

Possible Negative Effect of Pigmentation on Biosynthesis of Polyketide Mycotoxin Zearalenone in *Gibberella zea*

JUNG, SUNYO^{1,3}, JUNG-EUN KIM¹, SUNG-HWAN YUN², AND YIN-WON LEE^{1*}

¹School of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea

²Department of Biological Resources and Technology, Soonchunhyang University, Asan 336-745, Korea

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Abstract We investigated a possible coordination between the biosyntheses of two polyketides in the cereal head blight fungus *Gibberella zea*, zearalenone (ZEA) and aurofusarin (AUR), which are catalyzed by the polyketide synthases (PKS) PKS4/PKS13 and PKS12, respectively. To determine if the production of one polyketide influences that of the other, we used four different transgenic strains of *G. zea*; three were deficient for either ZEA or AUR or both, and one was an AUR-overproducing strain. The mycelia of both the wild-type and Δ PKS4 strain deficient for ZEA produced AUR normally, whereas the mycelia of both the Δ PKS12 and Δ PKS4:: Δ PKS12 strain showed no AUR accumulation. All the examined deletion strains caused necrotic spots on the surface of corn kernels and were found to produce the nonpolyketide mycotoxins trichothecenes to the same amount as the wild-type strain. In contrast, the AUR-deficient Δ PKS12 strains produced greater quantities of ZEA and its derivatives than the wild-type progenitor on both a rice substrate and a liquid medium; the AUR-overproducing strain did not produce ZEA on either medium. Furthermore, the expression of both PKS4 and PKS13 was induced earlier in the Δ PKS12 strains than in the wild-type strain, and there was no difference in the transcription of PKS12 between the two strains. Therefore, these results indicate that the ZEA biosynthetic pathway is negatively regulated by the accumulation of another polyketide (AUR) in *G. zea*.

Key words: Aurofusarin, zearalenone, polyketide synthase genes, *Gibberella zea*

Gibberella zea (anamorph: *Fusarium graminearum*) is an ascomycetous fungus with a ubiquitous geographic

*Corresponding author

Phone: 82-2-880-4671; Fax: 82-2-873-2317;

E-mail: lee2443@snu.ac.kr

³Present address: School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Korea

distribution that causes serious disease in cereal crops, such as corn, wheat, barely, and rice [15]. This fungus produces a broad range of secondary metabolites, including mycotoxins, antibiotics, and pigments [14, 16, 18]. Among these metabolites, the production of polyketides such as zearalenone (ZEA) and aurofusarin (AUR) is an important mycological characteristic of *G. zea*. ZEA is a mycotoxin that causes an estrogenic disorder in several commercially important animal species [14, 17, 21], and AUR is a dimeric naphthoquinone pigment that is produced in aerial mycelia [3, 4]. These two polyketides are synthesized by polyketide synthases (PKS) encoded by PKS genes in *G. zea*; in total, 16 putative PKS genes have been identified in the *G. zea* genome [10].

Using targeted deletions in the *G. zea* PKS genes, researchers have recently identified PKS4 and PKS13, both of which are required for ZEA biosynthesis [5, 9], and PKS12, which is required for AUR biosynthesis [7, 13]. ZEA deficiency caused by the deletion of either PKS4 or PKS13 leads to no significant alterations in other phenotypes of *G. zea*, such as hyphal growth, conidiation, perithecium formation, and pigmentation [9]. In contrast, a PKS12-deleted strain of *G. zea* that was devoid of pigmentation exhibited dramatic phenotypic changes, including enhanced hyphal growth and reduced ZEA production, compared with the wild-type strain [7, 13]. Although the influence of AUR deficiency on ZEA biosynthesis has not been completely demonstrated, the previous observations led us to focus on a possible connection between the biosyntheses of these two polyketides in *G. zea*.

Accordingly, the objectives of this study were to determine the possible effects of 1) AUR accumulation on the biosynthesis of ZEA and the expression of both PKS4 and PKS13, and 2) ZEA production on AUR production and the expression of PKS12 in *G. zea*.

MATERIALS AND METHODS

Strains and Media

Strain GZ3639 was used as the wild-type strain of *G. zeae*, which produces deoxynivalenol (DON) and ZEA [19]. The *G. zeae* strains T39ΔM2-1 and TdPKS4-2 are derived from GZ3639, where the former is a *mat1-2*-deleted self-sterile strain [12] that produces both ZEA and AUR, whereas the latter produces AUR, yet is deficient for ZEA because of the deletion of *PKS4* (designated Δ*PKS4*). For DNA isolation, the fungal strains were grown in 30 ml of a complete medium (CM) in 250-ml Erlenmeyer flasks [2] for 3 days at 25°C on a rotary shaker at 150 rpm. For the total RNA extraction and ZEA production, aerial mycelia of the strains were grown in 25 ml of a starch-glutamate (SG) liquid medium [9]. To observe the AUR production, the fungal strains were grown on either a potato-dextrose agar (PDA, Difco Laboratories, Detroit, MI, U.S.A.) or the SG medium at 25°C in the dark. Rice grains (50 g) in 250-ml Erlenmeyer flasks were also used as a solid medium for ZEA production [9]. A carrot agar was used for the sexual crosses [1].

Nucleic Acid Manipulations

The fungal genomic DNA and plasmid DNAs were extracted as previously described [6]. The total RNA was extracted from ground mycelia (0.1 g) in liquid nitrogen using 1 ml of a TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. Standard procedures were used for the restriction endonuclease digestion, ligation, agarose gel electrophoresis, and gel blotting [20]. ³²P-labeled probes were used in both DNA and RNA gel blot hybridizations. The PCR primers were obtained from the Bioneer oligonucleotide synthesis facility (Bioneer Co., Chungwon, Korea).

Double-Joint PCR and Fungal Transformation

A transforming DNA fragment carrying a hygromycin resistance gene (*hygB*) flanked by DNA sequences homologous to those located at the 5' and 3' flanking regions of the *PKS12* gene in *G. zeae* was amplified using a double joint (DJ)-PCR with modifications [22]. A 4.8-kb fusion PCR product was amplified for the genomic DNA of strain GZ3639; the primer pairs P12-5'f (5'-GGGGG-AGGCCATCTTTATCT-3')/P12-5'r (5'-CAGGTACACTT-GTTAGAGGAGAGGTAATGAAGCACAAT-3') and P12-3'f (5'-TCAATATCATCTTCTGTCGCTTTGTATTGCGC-TTCGTGTT-3')/P12-3'r (5'-CTAATATTCATCAGCTTTC-ACTCC-3') were used to amplify the 5'- and 3'-flanking regions of the *PKS12* ORF, and a nested primer pair (NP12-5'f, 5'-GATATCGCGACTGACGTAACAGG-TGG-3' and NP12-3'r, 5'-GCGACACGTATGGGCCAAA-GAATG-3') was used for the fusion product. The PCR conditions and further treatments of the PCR products were performed as previously described [7, 9].

For the fungal transformation, approximately 5 μg of the fusion PCR product obtained by the DJ-PCR was added directly with polyethyleneglycol into fungal protoplasts prepared by the treatment of young mycelia with Driselase (InterSpex Products, Inc., San Mateo, CA, U.S.A.), as previously described [11]. Stable transformants carrying the *hygB* gene were selected on a regeneration medium containing hygromycin B (75 μg/ml) and stored in 25% glycerol at -80°C.

Outcross and Virulence Test

A sexual cross of a transgenic *G. zeae* strain to T39ΔM2-1 was performed in a carrot agar medium, as previously described [12]. Two ascospores per perithecius formed on the mating plates were isolated for genetic analysis. The progenies from each cross were analyzed by thin-layer chromatography (TLC) for their ability to produce ZEA on a rice substrate and by pigmentation on PDA for AUR production.

To test the virulence, fungal mycelia were harvested from the strains grown on carrot agar plates for 2 weeks at 25°C by scraping the carrot agar cultures using a toothpick; they were then inoculated into corn by injecting the toothpick into a young corn ear. After inoculation, the corn samples were left for 1 week in a plastic box in which the humidity was maintained.

Chemical Analyses

The standards of ZEA, α-ZOL, and β-ZOL were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). To detect both ZEA and AUR in the SG culture of the fungal strains, both the culture filtrate and the dried liquid culture were extracted and analyzed by high-performance liquid chromatography (HPLC), as previously described [7, 9].

RESULTS

Targeted Deletions of *PKS* Genes in *G. zeae*

We employed a targeted gene replacement strategy to construct the transgenic *G. zeae* strains with the deletion of *PKS12* or both *PKS12* and *PKS4*. The entire open reading frame (ORF) for each *PKS* in the GZ3639 strain was deleted and replaced with a fungal selectable marker *hygB*, as previously described [7, 9]. The integration of the fusion PCR products, which carried both the 5' and 3' regions of the *PKS12* ORF fused to the marker, into the genome of the Δ*PKS4* strain (TdPKS4-2) [9] via a double homologous recombination between the fungal genome and the corresponding regions on the vector created Δ*PKS4::APKS12* (Fig. 1A). The Δ*PKS12* strain of GZ3639 was also constructed using the same strategy described above (data not shown). When probed with a 3'-flanking region of *PKS12*, the *StuI*-digested genomic DNA of both

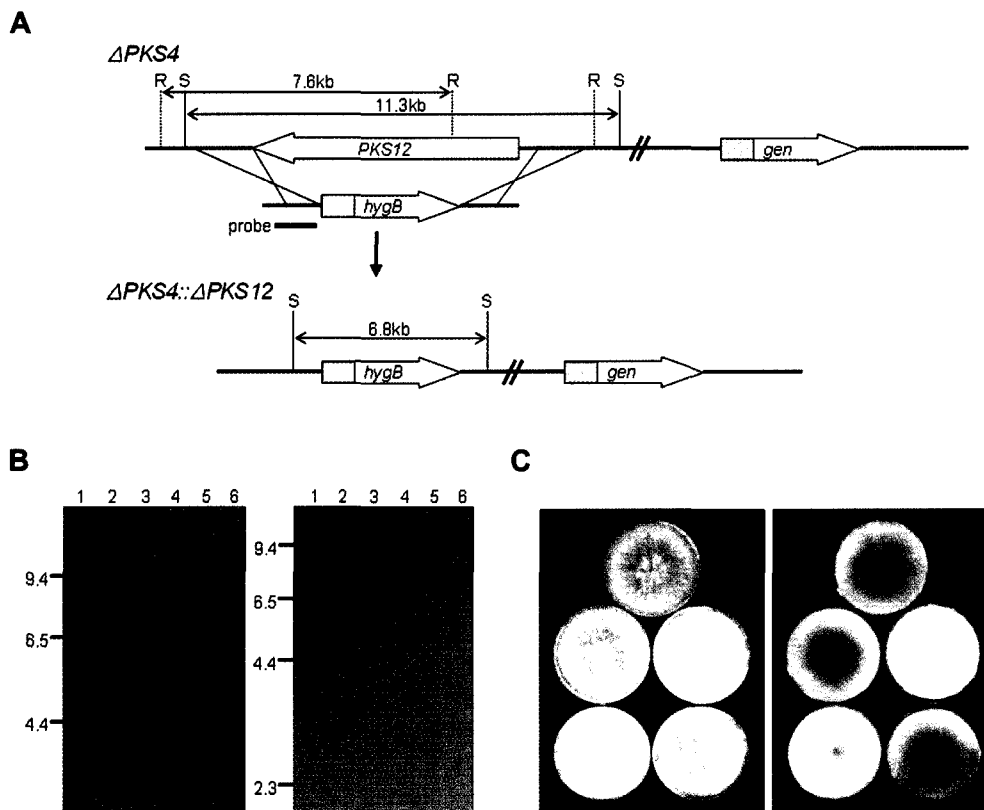


Fig. 1. Deletion of *PKS12* from the genome of *PKS4*-deleted GZ3639 strain of *G. zeae*.

A. Deletion strategy. R, EcoRI; S, StuI; *hygB*, hygromycin-B-resistance gene; *gen*, geneticin-resistance gene; //, discontinuity. **B.** Gel blots of genomic DNAs from the $\Delta PKS4$, $\Delta PKS12$, and $\Delta PKS4::\Delta PKS12$ strains digested with either StuI (left panel) or EcoRI (right panel). The 3'-flanking region or full ORF region of *PKS12* was used as a probe for DNA gel blot hybridizations. Lane 1, a wild-type strain (GZ3639); lane 2, a $\Delta PKS4$ strain (TdPKS4-2) [9]; lane 3, a $\Delta PKS12$ strain (TdPKS12-1); lanes 4 and 5, $\Delta PKS4::\Delta PKS12$ -deletion strains (TdPKS12/4-3 and TdPKS12/4-5); lane 6, a transformant carrying the transforming vector at an ectopic site (TdPKS12/4-10). The sizes of the lambda DNA standards (in kb) are indicated on the left of the blot. **C.** Pigmentation of the *PKS*-deletion strains. A top view of the aerial mycelia grown on the plates is to the left, whereas the undersurfaces of the corresponding plates are shown on the right. Top of both sides, GZ3639; middle left, TdPKS4-2; middle right, TdPKS12-1; bottom left, TdPKS12/4-3; bottom right, TdPKS12/4-10.

the $\Delta PKS12$ and $\Delta PKS4::\Delta PKS12$ strains exhibited a 6.8-kb band, instead of the 11.3-kb band in the wild-type GZ3639 and $\Delta PKS4$ strain (Fig. 1B). When probed with a *PKS12* fragment, the intact copy of the *PKS12* ORF was detected in both the GZ3639 and $\Delta PKS4$ strains, yet not in the $\Delta PKS12$ or $\Delta PKS4::\Delta PKS12$ strains (Fig. 1B). Three transgenic strains of each *PKS* deletion were used for further analyses.

Phenotypes of Transgenic *PKS*-Deletion Strains

The mycelia of the wild-type GZ3639 and $\Delta PKS4$ strains began to produce AUR on PDA after 5 days of incubation, turning carmine red (Fig. 1C). However, neither the $\Delta PKS12$ nor the $\Delta PKS4::\Delta PKS12$ strain was able to produce AUR during the entire time examined, remaining white at the top of the cultures and yellow at the bottom. The transgenic strains carrying the transforming DNA at ectopic positions exhibited a red pigmentation as did GZ3639. All the examined $\Delta PKS4$ strains were similar to their wild-type progenitor in mycelial growth, whereas the albino $\Delta PKS12$

and $\Delta PKS4::\Delta PKS12$ strains examined showed approximately 30% increases in mycelial growth compared with the GZ3639 or $\Delta PKS4$ strains (data not shown). In contrast to mycelial growth, all the *PKS*-deletion strains examined were similar to GZ3639 in their virulence on corn (Fig. 2); they produced nonpolyketide mycotoxins, *i.e.*, trichothecenes

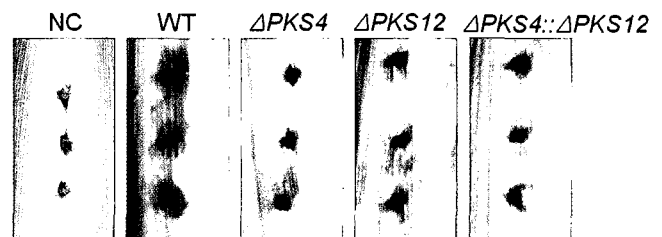


Fig. 2. Virulence of *PKS* deletion strains on corn ears. Necrotic symptoms occurred 3 days after inoculation. NC, uninoculated negative control; WT, wild-type strain GZ3639; $\Delta PKS4$, a *PKS4*-deletion strain; $\Delta PKS12$, a *PKS12*-deletion strain; $\Delta PKS4::\Delta PKS12$, a double deletion strain.

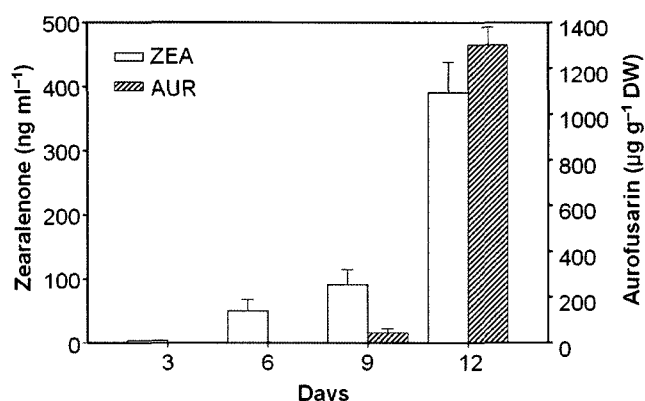


Fig. 3. Time course of both ZEA and AUR production by the wild-type strain GZ3639 in starch-glutamate (SG) liquid medium.

The data represent the mean±SD of three replicates.

(DON and its derivatives), in a manner similar to GZ3639 (data not shown).

Production of ZEA and AUR in Transgenic *G. zeae* Strains

In the SG liquid medium, the wild-type strain GZ3639 produced significant amounts of both ZEA and AUR after

12 days of incubation. However, the initiation of AUR production was delayed compared with that of ZEA. ZEA was first detected 3 days after inoculation and gradually increased until day 12, whereas AUR was first detected on day 9 (Fig. 3). The albino $\Delta PKS12$ strains produced ZEA with a similar pattern to GZ3639 in the same liquid medium. Moreover, the $\Delta PKS12$ strains produced approximately twice as much ZEA and β -ZOL as GZ3639 (Fig. 4A). The elevated ZEA production in the $\Delta PKS12$ strains also occurred on the rice substrate. The production of ZEA and β -ZOL increased gradually in both the GZ3639 and $\Delta PKS12$ strains during the 4-week cultivation period, yet the amounts of ZEA and β -ZOL in the $\Delta PKS12$ strains were 2 to 3 times greater than those in GZ3639 (Fig. 4B). ZEA and β -ZOL were not detected or were only present in trace amounts in the $\Delta PKS4$ and $\Delta PKS4::\Delta PKS12$ strains during the entire cultivation on both media (Fig. 4). Similarly, the AUR-overproducing strain Odzg2-1, which was derived from the Z03643 strain [8], did not produce ZEA under the same conditions (data not shown). A gradual increase in α -ZOL was observed in both the GZ3639 and $\Delta PKS12$ strains 2 weeks after inoculation, yet the amounts were negligible in comparison with ZEA or β -ZOL (data not shown). Interestingly, the SG medium exhibited a greater proportion of β -ZOL (75%) in the total

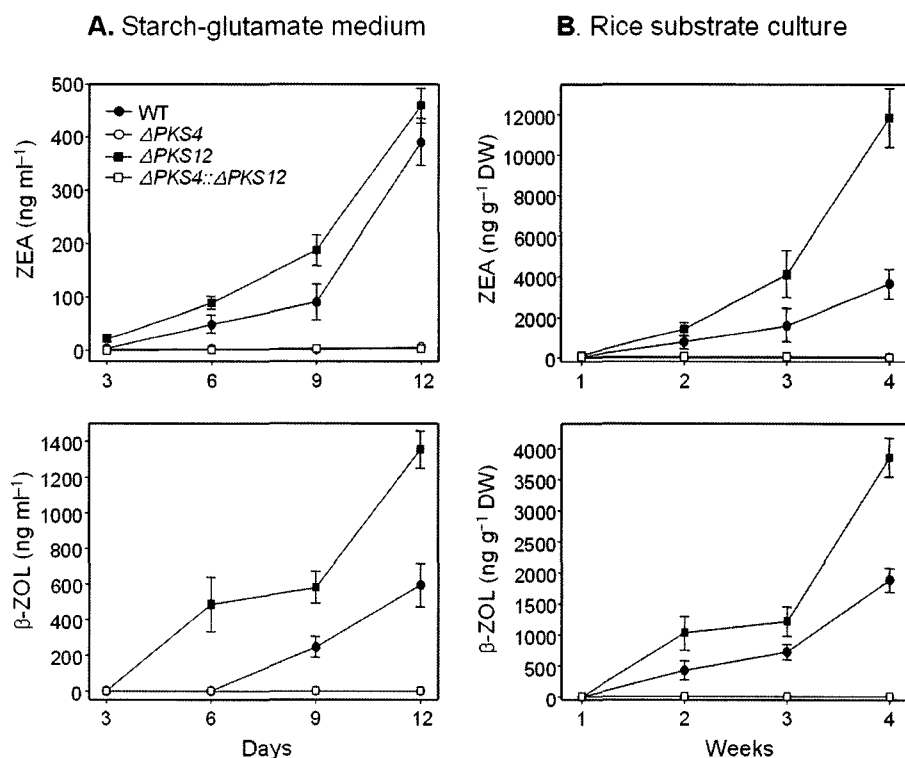


Fig. 4. Time course of ZEA and β -ZOL production in the wild-type strain GZ3639 and the *PKS*-deletion strains in SG medium (A) and on rice substrate (B).

WT, wild-type strain GZ3639; $\Delta PKS4$, a *PKS*-deletion strain; $\Delta PKS12$, a *PKS12*-deletion strain; $\Delta PKS4::\Delta PKS12$, a double deletion strain. The data represent the mean±SD of three replicates.

ZEA and β -ZOL than the solid rice medium (25%). Unlike the ZEA production, the AUR production was not altered in the cultures of the $\Delta PKS4$ strains, indicating that ZEA deficiency did not affect the AUR production in *G. zeae*.

Genetic Analysis of *PKS* Deletions

To verify the genetic linkage of ZEA- or AUR-deficiency with each *PKS* gene deletion in the transgenic strains, we outcrossed the *PKS* deletion strains to a self-sterile *mat1-2* deleted strain, T39 Δ M2-1 [12]. None of the progeny from the outcrosses of $\Delta PKS12$ (*hygB*⁻*aur*⁻) showed a recombinant phenotype between drug resistance and pigmentation, as previously described [7, 9]. Similarly, all the progeny in the outcross of the $\Delta PKS4::\Delta PKS12$ strain (*hygB*⁻*aur*⁻*gen*⁻*zea*⁻) to T39 Δ M2-1 showed genetic linkages between *gen* and *zea*⁻ and between *hygB* and *aur*⁻, indicating that the *zea*⁻ and *aur*⁻ mutations in all the strains examined were caused by the targeted deletions of the corresponding *PKS* genes from the GZ3639 genome. These genetic analyses could be used as an alternative for complementation analyses using an intact copy of each gene to confirm the gene function.

Expression Pattern of *PKS* Genes

As previously described [9], the expression pattern of the two *PKS* genes (*PKS4* and *PKS13*) required for ZEA biosynthesis differed from the production pattern of ZEA in the SG liquid culture of the wild-type strain GZ3639. The transcripts of both *PKS4* and *PKS13* were first detected in the RNA blots on day 6, increased up to day 9, and disappeared by day 12 (Fig. 5), whereas ZEA was first detected on day 3 and gradually increased until day 12 (Fig. 3). Both *PKS* transcripts in the $\Delta PKS12$ strain, however, were first detected 3 days earlier than those in GZ3639, and the band intensities of each *PKS* transcript seemed to be stronger (Fig. 5). This implies that the deletion of the *PKS12* gene is related with the regulation of ZEA production at the transcriptional level. The transcript level of *PKS13* in the $\Delta PKS4$ was significantly reduced compared with that in GZ3639, as previously reported [9]. Unlike the case of the ZEA *PKS* genes, the transcription

pattern of the *PKS12* gene did not differ between GZ3639 and the $\Delta PKS4$ strain and seemed to be correlated with the accumulation pattern of AUR in GZ3639. The expression of *PKS12* reached a maximum level 12 days after inoculation, at which time AUR was also highly accumulated (Figs. 3 and 5). The *PKS4* and *PKS12* transcripts were not detected in either the $\Delta PKS4$ or $\Delta PKS12$ strain or in the $\Delta PKS4::\Delta PKS12$ strain during the entire culture period, indicating successful deletion of each *PKS* gene in the transgenic strains. Interestingly, the reduced transcript level of *PKS13* in the $\Delta PKS4$ strain was recovered to the wild-type level in the $\Delta PKS4::\Delta PKS12$ strain (Fig. 5). Neither the *PKS4* nor the *PKS13* transcript was detected in the AUR-overproducing strain (data not shown).

DISCUSSION

The availability of the transgenic *G. zeae* strains deficient for either ZEA or AUR, or both, derived from GZ3639 that produced these polyketides more consistently than another standard *G. zeae* strain, Z03643 [7–9, 11], enabled an evaluation of a possible coordination between the biosyntheses of these two polyketides. It has been hypothesized that the production of the red pigment AUR should be reduced or inhibited for normal early vegetative growth by *G. zeae* [7]. In contrast, ZEA biosynthesis is initiated in the early hyphal stage (in this study, up to 9 days after inoculation in the SG liquid medium), during which time AUR biosynthesis has not yet been initiated. The detection of ZEA in the AUR-deficient *G. zeae* mutants grown under conditions that were unfavorable for ZEA production [13] also suggests the possible relationship between these two polyketide biosyntheses. However, our results confirmed that the relationship is not mutual, but unilateral in *G. zeae*. ZEA production was significantly elevated when the AUR biosynthetic pathway was blocked, yet AUR accumulation was not influenced by ZEA deficiency.

The earlier expression of both *PKS4* and *PKS13* required for ZEA biosynthesis in the $\Delta PKS12$ strain compared with

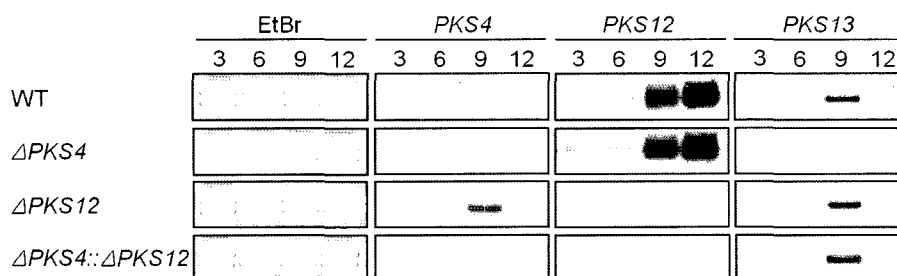


Fig. 5. RNA gel blots of the transgenic *PKS*-deletion strains of *G. zeae* grown in SG liquid medium for 12 days. The probe for each *PKS* gene is indicated above each blot. Ethidium bromide-stained agarose gels prior to blotting are shown in the left panel. WT, wild-type strain GZ3639; $\Delta PKS4$, a *PKS*-deletion strain; $\Delta PKS12$, a *PKS12*-deletion strain; $\Delta PKS4::\Delta PKS12$, a double deletion strain.

the wild-type strain could be attributed to the elevated production of ZEA by $\Delta PKS12$, indicating that AUR deficiency could be a better genetic background for *G. zeae* to produce ZEA. This suggests that ZEA biosynthesis may be negatively regulated by the AUR biosynthetic pathway in the wild-type *G. zeae* strain. This possible negative effect of AUR accumulation on ZEA production was also seen in the AUR-overproducing strain, where no ZEA was detected in either the rice or liquid culture of this strain during the entire period examined. A possible explanation for the effect of AUR deficiency has been proposed in that blocking the AUR biosynthetic pathway increases the availability of polyketide starters and extenders, acetyl and malonyl-CoA, into the remaining PKS pathways [13]. However, this hypothesis does not explain the small effect of ZEA deficiency on AUR production in *G. zeae*. A more reasonable speculation is that AUR biosynthesis is associated with a particular developmental stage, such as mycelial maturation or aging [8], whereas ZEA biosynthesis is not. AUR deficiency has a positive effect on vegetative growth, as well as on ZEA production in *G. zeae*, such as an increase in mycelial growth and conidiation [7, 13], whereas AUR overproduction is likely to act as a physiological stressor for mycelial growth in *G. zeae* [8]. Therefore, it is possible that AUR deficiency may keep the physiology of *G. zeae* more active, leading to greater activation of the ZEA biosynthetic pathway in the albino strain, which is not related to vegetative growth in *G. zeae*. The wild-type level expression of the *PKS13* gene in the $\Delta PKS4::\Delta PKS12$ strain confirms that the deletion of *PKS12* relieved the transcriptional repression of *PKS13*, which had been caused by $\Delta PKS4$ in *G. zeae*. This also indicates that the regulatory mechanism for *PKS12* gene expression in the AUR biosynthetic pathway is associated with the regulation of the ZEA gene cluster, including *PKS13*. However, further investigations are required to elucidate the specific regulatory network between these two polyketide biosynthetic pathways. In particular, the production patterns of other polyketides as well as nonpolyketide metabolites should be monitored in the AUR-deficient $\Delta PKS12$ strain.

Considering the negative effect of AUR accumulation on ZEA production, the AUR-deficient transgenic *G. zeae* strains examined in this study could be commercially useful for the large-scale production of ZEA. The derivative of ZEA, Zeranol, has been used as a bovine growth stimulant (Ralgro; Pitman-Moore, Terre Haute, IN, U.S.A.). The AUR deficiency causes an elevation in ZEA production by *G. zeae* and simplifies the purification steps that have been complicated by accumulated AUR [7]. We also expect further optimization of ZEA production through additional gene manipulation, such as the overexpression of both *PKS4* and *PKS13* in the $\Delta PKS12$ strain, along with the development of more favorable fermentation conditions.

Although the specific regulatory mechanism(s) between the biosyntheses of these two polyketides remains to be elucidated, both chemical and gene expression analyses using the *PKS*-deletion strains provide several lines of evidence that AUR accumulation is negatively coordinated with the regulation of the ZEA biosynthetic pathway in *G. zeae*. In addition, the present results may provide useful information for investigations of the coordinated regulatory network(s) for secondary metabolism in *G. zeae*.

Acknowledgments

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