

# *SnPKS19* Encodes the Polyketide Synthase for Alternariol Mycotoxin Biosynthesis in the Wheat Pathogen *Parastagonospora nodorum*

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**Alternariol (AOH) is an important mycotoxin from the *Alternaria* fungi. AOH was detected for the first time in the wheat pathogen *Parastagonospora nodorum* in a recent study. Here, we exploited reverse genetics to demonstrate that SNOG\_15829 (*SnPKS19*), a close homolog of *Penicillium aethiopicum* norlichexanthone (NLX) synthase gene *gsfA*, is required for AOH production. We further validate that *SnPKS19* is solely responsible for AOH production by heterologous expression in *Aspergillus nidulans*. The expression profile of *SnPKS19* based on previous *P. nodorum* microarray data correlated with the presence of AOH *in vitro* and its absence *in planta*. Subsequent characterization of the  $\Delta$ *SnPKS19* mutants showed that *SnPKS19* and AOH are not involved in virulence and oxidative stress tolerance. Identification and characterization of the *P. nodorum* *SnPKS19* cast light on a possible alternative AOH synthase gene in *Alternaria alternata* and allowed us to survey the distribution of AOH synthase genes in other fungal genomes. We further demonstrate that phylogenetic analysis could be used to differentiate between AOH synthases and the closely related NLX synthases. This study provides the basis for studying the genetic regulation of AOH production and for development of molecular diagnostic methods for detecting AOH-producing fungi in the future.**

Mycotoxins pose serious health risks to humans and animals (1, 2). Consumption of food and feed contaminated with mycotoxins can lead to various mycotoxicoses. Mycotoxins also cause significant economic losses in agriculture due to reduced crop value resulting from mycotoxin contamination and losses in animal productivity from mycotoxin-induced health problems (2). Furthermore, there are increasing concerns about mycotoxins in indoor environments (3), the contamination of water with mycotoxins in agricultural land affected by mycotoxin-producing plant pathogens (4), and the effect of climate change on mycotoxin contamination of pre- and postharvest food (5, 6).

Deciphering the molecular genetic basis of mycotoxin production in fungi will improve our understanding of its genetic regulation and facilitate the identification of potential mycotoxin-producing fungi in the environment (7, 8). There has been considerable interest in developing PCR-based diagnosis methods for detection of mycotoxin-producing fungi in agricultural commodities and in food and feed (9). Such diagnosis will also help determine if a fungal strain is suitable for use in the food industry or as a biocontrol agent. With the wide availability of genomic technologies, the genome of a fungal strain can be rapidly scanned for potential genes encoding mycotoxin biosynthesis. The genetic and molecular bases for biosynthesis of several important mycotoxins are well known (10), such as those for aflatoxins (11, 12), fumonisins (13, 14), zearalenone (15, 16), ergot alkaloids (17, 18), and trichothecenes (19, 20). Nonetheless, the molecular genetic bases for the production of many other mycotoxins, such as ochratoxins, altertoxins, and alternariol, remain elusive.

Alternariol (AOH) and alternariol-9-methyl ether (AME) are two important mycotoxins that are well known to be produced by fungi in the *Alternaria* genus, which are capable of infecting a variety of crop plants. The two mycotoxins are common contaminants of food such as grain and grain-based products, fruits/fruit juice, and vegetable products (in particular, tomato products) (21–23). They have been reported to exhibit cytotoxic, fetotoxic, teratogenic, and possible mutagenic and estrogenic effects (24–27)

and may contribute to development of human esophageal cancer (28). AOH has been demonstrated to inhibit cholinesterase (29) and topoisomerases I and II (30). Furthermore, it was shown that AOH induces DNA damage and cell cycle arrest *in vitro* in the murine macrophage RAW 264.7 cell line, and the treated cells exhibited an abnormal nuclear morphology (31, 32).

Recently, AOH was reported to be produced by *Parastagonospora nodorum* (synonymous to *Stagonospora nodorum* and *Phaeosphaeria nodorum*), an important wheat pathogen in Australia and worldwide. AOH was first detected in *P. nodorum* in a metabolomics study of a mutant lacking a short-chain dehydrogenase gene, *Sch1*, which accumulated AOH at a 200-fold-greater concentration than the wild type (WT) (33). *Sch1* was demonstrated to be regulated by G-protein signaling and is required for asexual sporulation (34). Subsequently, a transcription factor gene, *StuA*, has also been shown to be a positive regulator of AOH production (35). The discovery of AOH in *P. nodorum* raised new concerns about the health implication of this wheat pathogen.

Two polyketide synthase (PKS) genes have been recently inferred in an RNA-silencing knockdown study to be involved in AOH biosynthesis in *Alternaria alternata* (36). In this study, we

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have unequivocally identified the PKS gene responsible for the production of AOH in *P. nodorum* by both targeted genetic disruption and heterologous expression. The *P. nodorum* AOH PKS gene is different from that identified previously but is highly conserved with another PKS gene in *A. alternata*. Furthermore, identification of the *P. nodorum* PKS AOH gene has enabled the survey of homologs in other fungal genomes.

## MATERIALS AND METHODS

***P. nodorum* strains and culturing conditions.** The wild-type *P. nodorum* strain SN15 was obtained from Department of Agriculture and Food Western Australia (DAFWA) and had been previously deposited in American Type Culture Collection (ATCC MYA-4574) and the Fungal Genetics Stock Center (FGSC 10173). Both the wild-type and mutant strains generated in this study were maintained on V8-supplemented potato dextrose agar (V8-PDA) plates at 22°C under a 12-h dark/light regime, which induces sporulation (37). For screening of AOH and AME production in *P. nodorum* wild-type strain SN15 and  $\Delta$ SnPKS19 mutants, the strains were grown in defined minimal medium agar (MMA) (37) in the dark at 22°C for 7 days.

*Aspergillus nidulans* strain TNO2A3 (from the Fungal Genetics Stocks Centre, FGSC A1149) was maintained on *Aspergillus* glucose minimal medium (GMM) agar (38) supplemented with 0.5 mM pyridoxine, 0.01  $\mu$ g/ml riboflavin, 5 mM uridine, and 5 mM uracil. *Aspergillus nidulans* TNO2A3 and the transformed mutants were cultured in liquid GMM at 28°C with shaking 150 rpm.

**Metabolite profile analysis.** For the detection of AOH and AME in the *P. nodorum* SN15 extracts from various growth conditions, liquid chromatography-mass spectrometry (LC-MS) was performed on an Agilent 1200 LC system (Agilent, Santa Clara, CA, USA) coupled to an Agilent 6520 quadrupole time of flight (QToF) system with a Jetstream electrospray ionization (ESI) source to achieve higher sensitivity (see the methods in the supplemental material).

For comparison of the metabolite profiles of *P. nodorum*  $\Delta$ SnPKS19 strains against that of the wild type, agar blocks cut out from a 10-day-old MMA culture were extracted with an approximately equal volume of ethyl acetate (EtOAc). For *A. nidulans* expressing SnPKS19, 10 ml of liquid GMM cultures was sampled at 48 and 72 h postinoculation and extracted with an equal volume of EtOAc. The extracts were dried *in vacuo* and redissolved in methanol (MeOH). The metabolite profile analyses were performed on an Agilent 1200 LC system coupled to a diode array detector (DAD) and an Agilent 6120 quadrupole MS with an ESI source. Chromatographic separation and conditions were exactly as described previously (39). The authenticity of AOH was confirmed by comparing the *m/z* ratio, UV spectrum, and retention time with those of the corresponding commercial standards (LGC standards).

**Transformation and screening of *P. nodorum*  $\Delta$ SnPKS19 mutants.** A double-homologous SnPKS19 knockout (KO) cassette carrying a hygromycin resistance marker was assembled using the Gibson assembly method (40) on a pGEM-T easy vector backbone (Promega). The hygromycin resistance marker was amplified from pAN7-1 (41) using GPE1-F and TtrpC-R primers (see Table S1 in the supplemental material). The 5' and 3' homologous regions of SnPKS19 (1.6 kbp each) were amplified with the SnPKS19-KO-P2/P3 and SnPKS19-KO-P4/P5 primer pairs, respectively. The three overlapping DNA fragments and the linearized vector backbone were assembled using the Gibson assembly master mix (NEB) following the manufacturer's protocol. The resulting plasmid, pGEM-SnPKS19-KO, was sequenced to confirm the construct. Finally, the 5.8-kbp SnPKS19 KO cassette (see Fig. S3 in the supplemental material) was liberated by digesting the plasmid with NotI enzyme (NEB) and gel purified for *P. nodorum* transformation. Transformation of *P. nodorum* with the SnPKS19 KO cassette was achieved by the polyethylene glycol (PEG)-mediated protoplast transformation protocol as described previously (42). The integration of the KO cassette in the correct locus was confirmed by diagnostic PCR using two pairs of primers, SnPKS19-KO-

P1/Hyg-N-R and Hyg-C-F/SnPKS19-KO-P6, as described previously (43). The deletion of SnPKS19 was further confirmed by Southern blotting using a digoxigenin (DIG) DNA labeling and detection kit (Roche) according to the manufacturer's instructions.

**Characterization of the virulence and oxidative stress tolerance of *P. nodorum*  $\Delta$ SnPKS19 mutants.** A modified detached-leaf assay (DLA) was used to assess the virulence of *P. nodorum*  $\Delta$ SnPKS19 mutants against wheat. The protocol was as described previously (44), with the exception of using 0.25% gelatin instead of 0.2% Tween 20 for spore suspension. The oxidative tolerance of *P. nodorum*  $\Delta$ SnPKS19 mutants compared to wild type was determined by placing a 5-mm-diameter agar block on MMA containing 0, 2, 5, and 10 mM hydrogen peroxide in triplicates, as described previously (45).

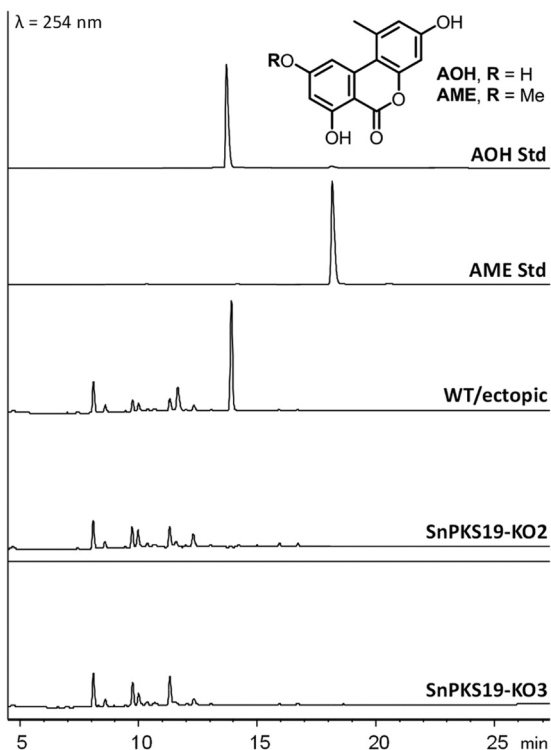
**Cloning and heterologous expression of SnPKS19 in *Aspergillus nidulans*.** The original annotations of the SnPKS19 translated protein in the GenBank and JGI databases lack the N-terminal starter unit:acyl carrier protein (ACP) transacylase (SAT) domain. The missing SAT domain conserved among nonreducing PKSs (NR-PKSs) was located by TBLASTX analysis with an additional 1,000 bp upstream of the SnPKS19 coding region added as a query sequence. The SAT domain was restored by manual annotation with the corrected annotation, placing the new ATG start codon 656 bp upstream of the original start codon.

SnPKS19 was cloned into the pBARGPE1-LIC expression plasmid. pBARGPE1-LIC was modified from pBARGPE1 (from FGSC) for ligation-independent cloning (LIC) as described previously (46). The plasmid contains an *gpdA* promoter and *trpC* terminator for gene expression and a *bar* gene for glufosinate resistance. The revised full-length SnPKS19 coding region, including introns (see Fig. S5 in the supplemental material), was PCR amplified from *P. nodorum* SN15 genomic DNA using primers LIC-SnPKS19-F\_new and LIC-SnPKS19-R (see Table S1 in the supplemental material). DNA assembly using LIC was performed as described previously (46). Transformation of *A. nidulans* TNO2A3 with the resulting plasmid, pBGP-SnPKS19, was performed using the PEG-mediated transformation protocol as described previously (47). For selection of glufosinate-resistant transformants, the glufosinate was prepared from the commercial herbicide Basta (Bayer CropScience) as described previously and added at 25  $\mu$ l/ml of *Aspergillus* glucose minimal medium with 10 mM ammonium tartrate as the sole nitrogen source (48).

The transcription of SnPKS19, *pkgA* (ANIA\_07071), and *pkgB* (ANIA\_07070) in the *A. nidulans* strain transformed with pBG-SnPKS19 was checked using reverse transcriptase PCR (RT-PCR), as described previously (46), with primer pairs SnPKS19-RT-F/R, AN7071-RT-F/R, AN7070-RT-F/R, which flank the last (3'-end) introns in the three respective genes (see Table S1 in the supplemental material).

**Phylogenetic analysis.** The conserved  $\beta$ -keto-acyl synthase (KS) domain sequences were used to infer the phylogenetic relationship between SnPKS19 and other fungal NR-PKSs. A total of 19 NR-PKSs characterized in previous studies were included as reference sequences, while seven additional characterized highly reducing PKSs (HR-PKSs)/PKS-NRPs were included as outgroup (see Table S2 in the supplemental material). To investigate the distribution of AOH and norlichexanthone (NLX) synthases, the phylogenetic analysis further included all NR-PKSs with KS domains that shared >80% identity with SnPKS19 from the NCBI GenBank and JGI Ascomycota (<http://genome.jgi-psf.org/ascomycota/ascomycota.info.html>) databases. Multiple-protein alignment was performed using CLUSTALX, and the resulting alignment was trimmed (corresponds to amino acids 418 to 821 of the corrected SnPKS19 protein sequence). The phylogenetic analysis was performed using the neighbor-joining method (49) embedded in the Geneious software 7.17 software (Biomatters Ltd.). The tree was constructed with 1,000 bootstrap replicates, and branches corresponding to partitions that were reproduced in fewer than 50% of bootstrap replicates are collapsed.

**Nucleotide sequence accession number.** The corrected annotation for SnPKS19 has been deposited in GenBank under accession no. KP941080.



**FIG 1** Comparison of metabolite profiles of the *P. nodorum* wild-type (WT) strain and  $\Delta$ *SnPKS19* mutants grown on minimal medium agar. UV chromatograms of *P. nodorum* WT/ectopic mutant and  $\Delta$ *SnPKS19* mutants are shown in comparison to AOH and AME standards.

## RESULTS

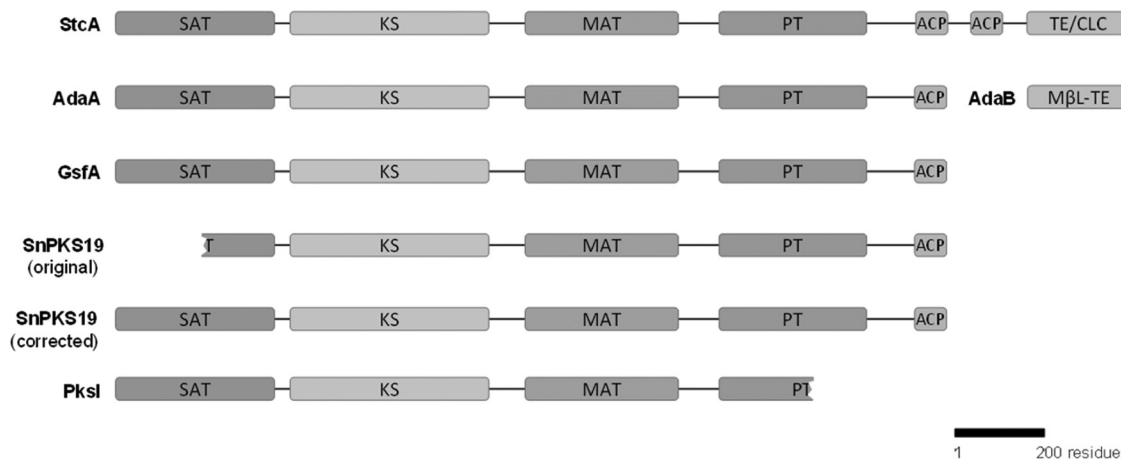
**Detection of alternariol under various growth conditions.** We first investigated for growth conditions that favor AOH production in the *P. nodorum* SN15 wild-type strain. The strain was cultured on different media and under different growth conditions and checked for AOH production by LC-DAD-MS. *P. nodorum* produced AOH ( $m/z$  259) when grown in defined minimal agar medium (MMA) at 22°C after 7 days (Fig. 1). AOH could also be

detected in shaking *P. nodorum* Fries medium cultures (see the methods and Fig. S1 in the supplemental material). To detect the possible production of AOH *in planta*, two experiments were performed, i.e., detached-leaf assay (DLA) and *in vivo* glume infection assay (see the methods in the supplemental material). However, we failed to detect any AOH in either the infected wheat leaves from DLAs or the infected wheat grains from glume infection assays (see Fig. S1 in the supplemental material).

### Bioinformatic analysis of putative PKS genes in *P. nodorum*.

We previously performed a detailed survey of the *P. nodorum* genome for secondary metabolite genes and have compiled the polyketide synthase (PKS) gene inventory for this wheat pathogen (50). To identify the PKS gene encoding AOH in *P. nodorum*, we first searched for *P. nodorum* PKSs that are homologous to PksH and PksJ, which were previously implied to be involved in AOH production in *A. alternata* by RNA interference (RNAi) silencing experiments (36). However, a BLASTP search did not identify any close homolog of *A. alternata* PksH and PksJ in *P. nodorum*.

To further expand the search for the AOH PKS, a genome-wide comparison of all the PKSs in *P. nodorum* and *A. alternata* (36) identified another PKS from *A. alternata* (PksI) sharing significant homology (86% identity) to SNOG\_15829 (abbreviated as SnPKS19) in *P. nodorum*. SnPKS19 is a typical nonreducing PKS (NR-PKS) (51, 52), consisting of a SAT domain, a  $\beta$ -keto-acyl synthase (KS) domain, a malonyl coenzyme A (malonyl-CoA): ACP transferase (MAT) domain, a product template (PT) domain, and an acyl carrier protein (ACP) domain. The original annotation of SnPKS19 in the GenBank and JGI databases lacked the SAT domain, but this N-terminal domain was restored by manual annotation (Fig. 2; see Fig. S4 in the supplemental material). PksI from *A. alternata*, however, is truncated at the 3' end based on the annotation by Saha et al., and the predicted PKS protein lacks the C-terminal ACP domain (Fig. 2) (36). The closest characterized homolog for the two PKSs in the GenBank protein database is the norlichexanthone (NLX) synthase (GsfA) in the griseofulvin pathway in *Penicillium aethiopicum* (synonym, *Penicillium lanosocoeruleum*) (53, 54), which share high percentages of protein identity (overall protein, 65% identity; KS domain, 83% identity). Both AOH and NLX are derived from a heptaketide



**FIG 2** Comparison of the SnPKS19 domain architecture with those of other nonreducing polyketide synthases (NR-PKSs). SAT, starter unit:ACP transacylase domain; KS,  $\beta$ -keto-acyl synthase domain; MAT, malonyl-CoA:ACP transferase domain; PT, product template domain; ACP, acyl carrier protein domain; TE/CLC, thioesterase/Claisen cyclase domain; M $\beta$ L-TE, metallo- $\beta$ -lactamase-like thioesterase. M $\beta$ L-TE is a discrete protein.

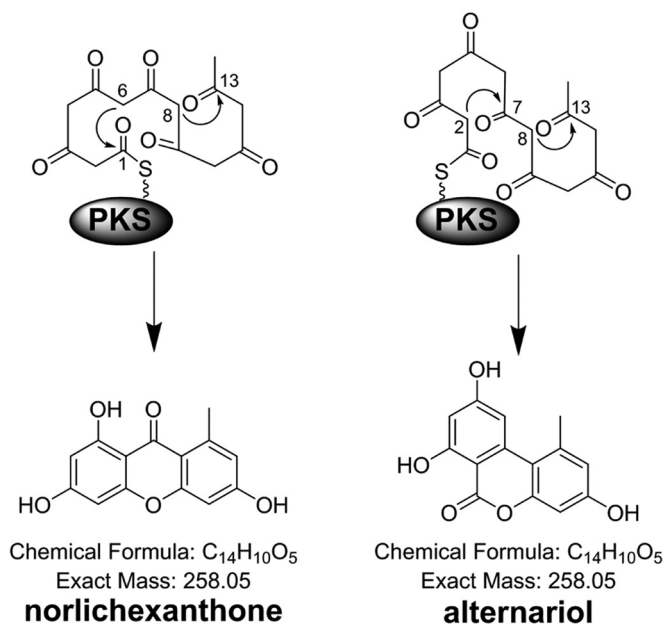


FIG 3 Biosynthesis of alternariol (AOH) in comparison to norlichexanthere (NLX) by polyketide synthases.

chain and involve a C-8–C-13 aldol cyclization in the biosynthesis, but the regiospecificity of the second cyclization diverges (Fig. 3). As AOH and NLX are structural isomers, we reasoned that it is highly likely that the PKS gene *SnPKS19* may encode the biosynthesis of AOH.

The genes in the vicinity of *SnPKS19* encode two putative *O*-methyltransferases, a putative  $\beta$ -lactamase, a putative short-chain dehydrogenase, a putative regulatory protein, and a putative transporter (see Fig. S2 in the supplemental material). We extracted the expression profiles of *SnPKS19* and its neighboring genes from previous microarray data (55, 56). The production of AOH in *P. nodorum* MMA culture and its absence in DLA samples correlated with the expression of *SnPKS19* during the growth *in vitro* and its silence *in planta* (see Fig. S2 in the supplemental material). One of the putative *O*-methyltransferase genes (SNOG\_15830), along with the colocated regulatory protein (SNOG\_15831) and the putative short-chain dehydrogenase (SNOG\_15832) genes, appears to be coregulated with *SnPKS19*, all with higher expression *in vitro* at 16 days postinoculation (dpi) than at 4 dpi, and all were silent *in planta*. Despite the presence of the putative *O*-methyltransferase gene in the gene cluster, we did not detect any *O*-methylated derivative of AOH in *P. nodorum* MMA culture (Fig. 1) and under the various other growth conditions tested.

**SnPKS19 is required for alternariol production in *P. nodorum*.** To confirm that the *SnPKS19* gene is involved in AOH biosynthesis, *P. nodorum*  $\Delta$ *SnPKS19* mutants were generated by polyethylene glycol (PEG)-mediated transformation with an *SnPKS19* knockout cassette. Diagnostic PCR identified three positive transformants where *SnPKS19* had been disrupted by double-homologous crossover recombination and replaced with a hygromycin resistance marker (see Fig. S3 in the supplemental material). The deletion of *SnPKS19* in two transformants (*SnPKS19*-KO2 and -KO3) was further confirmed by Southern blotting (see Fig. S4 in the supplemental material). No observable

growth defect or differences in the growth rate between the mutants and the wild-type (WT) *P. nodorum* were observed. The colony morphology of the mutants on V8-PDA and minimal medium plates were also indistinguishable from that of the WT.

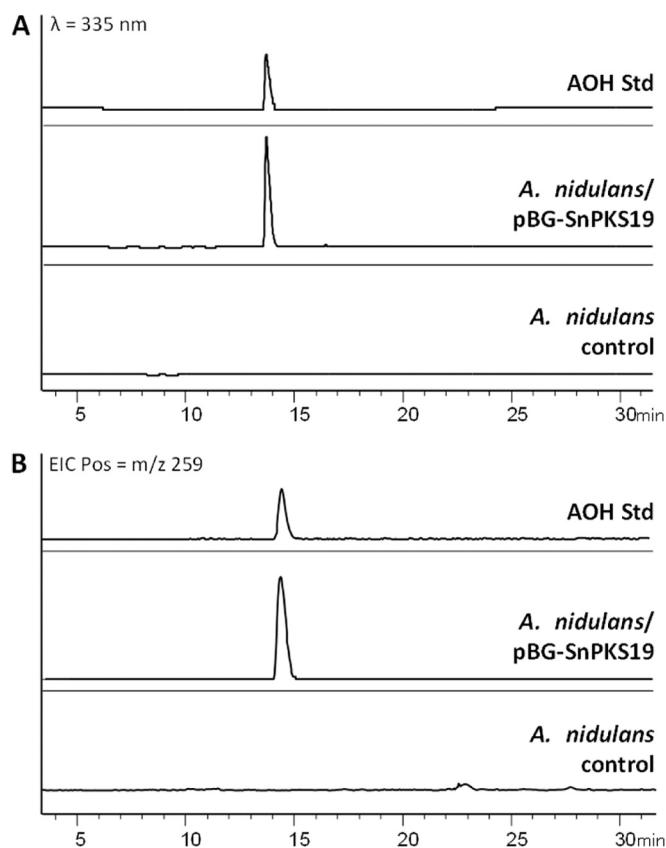
Five transformants, including three  $\Delta$ *SnPKS19* mutants and two ectopic mutants, were selected and grown on MMA under the above-mentioned condition. Comparative LC-MS metabolite profile analysis of the EtOAc extracts from the cultures showed that production of AOH was abolished in all the  $\Delta$ *SnPKS19* mutants but remained present in both ectopic mutants and the WT (Fig. 1). Thus, this confirmed that *SnPKS19* is required for AOH biosynthesis in *P. nodorum*.

**SnPKS19 alone is sufficient for biosynthesis of alternariol.** The predicted PKS protein encoded by *SnPKS19* lacks the polyketide product-releasing C-terminal thioesterase (TE)/Claisen cyclase (CLC) domain commonly found in fungal NR-PKSs (57, 58). Previous biosynthetic studies have shown that some fungal NR-PKSs lacking a releasing domain require an additional discrete metallo- $\beta$ -lactamase-like TE (M $\beta$ L-TE) protein to facilitate polyketide chain release or cyclization (Fig. 2) (54, 59–61). Thus, the putative  $\beta$ -lactamase gene (SNOG\_15826) in the vicinity of *SnPKS19* may be required for releasing of AOH via lactone formation. However, upon close inspection, SNOG\_15826 is seen to share little similarity to the characterized M $\beta$ L-TEs, as it belongs to a different family of  $\beta$ -lactamases. Furthermore, the previous microarray data showed that SNOG\_15826 was not coregulated with the PKS gene *SnPKS19* (see Fig. S3 in the supplemental material), which supported its noninvolvement in AOH biosynthesis.

To determine if *SnPKS19* alone was sufficient for AOH synthesis, *SnPKS19* with the restored 5' region was cloned into plasmid pBARGPE1-LIC for heterologous expression. The resulting plasmid, pBG-*SnPKS19*, was used to transform *A. nidulans* strain TNO2A3 (from FGSC). Two glufosinate-resistant transformants were verified to contain the *SnPKS19* gene by PCR and were cultured in *Aspergillus* minimal liquid medium for 72 h at 28°C. LC-DAD-MS analysis of the EtOAc extracts from the cultures showed that both transformants produced a unique peak with *m/z* (*M* + *H*)<sup>+</sup> of 259, corresponding to AOH, which was absent in the *A. nidulans* control strain transformed with the empty pBARGPE1-LIC plasmid (Fig. 4). The result showed that *SnPKS19* alone is sufficient for biosynthesis of AOH in *A. nidulans*.

In a previous study, promoter replacement of the NR-PKS gene *pkgA* (ANIA\_07071) and the M $\beta$ L-TE gene *pkgB* (ANIA\_07070) in *A. nidulans* resulted in production of AOH along with three other isocoumarins. To rule out that these two genes are involved in AOH production in the *A. nidulans* strains harboring *SnPKS19*, we performed an RT-PCR to determine the expression of *SnPKS19* and the two genes at 72 h postinoculation. RT-PCR showed that *SnPKS19*, but not *pkgA* and *pkgB*, was transcribed and spliced in the *A. nidulans*/pBG-*SnPKS19* strains (see Fig. S6 in the supplemental material).

**SnPKS19 is not involved in the virulence and oxidative stress protection of *P. nodorum*.** The ability of the  $\Delta$ *SnPKS19* mutant strains (*SnPKS19*-KO2 and -KO3) to cause disease was assessed using detached-leaf assays. No significant difference in disease symptoms was observed on detached wheat leaves sprayed with spores of  $\Delta$ *SnPKS19* mutants compared to the controls (wild-type and ectopic integration strains) (see Fig. S7A in the supplemental material).



**FIG 4** Heterologous production of AOH in *Aspergillus nidulans*. A UV chromatogram (A) and an extracted ion chromatogram (EIC) ( $m/z$  259) (B) of the culture extracts from *A. nidulans* transformed with pBG-SnPKS19 in comparison to a control and an AOH standard are shown.

Some secondary metabolites are known to possess antioxidant activities and are thought to serve as protectants against oxidative stress (62). However, we did not observe a significant change in the ability to tolerate increasing concentration of hydrogen peroxide between  $\Delta$ SnPKS19 mutant strains and wild-type strain SN15 (data not shown).

**Alternariol does not exhibit phytotoxic or antimicrobial activities.** The phytotoxic activity of AOH on wheat leaves was examined by leaf infiltration on whole plant wheat seedlings (see the methods in the supplemental material). No necrosis was observed on wheat leaves at concentrations up to 200  $\mu$ g/ml of AOH (see Fig. S7B in the supplemental material). The capacity of AOH to affect seed germination was also assessed. Unlike what we had observed for (*R*)-mellein in a previous study (39), AOH did not inhibit the germination of wheat and barrel medic seeds (data not shown). We have also tested the antimicrobial activity of AOH against *Escherichia coli*, *Saccharomyces cerevisiae*, *Zysoseptoria tritici*, and three environmental bacterial strains isolated from field wheat samples (*Bacillus cereus*, a *Flavobacterium* sp., and *Sphingobacterium multivorum*) (see the methods in the supplemental material). AOH did not inhibit the growth of any of the bacterial and fungal strains tested at concentrations up to 200  $\mu$ g/ml.

**Phylogenetic analysis can differentiate alternariol synthases from other fungal PKSs.** Previous studies have shown that fungal NR-PKSs (without a C-methyltransferase domain) can be classi-

fied by phylogeny into five major groups plus two additional groups that harbor a C-methyltransferase domain (59, 63). The two studies showed that the phylogenetic grouping correlated with the polyketide product chain length and cyclization regioselectivity of NR-PKSs. To determine the phylogenetic relationship of SnPKS19 with NLX synthase GsfA and in relation to other NR-PKSs, three PKSs from each group of group I to IV NR-PKSs, along with six PKS sequences from group V, were selected as reference sequences for phylogenetic analysis (see Table S2 in the supplemental material). The phylogenetic tree showed that the NR-PKSs were separated into the five respective groups (groups I to V), as expected (Fig. 5).

GsfA and SnPKS19 along with their close homologs grouped together with other group V NR-PKSs but formed a separate subclade. Within this subclade, SnPKS19 and GsfA homologs were further separated into two smaller subclades, referred to here as the AOH and NLX subclades, respectively. Within the NLX subclades, there are two other NR-PKSs that originated from known NLX/griseofulvin-producing fungi, i.e., *Penicillium canescens* (Penca1|243077) and *Penicillium raistrickii* (Penra1|289401) (Fig. 4) (64). Likewise, the AOH subclade is supported by NR-PKSs from AOH producers, including *A. alternata* (PksI) and *Talaromyces aculeatus* (Penac1|509622) (65). These analyses suggest that the NR-PKS homologs that fall in the AOH and NLX subclades may produce the respective compounds.

## DISCUSSION

Using both reverse genetics and heterologous expression, we recently identified the *P. nodorum* PKS gene responsible for production of (*R*)-(-)-mellein, which exhibited antigerminative activity (39). Here, using a similar approach, we unequivocally demonstrated that SnPKS19, which is highly similar to the *A. alternata* pksI product (86% protein identity), is solely responsible for the biosynthesis of AOH in *P. nodorum*. Saha et al. previously reported the involvement of pksH and pksJ in AOH biosynthesis in *A. alternata* based on RNAi silencing experiments (36). In contrast to PksH and PksJ, which are HR-PKSs commonly involved in biosynthesis of aliphatic compounds, both the SnPKS19 and PksI genes encode an NR-PKS that is typically involved in biosynthesis of aromatic polyketides (51). Curiously, the pksI in *A. alternata* is annotated as a truncated PKS gene (Fig. 2) (36). Quantitative real-time reverse transcriptase PCR (qRT-PCR) from this previous study by Saha et al. showed that pksI was transcribed and that its expression correlated to the timing of AOH production, suggesting that pksI is unlikely to be a pseudogene. Nevertheless, pksI was not investigated further (36). It is not uncommon that RNA silencing could result in off-target effects (66–68). Given that the highly conserved KS domain was targeted in the study by Saha et al., it is possible that the transcription of other PKS genes was knocked down as well due to the potential complementary nature of the nucleotide sequences. For example, transcription of both pksI and -J was silenced in the pksH knockdown strain (36), which may also explain the observed reduced AOH production if pksI is responsible for AOH production in *A. alternata*. It is possible that the presumed truncation of the pksI coding sequence is due to automated annotation error (like in the original SnPKS19 annotation) or frameshift errors in sequencing. Based on our results from this study, it is very likely that pksI is the gene responsible for AOH production in *A. alternata*. Further investigation is required to verify the identity of AOH synthase in *A. alternata*.

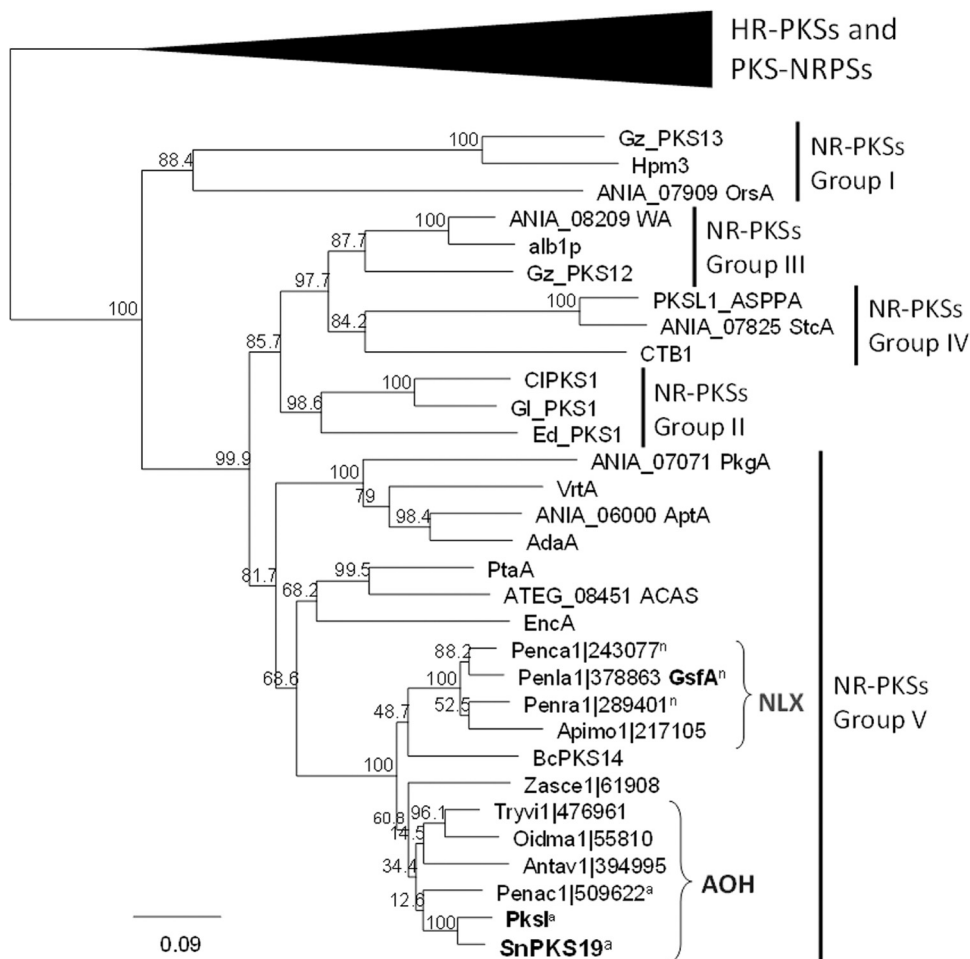


FIG 5 Phylogeny and distribution of AOH synthase homologs in fungi in relation to other fungal NR-PKSs. KS sequences used to construct the phylogenetic tree are listed in Table S2 in the supplemental material.

An NR-PKS gene (*pkgA*) from *A. nidulans* has been recently reported to produce AOH along with a mixture of several hexa- and heptaketide isocoumarin products, but only in the presence of the partner M $\beta$ L-TE gene (*pkgB*) (59). The abundances of the heptaketides citreoisocoumarin and dehydrocitreoisocoumarin are comparable to that of AOH in the *pkgA* and *-B* overexpression strain. Overexpression of the PKS gene *pkgA* alone by promoter exchange did not result in any observable product (59). Here, we used the *A. nidulans* system to characterize *SnPKS19* by heterologous expression and showed that the *SnPKS19*-expressing strains accumulated significant quantities of AOH at 72 h. No related isocoumarins identified in the previous study by Ahuja et al. (59) were detected in the *SnPKS19*-expressing *A. nidulans* strains, and the involvement of *pkgA* and *-B* is ruled out based on the RT-PCR results (see Fig. S6 in the supplemental material) and the absence of AOH in the control strain. Thus, we reasoned that AOH is the major and final product of *SnPKS19*, while AOH along with the hexaketide isocoumarins may be aberrant or derailed products of *PkgA* and *-B*, possibly due to overexpression of both genes at the same level (a nonoptimal gene/enzyme ratio) (69).

The PT domain of NR-PKSs mediates the cyclization of non-reduced polyketide chains into various aromatic systems (52, 70). All the isocoumarin products produced by *PkgA* and *-B* shared the

C-2–C-7 cyclized ring in AOH. In contrast, NLX shared the C-8–C-13 cyclized ring in AOH (Fig. 3). It is possible that the polyketide biosynthesis in *PkgA* involved PT domain-controlled C-2–C-7 cyclization followed by-product release via hydrolysis or lactonization catalyzed by *PkgB*. AOH was likely formed in the *pkgA*- and *B*-overexpressing strain via spontaneous aldol cyclization after polyketide release, whereas the PT domain of NLX and AOH synthases is likely to mediate the C-8–C-13 cyclization instead; the two pathways diverged during the second ring cyclization (C-6–C-1 Claisen cyclization or C-2–C-7 aldol cyclization) to yield NLX or AOH, respectively.

Previous phylogenetics analysis showed that the NLX synthase *GsfA* fell within the group V NR-PKSs (59). The present study showed that *GsfA* grouped together with group V NR-PKSs as well but formed a separate subclade with *SnPKS19* and other putative NLX/AOH synthases from the group V NR-PKSs that require a discrete M $\beta$ L-TE for product release, which include *ACAS* (60), *AptA* (71), *VrtA* (54), *AdaA* (61), *PkgA* (59), and *EncA* (72). Thus, the branching between these two subgroups of group V NR-PKSs could represent the point where the partnership with M $\beta$ L-TE was lost in the NLX/AOH synthases. It appears that the NLX/AOH synthases have evolved the ability to off-load PKS products by Claisen cyclization or hydrolysis/lactonization without C-termi-

nal appended TEs or discrete MBL-TEs. The molecular mechanism and the functional domain responsible for the polyketide release in these TE-less NLX/AOH synthases remain to be elucidated.

Based on the phylogenetic analysis, SnPKS19 and GsfA can be further divided into two smaller subclades consisting of putative AOH and NLX synthases, respectively. Both subclades are supported by two additional NR-PKSs from known AOH or NLX producers. Besides the high similarity shared between SnPKS19 and Penac1|509622 from the AOH/AME producer *T. aculeatus*, the two PKS gene clusters also encode a homologous O-methyltransferase (SNOG\_15830/Penac1|509623; 71% protein identity), suggesting that these two enzymes are likely involved in methylation of AOH. Although we did not detect any AME in *P. nodorum* under the growth conditions tested in this study, the ability of *P. nodorum* to produce AME cannot be excluded. Based on the gene cluster map reported by Saha et al., an O-methyltransferase is encoded next to *A. alternata pksI* as well (36), but the corresponding gene was not deposited in a public database and therefore cannot be compared. Similarly, the gene clusters containing the putative NLX synthase gene in the NLX/griseofulvin producers *P. canescens* and *P. raistrickii* encode all the homologous enzymes in the *P. aethiopicum gsf* gene cluster required for griseofulvin biosynthesis (53, 54). These data together strongly support that phylogenetic analysis can be used to predict whether an NR-PKS gene is responsible for production of AOH or NLX using SnPKS19 and GsfA as reference sequences.

It is difficult to determine the exact number of fungal genome sequences deposited in the NCBI GenBank database (312 are ascomycetes listed in NCBI Genome), but the search for AOH synthase homologs in the JGI database (<http://genome.jgi-psf.org/ascomycota/ascomycota.info.html>) included 259 ascomycetes. Although the AOH synthase gene does not appear to be ubiquitous, the orthologs are not restricted to a single ascomycete class but distributed across Dothideomycetes, Leotiomycetes, Eurotiomycetes, and Sordariomycetes (Fig. 5; see Table S2 in the supplemental material). It is less certain whether the two PKSs (*Botrytis cinerea* BcPKS14 and *Zasmidium cellare* Zasc1|61908) basal to the NLX and AOH clades are responsible for production of NLX or AOH. Interestingly, a dibenzopyrone botrallin, structurally related to AOH, has been reported from *Botrytis allii* (73, 74). There is also at least one instance where a lichenized fungus was reported to produce both AOH and NLX (75). Thus, the possible existence of such NR-PKSs that have the promiscuity to produce both structural isomers cannot be discounted. AOH had also been reported in an endophytic *Colletotrichum* sp. (76), but no close homolog of AOH synthase has been found among the sequenced *Colletotrichum* genomes.

Due to their occurrence in plant-pathogenic *Alternaria* species, AOH and AME had also been investigated for potential phytotoxic activities, but most reports are negative (77, 78). Interestingly, AME has been reported to suppress cyanobacterial growth by inhibiting the photosynthetic electron transport chain (79). In this study, we showed that the virulence of  $\Delta$ SnPKS19 against wheat leaves *in vitro* is comparable to that of the wild type; thus, AOH is unlikely to play a role in pathogenicity. This is not surprising given that the transcription of SnPKS19 is silent *in planta* based on previous microarray data and that we did not detect any AOH in infected wheat leaves or grains. Likewise, we did not detect any phytotoxic activity against wheat for AOH using leaf infiltration

and seed germination. We have tested AOH against common laboratory microorganisms and environmental isolates of bacteria and fungi as well, but we did not detect any growth-inhibitory activity. Thus, the biological and ecological roles of AOH remain a mystery.

AOHs are often detected in wheat, sorghum, and barley grains contaminated with *Alternaria* fungi and pose a health risk to humans and animals (23). However, an analysis of *P. nodorum*-infected grains in this study failed to detect any AOH. Furthermore, there has been no prior report of AOH in *P. nodorum*-contaminated grains, suggesting that AOH mycotoxin production is not part of the disease cycle. Given that *P. nodorum* has the genetic potential to produce AOH and has been shown to produce AOH under laboratory culture conditions, it cannot be discounted that the fungus may produce AOH on wheat grain when the conditions become favorable, especially in the face of changing climatic environments. Recently, it has shown that AOH production in *A. alternata* is affected by light conditions and partially regulated by the blue-light receptor LreA (80). The identification of the PKS gene encoding AOH production in this study opens up the opportunity for investigating the molecular genetic regulation of AOH in *P. nodorum* and other AOH-producing fungi. This discovery will also facilitate the assessment of the genetic potential for production of AOH in other fungi and the development of molecular diagnostic methods for detection and quantification of AOH-producing fungi in agricultural commodities and in food and feed.

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