SUPPLEMENTAL MATERIAL

SnPKS19 encodes the polyketide synthase for alternariol mycotoxin biosynthesis in the wheat pathogen *Parastagonospora nodorum*

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Running Head: Alternariol synthase from *Parastagonospora nodorum*

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SUPPLEMENTAL METHODS

Detection of alternariol (AOH) in various growth conditions

To achieve higher sensitivity for detection of AOH and AME in various growth conditions, LC-QToF/MS was used for the metabolite analyses. *P. nodorum* SN15 was grown in modified Fries liquid medium (30 g sucrose, 5 g ammonium tartrate, 1 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.13 g $CaCl_2$, 0.1 g NaCl) (1) in the dark at 22°C with shaking at 125 rpm for 15 days. Mycelia from the Fries liquid medium cultures were lyophilized and then macerated in EtOAc to extract the metabolites. The extracts were vacuum filtrated, dried *in vacuo* and resuspended in MeOH for metabolite analysis.

We used both detached leaf assays (DLA) and wheat glume infection assays to screen for AOH and AME production *in planta*. For DLA, leaves from 10-day-old seedlings were cut into 5 cm segments. The leaf segment tips were embedded in water-agar + 0.3% benzimidazole petri dishes forming bridges. Three droplets of 5µl of spore solution at 1×10^6 spores/ml in 0.2% Tween 20 were placed on the surface of the leaf bridges. Petri dishes containing the inoculated leaves were sealed with Parafilm® and incubated at 22˚C in a 12 hour light/dark cycle. The infected leaves were harvested 10 days after initial inoculation and extracted with EtOAc, dried *in vacuo* and resuspended in MeOH for metabolite analysis. For glume infection assays, wheat heads of 55-day-old wheat plants were inoculated with solution containing 2×10^6 spores/ml in 0.2% of Tween 20. The inoculated plants were watered for another 22 days and the seeds were collected after 10 days from when the watering stopped. To confirm successful infection of the wheat seeds, a small sample of wheat seeds were surfaced-sterilized and placed on tap water agar for re-isolation of the *P. nodorum* SN15 strain. The infected seeds were extracted with EtOAc, dried *in vacuo* and resuspended in MeOH for metabolite analysis.

LC-QToF/MS analysis was performed on an Agilent 1200 LC coupled to an Agilent 6520 quadrupole time-of-flight (QToF) system with Jetstream electrospray ionization (ESI) source. A 1µg/ml AOH and AME in MeOH was prepared from the 100µg/ml commercial standards (LGC standards) to be run by LC-MS. Chromatographic analyses were performed using a ZORBAX Eclipse XDB-C18 RRHD (2.1x150mm, 1.8µm) column at 40˚C. The gradient employed was: 2-10% B over 10 min, 10-25% B over 20 min, 50% B over 25 min 70% B over 30 min, 70% over 20min at a 200µl/min flow using H2O + 0.5% formic acid (HF) as solvent A and 90% MeCN in water + 0.5% HF as solvent B. The injection volume for all

runs was 5µl. The data were collected in the *m/z* range 100–1000 amu in positive mode. Results were processed with the Mass Hunter Workstation Qualitative Analysis B.04.00 software (Agilent).

Phytotoxicity, antigerminative and antibacterial activity assays

The phytotoxicity of AOH (Cayman Chemical) was assayed on the leaves of 11 day-old wheat seedlings that were grown at 22°C under a 12 h light/dark cycle regime, as described previously (2). Briefly, approximately 100 µL of each compound in 2% MeOH solution was infiltrated on the adaxial face of leaves at 50 and 100 µg/mL concentrations using a 1 mL volume syringe. Experiments were performed in six replicates and the infiltrated leaves were examined for the presence of necrosis or chlorosis after 24, 48 and 72 hours.

The ability of AOH to inhibit seed germination was assayed as described previously (2). Briefly, seeds of wheat (*Triticum aestivum* cv. Grandin) and Barrel medic (*Medicago truncatula* cv. Jemalong A17) are first surface-sterilized briefly in a solution containing 10% ethanol and 1% hydrogen peroxide. A single grain of the surface-sterilized seed was then placed on agar slants consist of 1.5 ml of tap water agar supplemented with either AOH and AME at 100 µg/mL final concentration (agar containing 2% methanol is used as control). The assays were performed in triplicates and the progress of seed germinations were observed and recorded for one week.

The antimicrobial activity of AOH was tested against *Escherichia coli*, *Saccharomyces cerevisiae*, *Zymoseptoria tritici*, and three environmental bacterial strains isolated from field wheat samples (*Bacillius cereus, Flavobacterium* sp. and *Sphingobacterium multivorum*). The antimicrobial activity was performed in 96 well plates with decreasing concentration of AOH (media containing 2% methanol was used as control). Bacteria were grown in lysogeny broth (LB), *Z. tritici* in potato dextrose broth (PDB), and *S. cerevisiae* in yeast peptone dextrose (YPD). Each well contains 0.5×10^4 cells or spores. The final concentrations of AOH in the wells were 200, 100, 50, 25, 12.5, 6.75, 3.37, and 1.65 µg/ml. The plates were incubated at 28 °C for *S. cerevisiae*, *Bacillius cereus* and *S. multivorum*, 22°C for *Z. tritici*, 37 °C for *E. coli* and *Flavobacterium* sp. The 96-well plates were observed daily until the cultures in control wells were growing.

SUPPLEMENTAL TABLES

Table S1. Primers used in this study.

Primer name	Sequence (5' to 3')
GPE1-F	CATGCGGAGAGACGGACGGAC
TtrpC-R	GGTCGAGTGGAGATGTGGAGT
SnPKS19-KO-P1	TGTTAGCTAGAATAGGCAGGC
SnPKS19-KO-P2	ATTCACTCGTCGTGTCGCCC-GATTTTTCGAGCCGATCCTC
SnPKS19-KO-P3	ACTCCACATCTCCACTCGACC-AGGCTTGCTTCAACCGTTCT
SnPKS19-KO-P4	GTCCGTCCGTCTCTCCGCATG-CGCAGTTTCATTTGGTATCA
SnPKS19-KO-P5	AATTCACGTCGTCGTCGCCC-ATTATAGAGAGTAGTAGAGC
SnPKS19-KO-P6	GTATAGCGAGCGATACCTAGC
Hyg-N-R	AGACGGCGTAACCAAAAGTCACA
Hyg-C-F	TTCTGGGTAAACGACTCATAGGAGA
LIC-SnPKS19-F_new	CTCGTCGTGTCGCCC-ATGACACGGGCCAAAGTTATC
LIC-SnPKS19-R	CGTCGTCGTCGCCCA-CTATCGAAGCAACGCCTTGAG
SnPKS19-RT-F	ACATACGCGTGGACCAACAA
SnPKS19-RT-R	CCTCGTAGAACACTGTCGCA
AN7071-RT-F	CTACTGCACCTGGACTCGTAC
AN7071-RT-R	GTATTCCGAGTGCCGTTTGC
AN7070-RT-F	GGCTACCTGTGCTCCCAGAA
AN7070-RT-R	CAGTGCGTTCTCTTCCTCCA

Table S2. KS domain sequences included in the phylogenetic analysis.

Sequence Name	**GenBank/ JGI Protein ID**	**Organism**	**PKS classification**	**Polyketide product (if known)**
Bref-PKS[o]	AIA58899	*Penicillium brefeldianum*	HR-PKS	Brefeldin precursor
Fma-PKS[o]	AGI05041	*Aspergillus fumigatus*	HR-PKS	fumagillin dodecapentaenoate
Fum1p[o]	AAD43562	*Fusarium verticillioides*	HR-PKS	Fumonisin precursor
LovF[o]	AAD34559	*Aspergillus terreus*	HR-PKS	Lovastatin diketide
PsoA[o]	ABS87601	*Aspergillus fumigatus*	PKS-NRPS	pseurotin A precursor
CcsA[o]	EAW09117	*Aspergillus clavatus*	PKS-NRPS	cytochalasin E/K precursor
LovB[o]	AAD39830	*Aspergillus terreus*	HR-PKS	lovastatin nonaketide
Gz_PKS13	ABB90282	*Gibberella zeae*	NR-PKS I	Zearalenone
Hpm3	ACD39762	*Hypomyces subiculosus*	NR-PKS I	Hypothemycin
ANID_07909 OrsA	AAC39471	*Aspergillus nidulans*	NR-PKS I	Orsellinic acid
ANIA_08209 WA	Q03149	*Aspergillus nidulans*	NR-PKS III	Naphthopyrone YWA1
Alb1p	AAC39471	*Aspergillus fumigatus*	NR-PKS III	Naphthopyrone YWA1
Gz_PKS12	AAU10633	*Gibberella zeae*	NR-PKS III	Bikaverin
PKSL1	Q12053	*Aspergillus parasiticus*	NR-PKS IV	Norsolorinic acid
ANIA_07825 StcA	CBF80184	*Aspergillus nidulans*	NR-PKS IV	Norsolorinic acid
CTB1	AAT69682	*Cercospora nicotiana*	NR-PKS IV	Cercosporin precurcor
ClPKS1	BAA18956	*Colletotrichum lagenarium*	NR-PKS II	Tetrahydroxy-naphthalene
GlPKS1	AAN59953	*Glarea lozoyensis*	NR-PKS II	Tetrahydroxy-naphthalene
EdPKS1	AAD31436	*Exophiala dermatitidis*	NR-PKS II	Tetrahydroxy-naphthalene
ANIA_07071 PkgA	CBF79143	*Aspergillus nidulans*	NR-PKS V	Citreoisocoumarin, alternariol, other isocoumarins
VrtA	ADI24926	*Penicillium aethiopicum* (*P. lanosocoeruleum*)	NR-PKS V	Viridicatumtoxin precursor
ANIA_06000 AptA	CBF70387	*Aspergillus nidulans*	NR-PKS V	Asperthecin
AdaA	AEN83889	*Aspergillus niger*	NR-PKS V	TAN-1612
PtaA	AGO59040	*Pestalotiopsis fici*	NR-PKS V	Pestheic acid diphenyl precursor
EncA	XP_746435	*Aspergillus fumigatus*	NR-PKS V	endocrocin
Penla1|378863 GsfA	ADI24953	*Penicillium aethiopicum* (*P. lanosocoeruleum*)[E]	NR-PKS V	Norlichexanthone
SnPKS19	**EAT76667**	***Parastagonospora nodorum* (*Stagonospora nodorum*)**[D]	**NR-PKS V**	**Alternariol**
PksI	AFN68300	*Alternaria alternata*[D]	NR-PKS V	Alternariol?

Table S2 (*continue*). KS domain sequences included in the phylogenetic analyses.

Sequence Name	GenBank/ JGI Protein ID	Organism	PKS classification	Polyketide product (if known)
BcPKS14	CCD48776	*Botrytis cinerea*[L]	NR-PKS V	Alternariol/ norlichexanthone?
Zasce1|61908	61908 (JGI)	*Zasmidium cellare*[D]	NR-PKS V	Alternariol/ norlichexanthone?
Penca1|243077	243077 (JGI)	*Penicillium canescens*[E]	NR-PKS V	Norlichexanthone?
Penra1|289401	289401 (JGI)	*Penicillium raistrickii*[E]	NR-PKS V	Norlichexanthone?
Apimo1|217105	217105 (JGI)	*Apiospora montagnei*[S]	NR-PKS V	Norlichexanthone?
Tryvi1|476961	476961 (JGI)	*Trypethelium eluteriae*[D]	NR-PKS V	Alternariol?
Oidma1|55810	55810 (JGI)	*Oidiodendron maius*[L]	NR-PKS V	Alternariol?
Penac1|509622	509622 (JGI)	*Talaromyces aculeatus*[E]	NR-PKS V	Alternariol?
Antav1|394995	394995 (JGI)	*Anthostoma avocetta*[S]	NR-PKS V	Alternariol?

Note: [O] indicates outgroup. [D], Dothideomycete; [L], Leotiomycete; [E], Eurotiomycete; [S], Sordariomycete.

SUPPLEMENTAL FIGURES

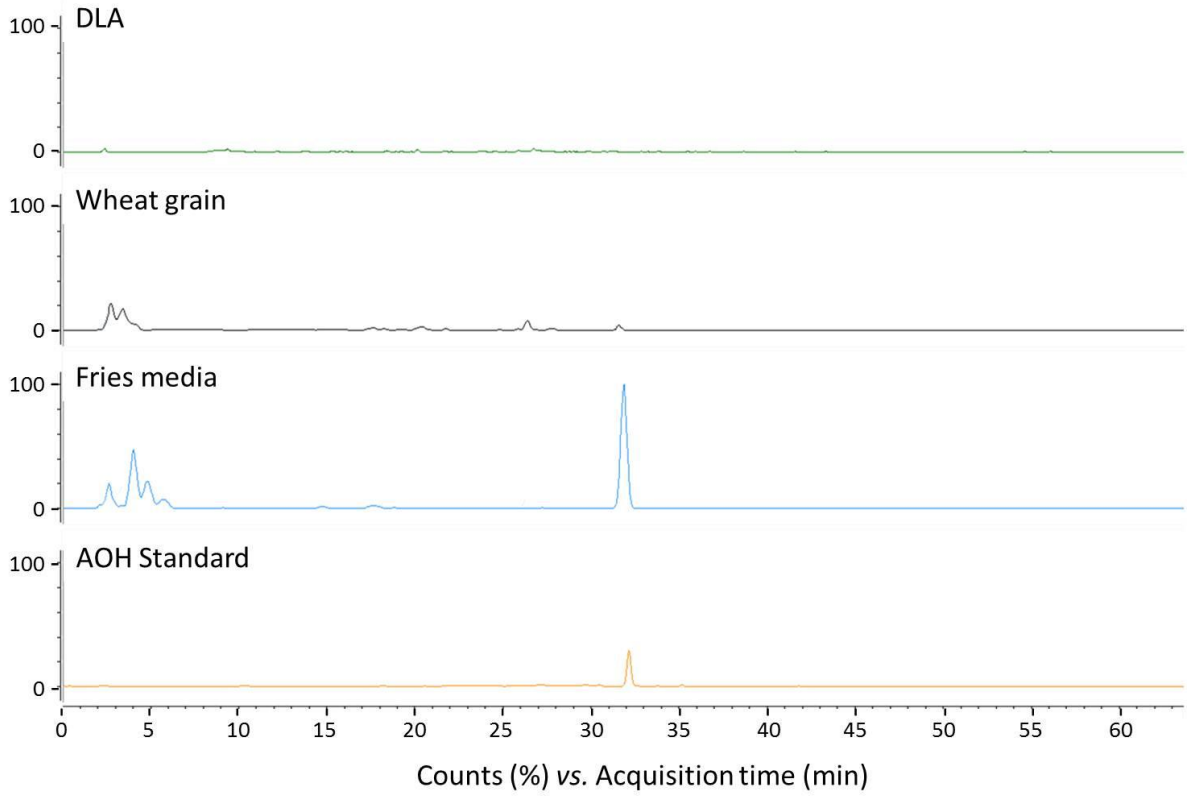
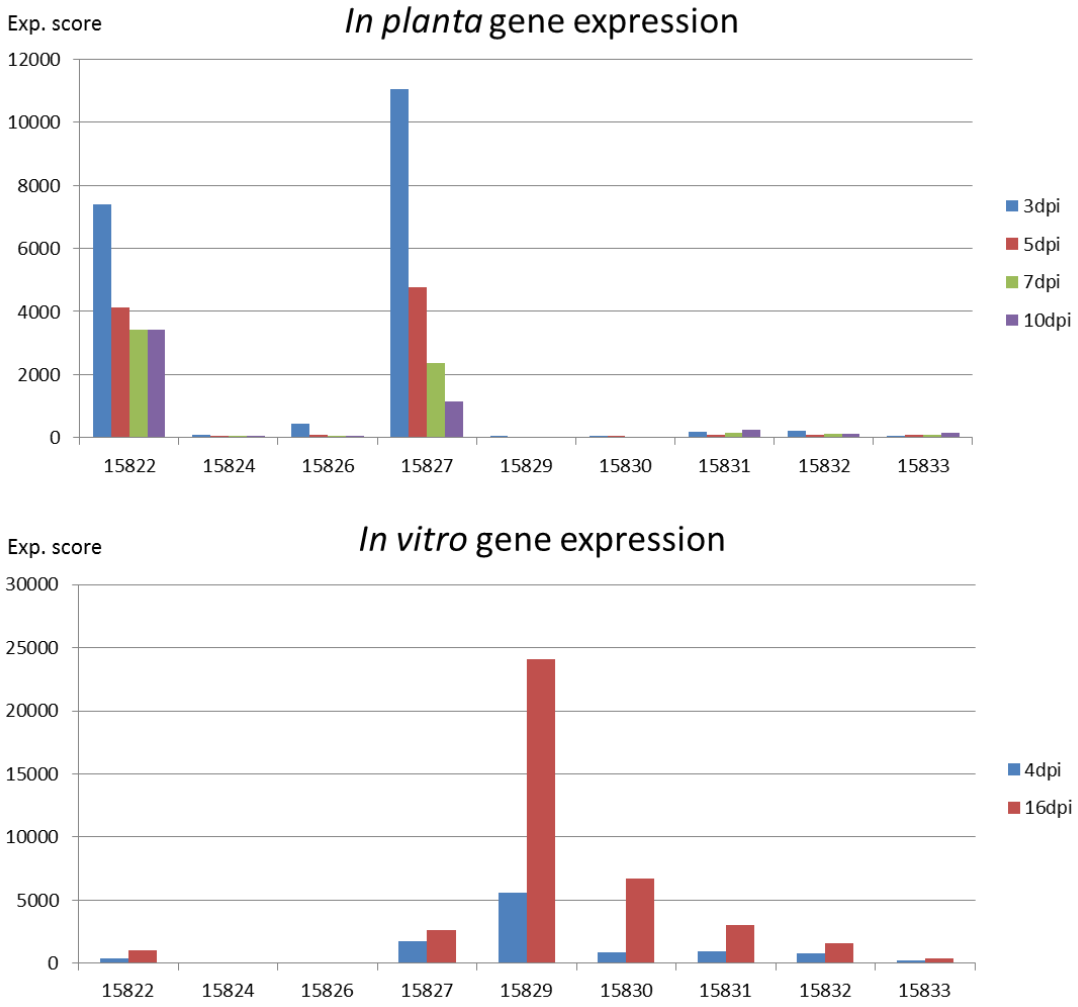


Figure S1. Detection of AOH in different growth conditions by LC-QToF/MS. Extracted Ion Chromatograms (EIC) of (AOH+H)+ (m/z=259.06) from detached leaf assays (DLA) of *P. nodorum* SN15 inoculated wheat leaves, SN15 infected wheat grain, SN15 mycelia of modified Fries media cultures and 1µg/ml AOH standard. AME is not detected in any of the samples above (data not shown).



Gene ID	Putative function
SNOG_15822	cyclohexanone monooxygenase
SNOG_15824	ABC multidrug transporter
SNOG_15826	beta-lactamase
SNOG_15827	O-methyltransferase
SNOG_15829*	alternariol polyketide synthase (SnPKS19)
SNOG_15830*	O-methyltransferase
SNOG_15831*	regulatory protein
SNOG_15832*	short-chain dehydrogenase
SNOG_15833	dioxygenase

* indicates gene that are co-regulated, i.e. low expression *in planta*, higher expression 16 dpi *in vitro*.

Figure S2. Gene-expression profile of *P. nodorum SnPKS19* gene cluster during growth *in planta* and *in vitro*. The expression data is extracted from the previous microarray study (3). An expression score of 500 or less essentially means no expression. 50000 or higher is out of the dynamic range (massive expression). dpi - days post inoculation.

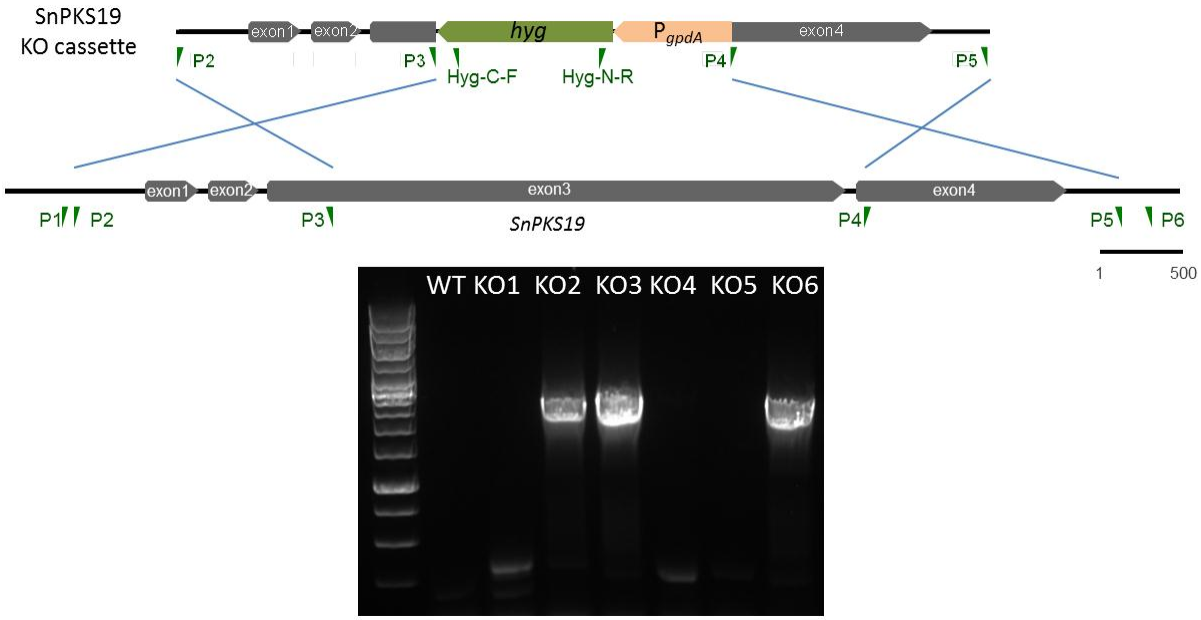


Figure S3. *SnPKS19* knockout scheme and diagnostic PCR for confirming *SnPKS19* deletion mutants. P1-P6 correspond to primer binding sites for SnPKS19-KO-P1 to -P6 (Table S1). Diagnostic PCR with SnPKS19-KO-P1/Hyg-C-F shown here.

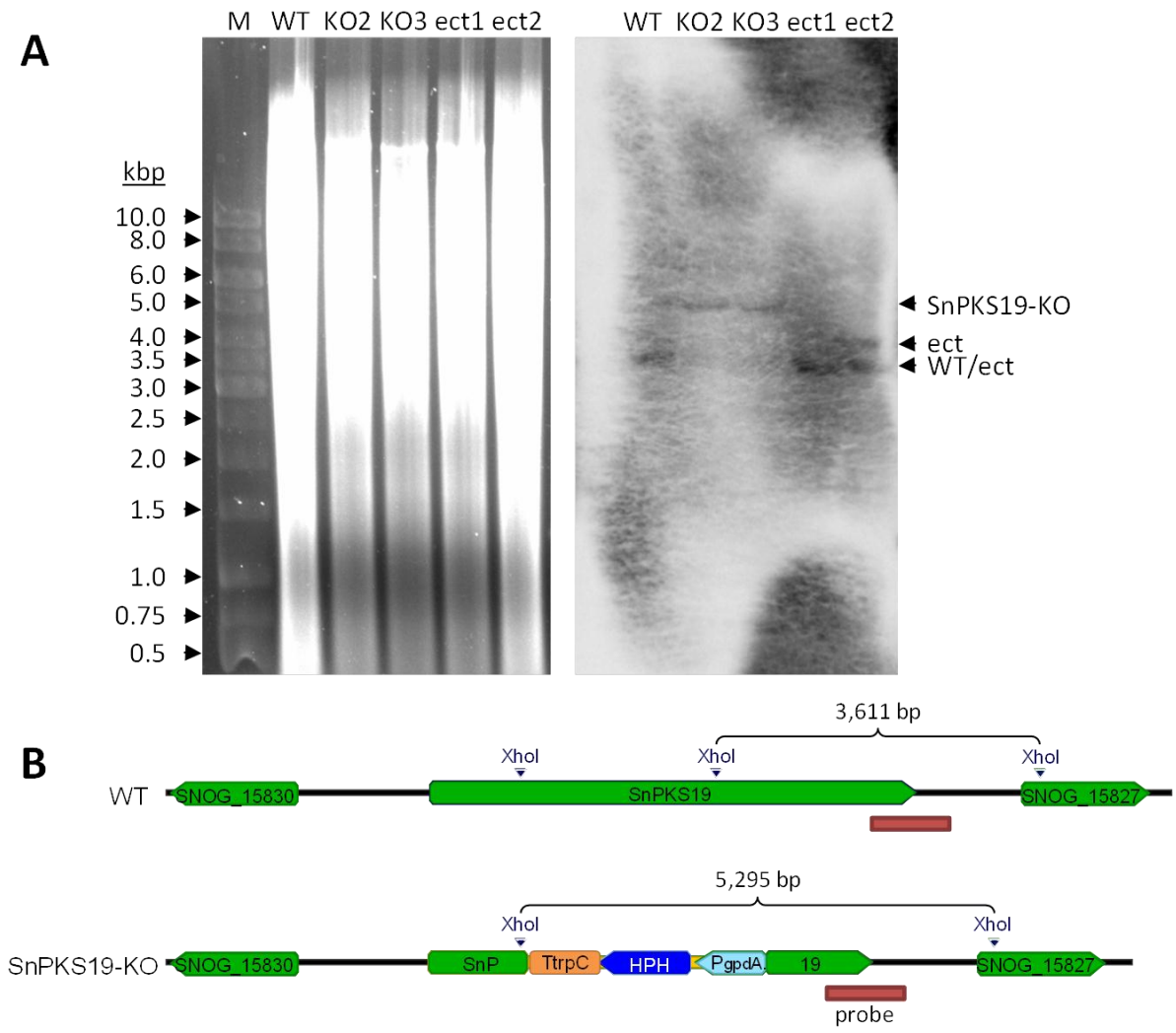


Figure S4. Southern blot confirmation of the *SnPKS19* knockout. (**A**) Southern blot with XhoI-digested genomic DNA (7-9 µg). (**B**) Knockout scheme showing the expected size for WT versus *SnPKS19*-KO and the location of the probe.

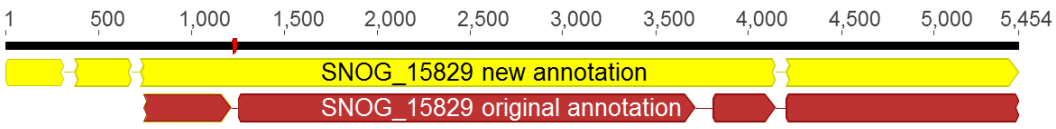


Figure S5. Comparison of the gene structure of the revised *SnPKS19* (SNOG_15829) with the original annotation. The newly revised annotation was deposited in GenBank accession number KP941080.

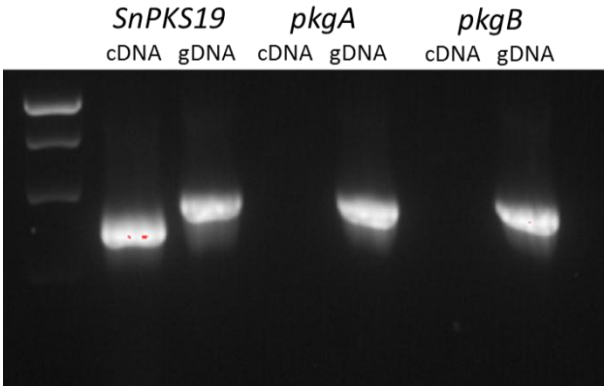


Figure S6. Reverse-transcriptase PCR of *SnPKS19* and *pkgA*/*B*. Primers flanking the last (3' end) intron in the respective genes were used for the PCR/RT-PCR. Product from cDNA is thus expected to be smaller than the gDNA due to intron splicing. The expected size for SnPKS19 product is 580 bp for gDNA and 520 for cDNA.

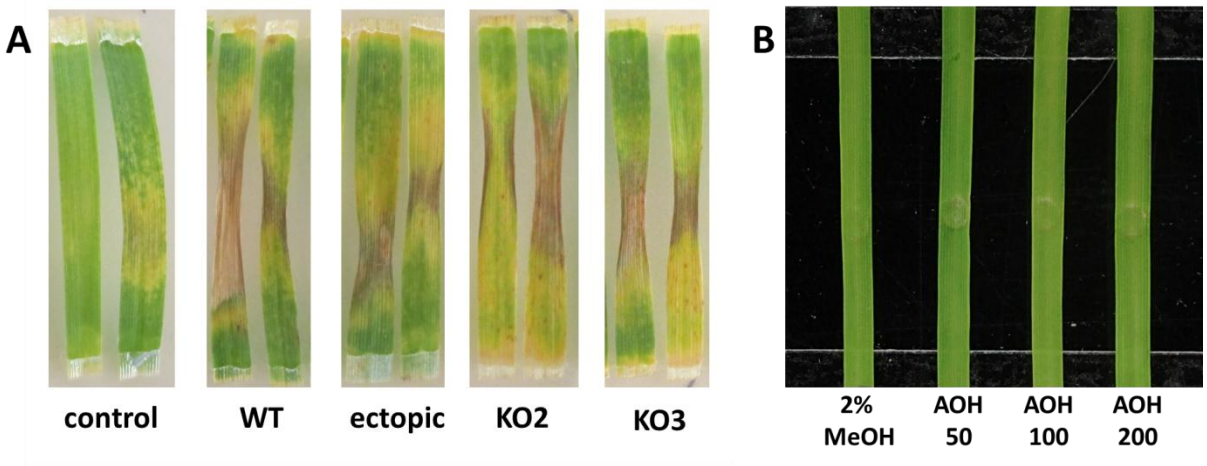


Figure S7. Wheat detached leaf assay of *P. nodorum* strains and phytotoxicity assay of alternariol (AOH). (**A**) Spray inoculated detached wheat leaves on agar, 10 days post inoculation. (**B**) Phytotoxicity assay of AOH as determined by leaf infiltration with 50, 100 and 200ppm AOH dissolved in 2% MeOH on whole plant wheat seedlings, 72 hours post infiltration. Both experiments were performed in six replicates.

SUPPLEMENTAL REFERENCES

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2. **Chooi YH, Krill C, Barrow RA, Chen S, Trengove R, Oliver RP, Solomon PS.** 2014. An in planta-expressed polyketide synthase produces (R)-mellein in the wheat pathogen *Parastagonospora nodorum*. Appl Environ Microbiol.
3. **Ipcho SV, Hane JK, Antoni EA, Ahren D, Henrissat B, Friesen TL, Solomon PS, Oliver RP.** 2012. Transcriptome analysis of *Stagonospora nodorum*: gene models, effectors, metabolism and pantothenate dispensability. Mol Plant Pathol **13:**531-545.