An In Planta-Expressed Polyketide Synthase Produces (R)-Mellein in the Wheat Pathogen *Parastagonospora nodorum*

Yit-Heng Chooi,a Christian Krill,b Russell A. Barrow,c Shasha Chen,c Robert Trengove,b Richard P. Oliver,d Peter S. Solomonb

Plant Sciences Division, Research School of Biology, The Australian National University, Canberra, Australian Capital Territory, Australia; Separation Science and Metabolomics Laboratory, Murdoch University, Murdoch, Western Australia, Australia; Research School of Chemistry, The Australian National University, Canberra, Australian Capital Territory, Australia; Centre for Crop and Disease Management, Department of Environment & Agriculture, Curtin University, Perth, Western Australia, Australia

*Parastagonospora nodorum* is a wheat pathogen that affects yields globally. Previous transcriptional analysis identified a partially reducing polyketide synthase (PR-PKS) gene, SNOG_00477 (*SN477*), in *P. nodorum* that is highly upregulated during infection of wheat leaves. Disruption of the corresponding *SN477* gene resulted in the loss of production of two compounds, which we identified as (R)-mellein and (R)-O-methylmellein. Using a *Saccharomyces cerevisiae* yeast heterologous expression system, we successfully demonstrated that *SN477* is the only enzyme required for the production of (R)-mellein. This is the first identification of a fungal PKS that is responsible for the synthesis of (R)-mellein. The *P. nodorum* Δ*SN477* mutant did not show any significant difference from the wild-type strain in its virulence against wheat. However, (R)-mellein at 200 μg/ml inhibited the germination of wheat (*Triticum aestivum*) and barrel medic (*Medicago truncatula*) seeds. Comparative sequence analysis identified the presence of mellein synthase (MLNS) homologues in several Dothideomycetes and two sodariomycete genera. Phylogenetic analysis suggests that the MLNSs in fungi and bacteria evolved convergently from fungal and bacterial 6-methylsalicylic acid synthases.

The dothideomycete plant pathogens are prolific producers of secondary metabolites (1). Many of these secondary metabolites serve as virulence mediators of plant pathogens (2, 3), as well as mycotoxins that are detrimental to human health (4). Thus, understanding of the secondary metabolites and their functions in plant pathogens is important for advancing our understanding of plant pathogenesis and may facilitate new prospects for controlling fungal diseases in the field. The availability of many dothideomycete plant pathogen genomes also provides a valuable resource for genome mining for novel secondary metabolites that can fuel the discovery of drugs and agrochemicals.

*Parastagonospora nodorum* (alternative names, *Phaeosphaeria nodorum* and *Stagonospora nodorum*) is a model dothideomycete necrotrophic pathogen and is the causative agent of brown spot on wheat (5). Its genome, sequenced in 2007 (6), has revealed a large number of secondary metabolite biosynthetic gene clusters. We recently surveyed the secondary metabolite genes in the *P. nodorum* genome and identified 23 polyketide synthase (PKS) genes, 14 nonribosomal peptide synthetase (NRPS) genes, 4 terpene synthase genes, and 2 prenyltransferase genes (7).

Along with the tailoring biosynthesis genes, they formed a total of 38 secondary metabolite gene clusters in *P. nodorum*. So far, none of the products of these secondary metabolite gene clusters have been identified, and only a few secondary metabolites have been identified in this species (7). These metabolites include (R)-mellein and derivatives (8, 9), secopters (10, 11), mycocphenolic acid (8), alternariol (12), and (+)-4’-methoxy-(2S)-methylbutyrophenone (13).

Polyketides belong to a major class of secondary metabolites that are assembled from the condensation of acetate and malonate units by PKS enzymes. They include important phytotoxins (e.g., host-specific T toxins and the light-activated toxin cercosporin) and mycotoxins (e.g., aflatoxins and fumonisins) (2, 14). To identify the *P. nodorum* polyketides that may potentially have a role in pathogen-host interaction and virulence, we mined the transcriptomic data from a previous microarray study for PKS genes that are significantly upregulated during infection on wheat leaves (15).

SNOG_00477 (herein abbreviated *SN477*) was identified from the transcriptomic data to be the most upregulated PKS gene in planta. We used a combination of reverse genetics, heterologous expression, and biological assays to characterize the product and function of *SN477*.

**MATERIALS AND METHODS**

*P. nodorum* strains and culturing conditions. The wild-type (WT) *P. nodorum* strain SN15 was obtained from the Department of Agriculture and Food Western Australia (DAFWA). Both the wild-type and mutant strains generated in this study were maintained on V8-supplemented potato dextrose agar (PDA) plates at 20°C under a 12-h dark/12-h near-UV light condition. For screening for mellein production, the *P. nodorum* wild-type and mutant strains were grown in 50 ml of defined minimal medium (30 g sucrose, 2 g NaNO₃, 1 g K₂HPO₄, 0.5 g KCl, 0.5 g MgSO₄ · 7H₂O, 0.01 g ZnSO₄ · 7H₂O, 0.01 g FeSO₄ · 7H₂O, 2.5 mg CuSO₄ · 5H₂O in 1 liter, adjusted to pH 6) or modified Fries medium (30 g sucrose, 5 g
ammonium tartrate, 1 g NH₄NO₃, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.13 g CaCl₂, 0.1 g NaCl) (16).

**Assays for *P. nodorum** virulence on wheat.** Detached leaf assays (DLAs) were performed as described previously (17). Briefly, sections of 11-day-old wheat leaves (*Triticum aestivum* cv. Calingiri) of approximately 5 cm in length were placed on tap water agar containing 75 mg/liter benzimidazole with the ends submerged in the agar with the adaxial side up. Each leaf was spot inoculated with 5 μl of 10⁶ spores/ml in 0.02% (vol/vol) Tween 20, and the DLA plates were incubated at 25°C in a 12-h light/12-h dark cycle.

Whole-plant spray assays were performed on 11-day-old leaves as described previously (18). Briefly, the plants were sprayed with 12 ml 10⁶ spores/ml suspended in 0.02% Tween 20 or 0.02% Tween 20 without spores (negative control). The plants were covered and kept in the dark at 20°C for 2 days, followed by a further 5 days under a 12-h dark/12-h light regime. Disease symptoms were assessed visually and scored on a scale from 0 (uninfected) to 9 (widespread necrosis).

**qRT-PCR.** Quantitative reverse transcriptase PCR (qRT-PCR) was performed on total RNA samples extracted from *P. nodorum*-infected wheat leaves from the DLAs at 3, 5, 7, and 10 days postinoculation (p.i.). Four leaves were collected at each time point and immediately snap-frozen. Isolation of total RNA was performed using the TRIzol reagent (Life Technologies, CA, USA). Possible DNA contamination was removed using a DNA-free reagent (Life Technologies) according to the manufacturer’s instructions.

cDNA was synthesized from extracted RNA using an iScript kit (Bio-Rad, CA, USA), according to the manufacturer’s instructions, in an Eppendorf Mastercycler thermal cycler (Eppendorf, Germany). Quantitative PCR (qPCR) was performed using iQ SYBR green Supermix (Bio-Rad, CA, USA) with the 00477RT-Flt primers (see Table S1 in the supplemental material) in a Rotor Gene (version 6) apparatus (Corbett Research, Australia). The thermal cycling program was as follows: 95°C for 3 min (initial denaturation), followed by 40 cycles of 94°C for 10 s (denaturation), 57°C for 20 s (annealing), and 72°C for 30 s (extension). Sample fluorescence was detected using a 470-nm excitation wavelength and a 510-nm detection wavelength. The proprietary Rotor Gene (version 6) software (Corbett Life Science) was used to process the data (comparative quantitation feature). All reactions were carried out in duplicate.

**Transformation and screening of *P. nodorum* mutants.** The SN477-knockout (KO) cassette was synthesized using a fusion PCR approach as described elsewhere (19). Upstream and downstream regions flanking SN477 were amplified with the primer sets 00477KO5-Flt and 00477KO3-Flt (see Table S1 in the supplemental material), and the phleomycin resistance cassette was amplified with the primers pan8-Flt from plasmid pAN8-1 (20). Transformation of *P. nodorum* with the SN477-knockout cassette was achieved by the polyethylene glycol-mediated protoplast transformation protocol described previously (21).

*P. nodorum* ΔSN477 mutants were screened by diagnostic PCR using the 00477Scr-Flt primers (see Table S1 in the supplemental material). The forward primers were designed to anneal outside the 5' flanking region of the KO constructs, while the reverse primers anneal within the resistance cassette. Gene deletion results in a 1-kb ampiclon, while ectopic mutants are not amplified. The genomic DNA used as the template for diagnostic PCR was extracted using a Retsch MM301 ball mill and a Qiagen Bio-Rad isogen. Isolation of total RNA was performed using the TRIzol reagent (Life Technologies, CA, USA). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out using TaKaRa polymerase (TaKaRa Bio Inc., Japan) in an Eppendorf Mastercycler ep thermocycler. The number of integrations in the ΔSN477 mutants was determined using a qPCR method described previously (22). The qPCR was performed in a Rotor Gene (version 6) apparatus using the PhloxPCRFlt primers (see Table S1 in the supplemental material), as follows: 95°C for 3 min (initial denaturation), followed by 40 cycles of 95°C for 10 s (denaturation), 57°C for 10 s (annealing), and 72°C for 20 s (extension). The proprietary Rotor Gene (version 6) software was used to preprocess the data (comparative quantitation feature). The results were imported into a spreadsheet to calculate the ratio of phleomycin to actin per sample. A ratio of 1 is diagnostic of single-copy integration. ΔSN477 mutants with single-copy integration were chosen for further experiments.

**Heterologous expression of SN477 in *Saccharomyces cerevisiae*.** The intronless SN477 gene was amplified directly from the genomic DNA of *P. nodorum* using primers xw-SN477_Fnew and xw-SN477_Rnew. The open reading frame is based on a corrected version of the genomic DNA sequence (deposited in GenBank under accession number KM365444), which contains an additional 65 amino acids at the N terminus compared to the original annotation in the NCBI GenBank and JGI databases. The forward and reverse primers contain a 40-bp overhang for direct cloning of SN477 into the *Saccharomyces cerevisiae* yeast expression plasmid YEpPlac-ADH2p yeast-*Esherichia coli* shuttle vector by in vivo yeast recombination. YEpPlac-ADH2p contains an autoinducible adh2 promoter for protein expression in *S. cerevisiae* (23). The resulting plasmid, YEpPlac-SN477n, was used to transform the engineered yeast strain *S. cerevisiae* BJ5464-NpgA, which is deficient in vacuolar proteases and harbors an integrated copy of *A. nidulans* npgA (24, 25).

The BJ5464-NpgA yeast harboring plasmid YEpPlac-SN477n was grown in 50 ml yeast extract-peptone-dextrose (YPD) broth in a 250-ml shake flask along with the control culture (an empty YEpPlac-ADH2p vector) at 28°C and 220 rpm. Five milliliters of the yeast cultures was sampled at 48 and 72 h and extracted with a mixed organic solvent containing ethyl acetate, methanol, and acetic acid (89:10:1). The organic layer was dried in vacuo and redissolved in 500 μl methanol for analysis by liquid chromatography (LC)-diode array detection (DAD)-mass spectrometry (MS).

**LC-MS analysis.** LC-MS metabolomics of the *P. nodorum* wild type and mutants were 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. The wild type and ΔSN477 mutants (SN477-KO26 and SN477-KO28) were grown in triplicate on various media and extracted with the ethyl acetate-methanol-acetic acid (89:10:1) solvent mixture. The crude extracts were dried in vacuo and redissolved in methanol for LC-MS analysis. Chromatographic separation was performed at 40°C using a Zorbax Eclipse RRHD C18 column (particle size, 1.8 μm; 2.1 mm [inside diameter] by 150 mm; Agilent) and an in-line filter. The mobile phase consisted of a linear gradient of 98% eluent A (0.1% [vol/vol] formic acid in deionized water) to 70% eluent B (0.1% [vol/vol] formic acid in 90% acetonitrile) over 30 min at a flow rate of 200 μl min⁻¹. The gradient was followed by a 20-min hold at 70% eluent B and was then back to 98% eluent A over 1 min, and a 14-min reequilibration was then performed. The data were collected in the m/z range from 100 to 1,000 atomic mass units in the positive mode. Data processing was performed with Mass Profiler Professional software (Agilent).

For the yeast cultures expressing the SN477 gene, the analysis of the metabolite profiles was performed on an Agilent 1200 LC system coupled to a diode array detector and an Agilent 6120 quadrupole MS with an ESI source. Chromatographic separation was performed at 30°C using a Kinetex C18 column (particle size, 2.6 μm; 2.1 mm [i.d.] by 100 mm; Phenomenex, Torrance, CA, USA). The compositions of eluents A and B used in the mobile phase were the same as those described above. The gradient consisted of a quick increment of eluent B from 5% to 30% over 5 min before it was gradually increased to 100% eluent B over 25 min at a flow rate of 200 μl min⁻¹. This was followed by a 10-min hold at 100% eluent B and was then back to 5% eluent B over 1 min, and a 15-min reequilibration was then performed. The metabolite profile of the yeast culture was compared with the profiles of purified (R)-mellein from *P. nodorum* (below) and an authentic (R)-mellein standard (Cyamn Chemical, MI, USA).

**Compound isolation and structural characterization.** For production of compounds, *P. nodorum* SN15 was grown in modified Fries liquid medium as described previously (8, 16). Briefly, about 10⁶ spores were inoculated in six 2-liter flasks containing 500 ml liquid medium and in

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cubated at 22°C for 48 h with shaking, followed by stationary incubation in the dark at 22°C for another 2 weeks. The culture was extracted with a mixed organic solvent containing ethyl acetate, methanol, and acetic acid (89:10:1), and the organic layer was dried in vacuo. The crude extract was then fractionated on a Reveleris flash chromatography system (Grace, MD, USA) using a hexane-ethyl acetate-methanol gradient. Eluting compounds were monitored with a UV detector (245 nm and 310 nm) and an evaporative light scattering detector (ELSD) coupled to the flash chromatography system. Fractions corresponding to single peaks were sampled and analyzed by LC-DAD-MS. Two of the fractions, identified to contain a single peak with m/z 179 and m/z 193, respectively, were dried in vacuo and redissolved in deuterated chloroform (CDCl3) for nuclear magnetic resonance (NMR) analysis on an Inova 500 MHz NMR system (Varian, CA, USA). The specific optical rotations of the two compounds were recorded on a model 343 polarimeter (PerkinElmer, MA, USA) at 589 nm (sodium D line). The specific optical rotation of (R)-mellein was also compared to that of an authentic standard (Cayman Chemical).

Phytotoxicity and antigerminative activity assays. The phytotoxicity of (R)-mellein and (R)-O-methylmellein was assayed on the leaves of 11-day-old wheat seedlings that were grown in 10-cm planting pots at 20°C under a 12-h light/12-h dark cycle regime. Briefly, approximately 80 μl of each compound in 2% methanol solution was infiltrated on the adaxial face of the leaves at various concentrations (25, 50, 100, 200 μg/ml) using a 1-ml-volume syringe. Experiments were performed in triplicate, and the infiltrated leaves were examined for the presence of necrosis or chlorosis after 24 and 48 h.

To test the ability of the compounds to inhibit seed germination, grains of wheat seed (Triticum aestivum cv. Lincoln) were first surface sterilized briefly in a solution containing 10% ethanol and 1% hydrogen peroxide. A single grain of the surface-sterilized wheat seed was then placed on agar slants containing 1.5 ml of tap water agar supplemented with either (R)-mellein or (R)-O-methylmellein at a 200-μg/ml final concentration (agar containing 2% methanol was used as a control). Barrel medic (Medicago truncatula cv. Jemalong A17) seeds were also tested using the same protocol to determine the host specificity of the compounds. The assays were performed in triplicate, and the progress of seed germinations was observed and recorded on a daily basis for 1 week.

Phylogenetic analysis. The phylogeny of the PKSs was inferred using conserved β-ketosynthase (KS) domains of the PKS protein sequences (corresponding to amino acids 67 to 463 of the corrected SN477 protein sequence). The PKSs used in the phylogenetic analysis included four characterized fungal 6-methysaliclic acid synthases (6MSAs) and all NCBI BLASTp hits (queried with the SN477 KS domain) from the GenBank database with a percent identity and score that were above those for the characterized 6MSA (ATX) from Aspergillus terreus (see Table S3 in the supplemental material). To investigate the distribution of SN477 homologs among Dothideomycetes, all BLASTp hits (queried with the SN477 KS domain) from the JGI Dothideomycetes genome database (http://genome.jgi-psf.org/Dothideomycetes/Dothideomycetes.info.html) with an E value of >10^{-100} were included in the phylogenetic analysis. Six characterized bacterial iterative PKSs known to produce 6-methysaliclic acid (6-MSA), orsellinic acid, naphthenic acids, and (R)-mellein were included for comparison, while seven selected fungal highly reducing (HR) PKSs and PKS-nonribosomal peptide synthetase (NRPS) hybrids were included for rooting of the trees (see Table S3 in the supplemental material). A total of 50 KS domain sequences were aligned using the MUSCLE alignment program embedded in Geneious (version 7.17) software (Bio-matters Ltd., Auckland, New Zealand) (26). The resulting multiple-sequence alignment was used for phylogenetic analysis.

The KS domain phylogenetic tree in Fig. 4 was constructed using the Geneious Tree Builder program embedded in Geneious (version 7.17) software with the neighbor-joining method (27). The tree was constructed with 1,000 bootstrap replicates, and branches corresponding to partitions that were reproduced in less than 50% of bootstrap replicates were collapsed. For comparison, a maximum likelihood tree was constructed using the RAxML (version 7.2.8) plug-in (28) in Geneious (version 7.17) software (see Fig. S2 in the supplemental material).

Nucleotide sequence accession number. The corrected SN477 coding sequence has been deposited in GenBank under accession number KM365454.

RESULTS
SNOG_00477 is the most highly expressed PKS gene in planta. Ipcho et al. previously used a custom microarray to obtain the global transcriptomic profile of 16,586 nuclear gene models of P. nodorum (15). The transcriptomic profiles of the genes expressed during infection of detached wheat leaves at time points spanning from early infection to sporulation (in planta) and during growth on defined minimal medium (in vitro) were compared. The study showed that 2,882 genes were expressed at a higher level in planta and 3,630 were expressed more highly in vitro. Using these transcriptome data, we extracted the gene expression profiles of all the 23 PKS genes in P. nodorum (see Table S2 in the supplemental material). The analysis showed that SNOG_00477 (abbreviated SN477) was the most upregulated PKS gene in planta compared to its level of expression in vitro. Although SN477 was expressed both in planta and in vitro, its expression in planta was, on average, 3-fold higher than that in vitro. The expression of SN477 was the highest at 3 days postinoculation (p.i.) on wheat leaves, prior to decreasing to approximately the same level as that in vitro on day 10 in planta (see Fig. S1A and Table S2 in the supplemental material).

The transcription of SN477 in planta, using the same detached leaf assay (DLA) used in the previous microarray study, was validated by quantitative reverse transcriptase PCR (qRT-PCR). The transcription of two additional PKS genes (SNOG_05791 and SNOG_011272) was included in the qRT-PCR experiments for comparison. The qRT-PCR results showed expression profiles similar to those observed in the microarray data for SNOG_05791 and SNOG_011272, but SN477 was expressed at the highest level at 5 days p.i. (see Fig. S1B in the supplemental material). The high level of SN477 expression in planta at the early infection stage suggests a possible role of the polyketide product in establishing infection and prompted us to further investigate the product and function of this PKS gene.

Analysis of the SN477 sequence showed that it encodes a typical partially reducing (PR) PKS with β-ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP) domains (7, 29–31). Among the characterized fungal PKSs, SN477 was the most similar to the partially reducing PKS ATX from Aspergillus terreus, which synthesizes 6-methysaliclic acid (6-MSA) (32). It shares 54% amino acid sequence identity with A. terreus ATX and has a domain architecture identical to that of A. terreus ATX. Thus, it was considered that SN477 likely encodes the biosynthesis of a partially reduced polyketide compound similar to 6-MSA.

Generation and characterization of P. nodorum ΔSN477 mutants. P. nodorum ΔSN477 mutants were generated by polyethylene glycol-mediated transformation with the SN477-knockout cassette described above. Diagnostic PCR identified five positive transformants where SN477 had been deleted by double homologous crossover recombination and replaced with a phleomycin resistance marker (ble). Two of the positive mutants, SN477-KO26 and SN477-KO28, were confirmed to contain only a single-copy integration by qPCR. No differences in the growth rate between the two mutants and the wild-type (WT) P. nodorum.
strains were observed. The growth rate and colony morphology on V8-PDA and minimal medium plates were indistinguishable from those of the WT. There was also no significant difference in sporulation. Thus, the two \( /H9004 \) \( SN477 \) mutants did not demonstrate any observable growth defect.

Metabolic profiling revealed that \( SN477 \) is involved in the synthesis of \((R)\)-mellein. On the basis of the previous microarray data, the \( SN477 \) gene showed modest expression on defined minimal medium agar at 4 and 16 days postinoculation. Since \textit{in planta} metabolite analysis would be complicated by the complex metabolite background in the wheat leaves, we first compared the metabolic profiles between the wild-type and the mutants grown on defined minimal medium in triplicate using LC-QTOF-MS in positive mode. Using subtractive metabolomics, we were able to identify two peaks (\( m/z \) 179.07 and \( m/z \) 193.08) in the \textit{P. nodorum} wild-type culture that were consistently absent in the \( \Delta SN477 \) mutants. However, these two peaks were present in only moderate to low abundance in the WT culture on defined minimal medium (data not shown).

Other media and culture conditions were tested to improve the production of the two compounds. It was found that these two peaks appeared as major metabolites in the \textit{P. nodorum} WT culture when grown in modified Fries liquid medium under stationary culture conditions, similar to the findings described previously (8, 16). As expected, the two peaks were absent in the corresponding cultures of the \( \Delta SN477 \) mutants (Fig. 1A and B).

**FIG 1** Comparative metabolomics analysis of \textit{P. nodorum} strains by LC-MS. (A, B) Base peak chromatogram (BPC) and extract ion chromatogram (EIC) of culture extracts from the \textit{P. nodorum} wild type (A) and \( \Delta SN477 \) mutant (B); (C) structural and molecular formulas of \((R)\)-mellein and \((R)\)-O-methylmellein.

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methylmellein. To determine if the reverse genetics and metabolic approaches described above confirmed that the annotated SN477 protein sequence has a slightly shorter N-terminus, which corresponds to other fungal 6MSASs (the corrected sequence is deposited in GenBank accession number KM365454). Recloning of the SN477 gene from S. cerevisiae BJ5464-NpgA into the YEplac-ADH2p vector for expression in the S. cerevisiae BJ5465-NpgA strain. This high-pressure liquid chromatography–DAD profile of the culture extract from S. cerevisiae BJ5465-NpgA expressing SN477 (YEplac-SN477n) compared to the profiles of the culture extracts from the empty vector control (YEplac-ADH2p) and the (R)-mellein standard is shown. mAU, milli-absorbance units.

To confirm the identities of the two compounds, the P. nodorum WT culture was scaled up to 3 liters and extracted after 21 days incubation. Using flash chromatography with a silica column, approximately 18 mg and 25 mg of the two compounds corresponding to m/z 179.07 and m/z 193.08, respectively, were isolated. NMR spectrum analysis established that the compound with m/z 179.07 [M + H]+ is mellein, while the compound with m/z 193.08 [M + H]+ corresponds to O-methylmellein (see Tables S4 and S5 and Fig. S3 and S4 in the supplemental material) (8, 33, 34). Optical rotation analysis showed that both compounds are the negative (−) enantiomers which have an R configuration at C-3 (8, 33). Together, the data established that the two compounds are (R)-(-)-mellein [(3R)-(−)-3,4-dihydro-8-hydroxy-3-methylisocoumarin] and (R)-O-methylmellein [(2R)-(−)-3,4-dihydro-8-methoxy-3-methylisocoumarin] (Fig. 1C). Both of these two compounds have been identified previously from P. nodorum (8). Hence, these data have confirmed that SN477 is the PKS gene involved in the biosynthesis of (R)-mellein and (R)-O-methylmellein.

Heterologous expression of SN477 in yeast resulted in the production of (R)-mellein. The reverse genetics and metabolic approaches described above confirmed that SN477 has a role in the synthesis of (R)-mellein. To determine if SN477 is soley responsible, we cloned the intron-less SN477 gene from P. nodorum into the YEplac-ADH2p vector for expression in the S. cerevisiae BJ5464-NpgA strain. This S. cerevisiae BJ5464-NpgA strain has been successfully used for the heterologous expression of large megasynthases from fungi (25, 29). Our initial attempt, based on the SN477 annotation in the NCBI and JGI databases, failed to produce (R)-mellein in the yeast strain (data not shown). Alignment of the protein sequence with other fungal 6MSASs showed that the annotated SN477 protein sequence has a slightly shorter N-terminus. A more detailed analysis identified another start codon 195 bp upstream of the originally annotated 5′ end, which corresponded to an additional 65 amino acids supported by homology to other 6MSASs (the corrected sequence is deposited in GenBank under accession number KM365454). Recloning of the SN477 coding sequence with the inclusion of the missing 195 bp resulted in the plasmid YEplac-SN477n. Transformation of S. cerevisiae BJ5464-NpgA with YEplac-SN477n resulted in the production of a new peak at 48 and 72 h upon inoculation of the seed culture in YPD liquid medium (Fig. 2). The new peak corresponded to the retention time, UV spectrum, and m/z of the purified (R)-mellein from P. nodorum and an authentic (R)-mellein standard. This result conclusively demonstrated that SN477 is a mellein synthase (MLNS) and is the only enzyme required for the production of (R)-mellein.

Do (R)-mellein and (R)-O-methylmellein play a role in virulence or affect plant development? The ability of the ΔSN477 mutant strains to cause disease was assessed using the whole-plant spray assay. No significant difference in disease symptoms between wheat leaves inoculated with ΔSN477 mutants and wheat leaves inoculated with the controls (wild-type and ectopic integration strains) was observed. Subsequent reanalysis using a detached leaf pathogenicity assay confirmed that SN477 does not have a role in virulence.

The phytotoxic activity of the purified (R)-mellein and (R)-O-methylmellein on wheat was then assayed. No necrosis was observed on wheat leaves treated with up to 200 μg/ml of (R)-mellein and O-methylmellein by leaf infiltration. The capacity of (R)-mellein and O-methylmellein to affect seed germination was also assessed. Complete inhibition of wheat seed germination was observed for (R)-mellein at 200 μg/ml (Fig. 3). However, (R)-O-methylmellein at the same concentration exhibited only moderate to low antigerminative activity. Likewise, 200 μg/ml of (R)-mellein completely inhibited the germination of barley medic seeds, while (R)-O-methylmellein exhibited only moderate inhibition. These data indicate that the inhibitory activity is neither host specific nor restricted to monocots or dicots.

Phylogeny of (R)-mellein synthase and its distribution among Dothideomycetes. Phylogenetic analysis of fungal PR-PKSs and bacterial iterative PKS was inferred by either the neigh-
bor-joining (27) or the randomized accelerated maximum likelihood (RAxML) (28) method and resulted in consensus trees with similar topologies (Fig. 4; see also Fig. S2 in the supplemental material). The separation of a clade containing bacterial iterative PKSs and fungal PR-PKSs from fungal highly reducing (HR) PKSs was well supported. The fungal PR-PKSs were divided into two large clades, one with SN477 and the other with the characterized 6MSASs. Four fungal PR-PKS genes have so far been matched to the polyketide products. All four of them, Penicillium griseofulvum MSAS (35), A. terreus ATX (32), Glarea lozoyensis PKS2 (36), and Aspergillus niger PKS48 (37), have been shown to encode the production of 6-MSA. Thus, SN477 is the first fungal PR-PKS shown to produce a polyketide compound other than 6-MSA that is instead linked to mellein. It is likely that most of the other fungi in the mellein clade may also produce this compound.

A previous phylogenetic study has inferred that the fungus acquired the 6MSAS-type PR-PKSs by horizontal gene transfer (HGT) from bacteria (38). Here, our analysis showed that SN477 and its homologues are more closely related to fungal 6MSASs than Saccharopolyspora erythraea SACE_5532, which synthesizes the same polyketide product, (R)-mellein (33). This suggests a PKS convergent evolution after the initial HGT, where the fungal MLNSs evolved from an ancestral fungal 6MSAS independently from the bacterial MLNS SACE_5532.

A homologous gene search and phylogenetic analysis indicate that SN477 homologues are widely but not uniformly distributed among the Dothideomycetes class of fungi. Thirteen of the 63 Dothideomycetes in the JGI Dothideomycetes genome database and 3 nondothideomyceteous (sordariomycete) species in the GenBank database contain a copy of the SN477 homologue (Fig. 4; see also Table S3 in the supplemental material). SN477 and its closest homologues (9 from Dothideomycetes and 3 from Sordariomycetes) formed a distinct clade, and an additional 4 dothideomycete homologues formed a closely related sister clade separated from the fungal 6-MSASs. Neofusicoccum parvum has previously been shown to produce (3R,4R)-(−)-4-hydroxymellein and (3R,4S)-(−)-4-hydroxymellein (39). Out of the three PR-PKSs in N. parvum, UCRNP2_6207, which grouped with SN477, is most likely to be the PR-PKS responsible for production of the mellein

**FIG 4** Phylogeny and distribution of (R)-mellein synthase homologues in fungi in relation to other fungal PR-PKSs and the bacterial counterparts. The KS domain consensus tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates (numbers at the nodes indicate bootstrap values). Branches corresponding to partitions that were reproduced in less than 50% of bootstrap replicates are collapsed. †, SNOG_00470 is (R)-mellein synthase characterized in this study; *, fungal 6-MSAs characterized in other studies; ‡, homologues from dothideomycete fungi (see Table S3 in the supplemental material). The tree is rooted with fungal HR-PKSs and hybrid PKS-NRPSs. A maximum likelihood tree with a similar topology is presented in Fig. S2 in the supplemental material.
precursor. This observation, in turn, supports the postulation that the 12 PR-PKSs (9 from Dothideomycetes and 3 from Sordariomycetes) in the same clade as SN477 are functional homologues of SN477 and likely synthesize mellein (Fig. 4).

**DISCUSSION**

In this study, we have functionally characterized SN477, the PKS gene most highly expressed in planta. SN477 was shown to encode a PR-PKS that synthesizes (R)-melline and, as such, is the first (R)-melline synthase identified in fungi. SN477 harbors a typical PR-PKS domain architecture (KS-AT-thiohydrolyase [TH]-KR-ACP) similar to that of 6MSAs (29–31). The biosynthesis of (R)-melline is highly parallel to that of 6-MSA but requires additional chain elongation and keto reduction steps (Fig. 5). The nascent pentaketide intermediate then undergoes an aldol cyclization and is aromatized via dehydration. The stereospecific lactonization and release of the mature polyketide product are likely catalyzed by the TH domain in MLNS, similar to that identified in 6MSAs (33, 40). The (R)-O-methylmelline isolated from *P. nodorum* in this study is most likely to be derived from (R)-melline via an additional methylation at the hydroxyl group. Interestingly, no O-methyltransferase gene is encoded in the vicinity of SN477 on the chromosome. Thus, the O-methylation is likely to be catalyzed by an endogenous O-methyltransferase encoded elsewhere in the genome of *P. nodorum*.

Since the discovery of the first 6MSA from *Penicillium patulum* (35), subsequent homologues of PR-PKSs characterized in fungi have been shown to produce 6-MSA (32, 36, 37). Thus, SN477 is the first PR-PKS that has been demonstrated to produce a polyketide compound other than 6-MSA. The discovery and characterization of SN477 as an MLNS hint that there may be remaining undiscovered chemical diversity in this group of fungal PKSs. Our phylogenetic analysis inferred that the fungal and bacterial MLNSs (sharing 37% amino acid sequence identity) have evolved independently from ancestral fungal and bacterial 6MSAs, respectively. A similar convergent evolution of bacterial and fungal type I PKSs has previously been observed in orsellinic acid biosynthesis. While the fungal orsellinic acid synthases are typical fungal nonreducing PKSs (41–43), the bacterial orsellinic acid synthases are related to bacterial iterative type I PKSs (homologous to fungal PR-PKSs) and are likely to have evolved from ancestral bacterial 6MSAs via mutations in the KR domain (44–46). From a protein engineering perspective, the convergent evolution of MLNSs implies that there is more than one way in nature to modify the chain length specificity and ketoreduction regioselectivity of iterative PKSs.

(R)-Melline (also known as ochracin) and its dihydroisocoumarin derivatives are widespread in fungi, particularly among the Dothideomycetes. (R)-Melline was first isolated from *Aspergillus melleus* in 1933 (47), and its structure and stereochemistry were later determined in 1955 and 1968, respectively (48, 49). Subsequently, melline and its derivatives have been isolated from a variety of fungi, including *Septoria nodorum* (synonym *Parastagonospora nodorum*) (8), *Cercospora taiwanensis* (50), *Botryosphaeria* spp. (51–53), *Phoma tracheiphila* (54), *Xylaria longijana* (55), *Microsphaeropsis* sp. (56), *Sphaeropsis sapinea* (57), a *Nigrospora* sp. (58), *Apiospora montagnei* (59), and *Pezicula livida*, a *Plectospherella* sp., and *Cryptosporiopsis* spp. (60). Despite the widespread occurrence of melline in fungi, this is the first time that a fungal PKS gene responsible for the synthesis of melline has been identified. Outside of the fungal kingdom, melline has been found in actinomycetes (33) and insects (61–63).

(R)-Melline and its derivatives have been shown to display a myriad of bioactivities. It is weak to moderately active against a range of bacteria and fungi (56, 58, 60). (R)-Melline has also been shown to exhibit antiviral activity against hepatitis C virus (64), antiparasitic activity against *Schistosoma mansoni* (59), and zootoxicity against *Artemia salina* larvae (54). It has been reported to be a trail pheromone of ants (61, 63), while the larvae of the parasitoid wasp *Ampulex compressa* apparently impregnate the cockroach host with (R)-melline as one of the antimicrobials for defense against entomopathogenic microbes (62). Above all, the most commonly reported bioactivity of melline and its derivatives is their non-host-specific toxicity against plants. (R)-Melline and hydroxymellines have been isolated as phytotoxins of the apple and grapevine pathogen *Botryosphaeria obtusa* (53, 65), the pine pathogen *S. sapinea* (57), the citrus pathogen *Phoma tracheiphila* (54), and the grapevine canker agent *N. parvum* (39). It has also been shown to induce phytotoxic symptoms on tomato cuttings when it is used at 100 μg/ml (54) and causes necrosis on detached grape (*Vitis vinifera*) leaf at concentrations as low as 3 μg/ml (65). On the other hand, a recent study showed that up to 500 μg/ml of (R)-melline is required to cause necrosis on the calli of *V. vinifera* and induces defense gene expression (66). Production of O-methylmelline was induced in an agar medium coculture with *Botryosphaeria obtusa* and *Eutypa lata*, the two wood-decaying fungi involved in esca disease of grapevine (34). The O-methylmelline isolated in the study showed a strong antigerminative effect against garden cress at a 0.001% (10 μg/ml) concentration. For wheat, Keller et al. reported that (R)-melline inhibits the growth of wheat embrylo culture at 50 μg/ml (67).

Here, we showed that the *P. nodorum ΔSN477* mutants did not have observable differences in virulence against wheat compared with that of the *P. nodorum* WT. The phytotoxic effects of (R)-melline and O-methylmelline were also tested by infiltration of the compounds on wheat leaves on whole plants, but neither compound induced any necrotic or chlorotic symptoms at concentrations up to 200 μg/ml. Thus, the results together rule out the possibility that (R)-melline and O-methylmelline play any impor-
tant role in lesion development on wheat. Curiously, when tested for antigerminative activity, (R)-mellein at 200 μg/ml completely inhibited the germination of wheat and barley medec seeds. The results suggest that (R)-mellein at 200 μg/ml may interfere with the cellular pathway involved in germination or hormone signaling in plants. This result is consistent with the previous observation that (R)-mellein suppressed the elongation of roots and coleoptiles of wheat seedlings at concentrations ranging from 50 to 200 μg/ml (16). In addition, it has been observed that mellein slows the cell cycle, with the cell having an extended mitotic phase (68). Interestingly, the biological sources of (R)-mellein are over-represented in fungal plant pathogens (39, 53, 54, 57, 65) and endophytes (51, 52, 58, 59). Whether mellein plays a role in this plant-fungus interaction and why SN477 is strongly upregulated in planta are questions that warrant future investigation.

The characterization of SN477 encoding a MLNS prompted us to survey its distribution in other fungal genomes, in particular, in those of the Dothideomycetes, many members of which are plant pathogens. The survey showed that about one-fifth of the dothideomycete genomes in the JGI database encode a close homolog of SN477. In contrast, among the nondotheomycete fungal genomes, SN477 homologues can be found in only two sordariomycete genera (Pestalotiopsis fici [PFICI] and Aplpr1, Botryosphaeria dothidea [UCNRPI2], Aplsporea prunicola [Applpr1], Botryosphaeria dothisae [Botdo1], Macrophomina phaseolina [MPH]) in the phylogenetic tree contain both MLNS and 6MSAS homologues. This suggests that the fungal MLNSs may have originated from a typical gene duplication and functional divergence from an ancestral Dothideomycetes fungus and both orthologues were retained in some of the Botryosphaeriaceae species. It also suggests that the PKS gene has frequently been lost from many taxa.

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