Real-Time Quantitative Expression Studies of the Zearalenone Biosynthetic Gene Cluster in *Fusarium graminearum*

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ABSTRACT

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The estrogenic mycotoxin zearalenone (ZON) produced by some *Fusarium* spp. causes reproductive problems and hyperestrogenic syndromes in mammals. In an effort to elucidate the molecular pathways of ZON production, we present a comparative real-time quantitative polymerase chain reaction expression study of seven contiguous genes in the ZON biosynthetic cluster on sterile rice and during wheat and oat infection. Under ZON production on rice, the polyketide synthase (PKS) genes *PKS4* and *PKS13*, alcohol oxidase FG12056 gene, and transcriptional regulator FG02398 gene showed similarly upregulated patterns, whereas

the nonribosomal peptide synthetase (NPS) FG02394, the K⁺ channel β subunit FG12015, and the protein kinase FG02399 displayed a variant pattern. During the same time period under wheat infection when no ZON was produced, the PKS genes and the NPS were downregulated relative to rice whereas the K⁺ channel β subunit gene FG12015 was markedly upregulated, suggesting that it may play a role in the infection process. This is the first expression study of ZON biosynthetic genes in planta. The results give insight into the regulation and activities of the ZON gene cluster under different experimental systems and suggest a connection between ZON and a K⁺ channel that could reveal a novel function for ZON in *Fusarium* spp.

Additional keywords: fungus, gene expression, plant pathogen.

Ascomycete fungi of the genus *Fusarium* are some of the most devastating plant pathogens known on grain crops, and major outbreaks have been recorded in the United States, Europe, Asia, New Zealand, and South America (8). Due to the severity and scope of such recent outbreaks, experts have called *Fusarium* infection a threat to the world's grain supply (12). Economic losses due to *Fusarium graminearum* (teleomorph *Gibberella zeae*) in the United States alone during the 1990s are estimated at U.S.\$3 billion (44). Not only does infection greatly reduce crop yield and quality but the pathogen also can produce quantities of mycotoxins, chemically and thermally stable secondary metabolites that cause poisoning at low doses in both animals and humans (4,8). Due to their stability, these toxins can survive processing and pass into feedstuffs and food for human consumption (5).

The most important *Fusarium* mycotoxins are considered to be trichothecenes, fumonisins, and zearalenone (ZON) (5,8). Exposure to ZON has been linked to hyperestrogenic effects in livestock, including infertility, abortion, and immune disturbances (4,8). ZON has also been shown to be genotoxic and carcinogenic in mice (30). Despite its structural dissimilarity to the steroidal estrogens, it is believed that binding to the estrogen receptor is the major site of ZON action (7). Therefore, exposure is of serious concern to public health. Most ZON-producing *Fusarium* spp. also produce trichothecenes; hence, good management practices should reduce levels of both classes of mycotoxins (6). Directives from the European Union set maximum allowable levels for ZON (Commission Regulation (EC) no. 856/2005) and other *Fusarium* mycotoxins.

In the 1990s, *Fusarium* toxin research entered the age of genomics, with the discovery of genes involved in trichothecene biosynthesis in *F. sporotrichioides* (15,16) and fumonisin biosynthesis in *F. verticillioides* (32), and in the synthesis of a number of other minor toxins (14,22,25,36,38). In 2003, the complete genome sequence of *F. graminearum* was published by the Broad Institute (available online) and, in 2004, the annotated Munich Information Centre for Protein Sequences (MIPS) *F. graminearum* Genome Database (FGDB) was presented (available online).

We and others have recently published the identification of genes involved in the biosynthesis of ZON (11,18,24), showing that four genes are essential for ZON production: the two polyketide synthases (PKSs) *PKS4* and *PKS13*, the alcohol oxidase FG12056, and the transcription factor FG02398. The genes are named according to the MIPS FGDB (13,27) and Kroken et al. (20). A detailed knowledge of the expression of the ZON biosynthetic genes during different life-cycle phases might suggest putative functions of this mycotoxigenic compound. In this study, we have investigated the transcription of the four core ZON genes as well as additional genes in the ZON cluster (Fig. 1): the nonribosomal peptide synthetase (NPS) FG02394, the K⁺ channel β subunit FG12015, and the protein kinase FG02399. We have used real-time polymerase chain reaction (PCR) to examine their gene expression in detail, where the term "gene expression" is understood to mean the measure of the mRNA abundance. Previous studies have focused on the expression of these genes during in vitro growth of *F. graminearum* (11,18). To our knowledge, no studies of these genes have been performed in planta. The purpose of our work was to identify, characterize, and study the differences in expression of ZON biosynthetic genes when selected *Fusarium* strains grow saprophytically on sterile rice compared with pathogenically during infection of developing cereal seed. An understanding of how toxin production is con-

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trolled in *Fusarium* spp. at the molecular level could prove to be a useful tool in the development of techniques for detecting and curtailing ZON production in the field and under subsequent storage and transport of harvested grain.

MATERIALS AND METHODS

Expression of ZON genes on sterile rice. The *F. graminearum* isolates 1104-14, 1166-9, and ΔPKS4-T9 were used in the rice study. The ZON-negative mutant ΔPKS4-T9 was made from 1104-14 by replacing the central part of the *PKS4* gene with a *hygB* resistance gene through double homologous recombination (24). Growth of all three isolates under ZON-producing conditions on sterile rice was carried out as previously described (24). At harvest, mycelia were scraped from the surface, ground under liquid nitrogen with mortar and pestle, and frozen at -80°C. We sampled the rice for the analysis from different flasks for each specific condition, with three replicate flasks per time point. Total

RNA was extracted from 50 mg of tissue using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Genomic DNA for testing primers and optimizing real-time quantitative (q)PCR reactions was extracted from 1104-14 cultures using Qiagen DNeasy kit (Qiagen). Total RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX) and visualized on an agarose gel to examine the RNA quality. All samples were tested for DNA contamination by reverse-transcriptase negative real-time PCR using β-tubulin primers (33). First-strand cDNA synthesis was carried out with 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Priming was done with random hexamers. Gene expression was determined with real-time PCR on an Applied Biosystems 7900HT instrument, with a standard 96-well block. We used 1 μl (from 100 μl) of cDNA, 12.5 μl of SYBR Green PCR Master Mix SYBR (Applied Biosystems), and gene-specific primers (Table 1). The BestKeeper software (29) was used to mathematically validate RNA integrity by comparing

Fig. 1. Genetic organization of the zearalenone (ZON) biosynthetic gene cluster with putative protein function and gene coordinates. The arrows indicate direction of transcriptions and relative size of the genes. The genes are located in four contigs. Genes previously shown to be required for ZON biosynthesis are listed in bold.

TABLE 1. Primers used in the gene expression study with quantitative polymerase chain reaction (qPCR) reaction efficiency and amplicon melting temperature

Primer pair	Sequence (5' ≥ 3')	Efficiency ^a	Temperature (°C)
FG12015f	GATTGCTCGTCTCAAGCCTGTA	1.93	80
FG12015r	GGTACGGTCGCGGTTAT	...	...
FG02394f	ATGCTCAACCTCTTTGATACCTACTG	1.95	80
FG02394r	CGACACATCACGGGGTATTCC	...	...
PKS13-PS.1 ^b	CCCCCAACTCGACGTCAAATCTAT	1.92	81
PKS13-PS.2 ^b	TTCTTCCCGCCGACTTCAAAACA	...	...
PKS4-PS.1 ^b	GTGGGCTTCGCTAGACCGTGAGTT	1.93	80
PKS4-PS.2 ^b	ATGCCCTGATGAAGAGTTTGAT	...	...
FG12056f	CCATTTGGTACACGAGCGACTA	1.82	84
FG12056r	TTTTCTCGCACTTGTTACACGAT	...	...
FG02398f	GGGATTAACCGCTGTGG	2.00	79
FG02398r	TAGGCATGCCCCGAAACCGAAAGT	...	...
FG02399f	GGCGAACTTTACAAACACCTAC	2.00	79
FG02399r	TGCAGCGCTGATCAACTT	...	...
UBC-PS.1 ^b	TCCCCCTACTCTGGCGGTGTC	1.95	82
UBC-PS.2 ^b	TTGGGGTGGTAGATGCGTGTAGT	...	...
EF1a-PS.1 ^b	GGCTTTACCGACTACCTCCTCT	1.91	85
EF1a-PS.2 ^b	ACTTCTCGACGGCCTTGATGACAC	...	...
FGtubf ^c	GGTCTCGACAGCAATGGTGTT	1.96	82
FGtubr ^c	GCTTGTGTTTTTCGTGGCAGT	...	...

^a Efficiency: E = 10^{-1/slope}.
^b Lysøe et al. (24).
^c Reischer et al. (33).

the deviation of the housekeeping genes threshold crossing point (C_T) values from sample to sample and in relation to each other. A significant upward shift in the C_T values of the housekeeping genes would indicate degradation of the original RNA. The housekeeping genes used (with average C_T , standard error and variance from all the samples at all time points) were translation elongation factor 1 α (EF1 α , FG08811) ($\mu = 34.35 \pm 1.6$; $\sigma^2 = 2.7$), ubiquitin conjugating enzyme (UBC, FG10805) ($\mu = 24.81 \pm 1.8$; $\sigma^2 = 3.5$), and β -tubulin (Btub, FG09530) (33) ($\mu = 24.20 \pm 1.8$; $\sigma^2 = 3.3$), chosen from different protein families in order to reduce the possibility of coregulation. These genes were shown to be stably expressed in this experiment as measured by BestKeeper (BK) (29), and the BK index was used to normalize target gene data. After normalization, the expression levels of all genes at all time points were compared against one value, the expression data from the gene FG12056 in 1104-14 after 7 days. This was done to compare the expression levels for each gene over time as well as the relative differences between the genes. The C_T /BK ratios in the replicate runs were used to calculate the coefficient of variation and the standard deviations. The REST software (available online) (28) was used to determine relative gene expression from C_T data and to calculate statistical significance using a pairwise fixed reallocation randomization test.

Expression of ZON genes during infection in wheat and oat.

The spring wheat cv. Zebra and oat cv. Lena were grown in greenhouse conditions of 14 h of light and 10 h of darkness; day and night temperatures of 25 and 18°C, respectively; and relative humidity 60%. The high temperatures were used to avoid tillering. After 5 weeks, the temperature was reduced to 20 and 15°C (day and night, respectively). At flowering, the wheat heads and oat panicles were spray inoculated with a spore suspension of a mixture of four strains of *F. graminearum* or four strains of *F. culmorum* (10^5 spores ml $^{-1}$, where each strain contributed one fourth of the spores). The following strains were used: *F. graminearum* FG187-4, FG44A-33D, FG46A-38A, and 1166-9 and *F. culmorum* FCMA03H02-1, FCUA03F02-103, FC57A, and FCUF03F02-11. The inoculated plants were covered with transparent plastic for 72 h. Uninoculated plants of the same cultivars were grown in the greenhouse under the same growth conditions. Developing kernels from randomly infected plants were collected 2, 6, 10, and 14 days after inoculation (dai), at approximately 4:00 p.m. The plants from the 10-day time point were used as a control. Total RNA was extracted from infected wheat and oat kernels using the FastRNA Pro Green Kit (Qbiogene, Inc., Morgan Irvine, CA), according to the manufacturer's instructions. The sampled kernels from 10 plants were pooled for each time point and RNA was extracted twice from 100 mg of tissue. For each RNA sample, cDNA was synthesized twice according to Stahlberg et al. (39). The four cDNAs were run in duplicate, giving eight real-time PCR reactions per time point. Pipetting of the plates was done by an epMotion 5070 robot (Eppendorf, Hamburg, Germany). The RNA was treated with the TURBO DNA-free kit (Ambion) and visualized on an agarose gel to examine its integrity. The RNA concentrations were measured by the GeneQuant RNA/DNA Calculator (Biochrom Ltd., Cambridge, UK). Total RNA was tested for the presence of DNA contamination with a PCR reaction without reverse transcriptase using primers for EF1 α . First-strand cDNA synthesis, real-time PCR, and analysis were done as described for the rice study, except using 5 μ l of cDNA in the SYBR Green reactions, five times as much as the rice study. This was done to see stable expression results from the different genes. The housekeeping genes were EF1 α (FG08811) ($\mu = 24.96 \pm 2.2$; $\sigma^2 = 4.8$) and UBC (FG10805) ($\mu = 28.39 \pm 2.3$; $\sigma^2 = 5.2$). The Btub primers were discarded because they did not work well in planta. None of the housekeeping genes or ZON-related genes gave amplicons in the control plants.

Validation and optimization of qPCR primer pairs. Primer Express (Applied Biosystems), Primer3 (34), and Primer Select

(DNASar, Madison, WI) were used to design primers. To ensure that each primer pair amplified a unique sequence in the *F. graminearum* genome, BLAST similarity searches were carried out with the e -cutoff value set to +100. Standard curves made to 10-fold dilution series of the template over five to six points were run at different primer concentrations until all performed to >90% efficiency. The only exception was FG12056 (82%). The reaction efficiencies ($E = 10^{-1/\text{slope}}$) were calculated by the Sequence Detection Systems 2.2.2 software incorporated into the ABI 7900HT real-time PCR instrument, which was also used to generate dissociation curves (melting temperature) (Table 1). In all assays, a single amplicon peak was detected. All the primer pairs used also gave amplicons on *F. culmorum* DNA. The standard deviation of C_T values for technical replicates was used as a measure of intrarun variability, which was <0.5. The degree of experimental error inherent in the system (interrun error) was <2.5%, using four runs of the β -tubulin reaction with template diluted over three logs (data not shown).

High-performance liquid chromatography analysis of ZON.

The rice cultures and wheat kernels were freeze dried and ground, and 0.1 g of each sample was extracted and prepared according to the method described by Smedsgaard (37). The extract was separated by reverse-phase chromatography on an Eclipse XDB-C18 column (150 by 4.6 mm) with a mobile phase of 55% acetonitrile in water with 0.1% phosphoric acid. ZON was detected with a diode array detector at $\lambda = 236$ nm and a bandwidth of 4 nm. Additional identification was done by collecting 2 UV spectra per second from 200 to 400 nm with a resolution of 2 nm. The amount of ZON in the extracts was calculated against an external linear calibration curve of ZON (Sigma-Aldrich, Z2125) in the range of 0.1 to 200 μ g/ml, which bracketed the samples.

Real-time PCR on genomic DNA from selected isolates of *Fusarium*. Genomic DNA was extracted from fungal cultures using Qiagen DNeasy kit (Qiagen). Real-time PCR was performed on single isolates of *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. avenaceum*, *F. poae* (two isolates), *F. langsethiae*, *F. sporotrichioides*, *F. sambucinum*, and *F. oxysporum*, using the same primers and methods as in the gene expression studies. Strong, weak, and absence of PCR amplicons were recorded.

RESULTS

Expression of ZON genes on sterile rice. To study expression of known and putative ZON biosynthetic genes, we performed real-time PCR on seven genes, all situated in a cluster (Fig. 1). Gene expression in the three *F. graminearum* strains 1104-14 and 1166-9 and the ZON-negative *PKS4* deletion mutant Δ PKS4-T9 (24) was first studied on sterile rice, and expression of the seven genes was monitored 4, 7, 11, and 14 days after inoculation. After normalization of the expression levels by BK, the data from all time points were compared against one value, the expression data from the gene FG12056 in 1104-14 after 7 days (Fig. 2A). This enabled visualization of the dynamics of the expression levels over time not only for individual genes but also between the genes. FG12056 was chosen as the calibration point because it consistently showed the lowest expression levels of the seven genes studied. Results showed that the NPS FG02394 generally had the highest level of expression, followed by *PKS13*, *PKS4*, the protein kinase FG02399, the K $^+$ channel β subunit FG12015, the transcription factor FG02398, and the alcohol oxidase FG12056. In the ZON-producing strains, *PKS13* showed slightly higher expression than *PKS4* at all time points: ($P = 0.01$), 4 days (1.4 $\times$), 7 days (1.3 $\times$), 11 days (1.3 $\times$), and 14 days (1.1 $\times$) for 1104-14 and 4 days (2.4 $\times$), 7 days (1.3 $\times$), 11 days (1.6 $\times$), and 14 days (1.4 $\times$) for 1166-9.

F. graminearum 1104-14 demonstrated its highest levels of expression in all seven genes after 11 days of growth (Fig. 2A). In

comparison, at 11 days of growth, the ZON-negative mutant Δ PKS4-T9 showed dramatically reduced expression in FG02394 (–40 $\times$), FG12015 (–37 $\times$), *PKS13* (–30 $\times$), FG02398 (–22 $\times$), and FG12056 (–17 $\times$), and somewhat reduced in FG02399 (–5 $\times$) and *PKS4* (–2) in comparison with its wild type, 1104-14. Observed by eye, both 1166-9 and Δ PKS4-T9 grew faster on sterile rice than 1104-14. The gene expression pattern in the two strains Δ PKS4-T9 and 1166-9 are similar and occur earlier than in 1104-14.

FG02394, FG12015, and FG02399 had a similar expression pattern in all three isolates, with a bell-shaped normal curve, although FG02399 showed a somewhat broader peak compared with the other two genes (Fig. 2B). The four genes *PKS13*, *PKS4*, alcohol oxidase FG12056, and transcription factor FG02398 also had a bell-shaped curve in 1104-14 but showed a different expression pattern in the mutant Δ PKS4-T9 and 1166-9. Here, the expression pattern started as a bell-shaped curve, followed by increased gene expression again at 14 days. The increase in gene

expression at 14 days was especially prominent for the transcriptional regulator FG02398.

Expression of ZON genes in planta. Infected glumes from *F. graminearum*- or *F. culmorum*-inoculated spikes were harvested 2, 6, 10, and 14 dai. After normalizing by the BK index, the relative gene expression was compared against one point, the expression of the alcohol oxidase gene FG12056 after 6 dai. In *F. graminearum*-infected wheat 2 dai, low expression levels in the genes could be detected in some but not all cDNA replicates (data not shown). At 6 dai, the four genes encoding the K⁺ channel β subunit FG12015, the alcohol oxidase FG12056, the transcription factor FG02398, and the protein kinase FG02399 showed low expression in all cDNA replicates (Fig. 3). All seven genes were expressed at 10 and 14 dai. Small differences in transcription were seen between 10 and 14 dai; only FG02394 clearly showed lower expression 10 dai. The expression of *PKS13* was slightly higher than *PKS4* at both 10 dai (1.5 $\times$) and 14 dai (2 $\times$), similar to the rice study.

Fig. 2. A, Relative gene expression of zearalenone (ZON)-related genes in *Fusarium graminearum* 1104-14 (wild type), *F. graminearum* 1166-9, and ZON-negative mutant Δ PKS4-T9 after 4, 7, 11, and 14 days of growth on sterile rice. Genes previously shown to be required for ZON biosynthesis are listed in bold. All gene expression data are normalized with the BestKeeper index and compared with the data from one point, the *F. graminearum* 1104-14–FG12056, 7 days. The levels of down- and upregulation of the genes in Δ PKS4-T9 compared with the wild type at the different time points are listed, adjusted to the nearest integer; nd = not detected. Error bars indicate standard deviations from three biological replicates. **B,** Generalized expression patterns over time of the genes from A where two different patterns emerge. The line drawings are for illustrative purposes only.

In *F. culmorum*-infected wheat, only low expression of the different genes could be detected in some of the cDNA replicates during all time points (results not shown). The *F. graminearum*-inoculated wheat showed clear disease symptoms by 10 and 14 dai; however, on wheat inoculated with *F. culmorum*, only weak symptoms could be seen. In *F. graminearum*- and *F. culmorum*-infected oat, only some of the cDNA replicates showed low expression of the genes during the investigated time frame from 2 to 14 dai (data not shown). No visible symptoms were seen on oat.

Comparison of rice and wheat experiments. To find relative differences in the expression of the ZON biosynthetic genes when the fungus is grown saprophytically on rice and pathogenically on wheat, we compared the results from *F. graminearum*-infected wheat from 10 dai to the rice from 11 days (Fig. 4). These were time points with high expression levels in both experiments. EF1 α showed much higher expression in wheat compared with rice, relative to the other genes. Therefore, we used only the UBC gene for comparison and normalization of the results. The amount of UBC mRNA was also $\approx$ 50-fold less in wheat compared with rice, which potentially could be related to the amount of total *F. graminearum* RNA in the wheat samples. The results from this analysis show that the genes encoding the two polyketide synthases *PKS4* and *PKS13* ($\approx$ 6.5-fold) and the NPS FG02394 ($\approx$ 8.5-fold) clearly are downregulated in wheat compared with rice. Together with the amount of *F. graminearum* RNA in wheat, this correlates well with the results from high-performance liquid chromatography (HPLC) analysis of ZON production, where no ZON could be found in the wheat samples (see next paragraph). The three genes alcohol oxidase (FG12056), transcription factor (FG02398), and protein kinase (FG02399) showed similar expression in wheat and rice. The K⁺ channel β subunit (FG12015) clearly was upregulated ($\approx$ 11-fold) in wheat, and seems to have a function during the infection process.

ZON production. The ZON production in the sterile rice and wheat studies was measured by HPLC. In rice, the accumulated ZON in 1104-14 followed the same bell-shaped curve as the real-time gene expression pattern from Figure 2, with an average of 220 ppm after 11 days of growth (Fig. 5). The 1166-9 showed a more linear increase over the whole time period, with the highest amount of accumulated ZON after 14 days of growth (average 800 ppm). The mutant Δ PKS4-T9 did not produce ZON during any conditions. In the wheat study, no ZON could be found in the *F. graminearum*-infected samples.

Specificity of the primers in other *Fusarium* spp. We tested the primers from this study on genomic DNA from other *Fusarium* spp. to examine their specificity (Table 2). Except from *F. graminearum*, only *F. culmorum* gave amplicons with all the ZON-related primers. *F. equiseti* gave amplicons with the three ZON genes *PKS13*, *PKS4*, and FG12056. The others did not give amplicons with any of the ZON-related primers, except for weak signals for FG12056 in *F. langsethiae* and *F. sambucinum*. The Btub primers generated strong signals with *F. graminearum* only, and weak signals from *F. culmorum*, *F. poae*, and *F. sporotrichioides* DNA. The EF1 α and UBC primers showed very strong signals for all species tested, except *F. oxysporum* (EF1 α) and *F. sambucinum* (EF1 α and UBC), where no amplicons could be detected.

DISCUSSION

The fact that both neighboring genes *PKS13* and *PKS4* are required for ZON production caused us to speculate about how ZON is synthesized. One possibility is that the enzyme PKS4 catalyzes the first 10 carbon additions and that this reduced

Fig. 4. Expression of the wheat samples from 10 days after inoculation (dai) compared with the rice samples from 11 days. Expression levels are normalized with ubiquitin conjugating enzyme only and the error bars indicate standard deviations from three biological replicates.

Fig. 3. Relative gene expression patterns of seven genes from the zearalenone cluster in *Fusarium graminearum* inoculated wheat 6, 10, and 14 days after inoculation (dai). All gene expression data are normalized relative to one point, FG12056 at 6 dai. Error bars indicate standard deviations from three biological replicates.

moiety is subsequently transferred to PKS13, where the remaining three rounds of C2 additions are catalyzed, similar to the proposed biosynthetic pathway of Gaffoor and Trail (11). Another possibility is that PKS13 and PKS4 function together as a multiprotein complex. There are several known examples of two PKSs being involved in the production of a polyketide; for example, in the biosynthesis of lovastatin (*Aspergillus terreus*) (17), compactin (*Penicillium citrinum*) (1), and T-toxin (*Cochliobolus heterostrophus*) (46). Our results show that *PKS13* has slightly higher gene expression than *PKS4* in the wild types, confirming the results from Northern blot analysis as presented by Kim et al. (18). This could suggest that the enzymes PKS13 and PKS4 work independently of each other and are not part of a multiprotein complex, or it could simply be due to the fact that the *PKS4* transcript is longer ($\approx 1.2\times$) than that of *PKS13*. According to Gaffoor and Trail (11), these two genes are transcribed from a putatively shared promoter, and transcripts from the smaller *PKS13* might consequently accumulate faster than the *PKS4* transcripts.

Interestingly, the expression pattern in the Δ PKS4-T9 mutant, although reduced for all cluster genes except *PKS4*, does not follow the overall pattern of its parent 1104-14. Rather, it shares striking similarity with the high-ZON-producing 1166-9 strain. Both Δ PKS4-T9 and 1166-9 give an early bell-shaped curve with peak expression at 7 days and increasing expression at 14 days. The 1104-14 gives a later, simple bell-shaped curve peaking at 11 days. In addition, Δ PKS4-T9 and 1166-9 grew faster than 1104-14. Notably, faster growth of a *PKS4* disruption mutant was also observed by Gaffoor and Trail (11), and this might explain the

Fig. 5. High-performance liquid chromatography analysis of zearalenone (ZON) production in *Fusarium graminearum* 1104-14 and *F. graminearum* 1166-9 after 4, 7, 11, and 14 days of growth on sterile rice. The samples are from the same material as the relative gene expression study in Figure 3. The numbers over the bars are the amount of ZON produced (ppm) and error bars indicate standard deviation from three biological replicates. The ZON mutant Δ PKS4-T9 showed no ZON production at any of the time points.

different gene expression pattern in the mutant compared with its wild type. It may be that ZON production in 1104-14 inhibits growth or that the nutritional resources used to produce ZON in the wild type are diverted to increase growth in Δ PKS4-T9. Δ PKS4-T9 produces no ZON, excluding the possibility that a ZON-initiated feedback loop controls the expression of the cluster genes. The expression pattern of *PKS4* in the Δ PKS4-T9 mutant follows the other three ZON-related genes. The Δ PKS4-T9 mutant was created by Lysøe et al. (24), in which a central portion of *PKS4* is replaced by a *hygB* resistance gene through double homologous recombination. The *hygB* expression cassette was inserted in the opposite direction (3'→5') compared with *PKS4* (5'→3'). The primers are located toward the 3' end of *PKS4* and, therefore, expression in the *PKS4* gene cannot be driven by the *hygB* promoter but is probably regulated by the intact *PKS4* promoter.

Results from Kim et al. (18) showed that only *PKS13*, *PKS4*, FG02398, and FG12056 were required for ZON production, but deletion of the transcription factor FG02398 also turned off expression of FG02394 and FG12015 and reduced the expression of FG02399. Our results demonstrate that only the four core genes required for ZON production seem to be coregulated, showing a similar expression pattern in 1166-9 and Δ PKS4-T9. It seems that FG02398 directly regulates the three genes *PKS4*, *PKS13*, and alcohol oxidase FG12056, and not the NPS FG02394, the K⁺ channel β subunit FG12015, and the protein kinase FG02399. The accumulated ZON in the rice samples also followed a pattern similar to that of the gene expression data of the transcription factor FG02398, with a bell-shaped curve in 1104-14 and an increasing curve in 1166-9, which could correspond to the increased expression of the four genes again after 14 days of growth.

The reduction in Δ PKS4-T9 expression of the genes encoding the NPS FG02394, the K⁺ channel β subunit FG12015, and the protein kinase FG02399 could be due to the lack of ZON and may not be a consequence of direct regulation by FG02398. In addition, northern results by Kim et al. (18) showed two different transcripts for the transcription factor FG02398, so there is a possibility of regulating the genes in the ZON cluster at different levels. The expression patterns of the three genes FG02394, FG12015, and FG02399 were similar to each other but different from those of the four core genes in 1166-9 and Δ PKS4-T9. Also, expression data for 11 other genes located elsewhere in the *F. graminearum* genome and not believed to be involved in ZON production had no similarity to the two expression patterns reported here for known and putative genes in the ZON cluster (23). In addition, expression of these 11 genes in Δ PKS4-T9 at 11 days of growth did not show any obvious downregulation compared with the wild type. Therefore, these three genes—FG02394, FG12015, and FG02399—might have a more indirect role in ZON biosynthesis or related functions in other ZON pathways.

TABLE 2. Real-time polymerase chain reaction amplification (PCR) of seven genes in the zearalenone biosynthetic cluster and the three housekeeping genes β -tubulin (Btub), elongation factor 1 α (EF1 α), and ubiquitin conjugating enzyme (UBC), using genomic DNA from different *Fusarium* spp. as template^a

Genes	FG	FC	FE	FA	FP	FL	FSP	FSA	FO
FG02394	++	+	nd	nd	nd	nd	nd	nd	nd
FG12015	++	++	nd	nd	nd	nd	nd	nd	nd
PKS13	++	++	+	nd	nd	nd	nd	nd	nd
PKS4	++	++	++	nd	nd	nd	nd	nd	nd
FG12056	++	++	++	nd	nd	+	nd	+	nd
FG02398	++	+	nd	nd	nd	nd	nd	nd	nd
FG02399	++	+	nd	nd	nd	nd	nd	nd	nd
Btub	++	+	nd	nd	+	nd	+	nd	nd
EF1 α	++	++	++	++	++	++	++	++	nd
UBC	++	++	++	++	++	++	++	nd	nd

^a Genes listed represent the primers used in this study: FG, *Fusarium graminearum*; FC, *F. culmorum*; FE, *F. equiseti*; FA, *F. avenaceum*; FP, *F. poae*; FL, *F. langsethiae*; FSP, *F. sporotrichioides*; FSA, *F. sambucinum*; and FO, *F. oxysporum*; ++, +, and nd = or strong, weak, or absence of PCR amplicon, respectively.

ZON genes in *F. graminearum*-infected wheat showed only low expression in some of the cDNA replicates 2 dai, and the four genes FG12015, FG12056, FG02398, and FG02399 were the only genes that gave PCR amplicons in all the cDNA replicates by 6 dai. Interestingly, the genes for the putative transcription factor FG02398 and the K⁺ channel β subunit FG12015 showed up-regulation in wheat earlier and at higher expression levels than the PKSs, something that never occurred in the rice study. All seven genes were active in wheat by 10 and 14 dai. Somewhere between 6 and 10 dai, the ZON-producing genes were turned on. The *F. graminearum*-inoculated wheat samples showed clear symptoms of infection 10 dai. In contrast, there were minimal symptoms of infection in *F. graminearum*-inoculated oat and in *F. culmorum*-infected wheat and oat, and the same genes showed only very low expression in some of the cDNA replicates. These results suggest that *F. graminearum* could produce more ZON than *F. culmorum* during infection of wheat. In addition, wheat seems to be more susceptible than oat to *F. graminearum* infection. This is the first published report on gene expression studies of ZON biosynthetic genes during the infection process of wheat and oat.

We compared our results with previously published gene expression data from the same gene cluster (11,18), where *Fusarium* spp. were grown under different conditions. For this comparison, the seven genes were sorted by decreasing level of gene expression (Table 3). One interesting observation is that expression levels of the four known ZON genes (*PKS13*, *PKS4*, FG12056, and FG02398) were stable in relation to one another in six of nine experiments. The other three experiments showed only small differences in the gene order. This also supports our previous results that from the seven genes investigated, only these four genes seem to be coregulated and directly involved in ZON production. Expression of the seven genes relative to each other on rice also showed identical gene order for the two isolates 1404-14 (11 days) and 1166-9 (7 days) when the ZON gene expression was at a maximum. This supports our observation that the expression in 1166-9 reached its maximum level earlier than in 1404-14. Comparison of the rice and wheat study suggests that the two polyketide synthases (ZON builders) have relatively low expression in wheat compared with rice. This was also supported by HPLC, where no ZON could be found in the wheat samples. The K⁺ channel β subunit gene FG12015 clearly showed an early upregulation relative to the other cluster genes in wheat (Fig. 4) that was absent on sterile rice, which suggests a role during wheat infection. The FG12015 had lower expression on starch-glutamate liquid medium, medium expression on rice, and maximum expression on wheat compared with the other genes. This result encouraged us to investigate this gene in the literature, revealing

findings that allow us to suggest a possible function for the estrogenic polyketide ZON.

From all the nine *Fusarium* spp. tested for primer specificity (Table 2), only *F. graminearum*, *F. culmorum*, and *F. equiseti* are considered to be ZON producers (26,40,42). This is supported by the specificity of the primers from the ZON cluster, where both *F. graminearum* and *F. culmorum* gave amplicons with all primers, and *F. equiseti* gave amplicons in the three genes *PKS4*, *PKS13*, and FG12056. The *PKS4* and FG12056 primers showed the highest specificity and have potential to be used in a diagnostic assay to find potential ZON producers in a grain sample. The EF1 α and UBC primers were also very good for most species tested but not for *F. sambucinum* (EF1 α) or *F. oxysporum* (EF1 α and UBC). Therefore, they could probably be used as house-keeping genes in real-time assays from many different *Fusarium* spp. The Btub primers could be used only for *F. graminearum* in accordance with the fact that these primers were developed by Reischer et al. (33), as part of an *F. graminearum*-specific TaqMan assay.

A proposed function for ZON in *Fusarium* spp. ZON resembles 17 β -estradiol, the principal estrogenic hormone produced by the human ovary, and has been associated with estrogenic syndromes in swine and experimental animals (4,6). ZON has a broad range of biological effects in mammals but it is believed that binding to the estrogen receptor is the major site of action (6). Previous studies have shown that 17 β -estradiol binds to a K⁺ channel β subunit and activates a Maxi-K channel (41). Several studies have discussed the interaction of 17 β -estradiol (3,10, 19,48) or other hormones (21) with K⁺ channels. In fungi, because genes involved in secondary metabolism are often clustered (31,47), the location of the K⁺ channel β subunit gene FG12015 in the same cluster as the ZON biosynthetic genes would suggest some related function. This gene was also turned off in the transcription factor mutant FG02398 by Kim et al. (18), indicating some kind of role in the ZON biosynthetic pathway. We postulate a possible function for ZON in *Fusarium* spp.: the binding to, and potentially the activation of, the K⁺ channel β subunit encoded by FG12015.

Kim et al. (18) showed that the alcohol oxidase deletion mutant FG12056 (*zeb1*) produced the ZON derivative β -zearealenol instead of ZON, and concluded that FG12056 is involved in an oxidation step from β -zearealenol to ZON. β -Zearealenol is 3.5 $\times$ more estrogenic than ZON (35), and the *zeb1* deletion mutant clearly showed an upregulation of the K⁺ channel β subunit gene FG12015 compared with the wild type. If β -zearealenol stimulates expression of FG12015 more than ZON, it could possibly have a stronger and more potent binding than ZON to the K⁺ channel β subunit encoded by FG12015. In *Fusarium* spp., ZON has many

TABLE 3. Expression of genes associated with zearealenone (ZON) production in different *Fusarium graminearum* strains on sterile rice, rice agar, and starch-glutamate (SG) liquid medium, and from infection studies in wheat^a

Strains ^b	Genes
Rice 1404-14 (7 days)	FG02394 = FG020399, PKS13 , PKS4 , FG12015, FG02398 , FG12056
Rice 1166-9 (11 days)	FG02394, FG020399, PKS13 , PKS4 , FG12015, FG02398 , FG12056
Rice 1166-9 (7 days)	FG02394, PKS13 , PKS4 , FG020399, FG12015, FG02398 , FG12056
Rice 1404-14 (11 days)	FG02394, PKS13 , PKS4 , FG020399, FG12015, FG02398 , FG12056
Rice agar PH1 ^c	FG02394, PKS13 = PKS4 = FG12015, FG12056 , FG02398
In wheat (6 dai)	FG02398 , FG02399, FG12015, FG12056
In wheat (10 dai)	FG02399, FG12015, FG02394 = PKS13 = FG02398 , PKS4 , FG12056
In wheat (14 dai)	FG02399 = FG02394, PKS13 , FG12015, FG02398 , PKS4 , FG12056
SG-GZ3639 (4 days) ^d	PKS13 , PKS4 = FG02399, FG02398 ^e , FG12056 = FG02394, FG12015
SG-GZ3639 (6 days) ^d	PKS13 , PKS4 = FG02399 = FG02394, FG02398 ^e , FG12056 , FG12015

^a Genes were sorted by decreasing levels of gene expression. Genes previously shown to be required for ZON biosynthesis are listed in bold.

^b Expression data were compared after a certain number of days or at specific days after inoculation (dai).

^c Results from Gaffoor and Trail (11); FG02399 was not included in their study.

^d Results from northern analyses of Kim et al. (18). Gene expression was here only visually observed from the autoradiogram; therefore, quantitative gene expression data are not available.

^e This gene showed two transcripts; the longest transcripts (used here) followed the transcription pattern of both polyketide synthase (*PKS*) genes.

derivatives differing in estrogenic activity (35), and this could suggest a regulation of K⁺ channel activity on different levels. ZON is a polyketide, and there is also some evidence that the polyketides FK506 and rapamycin cause K⁺ channel activation (2,43). It has further been shown that ZON and α -zearalenol bind to *Lactobacillus* cells (live, heat-treated, and acid-treated cells), and both were recovered from the bacterial pellet after binding (9). This also supports our theory of ZON binding to a K⁺ channel, because these channels are integrated into the cell membrane.

What is the purpose of ZON in *Fusarium* spp.? The previous hypothesis that ZON induces sporulation and enhances perithecia formation in *F. graminearum* has been disproven (11,24,45). Although it is interesting that the phytopathogenic *F. graminearum* genome has four genes encoding K⁺ channel β subunits while the saprophyte *Neurospora crassa* seems to have only one, infection studies have shown that ZON is not necessary for infection of wheat and barley (11,18,24). We also examined whether K⁺ concentrations had some influence on growth of *F. graminearum*. Cultivation on potato dextrose agar supplemented with KCl at concentrations ranging from 0 to 80 g liter⁻¹ showed no obvious differences in growth or phenotype between the wild type and the ZON-negative mutant Δ PKS4-T9 (data not shown). The putative binding of ZON to a K⁺ channel could have a function in a signal transduction pathway; it could regulate the potassium ions in the cell or be involved in ZON secretion. Because ZON is only produced by a few *Fusarium* spp., its function must be a secondary one. Future investigation will be needed in order to clarify the putative relationship between ZON and the K⁺ channel β subunit. Until then, although the present findings give some clues, its function in the cell can only be speculative.

This is the first expression study of ZON biosynthetic genes in planta, and also the first to suggest a possible function for ZON in *Fusarium* spp. The gene expression studies give insight into the regulation and activities of the ZON gene cluster under different experimental systems, and the suggested connection between ZON and a K⁺ channel system might initiate further research to develop strategies to combat the fungus or to inhibit the production of ZON.

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