNA extraction and real-time RT-PCR expression analysis

Total RNA extraction and purification were performed according to Lanubile et al. (2015). Real-time RT-PCR experiments were performed on kernels collected at 24, 48, 72 and 96 hpi using the 2x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the CFX-96 device (Bio-Rad). One microgram of total RNA was used for cDNA synthesis following the iScript cDNA synthesis kit protocol (Bio-Rad). Twenty nanogram of single strand cDNA, determined by fluorometric assay (Qubit, Invitrogen), were used for real-time RT-PCR. Relative quantitative analysis was performed under the following conditions: 95°C for 3 min and 40 cycles at 95°C 15 s, 57–63°C for 30 s. A melting curve analysis, from 60°C to 95°C with a 0.5°C increment each 5 s, was used to identify different amplicons, including nonspecific products. Three technical replicates (within each biological replicate) were employed for each tested sample and template-free negative controls. Gene-specific primers were downloaded from the literature or designed possibly within consecutive exons, separated by an intron, using Primer3 software (supplemental Table 1). Relative quantification of maize genes was normalized to the housekeeping gene \textit{β-actin} (supplemental Table 1) and fold change (FC) values in gene expression were analyzed by using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008).

To quantify fungal growth, the copy number of \textit{calmodulin} transcript was detected using real-time RT-PCR; thermal cycling conditions were same as those reported above. The \textit{calmodulin} number of copies is related to the ng of cDNA obtained from kernel tissues according to the equation of the linear regression reported in the technical manual (Bio-Rad). Fungal cDNA (20 ng) was serially diluted [1:1, 1:5, 1:5$^2$, 1:5$^3$, 1:5$^4$, 1:5$^5$] in sterile water and 20 ng of each kernel cDNA sample was compared to the dilution standard curve to determine fungal cDNA copy number.

2.4. Aflatoxin analysis

Aflatoxin content in kernels was measured according to Bertuzzi et al. (2012). Briefly, 5 g of kernel sample, homogenized at 96 hpi, was extracted for 60 min with 20 mL of acetone-water 7 + 3 v/v using a rotary-shaking stirrer. After purification through an immunoaffinity column, the extract was filtered (Millex HV 0.45 mm) before high-performance liquid chromatography–fluorescence detection analysis (Jasco Corporation, Tokyo, Japan). Detected aflatoxins B$_1$ (AFB$_1$) and B$_2$ (AFB$_2$) were expressed in µg kg$^{-1}$. The limit of detection was 0.1 µg kg$^{-1}$.

2.5. Statistics

Two-factor analysis of variance (ANOVA) was performed on all genes, considering the sampling time points (24, 48, 72 and
96 hpi) and treatments (aflatoxin-producing and atoxigenic-inoculated samples) as fixed factors, in order to test the significance (P ≤ .05) of sampling, treatments and their interactions. Differences between aflatoxin-producing and atoxigenic strains within the same time of sampling were performed using two-way ANOVA and considered to be significant at *P ≤ .05; **P ≤ .01; ***P ≤ .001.

3. Results and discussion

3.1. Fungal growth and aflatoxin accumulation in kernels challenged with toxigenic and atoxigenic strains of A. flavus

In order to determine differences in fungal colonization between the toxigenic (MPVP A2092) and atoxigenic (MUCL54911)-inoculated maize kernels from ears of the susceptible CO354, quantification of the fungal calmodulin transcript by real-time RT-PCR was carried out in this study (Table 1). Fungal growth of both A. flavus strains increased over the time course of 96 h, with the highest levels of calmodulin copy number at 72 hpi (818 vs. 900 copies for the toxigenic and atoxigenic strains, respectively) (Table 1). The decrease in calmodulin transcripts from 72 to 96 hpi probably coincided with the beginning of the necrosis of infected maize kernel. No significant differences were observed in the ability to colonize maize ears at 24 and 48 hpi between the two strains. On the other hand, in the later stages of infection the atoxigenic strain showed a significantly higher copy number compared to the toxigenic one (900 and 545 copies at 72 and 96 hpi, respectively, for the atoxigenic strain compared to 818 and 400 copies for the toxigenic one). Aflatoxin levels in kernels inoculated at 96 h with the toxigenic strain were 3900.2 and 3.1 μg kg⁻¹ for AFB₁ and AFB₂, respectively, while, as expected, no aflatoxins were produced by the atoxigenic strain. The toxigenic strain A2092 was reported as one of the best producers of AFB₁ on cocoanut-based medium (Giorni et al. 2007). Aflatoxin production in the Aspergilli is a complex process under a high degree of regulation through multiple mechanisms (Amaike & Keller 2011). Currently, the molecular mechanisms responsible for the loss of aflatoxin production in A. flavus are not well understood (Schmidt-Heydt et al. 2008). Atoxigenic A. flavus isolates were commonly deleted of a part or the entire aflatoxin gene cluster (Chang et al. 2005). The atoxigenic isolate MUCL54911 belongs to the VCG IT6 that was understood (Schmidt-Heydt et al. 2008). Atoxigenic strains within the same time of sampling were performed using two-way ANOVA and considered to be significant at *P ≤ .05; **P ≤ .01; ***P ≤ .001.

Table 1. Analysis of A. flavus infection steps.

|          | Copy N° | SD | Copy N° | SD | Copy N° | SD | Copy N° | SD |
|----------|---------|----|---------|----|---------|----|---------|----|
| 24 hpi   |         |    |         |    |         |    |         |    |
| Toxigenic| 200     | 15 | 600     | 45 | 818     | 70 | 400     | 23 |
| Atoxigenic| 150    | 12 | 545     | 31 | 900     | 65 | 525     | 34 |

Notes: Copy number (Copy N°) of transcripts for the constitutive gene calmodulin in kernels of the susceptible CO354 maize line inoculated with the toxigenic (MPVP A2092) and atoxigenic (MUCL54911) strains of A. flavus, over a time course of 96 hpi. Values represent the mean ± standard deviation (SD) of three pools of kernels for each time-point, where each pool derived from the mixing of three different ears. Same letters state not significant differences between means of toxigenic (Greek letters)- and atoxigenic (Latin letters)-inoculated samples, as resulting from Tukey's honest significant difference test (P ≤ .05). *Significant differences between atoxigenic and toxigenic-inoculated means within the same time of sampling, according to two-way ANOVA (*P ≤ .05; **P ≤ .01).

3.2. Time course expression analysis on selected candidate genes

Determination of mechanisms underlying maize host resistance to aflatoxin accumulation has been proved difficult due to the complex nature of this quantitative trait. Many genes were found to be involved in the maize host resistance (Kelley et al. 2012). The identification of genes having larger effects on the resistance or susceptibility is important to facilitate marker-assisted selection breeding of resistant maize lines. Without aflatoxin-resistant commercial varieties, farmers rely on cultural practices and lately biocontrols to manage A. flavus infection and to reduce aflatoxin contamination. Our study provides the first investigation of gene expression changes that occur when maize is inoculated with A. flavus by comparing toxigenic and atoxigenic interactions in the same genetic background, using the susceptible CO354 genotype. Eighteen potentially relevant genes related to maize defense to A. flavus infection were selected, and then real-time RT-PCR expression analysis was conducted to take a closer look at the highly expressed host plant genes and to strengthen our knowledge concerning novel details of infection by using two different host-pathogen combinations.

3.3. Expression of pathogenesis-related genes

Upon pathogen attack, plants react with complex defense responses including the production of pathogenesis-related (PR) proteins. Among the 17 PR protein families the expression profiles of five PR genes (PR1, PRm3, PR5, PRm6 and PR10) were investigated after inoculation of maize kernels with toxigenic and atoxigenic strains of A. flavus (Figure 1(a)–(e)). Compared to mock-inoculated control kernels, PR gene expression was induced by the inoculation of maize ears with both A. flavus strains as early as 24 up to 96 hpi. Interestingly, significantly higher FCs were observed in response to the atoxigenic strain for all tested genes starting from 48 hpi and 96 hpi. The atoxigenic strain caused drastic induction of PR1 and PR10 genes (201.4 and 34.4 FCs, respectively) at 48 hpi that declined in the further time-points (Figure 1(a) and 1(e)). Similarly, PR5 and PRm6 reached the highest level of induction at 48 hpi.
that remained high at 72 hpi, whereas declined at 96 hpi (Figure 1(c) and 1(d)). Conversely, the expression of PRm3 was different and peaked at 96 hpi with an FC value of 187.2 (Figure 1(b)). The most striking differences in the host response following inoculation with the atoxigenic and toxigenic strains of A. flavus were notably evident at 48 hpi, where the magnitude of induction was significantly higher in response to the atoxigenic one of 6.4 fold for PR10 gene, followed by a 5.1, 3.7 and 2.6 fold induction for PRm6, PR1 and PR5 genes, respectively. In the later time-points (72–96 hpi) less pronounced differences were observed, with the exception of PRm3 gene expression that was 5.5-fold significantly upregulated at 96 hpi with the atoxigenic strain compared to the toxigenic one (Figure 1(b)). Even though PR10 gene displayed the lower FC values compared to the other PR genes, however, the most marked significant differences between the atoxigenic and toxigenic strains were observed for this gene all over the considered time course. The expression of PR10 gene has been reported to be induced in fungal-infected maize seed (Cordero et al. 1994). Furthermore, studies by Chen et al. (2010) showed that PR10 has antifungal activity against A. flavus in vitro. They also showed that repression of maize PR10 by RNAi gene silencing resulted in increased susceptibility to A. flavus and aflatoxin production (Chen et al. 2010).

PRm6 was the gene with the highest induction values ranging from 128.7 to 4320.9 with the atoxigenic A. flavus and from 54.2 to 1771.7 with the toxigenic one over the time course of four days (Figure 1(d)). It is well known that plant β-1,3-glucanases, such as PRm6, play a role in plant defense against various pathogen invasions. Several studies have revealed the presence of a relationship between maize β-1,3-glucanase and A. flavus infection. It was reported that the growth of A. flavus was inhibited more by callus of a resistant maize genotype (Tex 6 x Mo17) than by a susceptible genotype (Pa21) (Lozovaya et al. 1998). This inhibition was correlated with the activity levels of β-1,3-glucanase in the callus and in the culture medium. Bedre and co-workers (2015) reported that two β-1,3-glucanases (BG1 and BG3) were highly induced by an atoxigenic strain of A. flavus on cotton seed, whereas another BG3 transcript was upregulated specifically by the toxigenic strain (Bedre et al. 2015). The hydrolytic enzymes β-1,3-glucanase and chitinase, such as PRm3, possess antifungal activity by degrading the fungal cell wall containing chitins (Cleveland et al. 2004). Plant chitinases possess lysozyme activity and are highly active in

Figure 1. FC of differentially expressed genes PR1 (a), PRm3 (b), PR5 (c), PRm6 (d) and PR10 (e), in kernels of the susceptible CO354 maize line at 24, 48, 72 and 96 hpi with aflatoxin-producing and atoxigenic strains of A. flavus (white and gray bar charts, respectively). Vertical bars indicate ±standard deviation. *Significant differences between aflatoxin-producing and atoxigenic-inoculated means within the same time of sampling, according to two-way ANOVA (*P ≤ .05; **P ≤ .01; ***P ≤ .001).
inhibiting fungal growth (Prasad et al. 2013). Different isoforms of chitinases were induced by infection with both atoxigenic and toxigenic *A. flavus* in cotton pericarp and seed (Bedre et al. 2015). Furthermore, a chitinase A positioned in bin 2.04 was associated with a large aflatoxin reducing QTL, as reported by Hawkins et al. (2015).

Overall, the induction and greater accumulation of PR genes confirmed their essential role in the response against *A. flavus* pathogen. Furthermore, these data provided the first evidence that the transcriptional response of maize to an atoxigenic strain of *A. flavus* was more pronounced in FC values compared to the toxigenic one.

### 3.4. Modulation of reactive oxygen species scavenging-related genes

Oxidative burst is one of the earliest defense responses of plants against pathogen infection and wounding, and it is associated with production of reactive oxygen species (ROS; Dickman & Fluhr 2013). ROS are involved not only in direct defense reaction by killing the pathogens, but also in the activation of defense-related genes through a signaling mechanism (Torres 2010). ROS can regulate the transcription factors (TFs) and produce antimicrobial phytoalexins and other secondary metabolites, which have inhibitory activity on pathogen growth (Torres 2010). The transcripts encoding *catalase* (CAT), *ascorbate peroxidase* (APX), *peroxidase* (POX) and *superoxide dismutase* (SOD), which are involved in ROS processing and scavenging, showed increased expression levels under *A. flavus* infection in maize (Figure 2(a)–(d)). In general, the susceptible line showed a stronger induction for almost all antioxidant genes analyzed in this study in response to the atoxigenic strain, even though at less extend levels, as previously reported for PR genes. POX was the most induced gene with FC values ranging from 2.6 to 6.5 and from 2.2 to 3.9 over the time course following atoxigenic and toxigenic strain inoculation, respectively (Figure 2(c)), and the atoxigenic *A. flavus* inoculation elicited a reaction of about two times higher at the later time-points (72–96 hpi) compared to the toxigenic one (Figure 2(c)). Less marked significant differences were observed in favor of atoxigenic strain for CAT gene between 48 and 72 hpi and SOD gene from 48 to 96 hpi (Figure 2(a) and 2(d)), while the expression patterns for APX gene were similar under the atoxigenic and toxigenic strain inoculation (Figure 2(b)). The observed enhanced expression level of antioxidant mechanisms against the atoxigenic strain in maize tissues leads to the hypothesis that the increase in defense responses to ROS-induced oxidative stress may correlate to resistance to *A. flavus* and aflatoxin reduction. Prior research exploring the roles of oxidative stress in regulating aflatoxin biosynthesis as well as recent discoveries into the upstream regulation of major pathway regulatory factors has begun to elucidate the biological function of aflatoxins (Roze et al. 2013). Numerous studies have shown that aflatoxin production can be exacerbated by ROS and their reactive products, including oxylipins (Gao & Kolomens 2009). Several works have demonstrated that *A. flavus* toxigenic isolates exhibited elevated oxygen consumption, greater mycelial ROS accumulation and greater peroxisome number than atoxigenic isolates, indicating a reasonable link between ROS and aflatoxin accumulation (Reverberi et al. 2012). Given the possible role of oxidative stress in the promotion of aflatoxin biosynthesis, the hypothesis has been proposed that aflatoxin may function as a form of antioxidant protection to toxigenic *Aspergilli* (Reverberi et al. 2010), and as a consequence the toxigenic strain could use aflatoxin to inhibit and reduce the expression level of maize oxidative stress-related responses. This would provide an explanation to the biological significance of aflatoxin, although the potential antioxidant mechanism of action of this mycotoxin has yet to be fully elicited.

### 3.5. Changes in genes encoding TFs

The expression of downstream genes in response to pathogen infection is normally controlled by TFs, direct or indirect targets of various signal transduction pathways. Many components of these pathways are regulated by TFs of a type known as WRKY TFs (Rushton et al. 2010). WRKY TFs are abundant in many model and crop species, such as *Arabidopsis* (74), rice (109), soybean (176) and maize (136) (Wu et al. 2005; Pandey & Somssich 2009; Wei et al. 2012; Song et al. 2016). In the present study the expression levels of three WRKY and one *Myb*-like *DNA-binding protein* TF genes were also examined over the time course following toxigenic and atoxigenic *A. flavus* inoculation (Figure 3(a)–(d)). The expression of WRKY1 increased in inoculated kernels from 24 to 48 hpi and thereafter for both strain treatments (Figure 3(a)), but the atoxigenic strain caused a stronger significant induction compared to the toxigenic one from 48 to 96 hpi. The highest expression levels were observed for WRKY29 gene where significant differences were displayed between the two strains at 24 and 72 hpi (FC of 1 vs. 4.2 and 9 vs. 24.7 in response to the toxigenic and atoxigenic strains, respectively; Figure 3(b)). No significant variation in the expression levels between the two strain treatments was detected for WRKY110 gene, except at 72 hpi, which showed a marginally significant induction in response to atoxigenic *A. flavus* inoculation (FC of 1.4 vs. 2.1 against the toxigenic and atoxigenic strains, respectively; Figure 3(c)). This is in agreement with previous results reported by Wei et al. (2012), that also found a gradual increasing trend in the expression levels of maize WRKY29 gene (designated as WRKY39) during the infection with *Ustilago maydis*. On the other hand no expression changes were observed for WRKY110 gene (designated as WRKY30), suggesting that it may respond primarily to environmental stress and not to pathogen infections (Wei et al. 2012). Recent transcriptional profiling studies have shown that 28 WRKY-related transcripts were differentially expressed under toxigenic and atoxigenic strains of *A. flavus* in cotton seed and pericarp tissues (Bedre et al. 2015). Most of the WRKY TFs were upregulated under the atoxigenic strain infection in pericarp and the toxigenic strain infection in seed, with the exception of WRKY75 and WRKY40 genes that were specifically upregulated under the atoxigenic strain in both tissues. Furthermore, Fountain et al. (2015) examined the expression of several WRKY TFs in both resistant and susceptible maize inbred lines in response to *A. flavus* inoculation under field conditions over a time course of 18 days. Interestingly, elevated expression levels of WRKY53.1 were observed in the susceptible line B73. The significant induction of this gene could be due to the presence of at least two paralogous copies of the gene in the genome of B73 or to a defect in feedback inhibition of expression mediated by the WRKY53.1 protein (Fountain et al. 2015). Identifying downstream
target genes of WRKY factors will be crucial in understanding their biological functions. Transient overexpression of *Petroselinum crispum* WRKY1 in parsley protoplast led to the activation of a reporter gene driven by the promoters of three potential target genes, namely *Pc Pathogenesis-related 1-1* (*PcPR1-1*), *PcWRKY1* and *PcWRKY3* (Eulgem et al. 1999). Consistent with this, a strong induction of *PR* genes was also observed in our study, more marked...
in response to the atoxigenic *A. flavus* strain, as discussed in the previous paragraph.

Among TFs a pivotal role in biotic responses is also played by *Myb* genes. In the current study, *A. flavus* inoculation induced the *Myb* expression from 24 to 96 hpi, and significant different expression patterns were found between the two strains all over the time course considered (Figure 3(d)). The strongest induction was detected against the atoxigenic strain and transcripts were from 2.5 to 5.8-fold higher than that in response to the toxigenic one (Figure 3(d)). The *Myb* TFs regulate the downstream expression of flavonoid genes, *PR* genes and genes involved in secondary metabolism (Ambawat et al. 2013). Taken together these results suggest that atoxigenic *A. flavus* may have an enhancing effect on maize kernel TFs, which in turn could determine the increase in downstream *PR* and ROS-related gene expression.

### 3.6. Expression of lipoxygenase genes

Functional and physiological analyses of lipoxygenases (LOXs) were first addressed to dicots and recent studies aimed to unravel the role of LOX in plant–pathogen crosstalk with the use of maize mutants (Gao et al. 2009; Christensen et al. 2014; Maschietto et al. 2015) and fungal mutants (Lanubile et al. 2013; Scala et al. 2014). In this work the expression levels of three selected 9-LOX genes (*Lox*3, *Lox*5 and *Lox*12), one 13-LOX gene (*Lox*10), and the plastidial *Lox*6 gene were analyzed by real-time RT-PCR after treatment with toxigenic and atoxigenic *A. flavus* pathogens (Figure 4(a)–(e)). The most striking significant differences between the atoxigenic and the toxigenic strain were given at 72 hpi for all assayed genes. At this time-point atoxigenic strain inoculation caused a higher induction in the expression level of 1.9-fold for *Lox*3 (Figure 4(a)), 2.4-fold for *Lox*5 (Figure 4(b)), 2.2-fold for *Lox*6 (Figure 4(c)), 2.6-fold for *Lox*10 (Figure 4(d)) and about 5-fold for *Lox*12 (Figure 4(e)), compared to the toxigenic one. The expression level of *Lox*3, *Lox*5, *Lox*6 and *Lox*10 genes peaked at 72 hpi in response to the atoxigenic strain and declined to the toxigenic strain levels at 96 hpi, with the exception of *Lox*10; significant variations of *Lox*10 were still observed between the two strains at 96 hpi (Figure 4(d)). Overall, *Lox*12 gene showed the strongest induction at 48 hpi against both pathogens, with FC values of 9.5 and 20.6 for the toxigenic and atoxigenic strains, respectively (Figure 4(e)). Previous studies reported the identification of three differentially expressed *Lox* genes (*Lox*1, *Lox*2 and *Lox*3) in pericarp cotton tissue under toxigenic and atoxigenic *A. flavus* interaction, suggesting their

![Figure 4](image-url)
role in the mechanism of resistance against this pathogen (Bedre et al. 2015). Prior research illustrated the role of lipids, particularly oxylipins, in the specific interaction between maize and A. flavus and in regulating aflatoxin biosynthesis (Gao & Kolomiets 2009). It was found that disruption of the ZmLOX3 caused enhanced resistance to F. verticillioides, Colletotrichum graminicola, Cochliobolus heterostrophus, and Exserohilum fellucidatum (Isaie et al. 2007; Gao et al. 2009). However, the maize lox3 mutant showed increased susceptibility to A. flavus and Aspergillus nidulans, indicating that this gene regulated disease resistance in a pathogen-specific manner (Gao et al. 2009). Fabbri et al. (1983) found that seeds of high-oil crops such as peanut supported higher levels of aflatoxin production by Aspergillus parasiticus than seeds of graminaceous plants, such as maize or wheat, which contain higher levels of starch. In addition, they found that culturing A. parasiticus amended with peroxidized lipids resulted in significantly elevated aflatoxin production with no significant effect on fungal biomass (Fabbri et al. 1983). These findings indicate that certain 9-oxylipins can play important roles in suppressing aflatoxin biosynthesis while other oxylipins may promote aflatoxin biosynthesis (Gao et al. 2009). Results of this study suggest that low expression levels of maize defense-associated genes, such as Lox genes, displayed in response to the toxigenic A. flavus strain, may be related to the negative impact of aflatoxin. As a consequence the mycotoxic could provide a competitive advantage for the aflatoxigenic strain and predispose the susceptible plant to a greater infection.

4. Conclusions

The contamination of agricultural crops with aflatoxins is a major concern for global food safety and security, particularly in developing countries and in climate change scenarios. Numerous studies have shown that the resistant maize inbred lines exhibited significantly low levels of aflatoxin accumulation. Determination of the mechanisms underlying such maize host resistance to aflatoxin contamination has been proved difficult due to the complex nature of this quantitative trait. Use of non-aflatoxigenic strains has emerged as the best management practice for reducing aflatoxin contamination, and has led to commercially registered products, first in cotton (2003), then in peanut (2004), and more recently in maize (2008). In this study, we conducted real-time RT-PCR on 18 genes that comprise the potential components of maize host resistance. Four different maize functional groups of genes were considered, including PR, oxidative stress-related, TFs, and Lox genes, in response to aflatoxigenic and toxigenic A. flavus strains over a time course of 96 h. The transcriptional analysis provided a clue to the molecular mechanisms of the interactions of a susceptible maize genotype with aflatoxigenic/toxigenic strains of A. flavus and more drastic and active reactions were observed in response to the toxigenic A. flavus for almost all assayed genes. These results suggest that aflatoxin could contribute to A. flavus virulence in maize. How the toxin enhances virulence of this fungus is not clear, however, it seems likely that the inhibitory effects of aflatoxins could impair plant defense responses. On the other hand, the more elevated significant levels of disease caused by the aflatoxin-nonproducing strain in the later stages of inoculation may demonstrate that other factors contribute to the ability of the A. flavus to cause disease and enhanced differential plant responses. Indeed, A. flavus produces other toxins (e.g. cyclopiazonic acid and G-aflatoxins) and factors that may contribute to the virulence of this pathogen. The differences observed in plant gene expression levels between the two strains cannot be exclusively attributed to the absence and/or presence of aflatoxin. It could be speculated that other deletions in regions outside the aflatoxin gene pathway cluster can be involved in the atoxigenic strain. Furthermore, in the cluster for aflatoxin biosynthesis the functions of 19 genes have been assigned, but for 6 of them the functions are still unassigned (Yu et al. 2004). The lack of these genes with unknown function may compromise or alter the functionality of additional downstream genes. For this reason the atoxigenic strain could reinforce other metabolic pathways in order to recover the lack of these genes. Further investigations on the behavior of these atoxigenic and toxigenic strains co-inoculated during crop infection will provide new information on the ability of the atoxigenic strain to inhibit aflatoxin production by toxigenic strain. Different biocontrol mechanisms may contribute to the definitively decreased aflatoxin contamination observed in previous co-inoculation experiments. It could be speculated that the physical exclusion and/or the competition for nutrients are insufficient elements to explain the reduction of aflatoxin in biological control, and other unknown factors perhaps involving interactions with the host, such as the elevated expression levels of plant defense genes, suppress aflatoxin synthesis in maize. For this reason, knowing which genes are typically expressed in response to A. flavus attack will lead to a better management of aflatoxin contamination and enhancement of host resistance to aflatoxin contamination through conventional breeding and biotechnology applications.

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