농학박사학위논문

*Fusarium graminearum*의 ZEB2 동형단백질에 의한 지랄레논 생합성 자가조절 연구

Autoregulation of zearalenone biosynthesis by ZEB2 isoforms in *Fusarium graminearum*

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농생명공학부 식물미생물전공

박 애 란

Autoregulation of zearalenone biosynthesis by ZEB2 isoforms in *Fusarium graminearum*

A dissertation submitted in partial
fulfillment of the requirement for
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by

Ae Ran Park

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Autoregulation of zearalenone biosynthesis by ZEB2 isoforms in *Fusarium graminearum*

UNDER THE DIRECTION OF DR. YIN-WON LEE

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

BY
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MAJOR IN PLANT MICROBIOLOGY
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
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ABSTRACT

Autoregulation of zearalenone biosynthesis by ZEB2 isoforms in *Fusarium graminearum*

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The ascomycete fungus *Fusarium graminearum* is the most common pathogen of Fusarium head blight (FHB), a devastating disease for major cereal crops worldwide. FHB causes significant crop losses by reducing grain yield and quality as well as contaminating cereals with trichothecenes and zearalenone (ZEA) that pose a serious threat to animal health and food safety. ZEA is a causative agent of hyperestrogenic syndrome in mammals and can result in reproductive disorders in farm animals. In *F. graminearum*, the ZEA biosynthetic cluster is composed of four genes, *PKS4*, *PKS13*, *ZEB1*, and *ZEB2*, which encode a reducing polyketide synthase, a nonreducing polyketide synthase, an isoamyl alcohol oxidase, and a transcription factor, respectively. Although it is known that ZEB2 primarily acts as a regulator of ZEA biosynthetic cluster genes, the

mechanism underlying this regulation remains undetermined. In this study, two isoforms (ZEB2L and ZEB2S) from the *ZEB2* gene in *F. graminearum* were characterized. It was revealed that ZEB2L contains a basic leucine zipper (bZIP) DNA-binding domain at the N-terminus, whereas ZEB2S is an N-terminally truncated form of ZEB2L that lacks the bZIP domain. Interestingly, ZEA triggered the induction of both *ZEB2L* and *ZEB2S* transcription. In ZEA producing condition, the expression of *ZEB2S* transcripts via alternative promoter usage was directly or indirectly initiated by ZEA. Physical interaction between ZEB2L and ZEB2L as well as between ZEB2L and ZEB2S was observed in the nucleus. The ZEB2S-ZEB2S interaction was detected in both the cytosol and the nucleus. ZEB2L-ZEB2L oligomers activated ZEA biosynthetic cluster genes, including *ZEB2L*. ZEB2S inhibited *ZEB2L* transcription by forming ZEB2L-ZEB2S heterodimers, which reduced the DNA-binding activity of ZEB2L. This study provides insight into the autoregulation of *ZEB2* expression by alternative promoter usage and a feedback loop during ZEA production.

KEY WORDS: *Fusarium graminearum*, Mycotoxin, Zearalenone, bZIP transcription factor, ZEB2, Autoregulation

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INTRODUCTION

The ascomycete fungus *Fusarium graminearum* is the causal agent of Fusarium head blight (FHB), a devastating disease for major cereal crops worldwide. FHB causes significant yield loss, but also reduces the quality and feeding value of the grain. The fungi that cause FHB may contaminate cereals with trichothecenes and zearalenone (ZEA) mycotoxins that pose a serious threat to animal health and food safety, thus dramatically lowering the market value of the crops.

Zearalenone (ZEA, 6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcyclic acid lactone) is a polyketide mycotoxin produced by several *Fusarium* species, including *F. graminearum*, *F. culmorum*, *F. equiseti*, and *F. crookwellense* (Zinedine *et al.*, 2007; Desjardins, 2006; Marasas *et al.*, 1984). ZEA induces hyperestrogenic effects in mammals, which may result in reproductive disorders in farm and laboratory animals (Desjardins, 2006). ZEA commonly coexists with the trichothecene mycotoxin deoxynivalenol in cereal crops infected with *F. graminearum*, which is the primary causal agent of Fusarium head blight of small-grain cereals worldwide (Desjardins, 2006; Marasas *et al.*, 1984). ZEA is also a regular contaminant of foodstuffs, including cornmeal, corn flakes, corn porridge, and beer; therefore, it is a threat to human health (Marasas *et al.*, 1984). Additionally, a synthetic commercial formulation of the reduced form of ZEA (zeranol) increases estrogenic activity and has been used to increase cattle growth in several regions throughout the world (Zhong *et al.*, 2011).

ZEA is synthesized by the head-to-tail condensation of acetate units via the

acetate-malonyl-coenzyme A (CoA) enzyme system through a polyketide pathway (Steele *et al.*, 1974; Mirocha *et al.*, 1978). The ZEA biosynthetic cluster genes *PKS4*, *PKS13*, *ZEB1*, and *ZEB2* encode a reducing polyketide synthase, a nonreducing polyketide synthase, an isoamyl alcohol oxidase, and a basic leucine zipper (bZIP) transcription factor (TF), respectively (Kim *et al.*, 2005b; Gaffoor and Trail, 2006; Lysøe *et al.*, 2006). The ZEA biosynthetic pathway is initiated by PKS4, which catalyzes the condensation of carbons from one acetyl-CoA and five malonyl-CoA molecules, resulting in the formation of an alkene. After this alkene is delivered to PKS13, the synthase continues to extend the ZEA chain by adding three malonyl-CoA molecules. Then, the unreduced ketones undergo intramolecular aromatic reactions, causing the formation of an aromatic ring and a macrolide ring structure containing a lactone bond (Gaffoor and Trail, 2006). The conversion of β-zearalenol to ZEA is catalyzed by ZEB1, with ZEB2 acting as a potential transcriptional activator of genes that regulate ZEA biosynthesis (Kim *et al.*, 2005b).

Although ZEB2 primarily acts as a regulator of ZEA biosynthetic cluster genes, the mechanism underlying this regulation remains undetermined. In a previous study, two potential *ZEB2* transcripts were found to be expressed under ZEA-producing conditions; however, the identities and functions of these transcripts remained unknown (Kim *et al.*, 2005b). Because these two *ZEB2* transcripts had different expression patterns with distinct maximal expression and termination times, I hypothesized that they are formed by alternative transcription events and that their protein products have different effects on ZEA production. Alternative splicing is a well-described biochemical process in which multiple

protein isoforms are formed from a single gene, thereby increasing proteomic and functional diversity in eukaryotes (Matlin *et al.*, 2005). Alternative splicing is tightly linked to other processes that lead to the expression of different mRNAs from one gene, such as transcription initiation using alternative promoters and alternative polyadenylation (Artamonova and Gelfand, 2007). In this study, I determined the identities of these two *ZEB2* transcripts, analyzed the effects of their protein products on ZEA biosynthesis, and identified their protein-interacting partners. Overall, this study provides important insights into the mechanisms that regulate ZEA biosynthesis.

MATERIALS AND METHODS

I. Fungal strains and culture conditions

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie, 1999) and mutants derived from this strain were maintained according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006) and were stored in 20% (v/v) glycerol at -70 ℃. The ZEA-deficient mutants Δ*zeb2*, Δ*pks4* and Δ*pks13* were obtained from a previous experiment (Kim *et al.*, 2005b). All strains used in this study are listed in Table 1. For genomic DNA isolation, fungal cultures grown in liquid complete medium (CM) for 5 days at 25 °C were harvested and lyophilized. The genomic DNA isolation procedures were performed as previously described (Leslie and Summerell, 2006). For total RNA and protein extraction and for ZEA production analysis, wild-type and mutant strains were grown in 25 ml of CM or SG liquid medium (Bacon *et al.*, 1977).

II. cDNA library construction

Total RNA was isolated from 6-day-old wild-type cultures grown in SG liquid medium using TRIzol (Invitrogen, Carlsbad, CA, USA). Then, 250 μg of total RNA was further purified using the Oligotex mRNA kit (Qiagen, Valencia, CA, USA). Approximately 0.5 μg of mRNA was reverse-transcribed using the EasyClone cDNA library construction kit (Dualsystems Biotech, Schlieren, Switzerland). The mRNA sample was purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). *Sfi*I-digested double-stranded cDNA was performed a size fractionation of the entire cDNA using a Chromaspin^{TM}-400

Table 1. *F. graminearum* strains used in this study.

Strain	Description and genotype	Reference or parents
Z-3639	*F. graminearum* wild-type strain	Bowden and Leslie, 1999
Δ*mat1*	Tester strain for outcross: Δ*mat1-1-1::Gen*^{R}	Lee et al., 2003
Δ*zeb2*	Deletion mutant of the *ZEB2* gene: Δ*zeb2::Gen*^{R}	Kim et al., 2005b
Δ*pks4*	Deletion mutant of the *PKS4* gene: Δ*pks4::Gen*^{R}	Kim et al., 2005b
Δ*pks13*	Deletion mutant of the *PKS13* gene: Δ*pks13::Gen*^{R}	Kim et al., 2005b
mat1g	Strain for outcross expressing histone H1 fused to GFP to visualize nuclei: Δ*mat1-1-1::Gen*^{R}; *hH1-GFP-Hyg*^{R}	Hong et al., 2010
mat1r	Strain for outcross expressing histone H1 fused to RFP to visualize nuclei: Δ*mat1-1-1::Gen*^{R}; *hH1-RFP-Gen*^{R};	Son et al., 2011a
FAZ03N	Complemented strain expressing ZEB2-HA in the deletion mutant: Δ*zeb2::Gen*^{R}; P_{ZEB2L}-*ZEB2-HA*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ003	Complemented strain expressing ZEB2L-HA in the deletion mutant: Δ*zeb2::Gen*^{R}; P_{ZEB2L}-*ZEB2L-HA*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ005	Strain expressing ZEB2S-Flag in Z-3639: P_{ZEB2S}-*ZEB2S-Flag*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ001(L)	Complemented strain overexpressing ZEB2L-HA in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ001C	Complemented strain overexpressing ZEB2L-HA in the deletion mutant for	FAZ001 × Δ*mat1*

	outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-*Hyg*^{R}	
FAZ002 (S)	Complemented strain overexpressing ZEB2S-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ002C	Complemented strain overexpressing ZEB2S-Flag in the deletion mutant for outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-*Hyg*^{R}	FAZ002 × Δ*mat1*
FAZ006 (L/S)	Complemented strain coexpressing ZEB2L-HA and ZEB2S-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-*Hyg*^{R}	FAZ001 × FAZ002C
FAZ007	Complemented strain overexpressing ZEB2L-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-Flag*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ008	Complemented strain overexpressing ZEB2S-Myc in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Myc*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ009	Complemented strain overexpressing ZEB2LM-HA in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM* (*ZEB2L* Δ338-363, aa 1-337)-*HA*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ010	Complemented strain overexpressing ZEB2LM-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM* (*ZEB2L* Δ338-363, aa 1-337)-*Flag*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ011	Complemented strain overexpressing ZEB2SM-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; P_{ZEB2S}- *ZEB2SM* (*ZEB2S* Δ1-206, aa 207-363)-*Flag*-T_{ZEB2}-*Hyg*^{R}	This study
FAZ012 (L/L)	Complemented strain coexpressing ZEB2L-HA and ZEB2L-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2L-Flag*-T_{ZEB2}-	FAZ001C × FAZ007

	Hyg^R	
FAZ013 (S/S)	Complemented strain coexpressing ZEB2S-Flag and ZEB2S-Myc in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-Hyg^R; $P_{EF1\alpha}$-*ZEB2S-Myc*-T_{ZEB2}-Hyg^R	FAZ002C × FAZ008
FAZ014 (L/LM)	Complemented strain coexpressing ZEB2L-HA and ZEB2LM-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-Hyg^R; $P_{EF1\alpha}$-*ZEB2LM-Flag*-T_{ZEB2}-Hyg^R	FAZ001C × FAZ010
FAZ015 (S/LM)	Complemented strain coexpressing ZEB2S-Flag and ZEB2LM-HA in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-Hyg^R; $P_{EF1\alpha}$-*ZEB2LM-HA*-T_{ZEB2}-Hyg^R	FAZ002C × FAZ009
FAZ016 (L/SM)	Complemented strain coexpressing ZEB2L-HA and ZEB2SM-Flag in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-Hyg^R; $P_{EF1\alpha}$-*ZEB2SM-Flag*-T_{ZEB2}-Hyg^R	FAZ001C × FAZ011
FAZ017	Complemented strain overexpressing ZEB2L-GFP in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-GFP*-T_{ZEB2}-Hyg^R	This study
FAZ018	Complemented strain overexpressing ZEB2S-RFP in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-RFP*-T_{ZEB2}-Hyg^R	This study
FAZ018C	Complemented strain overexpressing ZEB2S-RFP in the deletion mutant for outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-RFP*-T_{ZEB2}-Hyg^R	FAZ018 × Δ*mat1*
FAZ021	Complemented strain coexpressing ZEB2L-GFP and ZEB2S-RFP in the deletion	FAZ017 × FAZ018C

	mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-GFP*-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2S-RFP*-T_{ZEB2}-*Hyg*^{R}	
FAZ022	Complemented strain coexpressing ZEB2L-GFP and Histone H1-RFP in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-GFP*-T_{ZEB2}-*Hyg*^{R}; *hH1-RFP-Gen*^{R}	FAZ017 × mat1r
FAZ023	Complemented strain coexpressing ZEB2S-RFP and Histone H1-GFP in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-RFP*-T_{ZEB2}-*Hyg*^{R}; *hH1-GFP-Hyg*^{R}	FAZ018 × mat1g
FAZ024	Complemented strain overexpressing ZEB2L-YFP^N in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	This study
FAZ024C	Complemented strain overexpressing ZEB2L-YFP^N in the deletion mutant for outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	FAZ024 × Δ*mat1*
FAZ025	Complemented strain overexpressing ZEB2L-YFP^C in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	This study
FAZ026	Complemented strain overexpressing ZEB2S-YFP^N in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	This study
FAZ026C	Complemented strain overexpressing ZEB2S-YFP^N in the deletion mutant for outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	FAZ026 × Δ*mat1*
FAZ027	Complemented strain overexpressing ZEB2S-YFP^C in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	This study
FAZ028	Complemented strain overexpressing ZEB2LM-YFP^N in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	This study

FAZ028C	Complemented strain overexpressing ZEB2LM-YFP^{N} in the deletion mutant for outcross: Δ*mat1-1-1::Gen*^{R}; Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}	FAZ028 × Δ*mat1*
FAZ029	Complemented strain coexpressing ZEB2L-YFP^{N} and ZEB2L-YFP^{C} in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2L-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	FAZ024C × FAZ025
FAZ030	Complemented strain coexpressing ZEB2L-YFP^{N} and ZEB2S-YFP^{C} in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2L-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2S-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	FAZ024C × FAZ027
FAZ031	Complemented strain coexpressing ZEB2S-YFP^{N} and ZEB2S-YFP^{C} in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2S-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R}; $P_{EF1\alpha}$-*ZEB2S-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	FAZ026C × FAZ027
FAZ032	Complemented strain coexpressing ZEB2L-YFP^{N} and ZEB2LM-YFP^{C} in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R} $P_{EF1\alpha}$-*ZEB2L-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	FAZ028C × FAZ025
FAZ033	Complemented strain coexpressing ZEB2S-YFP^{N} and ZEB2LM-YFP^{C} in the deletion mutant: Δ*zeb2::Gen*^{R}; $P_{EF1\alpha}$-*ZEB2LM-Myc-YFP*^{N}-T_{ZEB2}-*Hyg*^{R} $P_{EF1\alpha}$-*ZEB2S-HA-YFP*^{C}-T_{ZEB2}-*Hyg*^{R}	FAZ028C × FAZ027

column (Clontech, Palo Alto, CA, USA) and was directly cloned into the pPR3-N library vector. The ligation mixture was transformed into DH10B Electromax cells (Invitrogen) for amplification of the cDNA library.

III. *ZEB2* transcript identification

The cDNA library described above was used for cDNA sequencing analysis. Primary PCR was performed with the Anchor-5N/PZ3-1 and Anchor-3N/PZ5-1 primer pairs. Diluted PCR products were reamplified under the same conditions with both anchor primers and the nested primers PZ5-2 and PZ3-2. The major bands resolved by agarose gel electrophoresis were gel-purified, cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced. The PCR primers (Table 2) used in this study were synthesized by the Bionics oligonucleotide synthesis facility (Seoul, Korea). DNA sequencing was performed by Macrogen Inc. (Seoul, Korea).

For Northern blot analyses, DNA probes specific to the N-terminus (PZ019-5/PZ189-3 for probe 1 and PZ187-5/PZ453-3 for probe 2) or C-terminus of *ZEB2* (PZ577-5/PZ790-3 for probe 3 and PZ919-5/PZ1086-3 for probe 4) were generated. Exogenous ZEA can induce ZEA biosynthetic cluster genes; therefore, SG liquid medium was supplemented with 1 ppm ZEA to increase the band intensity for Northern blot analyses (Lee *et al.*, 2011a). Total RNA was extracted from wild-type cultures grown in SG liquid medium containing or lacking 1 ppm ZEA. Gel electrophoresis, gel blotting, and hybridization with ^{32}P-labeled probes were performed according to standard procedures (Green and Sambrook, 2012).

Table 2. Primers used in this study.

Primer	Sequence (5'-3')	Purpose
Anchor-5N	GTCGAAAATTCAAGACAAGG	cDNA sequencing
Anchor-3N	AAGCGTGACATAACTAATTAC	cDNA sequencing
PZ5-1	ATGAGTCGTATTCTCAAGGTTATTCAGG	cDNA sequencing (position bp 574 of *ZEB2L*)
PZ5-2/ PZ577-5	AGTCGTATTCTCAAGGTTATTCAGGATG	cDNA sequencing and amplification of Northern blot probe c
PZ3-1	GGCGTAGATGGCTTCGGCTGATTTAGATAT	cDNA sequencing
PZ3-2/ PZ790-3	ATATCTCTTGTTGGTAATCATGTGCTTCA	cDNA sequencing and amplification of Northern blot probe c
PZ019-5	ATGCAATCTACCGAGTCGTTCCAT	Amplification of Northern blot probe a
PZ189-3	ATTCGACGTTTGGCATTGGTTGTTGAC	Amplification of Northern blot probe a
PZ187-5	AATTGGACCAGCCACGCACCCACAGAT	Amplification of Northern blot probe b
PZ453-3	TGAGCTATGATCGCCGATAGAAAGGCCT	Amplification of Northern blot probe b
PZ919-5	ATTGAGAACCATCTCCGACACGAACAG	Amplification of Northern blot probe d
PZ1089-3	TCAATTTTGCGACATACGGGAGGCTTGTAG	Amplification of Northern blot probe d
PEFp-5 *Apa*I	gatcgggcccGACTCACTATAGAATTCCGGTACCTATA	Amplification of overexpressed promoter ($P_{EF1\alpha}$)
PEFp-3 *Spe*I	gatcactagtTTTGAAGATTGGGTTCCTTTTGTG	Amplification of overexpressed

PZLp-5 *Apa*I	gatcgggcccAGTGCCAGGCTGTTGTCTACCATGTAT	promoter ($P_{EF1\alpha}$) Amplification of native *ZEB2L* promoter (P_{ZEB2L}, -1166 to -1)
PZLp-3 *Spe*I	gatcactagtTTAAGCTGAATTAAGGTCACGGCCGTT	Amplification of native *ZEB2L* promoter (P_{ZEB2L}, -1166 to -1)
PZSp-5 *Apa*I	gatcgggcccAACGGCCGTGACCTTAATTCAGCTTAA	Amplification of native *ZEB2S* promoter (P_{ZEB2S}, -27 to +604)
PZSp-3 *Spe*I	gatcactagtGTCGCGGAGGTCTTGGTTGCACAGATTAT	Amplification of native ZEB2S promoter (P_{ZEB2S}, -27 to +604)
PZLO-5 *Spe*I	gatcactagtATGACATTGGTTGACAATATGCAAT	Amplification of ZEB2L coding sequence
PZSO-5 *Spe*I	gatcactagtATGGTGCCCGAGGAGAAAATGAGTCGT	Amplification of ZEB2S coding sequence
PZLO-3 *BamH*I	gatcggatccATTTTGCGACATACGGGAGGCTTGTAG	Amplification of ZEB2 coding sequence
PZLO-5 *EcoR*I	gatcgaattcATGACATTGGTTGACAATATGCAAT	Insertion of ZEB2L into pET28a
PZSO-5 *EcoR*I	gatcgaattcATGGTGCCCGAGGAGAAAATGAGTCGT	Insertion of ZEB2S into pET28a
PZLO-3 *Xho*I	gatcctcgagATTTTGCGACATACGGGAGGCTTGTAG	Insertion of ZEB2 into pET28a
PZt-5 *Hind*III	gatcaagcttTGATGTAACTATATTGATGGACATGGG	Insertion of ZEB2 terminator into pGZT001
PZt-3 *Sac*I	gatcgtcgacTCGGCAACCGAACTATCAAGATGTC	Insertion of ZEB2 terminator into

		pGZT001
PL2-5	ATCTGTGCAACCAAGACCTCCGCGACcTGGTGCCCGAGGAGAAAcTGAGTCGTATTCT	Point mutation of two internal start codons of ZEB2L (M186L and M192L) to leucine
PL2-3	gTTTCTCCTCGGGCACCAgGTCGCGGAGGTCTTGGTTGCACAGA	Point mutation of two internal start codons of ZEB2L (M186L and M192L) to leucine
HA Link-5 *BamH*I	gatcggatccTACCCATACGACGTCCCAGACTACGCTTAA	Fusion of *HA* to *ZEB2*
HA Link-3 *Hind*III	gatcaagcttTTAAGCGTAGTCTGGGACGTCGTATGGGTA	Fusion of *HA* to *ZEB2*
Flag Link-5 *BamH*I	gatcggatccGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAGGATGACGATGACAAGTAA	Fusion of *Flag* to *ZEB2*
Flag Link-3 *Hind*III	gatcaagcttTTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTC	Fusion of *Flag* to *ZEB2*
Myc Link-5 *BamH*I	gatcggatccGAACAAAAACTCATCTCAGAAGAGGATCTGTGA	Fusion of *Flag* to *ZEB2*
Myc Link-3 *Hind*III	gatcaagcttTCACAGATCCTCTTCTGAGATGAGTTTTTGTTC	Fusion of *Flag* to *ZEB2*
Gly Link-5	GATCCGGGGGAGGCGGGGGTG	Fusion of *glycine* linker to *ZEB2*

Gly Link-3	GATCCACCCCCGCCTCCCCCG	Fusion of *glycine* linker to *ZEB2*
PGFP-5 *BamH*I	gatcggatccATGGTGAGCAAGGGCGAGGAGCTGT	Amplification of the *GFP* reporter gene
PRFP-5 *BamH*I	gatcggatccATGGTGAGCAAGGGCGAGGAGGT	Amplification of the *RFP* reporter gene
PGFP-3/PRFP-3 *Hind*III	gatcaagcttCTTGTACAGCTCGTCCATGCCG	Amplification of the *GFP* and *RFP* reporter genes
PYFPNE-5 *BamH*I	gatcggatccATGGAGCAAAAGTTGATTTCTGAGGAGGA	Amplification of the N-terminal *YFP* fused to *Myc* for the BiFC assay
PYFPNE-3 *Hind*III	gatcaagcttGGCCATGATATAGACGTTGTGGCTGTTGT	Amplification of the N-terminal *YFP* fused to *Myc* for the BiFC assay
PYFPCE-5 *BamH*I	gatcggatccATGTACCCATACGATGTTCCAGATTA	Amplification of the C-terminal *YFP* fused to *HA* for the BiFC assay
PYFPCE-3 *Hind*III	gatcaagcttCTTGTACAGCTCGTCCATGCCGAGAGT	Amplification of the C-terminal *YFP* fused to *HA* for the BiFC assay
PY001-5 *Sfi*I	aacgcagagaggccattacggccATGACATTGGTTGACAATATGCAAT	Yeast two-hybrid assay (aa 1 of ZEB2L)
PY363-3 *Sfi*I	aacgcagagaggccgaggcggccATTTTGCGACATACGGGAGGCTTGTAG	Yeast two-hybrid assay (aa 363 of ZEB2L)
PY129-5 *Sfi*I	aacgcagagaggccattacggccATGCCTAGCAGCCCGACCTCCACCAGCAA	Yeast two-hybrid assay (aa 129 of ZEB2L)
PY173-5 *Sfi*I	aacgcagagaggccattacggccCGACAGCACGATAATCTGTGCAA	Yeast two-hybrid assay (aa 173 of

	CCAAGACC	ZEB2L)
PY172-3 *Hind*III	gatcaagcttAGTTGGAAGACTCCCTTCTGTCGATCCA	Yeast two-hybrid assay (aa 172 of ZEB2L)
PY186-5 *Hind*III	gatcaagcttATGGTGCCCGAGGAGAAAATGAGTCGTATTCT	Yeast two-hybrid assay (aa 186 of ZEB2L)
PY185-3 *Hind*III	gatcaagcttGTCGCGGAGGTCTTGGTTGCACAGATTAT	Yeast two-hybrid assay (aa 185 of ZEB2L)
PY192-5 *Hind*III	gatcaagcttATGAGTCGTATTCTCAAGGTTATTCAGG	Yeast two-hybrid assay (aa 192 of ZEB2L)
PY186-5 *Sfi*I	aacgcagagaggccattacggccATGGTGCCCGAGGAGAAAATGAGTCGTATTCT	Yeast two-hybrid assay (aa 186 of ZEB2L)
PY192-5 *Sfi*I	aacgcagagaggccattacggccATGAGTCGTATTCTCAAGGTTATTCAGG	Yeast two-hybrid assay (aa 192 of ZEB2L)
PY207-5 *Sfi*I	aacgcagagaggccattacggccATGGATTCATTCATGACTGAGT	Yeast two-hybrid assay (aa 207 of ZEB2L)
PY320-3 *Sfi*I	aacgcagagaggccgaggcggccTCTCTGCATCATCGACTGTTCGTGT	Yeast two-hybrid assay (aa 320 of ZEB2L)
PY337-3 *Sfi*I	aacgcagagaggccgaggcggccACGAATGGTAGCCGGAAAGCTCGGAT	Yeast two-hybrid assay (aa 337 of ZEB2L)
PBTUp-5	AGGAAGCTACTGATCTCCAGGA	Amplification of the *β-tubulin* promoter

PBTUp-3	ATTGACGGCTGTAGATGTAATG	(-641 to -1)
PPKS4p1-5	CGTATCATGGAGAGAATC	Amplification of the *β-tubulin* promoter (-641 to -1)
PPKS4p1-3	CGCTCTTCAGAAGGTCCCAGA	Amplification of the *PKS4* promoter (-711 to -389)
PPKS4p2-5	GAAAAGGTGAGCAGCAATAAGA	Amplification of the *PKS4* promoter (-711 to -389)
PPKS4p2-3	TCGCTCATGGAGGATCCAACA	Amplification of the *PKS4* promoter (-388 to -1)
PPKS13p1-5	GCCTCATATTTCTTGGATTCATCA	Amplification of the *PKS4* promoter (-388 to -1)
PPKS13p1-3	TGTCAGATTTACAGTCAGCGTAGC	Amplification of the *PKS13* promoter (-777 to -411)
PPKS13p2-5	TGTTCCGCTTCTAAGCACATACA	Amplification of the *PKS13* promoter (-777 to -411)
PPKS13p2-3	GATCTCCGAAGAGAAGGATTGTTTTC	Amplification of the *PKS13* promoter (-410 to +40)
PZEB1p1-5	AAACATCAAGCTCTGGGAAG	Amplification of the *PKS13* promoter (-410 to +40)
		Amplification of the *ZEB1* promoter (-

		1124 to -558)
PZEB1p1-3	ACATTTGTTCCGTTTCTCCTCA	Amplification of the *ZEB1* promoter (-1124 to -558)
PZEB1p2-5	TGAGGAGAAACGGAACAAATGT	Amplification of the *ZEB1* promoter (-580 to -298)
PZEB1p2-3	ACGAACGTAAGCGTTATGGCT	Amplification of the *ZEB1* promoter (-580 to -298)
PZEB1p3-5	TTCCTCTAGATACTTCATTGTGCTCTTA	Amplification of the *ZEB1* promoter (-298 to +31)
PZEB1p3-3	CGGGTAAATATTTTGACGGCG	Amplification of the *ZEB1* promoter (-298 to +31)
PZEB2Lp1-5	CAGGCTGTTGTCTACCATGT	Amplification of the *ZEB2* promoter (-1161 to -615)
PZEB2Lp1-3	ATCGCGATTAGCAATGCCAACCTCGA	Amplification of the *ZEB2* promoter (-1161 to -615)
PZEB2Lp2-5	GGTTCAGTACCCAGTCTCTTGCAT	Amplification of the *ZEB2* promoter (-735 to -272)
PZEB2Lp2-3	AAGTGGCATAAGCTACCCTGATAA	Amplification of the *ZEB2* promoter (-735 to -272)
PZEB2Lp3-5	TGCGAATGTACCACCCAAGGTTAG	Amplification of the *ZEB2* promoter (-

		380 to -7)
PZEB2Lp3-3	TGAATTAAGGTCACGGCCGTT	Amplification of the *ZEB2* promoter (-380 to -7)
PZEB2Sp1-5	AACGGCCGTGACCTTAATTCA	Amplification of the *ZEB2S* promoter (-27 to +502)
PZEB2Sp1-3	TGAGCTATGATCGCCGATAGAAAGGCCT	Amplification of the *ZEB2S* promoter (-27 to +502)
PZEB2Sp2-5	CCAAACGTCGAATTGGACCAGCCACGCA	Amplification of the *ZEB2S* promoter (+226 to +625)
PZEB2Sp2-3	CATTTTCTCCTCGGGCACCATGTCGCGGA	Amplification of the *ZEB2S* promoter (+226 to +625)
PPKS4RT-5	TGTGGTATCCTTGTTTTGAAG	qRT-PCR of *PKS4* transcripts
PPKS4RT-3	TACCATGAGCCTCCACATACT	qRT-PCR of *PKS4* transcripts
PPKS13RT-5	CCAAGACCGTCGAGGATAAGAA	qRT-PCR of *PKS13* transcripts
PPKS13RT-3	TCGCTTAGCCAGCTTCTGCATA	qRT-PCR of *PKS13* transcripts
PZEB1RT-5	GGACGCACCACCTCAAAAACAT	qRT-PCR of *ZEB1* transcripts
PZEB1RT-3	TGGAAAGTGCAGAATGGCCACCT	qRT-PCR of *ZEB1* transcripts
PZEB2LRT-5	ATGCAATCTACCGAGTCGTTCCAT	qRT-PCR of *ZEB2L* transcripts
PZEB2LRT-3	ATTCGACGTTTGGCATTGGTTGTTGAC	qRT-PCR of *ZEB2L* transcripts
PZEB2RT-5	AGTCGTATTCTCAAGGTTATTCAGGATG	qRT-PCR of *ZEB2* transcripts

PZEB2RT-3	ATATCTCTTGTTGGTAATCATGTGCTTCA	qRT-PCR of *ZEB2* transcripts
PCYP1RT-5	TCAAGCTCAAGCACACCAAGAAGG	qRT-PCR of *CYP1* transcripts
PCYP1RT-3	GGTCCGCCGCTCCAGTCT	qRT-PCR of *CYP1* transcripts

For qRT-PCR analyses of ZEA biosynthetic cluster genes, total RNA was extracted from 5-day-old cultures of fungal strains constitutively expressing *ZEB2* genes grown in CM. Total RNA (5 μg) was converted into cDNA with the SuperScript™ III First-Strand Synthesis System (Invitrogen) using oligo(dT)$_{20}$ according to manufacturer's recommendations, and a 1:100 fraction (corresponding to 50 ng of reverse-transcribed RNA) was used for qRT-PCR. All reactions were performed in a volume of 20 μl using iQ SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and a CFX96™ Real-Time System (Bio-Rad) according to the manufacturer's instructions. The thermal profile was 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 10 s and 58 ℃ for 30 s. Three technical replicates were performed for all qRT-PCR reactions. The Ct values were normalized to the endogenous housekeeping gene cyclophilin (*CYP1*) for each sample.

IV. Verification of *ZEB2* promoter function

Fragments corresponding to bp -1166 to -1 (P_{ZEB2L}) and bp -27 to +604 (P_{ZEB2S}) of *ZEB2* were amplified from wild-type genomic DNA using the primer pairs PZLp-5 *Apa*I/PZLp-3 *Spe*I and PZSp-5 *Apa*I/PZSp-3 *Spe*I, respectively. Full-length *ZEB2* genomic DNA (1.2 kb) was amplified from wild-type genomic DNA using PZLO-5 *Spe*I and PZLO-3 *BamH*I. The *ZEB2S* (0.5-kb) ORF was amplified from the cDNA library using PZSO-5 *Spe*I and PZLO-3 *BamH*I. For the *ZEB2L* construct containing internal start codons that were point-mutated to leucine-encoding CTG codons (M186L and M192L), two fragments amplified using PZEB2Sp1-5/PL2-3 and PL2-5/PZt-3 *Sac*I were fused and reamplified using

nested primers (PZLO-5 *Spe*I and PZLO-3 *BamH*I). The *ZEB2* terminator (227 bp, +1207 to +1432 region) was PCR-amplified from Z-3639 genomic DNA using PZt-5 *Hind*III/PZt-3 *Sac*I. These PCR fragments were cloned into pGH-T (Lee *et al.*, 2009) to generate pGZT001, which was used to generate plasmids for protein expression in *F. graminearum*. HA and Flag tags were inserted into the *BamH*I and *Hind*III sites between the ORF and the terminator. After the verification of the inserted fragments by sequencing analyses, vectors containing the *ZEB2* variants were used for fungal transformation (Kim *et al.*, 2005a). To identify ZEB2 proteins encoded by alternative promoters, Western blot analysis was performed as described below ("Immunoblotting and immunoprecipitation"). The plasmids used in this study are listed in Table 3.

V. Yeast two-hybrid (Y2H) assays

Y2H assays were conducted using the DUALhunter System (Dualsystems Biotech) in accordance with the manufacturer's instructions. Full-length *ZEB2L* cDNA cloned into the pDHB1 vector was used as the bait vector, which was cotransformed with the generated Y2H cDNA library. *Saccharomyces cerevisiae* NMY51 (*MAT a his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexApo)$_4$-HIS3 ura3::(lexApo)$_8$-lacZ ade2::(lexApo)$_8$-ADE2 GAL4*) was used as a host. A total of 100 positive clones identified by selection on selective medium without Leu, Trp, His, or Ade (SD-L-T-H-A) were sequenced. Several truncated *ZEB2L* constructs were cloned into prey vectors, and full-length *ZEB2L* and *ZEB2S* inserted into pDHB1 were used as bait vectors. After cotransformation, a colony picked from

Table 3. Plasmid constructs used in this study.

Strain/plasmid	Genotype	Reference or parents
pGH-T	Binary vector containing a hygromycin resistance gene (*Hyg*^{R})	Lee et al., 2009
pET28a	Cloning vector for constructing N-terminal His_6 proteins	Novagen
pDHB1	Cloning vector for a bait in the yeast two-hybrid assay	Dualsystems Biotech
pPR3-N	Cloning vector for a prey and a library in the yeast two-hybrid assay	Dualsystems Biotech
pAI-Alg5	Positive control prey vector in the yeast two-hybrid assay	Dualsystems Biotech
pDL2-Alg5	Negative control prey vector in the yeast two-hybrid assay	Dualsystems Biotech
pYB01	pDHB1 derivative expressing ZEB2L (aa 1-363)	This study
pYB02	pDHB1 derivative expressing ZEB2S (aa 186-363 of ZEB2L)	This study
pYP03	pPR3-N derivative expressing ZEB2L (aa 1-363)	This study
pYP04	pPR3-N derivative expressing ZEB2LΔ1-185 (aa 186-363, ZEB2S)	This study
pYP05	pPR3-N derivative expressing ZEB2LΔ1-128 (aa 129-363)	This study
pYP06	pPR3-N derivative expressing ZEB2LΔ1-172 (aa 173-363)	This study
pYP07	pPR3-N derivative expressing ZEB2LΔ1-191 (aa 192-363)	This study
pYP08	pPR3-N derivative expressing ZEB2LΔ1-206 (aa 207-363, ZEB2SM)	This study
pYP09	pPR3-N derivative expressing ZEB2LΔ173-185 (aa 1-172 & 186-363)	This study
pYP10	pPR3-N derivative expressing ZEB2LΔ173-191 (aa 1-172 & 192-363)	This study
pYP11	pPR3-N derivative expressing ZEB2LΔ186-191 (aa 1-185 & 192-363)	This study

pYP12	pPR3-N derivative expressing ZEB2LΔ338-363 (aa 1-337, ZEB2LM)	This study
pYP13	pPR3-N derivative expressing ZEB2LΔ321-363 (aa 1-320)	This study
pYP14	pPR3-N derivative expressing ZEB2S Δ1-185 Δ338-363 (aa 186-337)	This study
pYP15	pPR3-N derivative expressing ZEB2S Δ1-185 Δ321-363 (aa 186-320)	This study
pBE001	For expression of ZEB2L containing C-terminal His_6, cloned into the *Eco*RI and *Xho*I sites of pET28a	This study
pBE002	For expression of ZEB2S containing C-terminal His_6, cloned into the *Eco*RI and *Xho*I sites of pET28a	This study
pBE003	For expression of ZEB2Δ1-191 containing C-terminal His_6, cloned into the *Eco*RI and *Xho*I sites of pET28a	This study
pGZT001	pGH-T derivatives containing the ZEB2 3'UTR (*Hind*III-*Sac*I fragment of the *ZEB2* terminator, T_{ZEB2})	This study
pGZ01N	pGZT001 derivatives expressing ZEB2-HA. The *ZEB2* gene (including the *ZEB2L* promoter, the *ZEB2* coding sequence, and a HA linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 (P_{ZEB2L}-*ZEB2-HA*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ02N	pGZT001 derivatives expressing ZEB2L-HA. The *ZEB2* gene (including *ZEB2L* promoter, *ZEB2L* coding sequence containing the mutation of two internal start sites [M186L and M192L], and a HA linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 (P_{ZEB2L}-*ZEB2L-HA*-T_{ZEB2}-Hyg^R).	This study

	Ampicillin and hygromycin resistance.	
pGZ03N	pGZT001 derivatives expressing ZEB2S-Flag. The *ZEB2S* gene (including *ZEB2S* promoter, the *ZEB2S* coding sequence and a Flag linker) was cloned into *Apa*I and *Hind*III sites of pGZT001 (P_{ZEB2S}-*ZEB2S-Flag*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ003	pGZT001 derivatives overexpressing ZEB2L-HA. The *ZEB2* gene (including the elongation factor 1α promoter [$P_{EF1\alpha}$], the *ZEB2L* coding sequence containing the mutation of two internal start sites [M186L and M192L], and a HA linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2L-HA*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ004	pGZT001 derivatives overexpressing ZEB2L-Flag. The *ZEB2* gene (including $P_{EF1\alpha}$, the *ZEB2L* coding sequence containing the mutation of two internal start sites [M186L and M192L], and a Flag linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2L-Flag*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ005	pGZT001 derivatives overexpressing ZEB2S-Flag. The *ZEB2S* gene (including $P_{EF1\alpha}$, the *ZEB2S* coding sequence, and a Flag linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2S-Flag*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ006	pGZT001 derivatives overexpressing ZEB2S-Myc. The *ZEB2S* gene	This study

	(including $P_{EF1\alpha}$, the *ZEB2S* coding sequence, and a Myc linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2S-Myc*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	
pGZ007	pGZT001 derivatives overexpressing ZEB2LM-Flag. The *ZEB2LM* gene (including $P_{EF1\alpha}$, the *ZEB2LM* coding sequence [ZEB2LΔ338-363], and a Flag linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2LM-Flag*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ012	pGZT001 derivatives overexpressing ZEB2LM-HA. The *ZEB2LM* gene (including $P_{EF1\alpha}$, the *ZEB2LM* coding sequence [ZEB2LΔ338-363], and a HA linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2LM-HA*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ013	pGZT001 derivatives overexpressing ZEB2SM-Flag. The *ZEB2SM* gene (including $P_{EF1\alpha}$, the *ZEB2SM* coding sequence [ZEB2LΔ1-206], and a Flag linker) was cloned into the *Apa*I and *Hind*III sites of pGZT001 ($P_{EF1\alpha}$-*ZEB2SM-Flag*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ014	pGZT003 derivatives overexpressing ZEB2L-GFP. The *GFP* gene (including a glycine linker and the *GFP* reporter gene) was cloned into the *BamH*I and *Hind*III sites of pGZT003 ($P_{EF1\alpha}$-*ZEB2L-GFP*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ015	pGZT005 derivatives overexpressing ZEB2S-RFP. The *RFP* gene (including a	This study

	glycine linker and the *RFP* reporter gene) was cloned into the *BamH*I and *Hind*III sites of pGZT005 ($P_{EF1\alpha}$-*ZEB2S-RFP*-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	
pGZ018	pGZT003 derivatives overexpressing ZEB2L-YFP^N. The YFP^N gene (including a glycine linker and the N- terminal portion of *YFP* fused to *Myc*) was cloned into the *BamH*I and *Hind*III sites of pGZT003 ($P_{EF1\alpha}$-*ZEB2L-Myc-YFP*^{N}-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ019	pGZT003 derivatives overexpressing ZEB2L-YFP^C. The YFP^C gene (including a glycine linker and the C- terminal portion of *YFP* fused to *HA*) was cloned into the *BamH*I and *Hind*III sites of pGZT003 ($P_{EF1\alpha}$-*ZEB2L-HA-YFP*^{C}-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ020	pGZT005 derivatives overexpressing ZEB2S-YFP^N. The YFP^N gene (including a glycine linker and the N- terminal portion of *YFP* fused to *Myc*) was cloned into the *BamH*I and *Hind*III sites of pGZT005 ($P_{EF1\alpha}$-*ZEB2S-Myc-YFP*^{N}-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study
pGZ021	pGZT005 derivatives overexpressing ZEB2S-YFP^C. The YFP^C gene (including a glycine linker and the C-terminal portion of *YFP* fused to *HA*) was cloned into the *BamH*I and *Hind*III sites of pGZT005 ($P_{EF1\alpha}$-*ZEB2S-HA-YFP*^{C}-T_{ZEB2}-Hyg^R). Ampicillin and hygromycin resistance.	This study

pGZ022	pGZT007 derivatives overexpressing ZEB2LM-YFP^{C}. The *YFP^{C}* gene (including a glycine linker and the C-terminal portion of *YFP* fused to *HA*) was cloned into the *BamH*I and *Hind*III sites of pGZT007 ($P_{EF1\alpha}$-*ZEB2LM-HA-YFP^{C}*-T_{ZEB2}-*Hyg^{R}*). Ampicillin and hygromycin resistance.	This study

the SD-Leu-Trp plates was grown in liquid SD-Leu-Trp, subjected to four-fold serial dilutions, and spotted on selective plates. Empty vector and pDL20-Alg5 (-) were included as negative controls, and pAl-Alg5 (+) was included as a positive control.

VI. Immunoblotting and immunoprecipitation

ZEB2 variants fused to *HA*, *Flag*, or *Myc* were cloned into the *Spe*I-*Hind*III site in pGZT001. For the constitutive expression of the *ZEB2* gene in CM, the elongation factor 1α promoter ($P_{EF1\alpha}$) was amplified from pSKGEN (Lee *et al.*, 2011b) using the primers PEFp-5 *Apa*I and PEFp-3 *Spe*I; then, $P_{EF1\alpha}$ was inserted into each vector.

For the coimmunoprecipitation assay, fungal strains coexpressing different tagged ZEB2 variants were generated by outcrossing (Table 1). Filtered fungal mycelia cultures were homogenized to a fine powder under liquid nitrogen using a mortar and pestle. Powdered fungal cultures (50 mg) were sonicated in 250 μl of lysis buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5% glycerol; 1% NP-40; 1 mM EDTA; and protease inhibitors), boiled for 5 min in loading buffer, and subjected to SDS-PAGE analysis. After membrane transfer, the nitrocellulose membranes were immunoblotted with rabbit anti-HA (Abcam, Cambridge, UK, 1:4,000), mouse anti-Flag (Sigma-Aldrich, 1:4,000), and rabbit anti-β-tubulin (Abcam, 1:2,000) primary antibodies followed by horseradish peroxidase-linked secondary antibodies (GE Healthcare, Buckinghamshire, UK, 1:4,000). The target proteins were visualized using ECL Select^{TM} Western Blotting Detection Reagent

(GE Healthcare) according to the manufacturer's protocol.

For immunoprecipitation, the lysates were incubated in magnetic beads conjugated to a mouse anti-Flag antibody (Sigma-Aldrich) at 4 ℃ overnight and were washed five times with lysis buffer prior to resolution via SDS-PAGE analysis (12.5%). Western blot analysis was performed according to the techniques described above.

VII. Mycotoxin analyses

The ZEA extraction and quantification procedures were performed as previously documented (Kim *et al.*, 2005b). Briefly, a filtrate extracted with ethyl acetate was concentrated to dryness and dissolved in methanol-water (65:35, v/v) for HPLC analysis or in methanol for TLC analysis. A Shimadzu LC-10 AD HPLC equipped with an RF-10A fluorescence detector (Shimadzu, Kyoto, Japan) was used for the analysis. A Symmetry C_{18} column (15 mm × 4.6 mm; particle size, 5 μm; Waters Corp., Milford, MA, USA) was used at a detection wavelength of 274-466 nm. The mobile phase was 65% aqueous methanol, and the flow rate was 1.3 ml min^{-1}. The methanol aliquot of each sample was separated in the developing solvent, chloroform-methanol (9:1, v/v), on silica gel-coated TLC plates (Kiesel Gel 60, Merck, Darmstadt, Germany). Photographs were obtained after exposure to UV radiation (254-364 nm).

VIII. ZEB2 isoform subcellular localization

GFP and *RFP* were amplified from pIGPAPA and pLC25 using the primer

pairs PGFP-5 *BamH*I/PGFP-3 *Hind*III and PRFP-5 *BamH*I/PRFP-3 *Hind*III, respectively. The resulting PCR products and 6× glycine linkers were cloned into the corresponding enzyme sites of the pGZ003 and pGZ005 plasmids to generate pGZ014 and pGZ015, respectively. These plasmids were transformed into the *ZEB2* truncation mutants. The resulting transformants (FAZ017 and FAZ018) were outcrossed with mat1g or mat1r to generate FAZ22 and FAZ23, respectively, for the visualization of the nuclei using GFP or RFP (Son *et al.*, 2011a; Hong *et al.*, 2010). To assess the colocalization of ZEB2L-GFP and ZEB2S-RFP, the Δ*mat1* strain (Lee *et al.*, 2003) was fertilized with FAZ018 to generate FAZ018C. Next, the FAZ017 strain was outcrossed with FAZ018C to generate FAZ021. The heterothallic Δ*mat1* strain carrying a *MAT1-1-1* deletion was used as a tester strain for the outcross. Microscopic examinations were performed using a DE/Axio Imager A1 microscope (Carl Zeiss, Jena, Germany) equipped with a 38HE filter set (excitation 470/40; emission 525/50) for GFP and a 15 filter set (excitation 546/12; emission 590) for RFP.

IX. Bimolecular fluorescence complementation (BiFC) assay

YFP^N and YFP^C were amplified from pUC-SPYNE and pUC-SPYCE (Walter *et al.*, 2004) using the primer pairs PYFPNE-5 *BamH*I/PYFPNE-3 *Hind*III and PYFPCE-5 *BamH*I/PYFPCE-3 *Hind*III, respectively. The resulting PCR products and 6× glycine linkers were cloned into the *BamH*I-*Hind*III sites of pGZ003, pGZ005, and pGZ007. The resulting plasmids were transformed into the *ZEB2* truncation mutants. The normal expression of the ZEB2 fusion variants by

each transformant was confirmed by Western blot analysis. Various outcrosses were applied to generate strains coexpressing both ZEB2 fusion variants (Table S1). Microscopic examinations were performed using a DE/Axio Imager A1 microscope (Carl Zeiss) equipped with the 46 filter set (excitation 500/20; emission 535/30) for YFP.

X. Electrophoretic mobility shift assay (EMSA)

ZEB2L, *ZEB2S*, or *ZEB2* Δ1-191 cDNA was cloned into the pET28a vector (Novagen, Madison, WI, USA) and expressed as a His_6-fusion protein in the *E. coli* BL21-CodonPlus (DE3)-RIL strain (Stratagene, La Jolla, CA, USA) by adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM. Recombinant proteins were purified using a Ni Sepharose^{TM} 6 Fast Flow column (GE Healthcare). *PKS4*, *PKS13*, *ZEB1*, and *ZEB2* double-stranded DNA probes (2 pmol) were labeled using T4 polynucleotide kinase (Takara Korea Biomedical Inc., Seoul, Korea) and 20 μCi [γ-^{32}P]ATP (6,000 Ci mmol^{-1}, Perkin Elmer, Boston, MA, USA). EMSA binding reactions were performed in 15-μl volumes. In total, 15–120 nM of purified recombinant protein and labeled probes (0.04 pmol) were incubated for 20 min at room temperature in a buffer containing 10 mM Tris-HCl (pH 7.5), 4 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM $MgCl_2$, 0.54 mM EDTA, 8% (v/v) glycerol, 0.1 mM DTT, 40 μg of BSA, and 2 μg of poly(dI-dC). The reactions were electrophoresed on 4% polyacrylamide gels (acrylamide:Bis ratio of 29:1 w/w; Bio-Rad). The DNA complex bands were visualized with a BAS-2500 Bio-image Analyzer (Fujifilm, Tokyo, Japan).

For competitive binding experiments, 50-fold molar excess of nonlabeled DNA elements were included in the reaction solution prior to incubation in the purified proteins. The *β-tubulin* promoter region was used as a nonspecific competitor. DNA fragments containing identical nucleotide sequences to the probes were used as specific competitors. The *PKS4*, *PKS13*, and *ZEB1* promoters were incubated in 15 nM ZEB2 protein. The *ZEB2* promoters were incubated in 30 nM ZEB2 protein.

For competitive EMSA assays, the *ZEB2L* promoter-derived probe was incubated in 40 nM ZEB2L for 20 min, followed by the application of increasing concentrations of ZEB2S for an additional 20 min at room temperature. ZEB2Δ1-191 was used as a negative control for competitive EMSA. The complexes were visualized as described above.

XI. 2-D native/SDS-PAGE

Native separating gels were prepared using 5–15% acrylamide (acrylamide:Bis ratio of 37.5:1 w/w; Bio-Rad) in 0.375 M Tris-HCl (pH 8.8). The samples were electrophoresed at 25 mA at 4 ℃ in 1× gel buffer (10 mM Tris-glycine, pH 8.7). The channels to be used for SDS-PAGE were carefully sliced from the native gel; equilibrated for 15 min in 5 ml of a buffer containing 0.05 M Tris-HCl (pH 6.8), 2.3% SDS, 10% glycerol, and 5% β-mercaptoethanol; and heated in a microwave for 20 s. Electrophoresis was performed at 20 mA for approximately 2.5 h. The NativeMARK Unstained Protein Standard (Invitrogen) was used to determine the molecular weights of the proteins on native PAGE.

ZEB2 expression was detected by Western blot analysis with an anti-HA antibody.

XII. Size-exclusion chromatography

Fungal lysates were extracted from the FAZ012 (ZEB2L-HA/ZEB2L-Flag), FAZ006 (ZEB2L-HA/ZEB2S-Flag), and FAZ013 (ZEB2S-Myc/ZEB2S-Flag) strain CM cultures with lysis buffer; purified using an anti-Flag column; and dialyzed with FPLC running solution (0.1 M glycine, pH 3.5; 0.01% NP-40; 1 mM EDTA; and 5% glycerol). The oligomeric states of the purified fungal ZEB2 proteins were analyzed via AKTA FPLC using a Superdex^{TM} 200 10/300 GL column (GE Healthcare). The results were confirmed by Western blot analyses.

XIII. Induction of ZEB2 transcription by exogenous ZEA treatment

The wild-type strain Z-3639 and the ZEA-deficient mutants Δ*pks4* and Δ*pks13* were grown in 25 ml of SG medium supplemented with 0, 0.1, or 1 ppm ZEA. The mycelia of each strain after 0, 2, 4, 6, 8, 10, and 12 days of incubation were harvested by filtration through Whatman No. 2 filter paper. Northern blot and TLC analyses of each culture were performed as described above. DNA probes specific to the C-terminus of *ZEB2* (PZ577-5/PZ790-3 for probe 3) were used for Northern blot analyses.

XIV. Nucleotide sequence accession numbers

The *ZEB2L* and *ZEB2S* exon sequences were deposited in the GenBank

database under accession numbers KM382273 and KM382274, respectively.

RESULTS

I. Characterization of two transcripts of the *ZEB2* gene

Our previous study suggested that two distinct *ZEB2* transcripts are formed during ZEA production (Kim *et al.*, 2005b). I performed cDNA sequencing analyses of *ZEB2* using the *F. graminearum* cDNA library to confirm these results and found that *ZEB2* contains two introns and multiple alternative transcription start sites. The proteins translated from the longer 1.2-kb and shorter 0.7-kb *ZEB2* transcripts were designated ZEB2L and ZEB2S, respectively (Fig. 1 and Fig. 2A). 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.

Next, I performed Northern blot analyses with various probes derived from the upstream (probes 1 and 2) and downstream (probes 3 and 4) regions of the translation start site (+605) of the *ZEB2S* gene. The results verified the presence of the two *ZEB2* transcripts (Fig. 2B). The exogenous supplementation of ZEA with starch-glutamate (SG) liquid medium enhanced the transcription of the *ZEB2* gene, as previously reported (Lee *et al.*, 2011a). During ZEA biosynthesis, the expression of *ZEB2L* transcripts was detected at 3 days of incubation, increased up to 6 days, and disappeared by 10 days. In contrast, *ZEB2S* transcripts were detected after 5 days of incubation and remained until 10 days (Fig. 3).

I hypothesized that *ZEB2* gene expression is regulated by alternative promoters based on the presence of multiple transcription start sites and an N-terminally truncated isoform. Therefore, I examined whether the putative promoter regions of the ZEB2 isoforms are functional. Fungal transformants (FAZ03N)

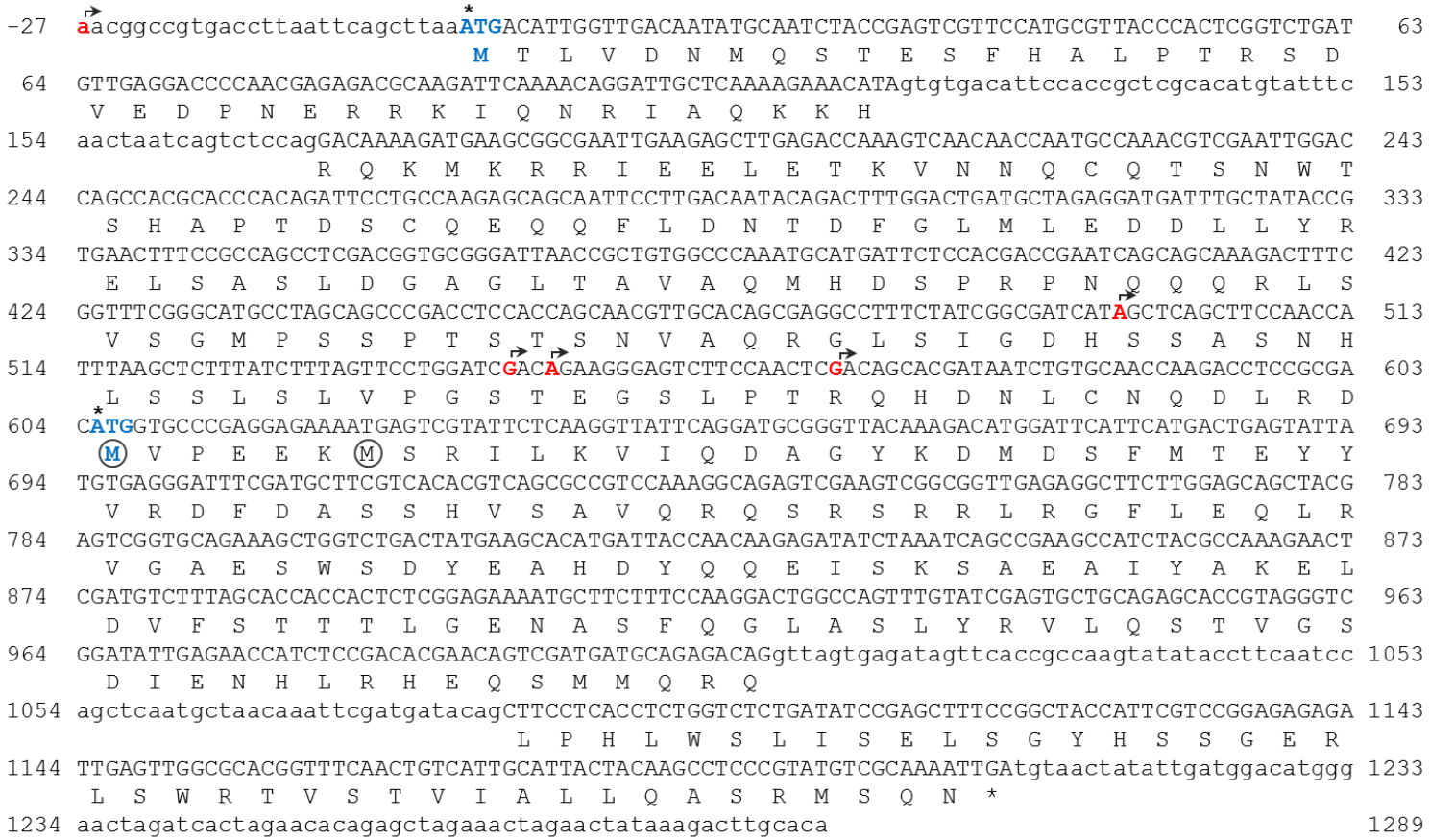
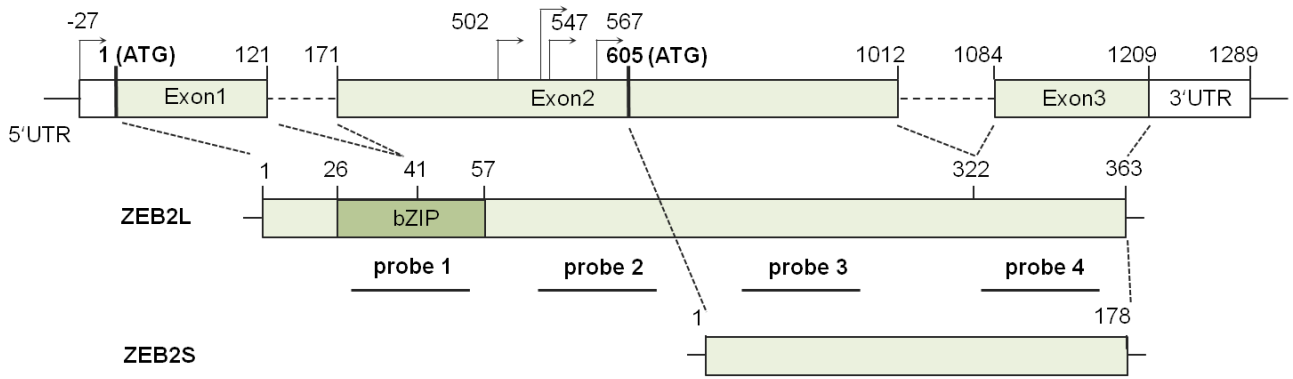

-27 aacggccgtgaccttaattcagcttaaATGACATTGGTTGACAATATGCAATCTACCGAGTCGTTCCATGCGTTACCCACTCGGTCTGAT 63
M T L V D N M Q S T E S F H A L P T R S D
64 GTTGAGGACCCCAACGAGAGACGCAAGATTCAAAACAGGATTGCTCAAAAGAAACATAgtgtgacattccaccgctcgcacatgtatttc 153
V E D P N E R R K I Q N R I A Q K K H
154 aactaatcagtctccagGACAAAAGATGAAGCGGCGAATTGAAGAGCTTGAGACCAAAGTCAACAACCAATGCCAAACGTCGAATTGGAC 243
R Q K M K R R I E E L E T K V N N Q C Q T S N W T
244 CAGCCACGCACCCACAGATTCCTGCCAAGAGCAGCAATTCCTTGACAATACAGACTTTGGACTGATGCTAGAGGATGATTTGCTATACCG 333
S H A P T D S C Q E Q Q F L D N T D F G L M L E D D L L Y R
334 TGAACTTTCCGCCAGCCTCGACGGTGCGGGATTAACCGCTGTGGCCCAAATGCATGATTCTCCACGACCGAATCAGCAGCAAAGACTTTC 423
E L S A S L D G A G L T A V A Q M H D S P R P N Q Q Q R L S
424 GGTTTCGGGCATGCCTAGCAGCCCGACCTCCACCAGCAACGTTGCACAGCGAGGCCTTTCTATCGGCGATCATAGCTCAGCTTCCAACCA 513
V S G M P S S P T S T S N V A Q R G L S I G D H S S A S N H
514 TTTAAGCTCTTTATCTTTAGTTCCTGGATCGACAGAAGGGAGTCTTCCAACTCGACAGCACGATAATCTGTGCAACCAAGACCTCCGCGA 603
L S S L S L V P G S T E G S L P T R Q H D N L C N Q D L R D
604 CATGGTGCCCGAGGAGAAAATGAGTCGTATTCTCAAGGTTATTCAGGATGCGGGTTACAAAGACATGGATTCATTCATGACTGAGTATTA 693
M V P E E K M S R I L K V I Q D A G Y K D M D S F M T E Y Y
694 TGTGAGGGATTTCGATGCTTCGTCACACGTCAGCGCCGTCCAAAGGCAGAGTCGAAGTCGGCGGTTGAGAGGCTTCTTGGAGCAGCTACG 783
V R D F D A S S H V S A V Q R Q S R S R R L R G F L E Q L R
784 AGTCGGTGCAGAAAGCTGGTCTGACTATGAAGCACATGATTACCAACAAGAGATATCTAAATCAGCCGAAGCCATCTACGCCAAAGAACT 873
V G A E S W S D Y E A H D Y Q Q E I S K S A E A I Y A K E L
874 CGATGTCTTTAGCACCACCACTCTCGGAGAAAATGCTTCTTTCCAAGGACTGGCCAGTTTGTATCGAGTGCTGCAGAGCACCGTAGGGTC 963
D V F S T T T L G E N A S F Q G L A S L Y R V L Q S T V G S
964 GGATATTGAGAACCATCTCCGACACGAACAGTCGATGATGCAGAGACAGgttagtgagatagttcaccgccaagtatataccttcaatcc 1053
D I E N H L R H E Q S M M Q R Q
1054 agctcaatgctaacaaattcgatgatacagCTTCCTCACCTCTGGTCTCTGATATCCGAGCTTTCCGGCTACCATTCGTCCGGAGAGAGA 1143
L P H L W S L I S E L S G Y H S S G E R
1144 TTGAGTTGGCGCACGGTTTCAACTGTCATTGCATTACTACAAGCCTCCCGTATGTCGCAAAATTGAtgtaactatattgatggacatggg 1233
L S W R T V S T V I A L L Q A S R M S Q N *
1234 aactagatcactagaacacagagctagaaactagaactataaagacttgcaca 1289

Fig. 1. ZEB2 nucleotide and deduced amino acid sequences. The nucleotide positions are provided relative to the first translational start site of *ZEB2L*. The blue letters with asterisks indicate two different translation start sites. The red letters with bent arrows indicate the transcription start sites. The capital letters below the nucleotide sequences represent the amino acid sequences. The two "M"s in circles indicate the start codon sites that were point-mutated to leucine in the *ZEB2L* constructs (M186L and M192L).

A


-27
1 (ATG)
121
171
502
547
567
605 (ATG)
1012
1084
1209
1289
Exon1
Exon2
Exon3
3'UTR
5'UTR
1
26
41
57
322
363
ZEB2L
bZIP
probe 1
probe 2
probe 3
probe 4
1
178
ZEB2S

B

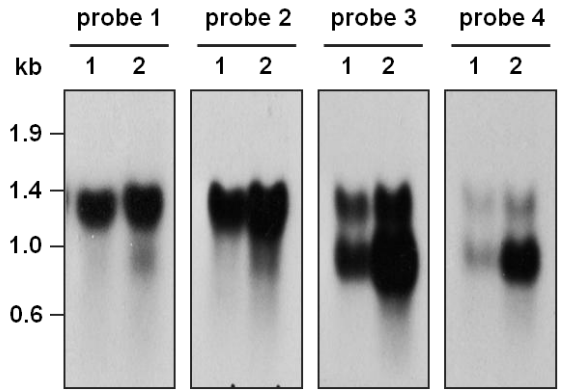

probe 1
probe 2
probe 3
probe 4
kb
1 2
1 2
1 2
1 2
1.9
1.4
1.0
0.6

Fig. 2. *ZEB2* gene expression is mediated by alternative promoter usage.

A. Schematic representation of the *ZEB2* gene and protein structures. *ZEB2* comprises two introns (dashed lines) and multiple transcription initiation sites (bent arrows). The resulting transcripts encode two proteins: ZEB2L and ZEB2S. Only ZEB2L contains a bZIP DNA-binding domain. Each Northern blot analysis probe used in B is illustrated as a black bar corresponding to the cDNA region.

B. Verification of the two *ZEB2* transcripts by Northern blot analysis. Total RNA was extracted from 6-day-old wild-type cultures in SG medium lacking (lane 1 for each blot) or containing 1 ppm ZEA (lane 2 for each blot). The utilized probe is indicated above each blot.

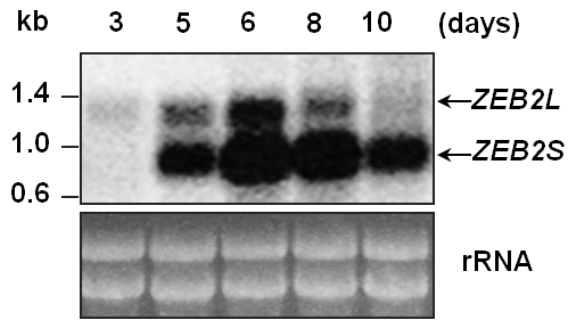


Fig. 3. *ZEB2* transcript profile throughout ZEA biosynthesis. Total RNA was extracted from cultures grown in SG medium for 3-10 days. Ethidium bromide-stained rRNA was included as a loading control.

carrying intact *ZEB2* open reading frame (ORF) genomic fragments under the control of the putative *ZEB2L* promoter region (P_{ZEB2L}) expressed both ZEB2L (~45 kDa) and ZEB2S (~23 kDa) (Fig. 4A). When the two internal start codons were substituted with leucine, the resulting strain (FAZ003) only produced the ZEB2L isoform (Fig. 4B). The region between positions –27 and +604 (designated the *ZEB2S* promoter [P_{ZEB2S}]) served as a regulatory element that enabled the expression of ZEB2S, suggesting that P_{ZEB2S} is a functional alternative promoter (Fig. 4C).

II. Identification of ZEB2 interaction partners *in vitro* and *in vivo*

I used a yeast two-hybrid (Y2H) system to screen for proteins associated with ZEB2L because bZIP TFs typically function as dimers. In a screen of 1×10^7 yeast cells cotransformed with a bait vector expressing ZEB2L and a *F. graminearum* cDNA library, the only positive clone possessed the ZEB2S-encoding sequence. Additional Y2H analyses revealed that both ZEB2 isoforms strongly interact with each other (Fig. 5). I mapped the ZEB2L- and ZEB2S-interaction domains by generating several deletion mutants (Fig. 5 and Fig. 6). Two interaction domains, ID1 (173-206) and ID2 (338-363), were found to be required for ZEB2L-ZEB2L, ZEB2L-ZEB2S, and ZEB2S-ZEB2S dimerization. In ZEB2L, the N-terminal region (173-191) of ID1 was important for these interactions; however, its C-terminal region (186-206) in ZEB2S was necessary for these interactions (see data concerning the ZEB2L-truncated forms Δ173-185 and Δ173-191 in Fig. 6). ID2 in both ZEB2 isoforms was important for their dimerization.

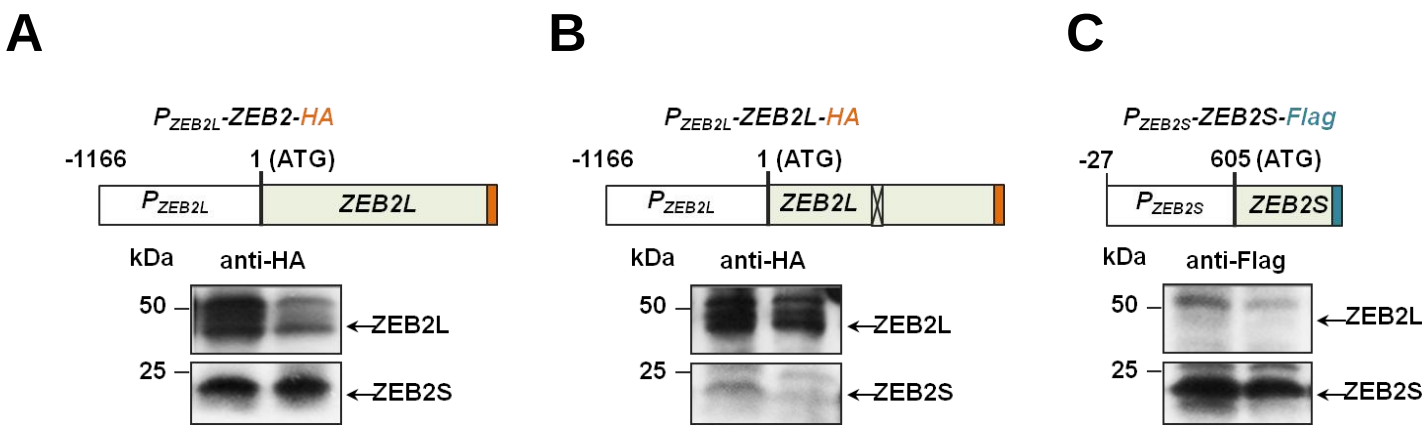


Fig. 4. ZEB2 isoform expression driven by the native promoters. Western blot analyses with anti-HA and anti-Flag antibodies were performed to verify the production of the corresponding proteins. An illustration of the transformed fungal construct used is included above each blot. In the P_{ZEB2L}-*ZEB2L-HA* construct, the internal start codons were mutated to leucine-encoding CTG codons (M186L and M192L). P_{ZEB2L} (-1166 to -1) and P_{ZEB2S} (-27 to +604) were the functional promoters for *ZEB2L* and *ZEB2S*, respectively. The molecular weight standards for RNA (in kb) and protein (in kDa) are indicated to the left of each blot.

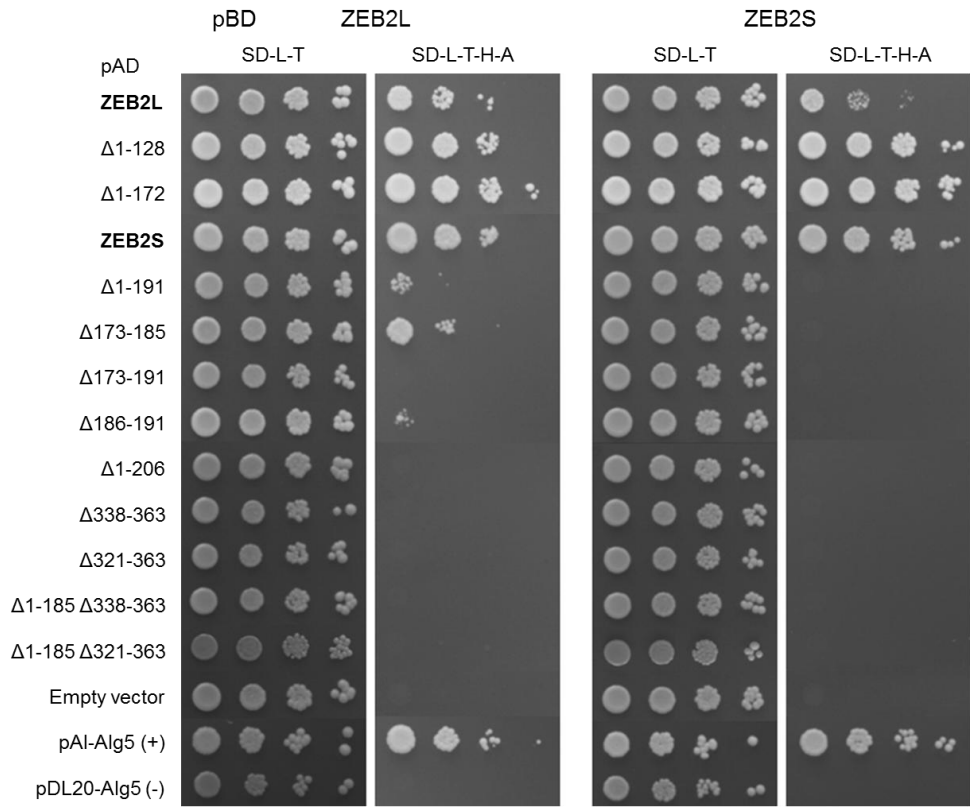


Fig. 5. Yeast coexpression assays. A series of *ZEB2L*-deleted derivatives was used for the Y2H assays. Cell growth on selective medium without Leu, Trp, His, or Ade (SD-L-T-H-A) indicates positive interactions. Each strain was spotted in four-fold serial dilutions. pDHB1 was used as the bait vector (pBD), and pPR3-N was used as the prey vector (pAD). Empty vector and pDL20-Alg5 (-) were included as negative controls, and pAl-Alg5 (+) was included as a positive control.

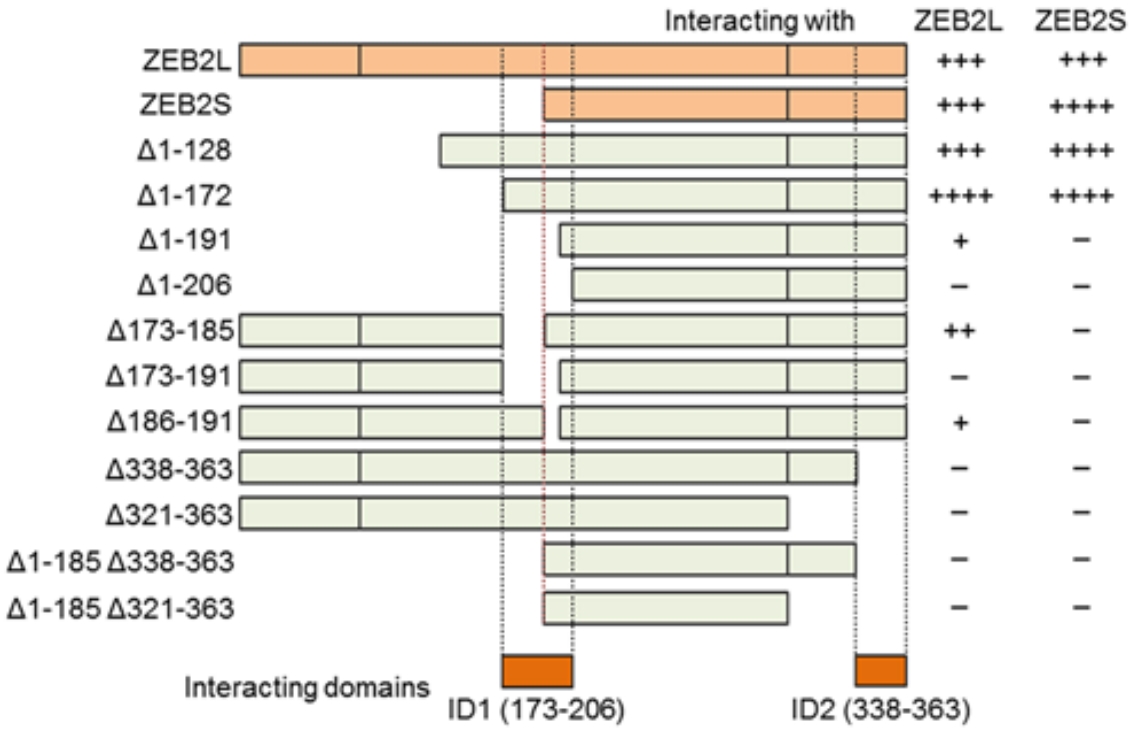
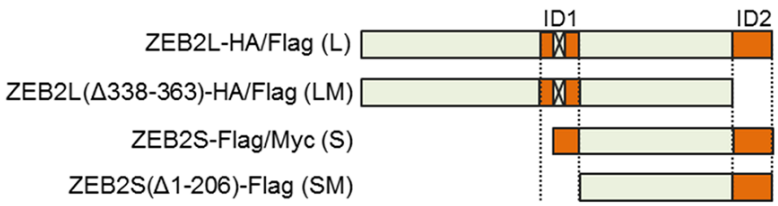

Interacting with
ZEB2L
ZEB2S
ZEB2L
+++
+++
ZEB2S
+++
++++
Δ1-128
+++
++++
Δ1-172
++++
++++
Δ1-191
+
–
Δ1-206
–
–
Δ173-185
++
–
Δ173-191
–
–
Δ186-191
+
–
Δ338-363
–
–
Δ321-363
–
–
Δ1-185 Δ338-363
–
–
Δ1-185 Δ321-363
–
–
Interacting domains
ID1 (173-206)
ID2 (338-363)

Fig. 6. Map of ZEB2-interacting domains. A series of ZEB2L truncation constructs was used in Y2H assays. ID1 and ID2 indicate the locations of the protein-protein interaction domains. Interactions were scored according to the number of positive clones (NPC) on the selection plates (-, no growth or NPC < 10; +, 10 < NPC < 10^2; ++, 10^2 < NPC < 10^3; +++, 10^3 < NPC < 10^4; ++++, NPC > 10^4).

To confirm that the ID1 and ID2 regions are critical for protein-protein interactions among ZEB2 isoforms *in vivo*, I generated *F. graminearum* strains coexpressing different ZEB2 variants tagged with HA, Flag, or Myc via fungal transformation and outcrossing (Table 1 and Fig. 7A). Using protein extracts from those strains, I found that ZEB2L and ZEB2S coimmunoprecipitated, supporting the findings from the Y2H analyses (Fig. 7B). As predicted, no interactions were detected between the ZEB2 isoforms and the ZEB2-truncated derivatives that lacked an interaction domain (LM and SM, shown in Fig. 7A).

To investigate the subcellular localization of the ZEB2 isoforms, I generated *F. graminearum* strains overexpressing the ZEB2 isoforms fused to green or red fluorescent protein (GFP or RFP, respectively). The ZEB2L-GFP fusion proteins localized exclusively to the nucleus, whereas the ZEB2S-RFP fusion proteins localized to both the cytosol and the nucleus (Fig. 8). To analyze the interactions between these proteins *in vivo*, bimolecular fluorescence complementation (BiFC) analysis was employed. Multiple outcrosses were utilized to generate *F. graminearum* strains possessing ZEB2 fusion proteins containing both halves of YFP (Table 1). Each transformant expressed ZEB2 proteins fused to the N or C terminus of YFP (YFP^N or YFP^C, respectively), as determined by Western blot analyses (Fig. 9). The BiFC analyses revealed interactions between ZEB2L and ZEB2L, as well as between ZEB2L and ZEB2S, in the nucleus (Fig. 10). In accordance with the subcellular localization of ZEB2S, the ZEB2S-ZEB2S interaction was detected in both the cytosol and the nucleus. Strains expressing ZEB2-truncated derivatives lacking an interaction domain did not show any detectable signal. Overall, these results suggested that these ZEB2 isoforms form

A


ID1
ID2
ZEB2L-HA/Flag (L)
ZEB2L(Δ338-363)-HA/Flag (LM)
ZEB2S-Flag/Myc (S)
ZEB2S(Δ1-206)-Flag (SM)

B

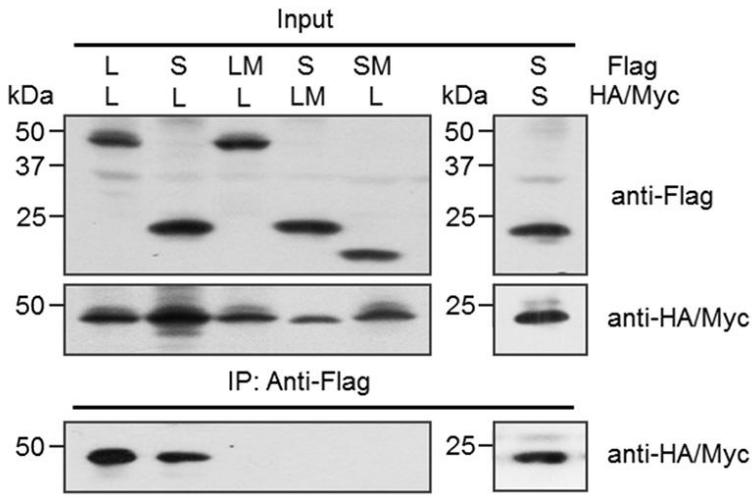

Input
L S LM S SM
kDa L L L LM L
50
37
25
50
kDa S
S
Flag
HA/Myc
50
37
25
anti-Flag
50
25
anti-HA/Myc
IP: Anti-Flag
50
25
anti-HA/Myc

Fig. 7. Identification of ZEB2-interacting proteins.

A. Schematic representation of the ZEB2 proteins used in the immunoprecipitation assays. The two internal start codons of the "L" and "LM" constructs were mutated to leucine-encoding CTG codons (M186L and M192L). Each ZEB2 recombinant was tagged with HA, Flag, or Myc. The interacting domains (ID1 and ID2) are indicated.

B. ZEB2 immunoprecipitation assay. Each transformed strain lysate carrying the corresponding ZEB2 protein was analyzed by immunoprecipitation using the anti-Flag antibody, followed by immunoblotting with the anti-HA or anti-Myc antibody. The protein molecular weight standards (in kDa) are indicated on the left of each blot.

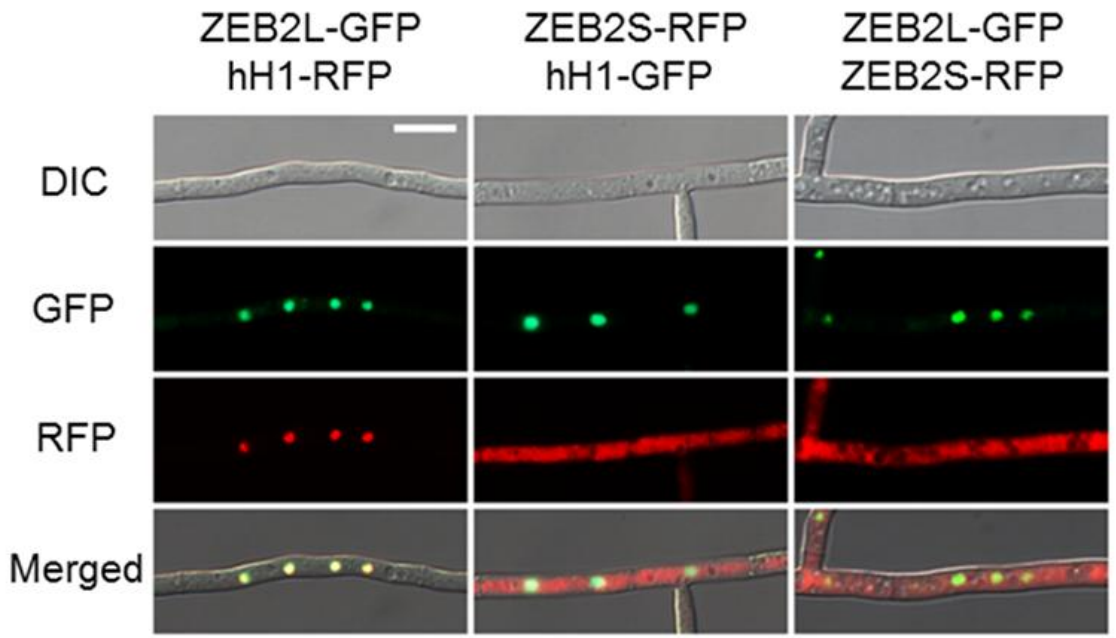


Fig. 8. Subcellular localization of ZEB2 isoforms. ZEB2 isoforms and histone H1 were fused to GFP or RFP. Scale bars: 10 μm.

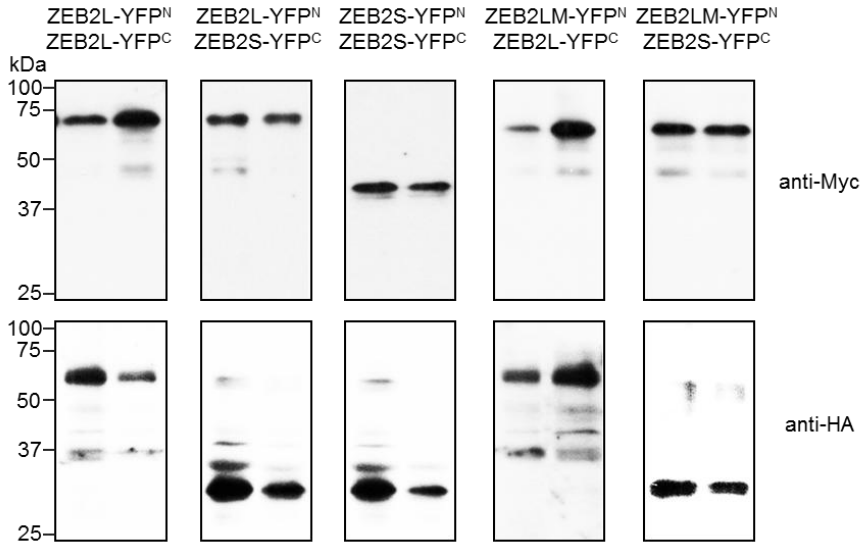


Fig. 9. Verification of ZEB2 fusion protein expression in the transformants used for BiFC. ZEB2 proteins fused to the N- or C-terminus of YFP (YFP^N or YFP^C) were immunoblotted using anti-Myc and anti-HA antibodies, respectively. The protein molecular weight standards (in kDa) are indicated on the left of the blot.

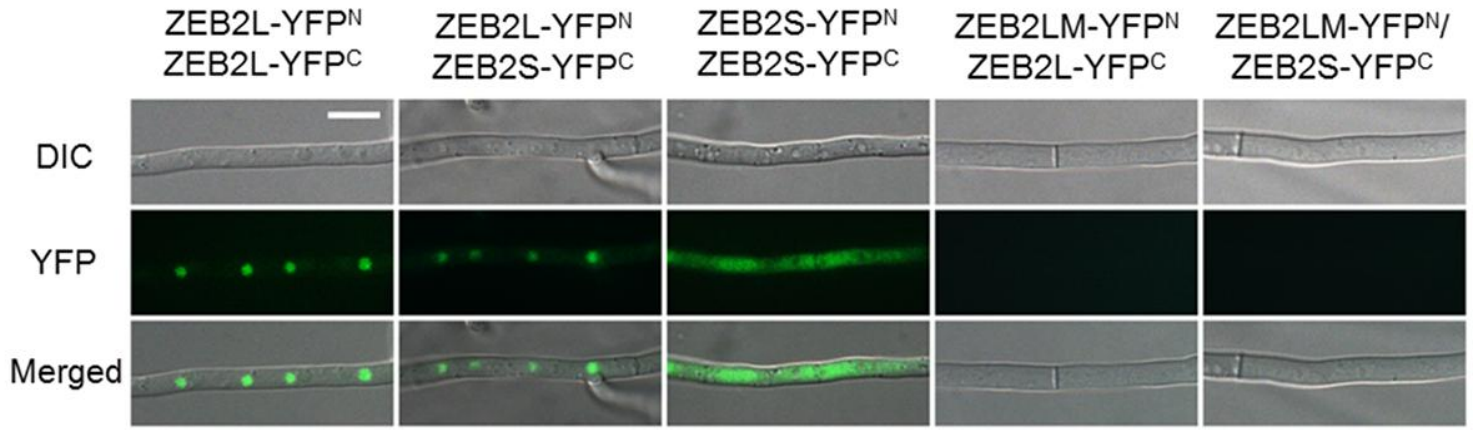


Fig. 10. BiFC visualization of ZEB2 dimerization in *F. graminearum*. YFP fluorescence, DIC, and merged images are shown for each transformation. Scale bars: 10 μm.

ZEB2L-ZEB2L, ZEB2L-ZEB2S, and ZEB2S-ZEB2S complexes.

III. ZEB2 isoforms bind to ZEA biosynthetic cluster gene promoters, and ZEB2L expression promotes ZEA biosynthesis

Using an electrophoretic mobility shift assay (EMSA) approach, I assessed whether ZEB2L directly binds to the promoter regions of *PKS4*, *PKS13*, *ZEB1*, and *ZEB2*. The putative promoter regions of these biosynthetic cluster genes were subdivided into several DNA fragments and were tested (Fig. 11). Purified recombinant ZEB2L, ZEB2S, and ZEB2Δ1-191 proteins were used for this assay (Fig. 12). ZEB2L formed shifted complexes with the *PKS4*, *PKS13*, *ZEB1*, and *ZEB2L* promoters, but it did not interact with the *ZEB2S* promoter (P_{ZEB2S}) under these conditions. ZEB2S did not bind to any of the tested promoter regions (Fig. 13). These results confirmed that ZEB2L directly binds to the putative promoters of ZEA biosynthetic cluster genes. Moreover, the results suggested that ZEB2L forms more stable complexes with the *PKS4*, *PKS13*, and *ZEB1* promoters than with the *ZEB2L* promoter *in vitro*, as smeared signals often appear when protein-DNA complexes are unstable. Next, I used competitive EMSA to examine the effect of the ZEB2L-ZEB2S interaction on ZEB2L DNA-binding activity (Fig. 14). The mobility shift caused by incubating ZEB2L in the presence of the *ZEB2L* promoter markedly decreased as the ZEB2S concentration increased, suggesting that ZEB2S inhibits the DNA-binding activity of ZEB2L on the *ZEB2L* promoter. When ZEB2Δ1-191, the truncated form of ZEB2S, was used as a nonspecific negative control, the shifted signal bands were not altered at the examined concentrations of

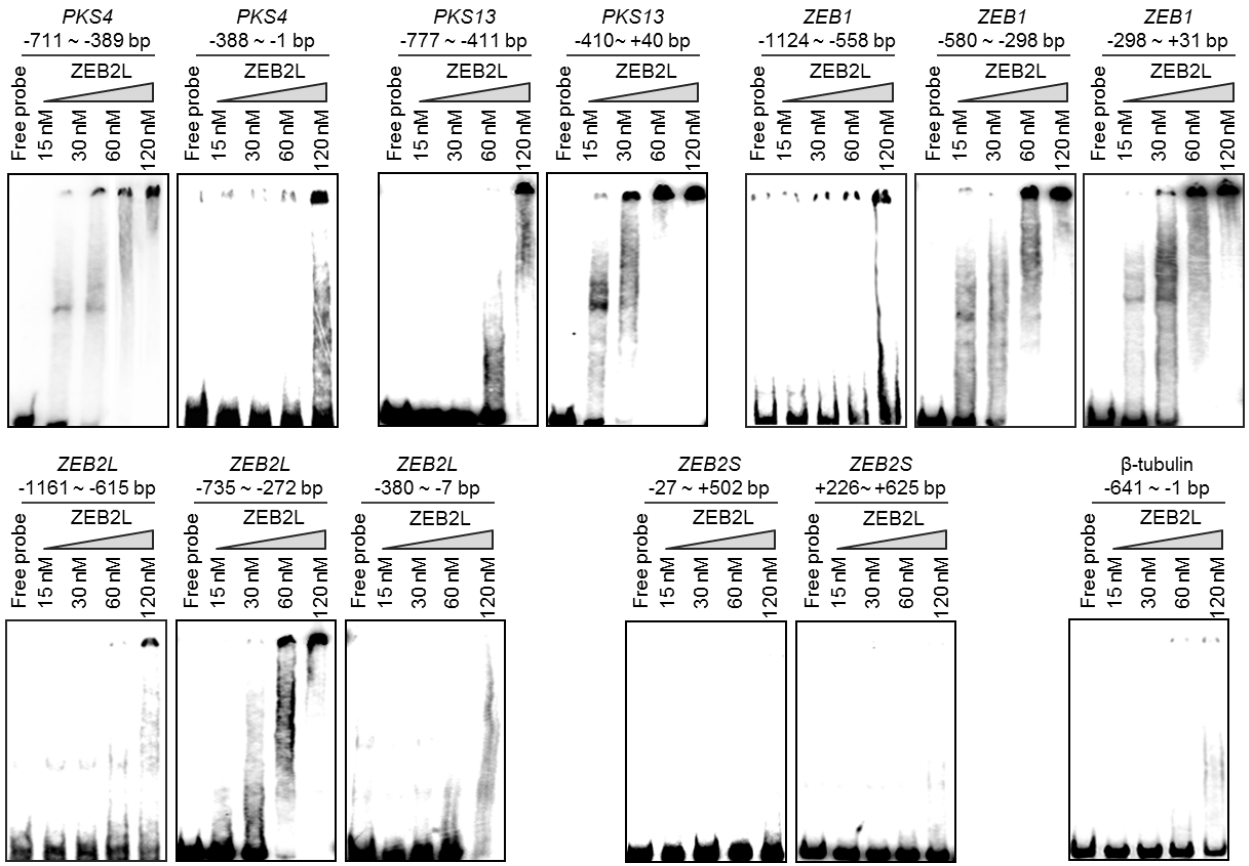
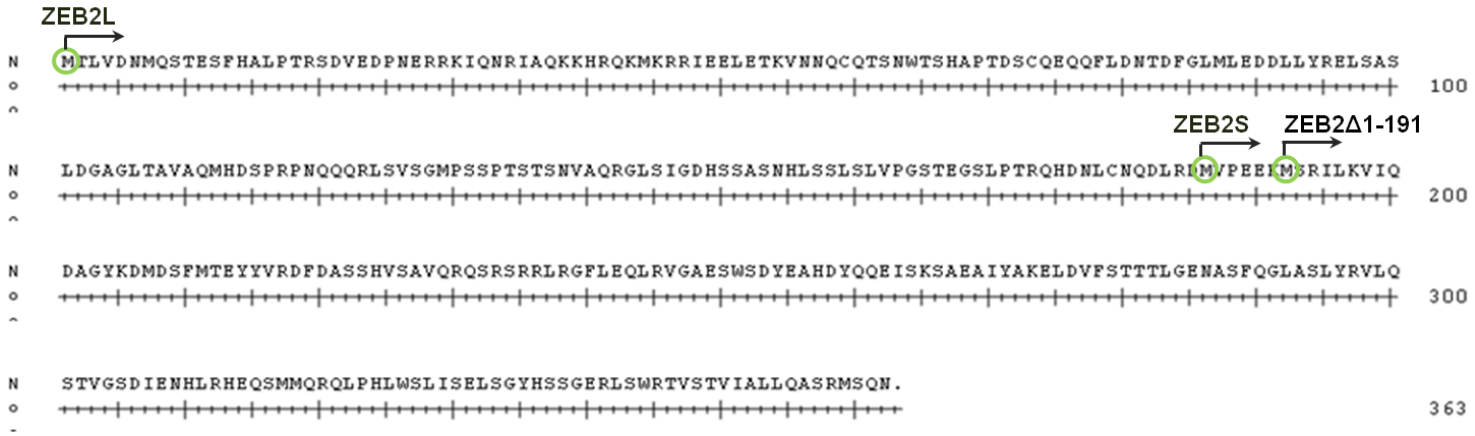

PKS4
-711 ~ -389 bp
PKS4
-388 ~ -1 bp
PKS13
-777 ~ -411 bp
PKS13
-410~ +40 bp
ZEB1
-1124 ~ -558 bp
ZEB1
-580 ~ -298 bp
ZEB1
-298 ~ +31 bp
ZEB2L
-1161 ~ -615 bp
ZEB2L
-735 ~ -272 bp
ZEB2L
-380 ~ -7 bp
ZEB2S
-27 ~ +502 bp
ZEB2S
+226~ +625 bp
β-tubulin
-641 ~ -1 bp
ZEB2L
Free probe
15 nM
30 nM
60 nM
120 nM

Fig. 11. EMSA analyses to identify ZEB2L DNA-binding regions. EMSA analyses of ^{32}P-labelled *PKS4*, *PKS13*, *ZEB1*, and *ZEB2* putative promoter fragments were conducted using increasing concentrations of purified ZEB2L. The gene name and the promoter region are indicated above each blot. *β-tubulin* gene promoter fragments were used as negative controls.


ZEB2L
ZEB2S
ZEB2Δ1-191
MTLVDNMQSTESFHALPTRSDVEDPNERRKIQNRIAQKKHRQKMKRRIEELETKVNNQCQTSNWTSHAPTDSCQEQQFLDNTDFGLMLEDDLLYRELSAS
100
LDGAGLTAVAQMHDSPRPNQQQRLSVSGMPSSPTSTSNVAQRGLSIGDHSSASNHLSSLSLVPGSTEGSLPTRQHDNLCNQDLRMVPEEMGRILKVIQ
200
DAGYKDMDSFMTEYYVRDFDASSHVSAVQRQSRSRRLRGFLEQLRVGAESWSDYEAHDYQQEISKSAEAIYAKELDVFSTTTLGENASFQGLASLYRVLQ
300
STVGSDIENHLRHEQSMMQRQLPHLWSLISELSGYHSSGERLSWRTVSTVIALLQASRMSQN.
363

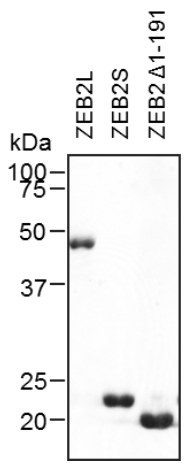

ZEB2L
ZEB2S
ZEB2 Δ1-191
kDa
100
75
50
37
25
20

Fig. 12. Purified recombinant ZEB2 proteins. Purified proteins were analyzed via 12.5% SDS-PAGE. ZEB2Δ1-191 represents the truncated isoform of ZEB2S. The protein molecular weight standards (in kDa) are indicated to the left of the blot.

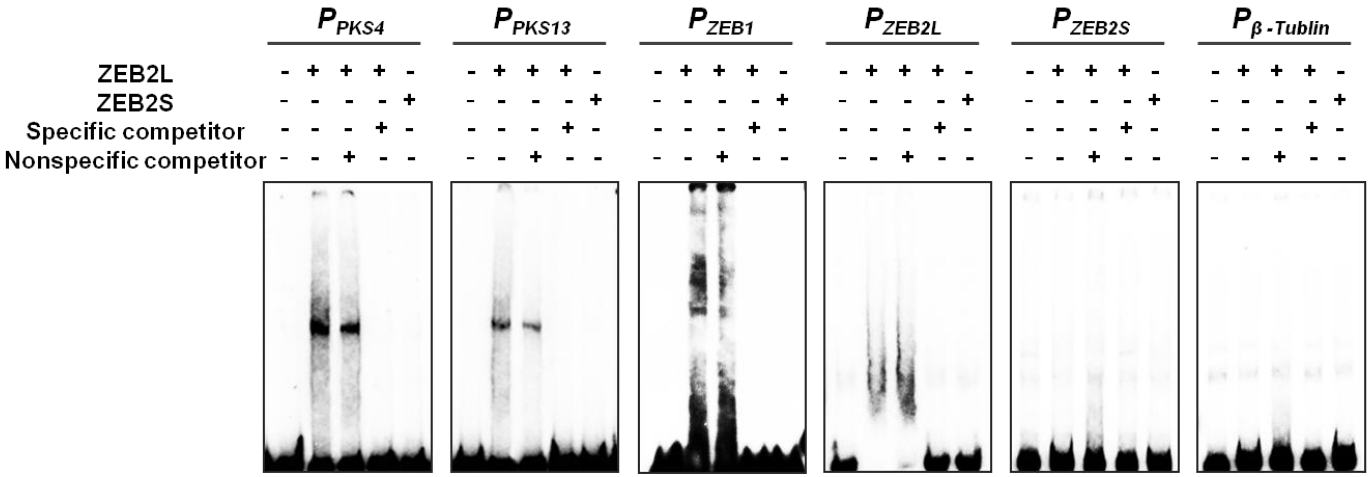


Fig. 13. EMSA analyses of ZEB2 isoforms and ZEA biosynthetic cluster gene promoters. Where indicated, a 50-fold excess of non-radiolabeled promoter fragments and *β-tubulin* gene promoter fragments were added as specific and nonspecific competitors, respectively.

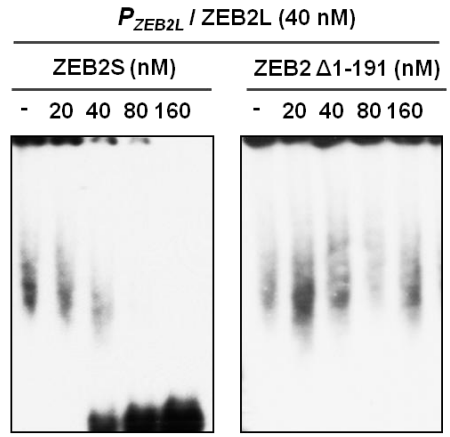


Fig. 14. Effect of ZEB2S on the DNA-binding activity of ZEB2L. ZEB2 isoform mixtures at specific concentrations were used for competitive EMSA analyses with the *ZEB2L* promoter fragments. ZEB2Δ1-191 protein was added to the competitive EMSA reaction with ZEB2L as a negative control.

ZEB2Δ1-191.

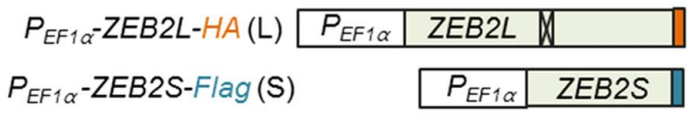
To determine the roles of each ZEB2 isoform in ZEA biosynthesis, I produced strains overexpressing ZEB2L (FAZ001), ZEB2S (FAZ002), or both isoforms (FAZ006). The expression of the ZEB2 isoform in each strain was verified by Western blot analysis and quantitative real-time PCR (qRT-PCR). ZEA production was tested using thin-layer chromatography (TLC). The FAZ001 strain produced ZEA (L in Fig. 15), whereas the FAZ002 strain did not produce ZEA (S in Fig. 15). No ZEA production was detected in the strain (FAZ006) that coexpressed ZEB2L and ZEB2S (L+S in Fig. 15). These results are representative of three independent transformants for each construct (Fig. 16).

Transcripts of ZEA biosynthetic cluster genes (*PKS4*, *PKS13*, and *ZEB1*) were detectable by qRT-PCR only in the strains expressing ZEB2L alone (Fig. 17). Taken together, these results suggested that ZEB2L is an activator and that ZEB2S is an inhibitor of ZEA biosynthesis.

IV. ZEB2 isoform profiles during ZEA production

To analyze the correlation between ZEB2 isoform accumulation and ZEA production, I examined the levels of ZEA produced by a fungal strain (FAZ03N) carrying HA-tagged intact ZEB2 proteins under the control of the native *ZEB2* promoter in SG liquid medium (Fig. 18). ZEA was initially detected at 2 days of incubation (21 ng ml^{-1}). The ZEA level increased until day 10 (598 ng ml^{-1}), and the graph of ZEA production over time exhibited a sigmoidal shape. The same tendency was shown between the HA-tagged fungal strain (FAZ03N) and the wild-

A


$P_{EF1\alpha}$-ZEB2L-HA (L)
$P_{EF1\alpha}$
ZEB2L
$P_{EF1\alpha}$-ZEB2S-Flag (S)
$P_{EF1\alpha}$
ZEB2S

B

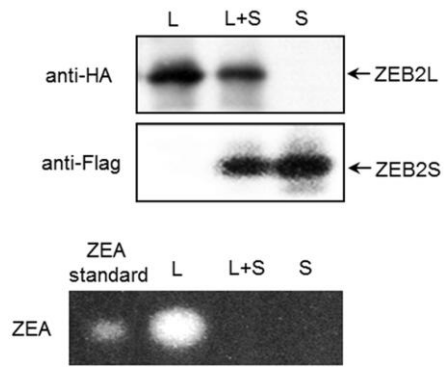

L
L+S
S
anti-HA
ZEB2L
anti-Flag
ZEB2S
ZEA standard
L
L+S
S
ZEA

Fig. 15. ZEB2 isoform coexpression inhibits ZEA production.

A. Expression of ZEB2 isoforms. Western blot analyses with anti-HA and anti-Flag antibodies were performed to verify the production of the corresponding proteins. An illustration of the construct used for fungal transformation is located above the blots. In the $P_{EF1\alpha}$-*ZEB2L-HA* construct, the internal start codons were mutated to leucine-encoding CTG codons (M186L and M192L).

B. ZEA production. Each construct expressed by the corresponding fungal strain is indicated above each blot. ZEA production was detected by TLC analysis. The results are representative of three independent transformants for each construct.

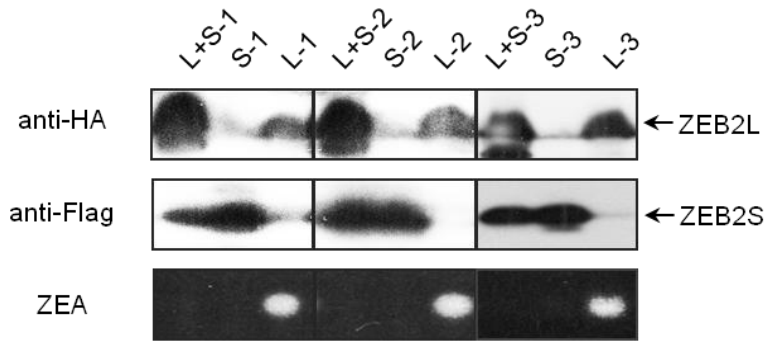


Fig. 16. Expression of ZEB2 isoforms and ZEA production. L, S, and L+S indicate *F. graminearum* strains overexpressing ZEB2L (FAZ001), ZEB2S (FAZ002), or both ZEB2 isoforms (FAZ006), respectively. Western blot analyses using anti-HA and anti-Flag antibodies were performed to verify the production of the corresponding proteins. ZEA production was detected by TLC analysis. Three independent transformants for each construct showed the identical results of ZEA production.

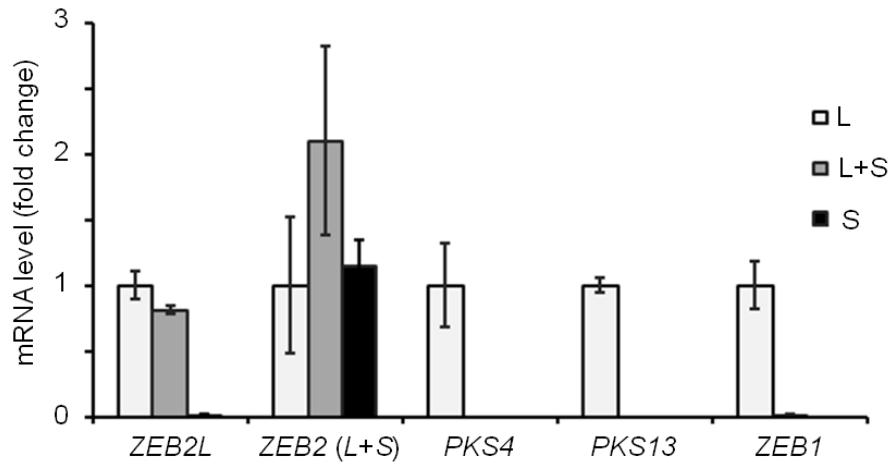


Fig. 17. Expression of ZEA biosynthetic cluster genes in *F. graminearum* strains overexpressing ZEB2L, ZEB2S, or both ZEB2 isoforms. The transcript levels of *ZEB2L*, *ZEB2* (*L+S*), *PKS4*, *PKS13*, and *ZEB1* were analyzed by qRT-PCR in the FAZ001 (L), FAZ006 (L+S), and FAZ002 (S) strains. The expression of the transcripts was normalized to that of the housekeeping gene *CYP1*. The transcript levels in the fungal strain overexpressing only ZEB2L were arbitrarily normalized to 1 unit.

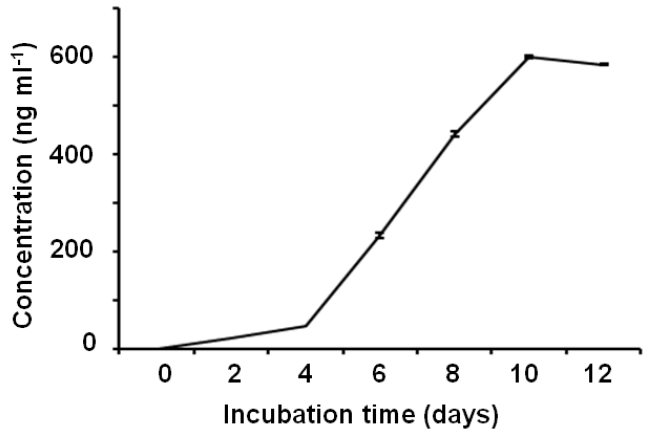


Fig. 18. ZEA production by the fungal strain (FAZ03N). The time course of ZEA production was monitored by HPLC analysis.

type strain (Z-3639) (Fig. 19). Next, I performed Western blot analyses to profile the ZEB2 isoforms present during ZEA production (Fig. 20). The ZEB2L levels decreased as ZEB2S appeared at 6 and 8 days of incubation, and ZEB2L was barely detectable by day 10. In contrast, ZEB2S was not detected until 5 days of incubation, when its levels were low. Then, the ZEB2S levels were increased at 6, 8, and 10 days. These results suggested that ZEA production is initiated by ZEB2L but is terminated by high levels of ZEB2S, which exerts a negative effect on ZEA biosynthesis by reducing the ZEB2L protein levels.

V. Oligomeric states of ZEB2 isoforms

To investigate the exact oligomeric states of ZEB2 isoforms during ZEA production, I utilized a two-dimensional (2-D) electrophoresis system involving native PAGE followed by SDS-PAGE. When ZEB2L alone accumulated in the early stage (3 days of incubation) of ZEA production (Fig. 20), ZEB2L existed either as oligomers (possibly homotetramers [~180 kDa]) or monomers (~45 kDa) (Fig. 21). During the middle stage of ZEA production (from days 6 to 8), ZEB2L formed heterodimers with ZEB2S (~70 kDa). ZEB2S homodimers (~45 kDa) were detected after day 8. During the late stage of ZEA production, only ZEB2S homodimers (~45 kDa) accumulated to detectable levels.

I also performed size-exclusion chromatography analyses using strains that expressed both ZEB2 isoforms (L+S, FAZ006) or ZEB2S alone (S, FAZ013). The elution peaks of the lysates from these two strains were slightly shifted to approximately the 66-kDa marker (Fig. 22A). ZEB2L-ZEB2S heterodimers were

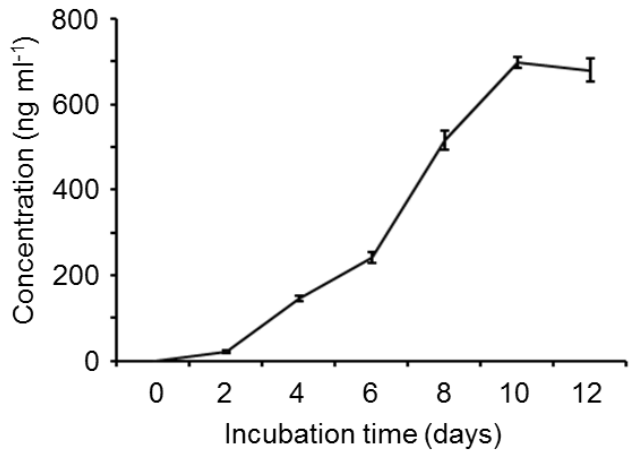


Fig. 19. ZEA production in wild-type strains. The time course of ZEA production was monitored via HPLC analysis.

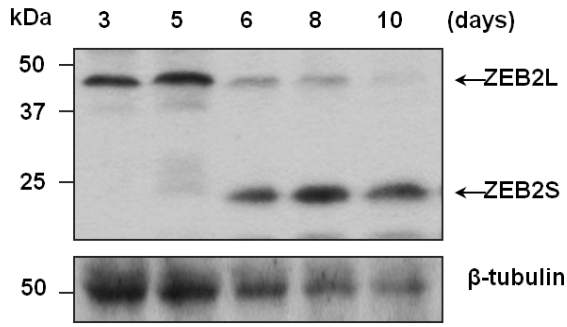


Fig. 20. Profile of ZEB2 isoform expression during ZEA biosynthesis. Western blot analyses using the anti-HA antibody were performed to profile ZEB2 isoform production during ZEA biosynthesis. β-tubulin was used as a loading control for the extracts. The incubation times are indicated above the blot. The protein molecular weight standards (in kDa) are indicated to the left of the blot.

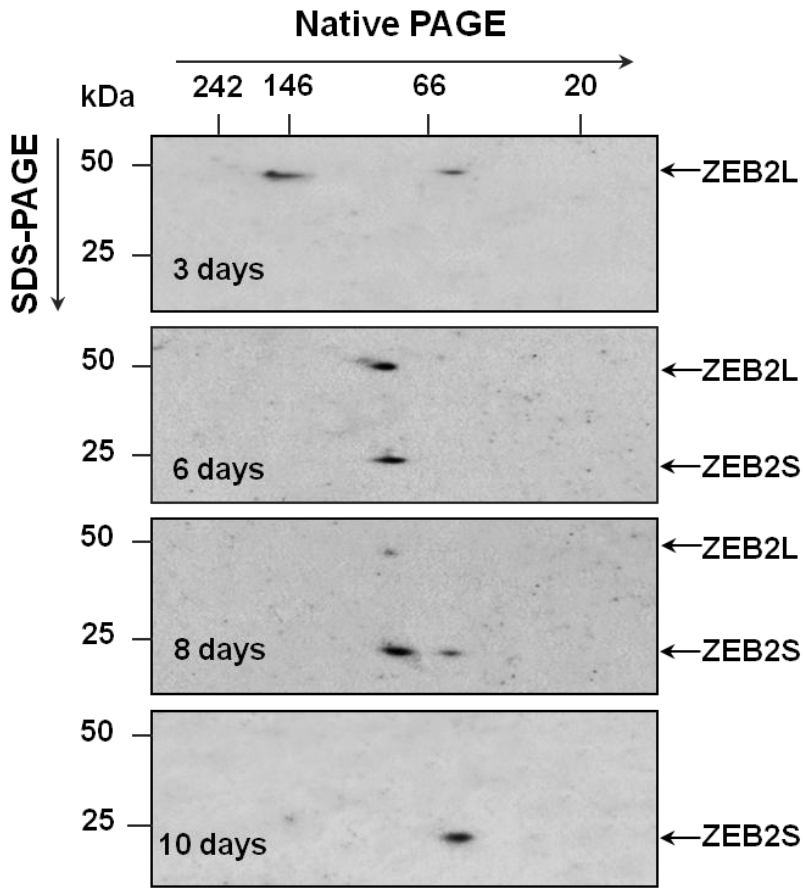
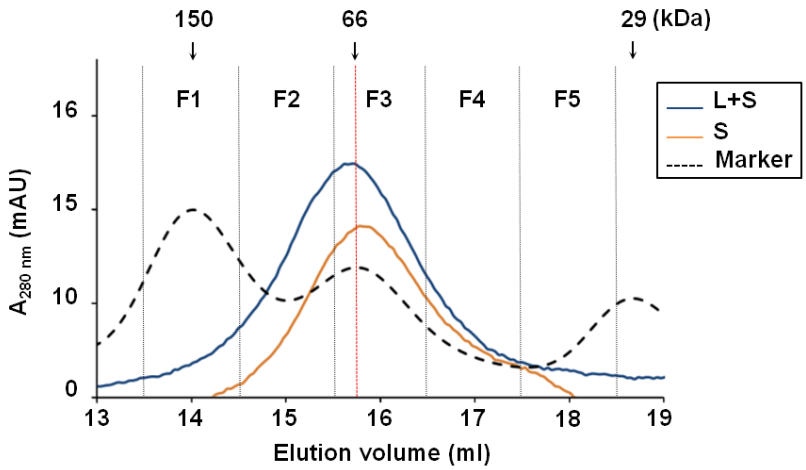

Native PAGE
242
146
66
20
kDa
SDS-PAGE
50
25
3 days
ZEB2L
50
25
6 days
ZEB2L
ZEB2S
50
25
8 days
ZEB2L
ZEB2S
50
25
10 days
ZEB2S

Fig. 21. Western blot analyses of native ZEB2 multi-oligomeric complexes by 2-D electrophoresis. A fungal strain (FAZ03N) producing both ZEB2 isoforms was used for the analysis. Total lysates were separated by native PAGE (5–15% acrylamide gradient). Next, 1-D gel products were subjected to denatured SDS-PAGE, followed by Western blot analysis with the anti-HA antibody. The protein molecular weight standards (in kDa) are indicated to the left and above each blot.

A


150
66
29 (kDa)
F1
F2
F3
F4
F5
L+S
S
Marker
16
15
10
0
$A_{280\ nm}$ (mAU)
13
14
15
16
17
18
19
Elution volume (ml)

B

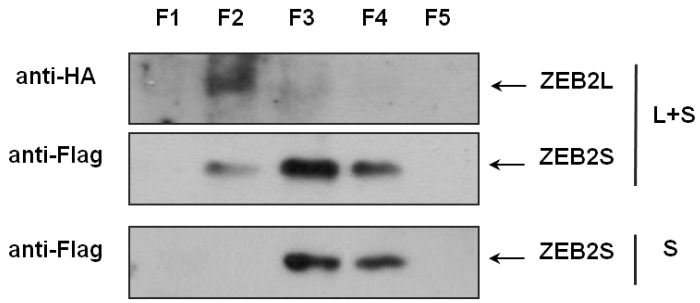

F1
F2
F3
F4
F5
anti-HA
ZEB2L
L+S
anti-Flag
ZEB2S
anti-Flag
ZEB2S
S

Fig. 22. Multi-oligomeric states of the ZEB2 complexes.

A. Size-exclusion chromatography profiles of ZEB2 multi-oligomeric complexes. Fungal strains expressing both ZEB2 isoforms (L+S, FAZ006) or ZEB2S alone (S, FAZ013) were used. Size-exclusion chromatography elution profiles are shown for the homo-oligomeric ZEB2S complex (orange line) and the mixed oligomeric ZEB2L-ZEB2S or ZEB2S-ZEB2S complexes (blue line). Arrows and dashed lines indicate molecular markers.

B. Western blot analyses of the fractions collected via size-exclusion chromatography. F1–5 represent the fractions used for Western blot analyses in A.

primarily detected in the F2 fraction of the FAZ006 strain. ZEB2S homodimers were detected in the F3 and F4 fractions of both strains (Fig. 22B). These data support the findings regarding the native oligomeric states of these ZEB2 isoforms. ZEB2L homo-oligomers could not be purified due to poor solubility.

VI. Induction of ZEB2 transcription by exogenous addition of ZEA

To investigate effects of ZEA on *ZEB2* expression, I added ZEA to the wild-type cultures. Exogenous treatment of ZEA (0.1 ppm) induced the expression of both the *ZEB2L* and *ZEB2S* transcripts at the early stage of ZEA production compared to the untreated control (Fig. 23). The transcript levels of *ZEB2S* tended to be more markedly increased than those of *ZEB2L* under the conditions of ZEA supplementation. At 8 days of incubation, the transcript levels of *ZEB2S* were higher in the presence of ZEA treatment than in the absence of ZEA treatment; alternatively, the transcript levels of *ZEB2L* were nearly undetectable in the presence of ZEA, in contrast to the untreated control. Similar to the *ZEB2L* transcript levels, ZEA accumulated more rapidly upon ZEA supplementation compared to the untreated control.

I also used the ZEA-deficient strains to verify the effect of ZEA on *ZEB2* expression. In accordance with a previous report (Kim *et al.*, 2005b), *ZEB2* transcripts were rarely detected in Δ*pks4* and Δ*pks13* in the absence of ZEA supplementation (Fig. 24). However, both mutant strains produced similar transcript levels of these two ZEB2 isoforms to the wild-type strain upon ZEA supplementation until 4 days of incubation (Fig. 24). Similar to those in the wild-type strain, the transcript levels of *ZEB2L* were dramatically decreased at 4 days, when the *ZEB2S* transcript levels were high. Taken

together, these results suggested that ZEA itself plays a role in an autoregulatory mechanism controlling *ZEB2* expression by activating *ZEB2* transcription.

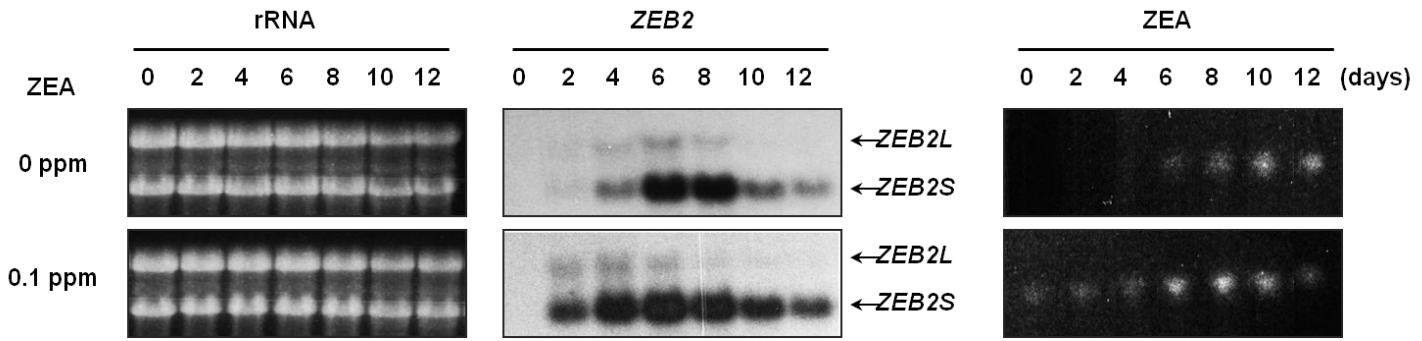


Fig. 23. Effect of ZEA treatment on the *ZEB2* transcription and ZEA production profiles. The concentration of supplemented ZEA is denoted on the left.

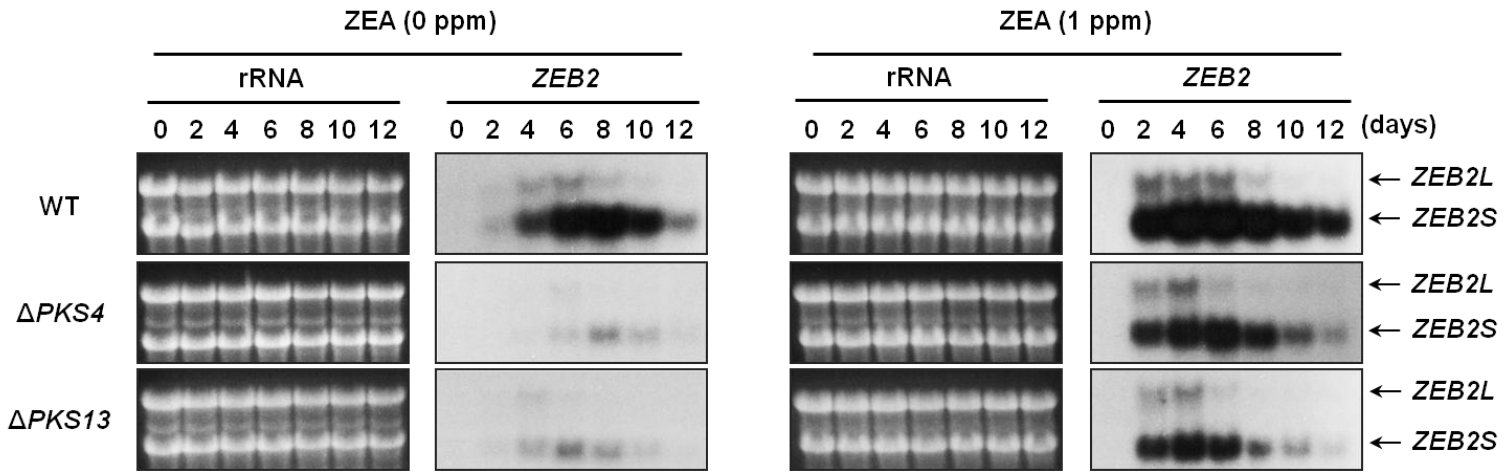


Fig. 24. ZEA triggers the induction of *ZEB2* transcription. Induction of *ZEB2* transcription by ZEA treatment in the transformed ZEA-deficient strains. ZEA and total RNA were extracted from *F. graminearum* cultures with or without ZEA supplementation for 0-12 days. ZEA production was detected by TLC analysis. The incubation times are indicated above each blot. Ethidium bromide-stained rRNA was included as a loading control.

DISCUSSION

Alternative promoter usage and alternative splicing mechanisms contribute to protein diversity in the eukaryotic genome (Keren *et al.*, 2010). Alternative promoters have different tissue specificities and developmental activities, and several alternative promoters enable the formation of various feedback loops (Pozniak *et al.*, 2000; Molina *et al.*, 1993). For example, inducible cAMP early repressor (ICER, a cAMP-responsive element modulator isoform) is expressed via an alternative promoter and represses the activity of its own promoter through a negative autoregulatory mechanism in human cells (Molina *et al.*, 1993). Although up to 58% of human genes are regulated by alternative promoters, the functional significance and biological roles of alternative promoters remain largely unexplored (Kimura *et al.*, 2006). Lower levels of alternative transcripts are predicted to be present in fungi than in animals (Loftus *et al.*, 2005), but few studies have examined fungal alternative promoters and their biological functions.

In this study, I demonstrated that the ZEB2S isoform plays a functional role in the inhibition of ZEA biosynthesis. I propose a novel regulatory mechanism for ZEA biosynthesis that involves the dimerization of the ZEB2L and ZEB2S isoforms under conditions favoring ZEA production (Fig. 25). In the early stage of the ZEA biosynthetic pathway, ZEB2L oligomers activate the ZEA biosynthetic cluster genes and *ZEB2* itself, resulting in the initiation of ZEA biosynthesis. ZEB2L is induced by environmental stimuli such as temperature, humidity, and nutrition (Naik *et al.*, 1978; Hesseltine, 1976). As ZEA biosynthesis progresses, ZEA affects the expression of *ZEB2S* transcripts directly or indirectly, and *ZEB2S*

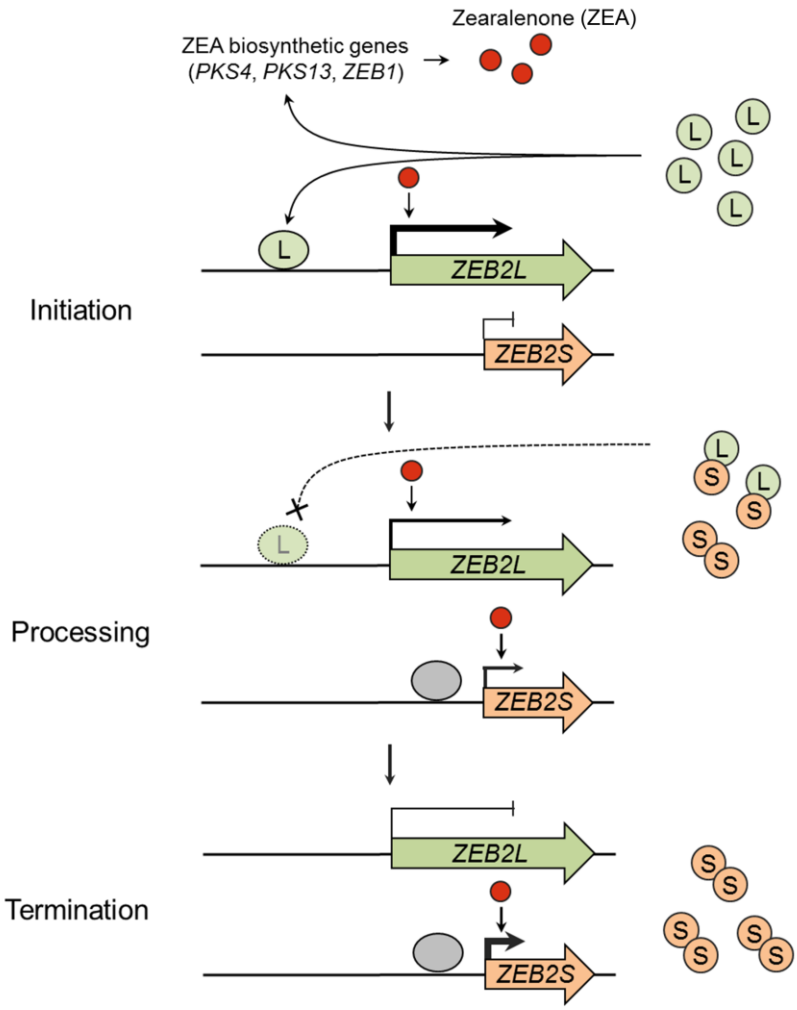

Zearalenone (ZEA)
ZEA biosynthetic genes
(PKS4, PKS13, ZEB1)
Initiation
ZEB2L
ZEB2S
Processing
ZEB2L
ZEB2S
Termination
ZEB2L
ZEB2S

Fig. 25. Proposed model of *ZEB2* autoregulation. After the initial induction of ZEB2, ZEB2L (L in green circle) is produced. The resulting homo-oligomers of ZEB2L (L in the green oval) directly activate ZEA biosynthetic cluster genes and *ZEB2L* during the early stage of ZEA production (Initiation). ZEA (red circle) affects the expression of *ZEB2* directly or indirectly, and *ZEB2S* transcripts are expressed by an alternative promoter. More *ZEB2S* transcripts are induced than *ZEB2L* transcripts as the ZEA level increases. During the processing step, ZEB2S (S in the orange circle) begins to accumulate, and the DNA-binding activities of the ZEB2L homo-oligomers are gradually eliminated by the production of ZEB2 heterodimers (ZEB2L-ZEB2S). Finally, *ZEB2L* transcription is completely terminated; only ZEB2S dimers remain. The green oval with a dotted line represents gradually reduced ZEB2L homo-oligomer levels. The grey ovals represent undefined activators responsible for the direct induction of *ZEB2S* transcription.

transcripts are expressed by an active alternative promoter. ZEA enhances both *ZEB2* transcripts, but more *ZEB2S* transcripts are formed than *ZEB2L* transcripts as the concentration of ZEA increases. ZEB2S inhibits *ZEB2L* transcription by forming ZEB2L-ZEB2S heterodimers, which reduce the DNA-binding activity of the ZEB2L oligomers. Because only a small portion of total ZEB2S is localized to nuclei (Fig. 2D), *ZEB2L* expression and ZEA production are not shut down as soon as ZEB2S transcription is induced. ZEA continues to be produced at this stage; however, the rate of ZEA production gradually decreases due to reduced ZEB2L levels. Finally, *ZEB2L* transcription is terminated due to the ZEA-induced increase in the ZEB2S levels, which ceases ZEA production.

The ZEA biosynthesis pathway is an archetypal example of autoregulation via the TF protein diversity caused by alternative promoter usage and feedback loop mechanisms, in which ZEA upregulates *ZEB2S* expression to maintain the appropriate levels of ZEA in the fungal cell. However, in contrast to previously characterized cases, such as human ICER, ZEB2S does not possess DNA-binding activity. Rather, ZEB2S interacts with ZEB2L to form a ZEB2L-ZEB2S heterodimer that inhibits ZEB2L from binding to the promoter of the *ZEB2L* gene.

In filamentous fungi, the CPCA of *Aspergillus nidulans* is an example of a homologous transcriptional regulator. CPCA directly binds to the CPCA recognition element in the *CpcA* promoter and activates its transcription under amino acid starvation conditions (Hoffmann *et al.*, 2001). *Frequency* (*frq*), a component of the circadian clock in *Neurospora crassa*, is negatively autoregulated by its own gene product, thus creating a feedback loop (Aronson *et al.*, 1994). However, both the ZEB2L and ZEB2S isoforms participate in the autoregulation of

ZEB2 as an activator and an inhibitor, respectively. To my knowledge, no autoregulatory mechanism for *ZEB2* expression has been reported in filamentous fungi.

In general, bZIP TFs form hetero- and/or homo-oligomers that affect DNA-binding specificity, affinity for promoter elements, and nuclear localization (Vinson *et al.*, 2006; Husberg *et al.*, 2003). Heterodimerization has been reported in various human bZIP TF families, including Fos/Jun, ATF/CREB, and C/EBP (Cao *et al.*, 1991; Smeal *et al.*, 1989; Karpinski *et al.*, 1992). Fos/Jun heterodimers have higher DNA-binding activity than Jun homodimers (Smeal *et al.*, 1989). ATF-1/CREB heterodimers activate transcription for different periods compared to ATF-1 homodimers (Karpinski *et al.*, 1992; Kobayashi and Kawakami, 1995). Common plant regulatory protein 1 (CPRF1), a bZIP TF in parsley that has been implicated in light-responsive element-mediated mechanisms, selectively forms heterodimers with CPRF4a (Kircher *et al.*, 1998). Class III homeodomain-leucine zipper (HD-ZIPIII) TFs regulate leaf polarity and vascular development in *Arabidopsis thaliana* and interact with LITTLE ZIPPER (ZPR) proteins as competitive inhibitors, resulting in the formation of nonfunctional heterodimers (Wenkel *et al.*, 2007). Little is known about bZIP TF heterodimerization in fungi. The yeast bZIP TFs Met4 and Met28 regulate the sulfur amino acid pathway to produce heterodimeric protein complexes (Newman and Keating, 2003). In *A. nidulans*, heterodimerization between bZIP TFs has been detected using an *in vitro* assay (Yin *et al.*, 2013), but the underlying regulatory mechanism remains unclear.

In *Saccharomyces cerevisiae*, one of the best-characterized bZIP TFs is GCN4 (Fernandes *et al.*, 1997). GCN4 directly binds to the optimal bZIP binding

site (TGAC/GTCA). However, the binding sequence of the other yeast bZIP protein, Yap1, differs at two of the five highly conserved residues in its GCN4 binding sites. Yap1 directly regulates the transcription of the γ-glutamyl cysteine synthetase (*GSH1*), thioredoxin (*TRX2*), ATP-binding transporter (*YCF1*), and glutathione reductase (*GLR1*) genes, which are involved in the cellular oxidative stress response. The Yap1 recognition site in *GSH1* is TGACTAA; this sequence differs from the GCN4 binding site by a single base pair. Alternatively those in the *TRX2*, *YCF*, and *GLR1* promoters differ at two positions from the GCN4 binding site (TTACTAA). I found a GCN4 motif only in the promoter of *ZEB1* (from -177 to -171) but did not detect the typical consensus bZIP binding sequence in the promoter region of *PKS4*, *PKS13*, or *ZEB2*. It appears that ZEB2L may bind to the unusual and distinct bZIP binding sites in the promoters of these four cluster genes, such as yeast Yap1. Further investigation is necessary to identify the bZIP binding sequences in the promoter region of ZEA biosynthetic cluster genes.

Fungal secondary metabolites are typically synthesized by genes that are grouped into contiguous clusters (Keller *et al.*, 2005). Pathway-specific regulators, which are often found in these clusters, positively regulate the expression of secondary metabolite biosynthetic pathway genes, such as *TRI6* for trichothecenes (Proctor *et al.*, 1995), *GIP2* for aurofusarin (Kim *et al.*, 2006), and *AflR* for aflatoxin and sterigmatocystin (Woloshuk *et al.*, 1994; Fernandes *et al.*, 1998). Most of the potential regulators of fungal polyketide synthase (PKS)-encoding cluster genes belong to the zinc cluster family (Brakhage, 2012). ZEB2 is the only known bZIP TF that is encoded by a secondary metabolite biosynthetic cluster gene and that regulates genes within that cluster in fungi.

bZIP TFs are typically involved in developmental and physiological processes and are responsive to environmental stress in eukaryotes (Tian *et al.*, 2011). In *Saccharomyces cerevisiae*, bZIP TFs have been implicated in responses to oxidative and osmotic stress, DNA damage, amino acid starvation, and alternative carbon source utilization (Tan *et al.*, 2008; Workman *et al.*, 2006; Rep *et al.*, 2001; Hinnebusch, 2005). In filamentous fungi, bZIP TFs respond to amino acid starvation, sulfur sources, oxidative stress, and virulence (Tian *et al.*, 2011; Ebbole *et al.*, 1991; Guo *et al.*, 2011). The association between bZIP proteins and secondary metabolite biosynthesis was recently proposed as a novel function of bZIP TFs in filamentous fungi. The overexpression of the *A. nidulans* Yap-like bZIP *RsmA* increased sterigmatocystin production by activating the zinc-finger TF *AflR* (Yin *et al.*, 2013). Moreover, *F. graminearum FgAP1* mediates trichothecene biosynthesis (Montibus *et al.*, 2013), and *A. fumigatus FlbB* is required for gliotoxin production (Xiao *et al.*, 2010).

In-depth analyses of TFs in *F. graminearum* have revealed that several bZIP TFs, with the exception of the *ZEB2* gene, affect ZEA production (Son *et al.*, 2011b). However, most of the characterized fungal bZIP TFs are not located in a contiguous cluster and do not directly activate secondary metabolite biosynthetic cluster genes. In *A. fumigatus*, *FlbB* encodes two bZIP domain proteins from a single gene, in contrast to *A. nidulans* (Xiao *et al.*, 2010). These two proteins are hypothesized to be induced by the alternative initiation of transcripts in a process similar to that for ZEB2; however, the underlying regulatory mechanism has not been determined.

In conclusion, ZEB2L forms heterodimers *in vivo* with the truncated

isoform ZEB2S, resulting in the autoregulation of ZEA biosynthesis via the attenuation of activator gene transcription. This study is the first to demonstrate the functional roles of bZIP TF heterodimers in secondary metabolite biosynthesis in a eukaryotic microorganism. I propose a novel regulatory mechanism that is self-controlled by fungal alternative isoforms, similar to the mechanisms observed in higher eukaryotes. These findings provide mechanistic insights into the regulation of secondary metabolite biosynthesis due to alternative promoter usage by TFs in filamentous fungi.

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*Fusarium graminearum*의 ZEB2 동형단백질에 의한 지랄레논 생합성 자가조절 연구

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초 록

자낭균인 붉은곰팡이(*Fusarium graminearum*)는 전세계적으로 주요 곡류에 막대한 경제적 손실을 일으키는 이삭마름병의 원인균이다. 지랄레논(zearalenone)은 붉은곰팡이가 생산하는 폴리케티드 곰팡이독소로서 인축에 생식능력저하 등의 과에스트로겐 증상을 보이는 중독현상을 유발한다. 지랄레논의 생합성은 붉은곰팡이 내에서 몇몇의 인접한 유전자 군에 의해 이루어지는데, 환원 폴리케티드 생성효소, 비환원 폴리케티드 생성효소, 이소아밀 알코올 산화효소, basic leucine zipper (bZIP) 전사조절인자 단백질을 각각 암호화하는 *PKS4*, *PKS13*, *ZEB1*, *ZEB2* 유전자가 그 안에 포함되어 있다. 본 연구에서는 지랄레논 생합성 유전자 군의 전사를 조절하는 핵심유전자 *ZEB2*의 기능과 그로 인한 지랄레논 생합성 조절기작을 연구하였다. 그 결과 ZEB2는 alternative promoter의 조절을 통해 두 개의

ZEB2 동형단백질(ZEB2L과 ZEB2S)을 생산하며 이들이 지랄레논 생합성 조절에 관여함을 발견하였다. ZEB2S는 bZIP DNA 결합부위가 존재하는 ZEB2L단백질의 아미노기 말단이 완전히 절단된 동형단백질로, ZEB2L과 헤테로다이머를 형성하고 *ZEB2L* 전사를 촉진하는 ZEB2L 호모다이머의 프로모터 결합을 방해하여 ZEB2L의 전사조절인자로서의 기능을 저해함을 밝혀냈다. 또한 붉은곰팡이 세포내의 지랄레논이 *ZEB2L*과 *ZEB2S*의 전사를 유도하여 ZEB2 발현자가조절에 관여하며 이를 통하여 지랄레논의 생합성에 영향을 줄 수 있음을 확인하였다. 이 연구는 alternative promoter에 의해 발현된 ZEB2 동형단백질의 서로 다른 기능과 지랄레논의 상호작용에 의한 *F. graminearum ZEB2* 발현의 자가조절과 그로 인한 지랄레논 생합성의 피드백 루프 조절기작을 설명하고 있으며 이는 전사조절인자에 의한 곰팡이 이차대사산물의 생합성 조절연구을 위한 초석이 될 것이다.

주요어: 붉은곰팡이, 진균독소, 지랄레논, bZIP 전사조절인자, ZEB2, 자가조절기작

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