Biosynthetic mechanism and regulation of zearalenone in *Fusarium graminearum*

Jung-Eun Kim¹, Hokyoung Son¹, Yin-Won Lee¹

¹Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea

**Abstract**

Zearalenone (ZEA) is an estrogenic polyketide-derived mycotoxin that is produced by several *Fusarium* spp. This mycotoxin is often found in *F. graminearum*-infected cereals and causes a number of illnesses in animals, including humans. The genetic information of the ZEA biosynthetic pathway has been characterized in *F. graminearum* using forward and reverse genetic approaches. Four genes responsible for ZEA biosynthesis, including two polyketide synthase (PKS) genes, are located in the ZEA biosynthetic gene cluster. In addition to two PKSs, *ZEB1* and *ZEB2* encode an isoamyl alcohol oxidase and a transcription activator carrying a basic leucine zipper (bZIP) DNA-binding domain, respectively. *ZEB2* produces two isoforms (*ZEB2L* and *ZEB2S*) via an alternative promoter. *ZEB2L* and *ZEB2S* interact with each other and participate in the autoregulation of *ZEB2* expression as an activator and an inhibitor, respectively, during ZEA biosynthesis. The catalytic and regulatory subunits of the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway in *F. graminearum*, *CPK1* and *PKR*, negatively regulate ZEA biosynthesis. In particular, it was found that the PKA pathway regulates *ZEB2L* transcriptionally and post-transcriptionally during ZEA production. These results increase our understanding of regulatory mechanisms underlying ZEA biosynthesis in *F. graminearum*.

**Keywords**

alternative promoter; autoregulation; *Fusarium graminearum*; PKA pathway; zearalenone (ZEA)

**Correspondence**

Hokyoung Son and Yin-Won Lee, Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Korea
E-mail: hogongi7@snu.ac.kr (Hokyoung Son) lee2443@snu.ac.kr (Yin-Won Lee)

(Received December 01, 2017, accepted December 08, 2017)

**Introduction**

Zearalenone ([ZEA, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone](https://doi.org/10.2520/myco.68-1-2)) is a mycotoxin that is produced by several *Fusarium* species, especially *Fusarium graminearum*, *F. culmorum*, and *F. crookwellense*. Together with trichothecene mycotoxins, ZEA is frequently detected in cereal crops infected by *F. graminearum*. Although ZEA is primarily a field contaminant, it is also found in foodstuffs such as cornmeal, corn flakes, corn porridge, and beer⁴. The acute toxicity of ZEA after oral administration is relatively low, but ZEA exhibits an estrogenic effect by binding to the mammalian estrogen receptor and therefore causes hyperestrogenism resulting in reproductive dysfunction in farm-raised and laboratory animals⁴. In particular, swine are the most sensitive animal to ZEA³.⁴. The genetic information of ZEA production has been studied intensively with the goal of developing strategies to reduce ZEA contamination in food and feed. The completion of fungal genome sequences and advanced molecular techniques, such as targeted gene deletion, overexpression, and gene fusion to fluorescent proteins (FPs), have uncovered mechanisms related to pathogenicity, sexual development, and secondary metabolite biosynthesis, including ZEA, in *F. graminearum*⁸.⁹.¹⁰.¹¹. This review is focused on the recent findings about the biosynthesis and regulation of ZEA in *F. graminearum*.

1. **ZEA biosynthesis pathway**

ZEA is a polyketide metabolite, synthesized by sequential reactions catalyzed by large multienzyme protein complexes that contain polyketide synthases (PKSs)¹²,¹³. Genome sequencing has revealed that *F. graminearum* has 15 PKSs, but the function of only 8 PKSs has been identified¹³. Among them, one reducing (PKS4) and one non-reducing (PKS13) PKSs are essential for ZEA production⁴,¹⁵. Fungal PKS genes generally reside in clusters of
genes that encode transcription factors, metabolic enzymes, and transporters\(^9\). Four neighboring genes (PKS4, PKS13, ZEB1, and ZEB2) are required for ZEA biosynthesis and constitute the ZEA biosynthetic gene cluster\(^8\). ZEB1 encodes an isoamyl alcohol oxidase that is required for the conversion of β-zearalenol into ZEA. ZEB2 is a transcription factor that carries a basic leucine zipper (bZIP) DNA-binding domain and regulates the activity of other ZEA biosynthetic cluster genes. Based on the results of molecular genetic analyses of these genes, it is proposed that the ZEA biosynthetic pathway is initiated by PKS4, which catalyzes the condensation of carbons from a single acetyl-CoA and five malonyl-CoA molecules, resulting in the formation of a hexaketide (Fig. 1)\(^8,15\). In the next step, PKS13 completes three iterations extending the ZEA chain by adding three malonyl-CoA molecules, resulting in a nonaketide. Then, the unreduced ketones undergo two rounds of intramolecular aromatic reactions, causing the formation of an aromatic ring and a macrolide ring structure containing a lactone bond. The final conversion of zearalenol to ZEA is catalyzed by ZEB1. ZEA biosynthetic genes are not essential for other traits of *F. graminearum* such as hyphal growth, conidiation, pigmentation, trichothecene production, virulence or sexual reproduction\(^8\).

2. Regulation of the ZEA biosynthesis pathway

2.1. Autoregulation of ZEA production by alternative promoter usage

We found that there is an autoregulation of the ZEA biosynthesis pathway via the transcription factor protein diversity caused by alternative promoter usage and feedback loop mechanisms. Two ZEB2 transcripts, ZEB2L and ZEB2S, were first found via northern blot analyses (Fig. 2A)\(^17\). We performed cDNA sequencing of the ZEB2 transcript and confirmed that ZEB2L contains a bZIP DNA-binding domain at the N-terminus, whereas ZEB2S is an N-terminally truncated form of ZEB2L that lacks the bZIP domain (Fig. 2B). Further subsequent western blot analyses revealed that both ZEB2L and ZEB2S transcripts produced functional proteins (Fig. 2C). When the two internal start codons of ZEB2S were mutated, only the ZEB2L protein was detected (Fig. 2D); the ZEB2S protein was successfully produced with a cryptic ZEB2S promoter, suggesting that ZEB2S production is regulated by alternative promoter usage (Fig. 2E).

![Fig. 1 Model for the ZEA biosynthetic pathway. The biosynthesis is initiated by PKS4 that catalyzes one acetyl-CoA and five malonyl-CoA. PKS13 functions as an extender unit that completes the polyketide backbone and is responsible for the cyclization and aromatization. As the final step, β-zearalenol is converted to zearalenone by ZEB1, which is the isoamyl alcohol oxidase. This scheme was drawn based on the results previously reported\(^8,15\).](image-url)
Fig. 2 ZEB2 transcripts produced by alternative promoter usage. (A) ZEB2 gene expression during ZEA biosynthesis. Two transcripts, ZEB2L and ZEB2S, were detected in total RNA of F. graminearum grown in starch glutamate (SG) medium with exogenous supplementation of ZEA for 3–10 days. (B) Illustrated putative promoter regions of ZEB2L (P<sub>ZEB2L</sub>) and ZEB2S (P<sub>ZEB2S</sub>). (C)–(E) Both ZEB2 isoforms, ZEB2L and ZEB2S, were produced when ZEB2 was under the control of the putative ZEB2L promoter region (P<sub>ZEB2L</sub>). Only ZEB2L, and no ZEB2S, was detected from the P<sub>ZEB2L</sub>-ZEB2L-HA construct in which the internal start codons were mutated to leucine. P<sub>ZEB2S</sub> was necessary for ZEB2S expression. An illustration of the transformed fungal construct used is included above each blot. These data came from a previous report with slight modifications.  

Yeast two-hybrid and immunoprecipitation assays were performed to identify proteins associated with ZEB2L. ZEB2L strongly interacted with both ZEB2L and ZEB2S, and two interaction domains of ZEB2 were found to be required for ZEB2L-ZEB2L, ZEB2L-ZEB2S, and ZEB2S-ZEB2S dimerizations. These results led us to examine the different roles of ZEB2 complexes as transcriptional regulators. The direct binding of ZEB2L to the promoter regions of the ZEA biosynthetic cluster genes, PKS4, PKS13, ZEB1, and ZEB2, was confirmed using an electrophoretic mobility shift assay (EMSA). As expected, ZEB2S did not bind to the promoter regions of the tested genes because ZEB2S lacks the bZIP DNA-binding domain. Interestingly, the DNA-binding affinity of ZEB2L was markedly decreased in the presence of ZEB2S. Furthermore, the fungal strain expressing both ZEB2L and ZEB2S did not produce ZEA, whereas the mutant solely expressing ZEB2L displayed high levels of accumulated ZEA. Taken together, we demonstrated a distinct role of ZEB2S as an inhibitor for the transcriptional activator (ZEB2L) for ZEA biosynthetic genes. 

The subcellular localization of the ZEB2 isoforms was confirmed by F. graminearum strains overexpressing the ZEB2 isoforms fused to green or red fluorescent proteins. ZEB2L exclusively localized in nuclei and ZEB2S localized to both cytosol and nuclei (Fig. 3A). In vivo interactions between ZEB2 isoforms were validated by the bimolecular fluorescence complementation (BiFC) technique. ZEB2L-ZEB2L and ZEB2L-ZEB2S interactions were detected in nuclei, whereas interaction between ZEB2S and ZEB2S was observed in both cytosol and nuclei (Fig. 3B). Likewise, ZEB2L-ZEB2S interactions in the nuclei inhibited ZEB2L function for ZEA production.

ZEA was initially detected after 2 days of incubation in a strain carrying HA-tagged intact ZEB2 under the control of the native ZEB2 promoter, and the level of ZEA increased until 10 days of incubation (Fig. 4A). Western blot analyses to profile the ZEB2 isoforms during ZEA production showed that the ZEB2L levels decreased as ZEB2S appeared after 6 days of incubation (Fig. 4B). In contrast, ZEB2S was not detected until 5 days of incubation, and its levels were increased after day 6. These results demonstrated that both ZEB2L and ZEB2S participated in the autoregulation of ZEA biosynthesis; ZEB2L initiated ZEA production and its production was terminated by high levels of ZEB2S. We also discovered that one of the factors regulating ZEB2S expression was the accumulation of ZEA.

Oligomeric states of ZEB2 isoforms during ZEA production were determined by the use of a two-dimensional electrophoresis system. ZEB2L existed as either oligomers or monomers in the early stages of ZEA production, and ZEB2L formed heterodimers with ZEB2S in the mid-stage. ZEB2S homodimers were detected after day 8 along with ZEB2L-ZEB2S heterodimers, but only ZEB2S homodimers were highly accumulated in the last stages.
Based on these results, we proposed a novel regulatory mechanism for ZEA biosynthesis (Fig. 5)\(^7\). ZEA biosynthesis is initiated by ZEA2L oligomers that activate the ZEA biosynthetic cluster genes and ZEB2 itself. As ZEA production progresses, an increased ZEA concentration induces the expression of ZEB2S transcripts directly or indirectly, and ZEB2S transcripts are expressed via an active alternative promoter usage. ZEA enhances the production of both ZEB2 transcripts, but more ZEB2S transcripts than ZEB2L transcripts are produced as the concentration of ZEA increases. ZEB2S interacts with ZEB2L and forms ZEB2L-ZEB2S heterodimers that inhibit ZEB2L function. As the levels of ZEB2S transcripts increase, the rate of ZEA production gradually decreases, and ZEB2L transcription is ultimately terminated. To our knowledge, this was the first description of an autoregulatory mechanism of transcription factors in filamentous fungi.

### 2.2. Transcriptional and posttranscriptional regulation of ZEB2 by the protein kinase A (PKA) pathway

The cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway is one of the major signal transduction pathways that is involved in various cellular processes in eukaryotes\(^{18,19,20}\). Previous studies have revealed that the biosynthesis of many secondary metabolites are under the control of the G-protein-cAMP-PKA signaling pathway in filamentous fungi\(^{21,22,23}\). In F. graminearum, G-protein complex subunits as well as regulators of G protein signaling genes are involved in ZEA production\(^{24}\). F. graminearum has two genes for PKA catalytic subunits, CPK1 and CPK2, and one PKR gene encoding the PKA regulatory subunit\(^{25}\). We investigated the regulatory role of the PKA pathway in ZEA biosynthesis\(^{26}\). The deletion of CPK1 and PKR resulted in positive and negative regulation of ZEA production, respectively, but the deletion of CPK2 showed no effect on ZEA production\(^{26}\). Disruption of the CPK1 component of the PKA pathway induced high levels of...
**Fig. 5** Proposed model of ZEB2 regulation. ZEB2L and ZEB2S are presented by L in a green and orange circle, respectively. Homooligomers of ZEB2L are represented by L in the green oval and a red circle representing the ZEA molecule. The green oval with a dotted line represents gradually reduced ZEB2L homo-oligomer levels. The gray ovals represent undefined activators responsible for the direct induction of ZEB2S transcription. These figures originated from a previous study.$^{17}$

**3. Conclusions and Perspectives**

One of the goals of mycotoxin research is to reduce mycotoxins in cereal crops. With that in mind, we have intensively studied the biosynthesis and regulation of the ZEA pathway in *F. graminearum*. These results provide novel mechanistic insights into the autoregulation of secondary metabolite biosynthesis by a transcription factor that produces heterodimers via an alternative promoter usage. Further investigation
is necessary to identify the bZIP binding sequences in the promoter regions of ZEA biosynthetic cluster genes and unknown ZEB2 regulons. In addition, molecular mechanisms underlying reprogramming of alternative transcription of ZEB2 via the PKA pathway should be uncovered. The biological functions of ZEA are still unclear and our future works will focus on these topics.

4. Acknowledgements

This work was supported by the National Research Foundation of Korea (2013R1A6A3A04059121) and Research Resettlement Fund for the new faculty of Seoul National University.

References

1) Wolf-Hall, C.E.: Mold and mycotoxin problems encountered during malting and brewing. Int J Food Microbiol, 119, 89-94 (2007)
2) Yazar, S., Omurtag, G.: Fumonisins, trichothecenes and zearalenone in cereals. Int J Mol Sci, 9, 2062 (2008)
3) Golinski, P., Waskiewicz, A., Gromadzka, K.: "Mycotoxins in Food, Feed and Bioweapons" (eds. Rai M., Varma, A.), pp. 113-129 (2010), Springer Berlin Heidelberg, Berlin, Germany.
4) Zinedine, A., Soriano, J.M., Molto, J.C., Manes, J.: Review of the toxicology, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol, 45, 1-18 (2007)
5) Tiemann, U., Dänicke, S.: In vivo and in vitro effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: a review. Food Addit Contam, 24, 306-314 (2007)
6) Desjardins, A.E.: Fusarium mycotoxins: chemistry, genetics and biology. (2006), The American Phytopathological Society, St. Paul, MN, U.S.A.
7) Minervini, F., Dell’Aquila, M.E.: Zearalenone and reproductive function in farm animals. Int J Mol Sci, 9, 2570-2584 (2008)
8) Kim, Y.-T., Lee, Y.-R., Jin, J., Han, K.-H., Kim, H., Kim, J.-C., Lee, T., Yun, S.-H., Lee, Y.-W.: Two different polyketide synthase genes are required for synthesis of zearalenone in Gibberella zeae. Mol Microbiol, 58, 1102-1113 (2005)
9) Kim, J.-E., Jin, J., Kim, H., Kim, J.-C., Yun, S.-H., Lee, Y.-W.: GIP2, a putative transcription factor that regulates the aurofusarin biosynthetic gene cluster in Gibberella zeae. Appl Environ Microbiol, 72, 1645-1652 (2006)
10) Kim, J.-E., Han, K.-H., Jin, J., Kim, H., Kim, J.-C., Yun, S.-H., Lee, Y.-W.: Putative polyketide synthase and laccase genes for biosynthesis of aurofusarin in Gibberella zeae. Appl Environ Microbiol, 71, 1701-1708 (2005)
11) Son, H., Seo, Y.-S., Min, K., Park, A.R., Lee, J., Jin, J.-M., Lin, Y., Cao, P., Hong, S.-Y., Kim, E.-K., Lee, S.-H., Cho, A., Lee, S., Kim, M.-G., Kim, Y., Kim, J.-E., Kim, J.-C., Choi, G.J., Yun, S.-H., Lim, J.Y., Kim, M., Lee, Y.-H., Choi, Y.-D., Lee, Y.-W.: A phenome-based functional analysis of transcription factors in the cereal head blight fungus, Fusarium graminearum. PLoS Pathog, 7, e1002310 (2011)
12) Crawford, J.M., Townsend, C.A.: New insights into the formation of fungal aromatic polyketides. Nature Reviews Microbiology, 8, 879 (2010)
13) Hansen, F.T., Sørensen, J.L., Giese, H., Sondergaard, T.E., Frandsen, R.J.N.: Quick guide to polyketide synthase and nonribosomal synthetase genes in Fusarium. Int J Food Microbiol, 155, 128-136 (2012)
14) Cuomo, C.A., Güldener, U., Xu, J.-R., Trail, F., Turgeon, B.G., Di Pietro, A., Walton, J.D., Ma, L.-J., Baker, S.E., Rep, M., Adam, G., Antoniw, J., Baldwin, T., Calvo, S., Chang, Y.-L., DeCaprio, D., Gale, L.R., Gnerre, S., Goswami, R.S., Hammond-Kosack, K., Harris, L.J., Hilburn, K., Kennell, J.C., Kroken, S., Magnuson, J.K., Mannhart, G., Mauceli, E., Mewes, H.-W., Mitterbauer, R., Muehlbauer, G., Münsterkötter, M., Nelson, D., O’Donnell, K., Que, T., Qin, W., Quevillon, E., Roncero, M.I.G., Seong, K.-Y., Tetko, I.V., Urban, M., Waalwijk, C., Ward, T.J., Yao, J., Birren, B.W., Kistler, H.C.: The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science, 317, 1400-1402 (2007)
15) Gaffoor, I., Trail, F.: Characterization of two polyketide synthase genes involved in zearalenone biosynthesis in Gibberella zeae. Appl Environ Microbiol, 72, 1793-1799 (2006)
16) Brown, D.W., Butchko, R.A.E., Baker, S.E., Proctor, R.H.: Phylogenomic and functional domain analysis of polyketide synthases in Fusarium. Fungal Biol, 116, 318-331 (2012)
17) Park, A.R., Son, H., Min, K., Park, J., Goo, J.H., Rhee, S., Chae, S.-K., Lee, Y.-W.: Autoregulation of ZEB2 expression for zearalenone production in Fusarium graminearum. Mol Microbiol, 97, 942-956 (2015)
18) Thevelein, J.M., De Winde, J.H.: Novel sensing mechanisms and targets for the cAMP–protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol Microbiol, 33, 904-918 (1999)
19) Choi, J., Jung, W.H., Kronstad, J.W.: The cAMP/protein kinase A signaling pathway in pathogenic basidiomycete fungi. Connections with iron homeostasis. Journal of Microbiology, 53, 579-587 (2015)
20) Neves, S.R., Ram, P.T., Jyengar, R.: G protein pathways. Science, 296, 1636-1639 (2002)
21) Shimizu, K., Keller, N.P.: Genetic involvement of a CAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics, 157, 591-600 (2001)
22) Studt, L., Humph, H.-U., Tudzynski, B.: Signaling governed by G proteins and cAMP is crucial for growth, secondary metabolism and sexual development in Fusarium fujikuroi. PLoS One, 8, e58185 (2013)
23) Yu, X., Liu, H., Niu, X., Akhberdi, O., Wei, D., Wang, D., Zhu, X.: The Ga1-CAMP signaling pathway controls conidiation, development and secondary metabolism in the taxol-producing fungus Pestalotiopsis microspora. Microbiol Res, 203, 29-39 (2017)
24) Yu, H.-Y., Seo, J.-A., Kim, J.-E., Han, K.-H., Shim, W.-B., Yun, S.-H., Lee, Y.-W.: Functional analyses of heterotrimeric G protein Gα and Gβ subunits in Gibberella zeae. Microbiology, 154, 392-401 (2008)
25) Hu, S., Zhou, X., Gu, X., Cao, S., Wang, C., Xu, J.-R.: The cAMP-PKA pathway regulates growth, sexual and axenial differentiation, and pathogenesis in Fusarium graminearum. Mol Plant Microbe Interact, 27, 557-566 (2014)
26) Park, A.R., Fu, M., Shin, J.Y., Son, H., Lee, Y.-W.: The protein kinase A pathway regulates zearalenone production by modulating alternative ZEB2 transcription. J Microbiol Biotechnol, 26, 967-974 (2016)