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High Trap Formation and Low Metabolite Production by Disruption of the Polyketide Synthase Gene Involved in the Biosynthesis of Arthrosporols from Nematode-Trapping Fungus *Arthrobotrys oligospora*

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Supporting Information

ABSTRACT: A group of morphology regulatory arthrosporol metabolites have been recently characterized from carnivorous fungus *Arthrobotrys oligospora* that can develop trapping networks to capture their prey. A combination of genetic manipulation and chemical analyses was applied to characterize the function of one polyketide synthase (PKS) gene *AOL_s00215g283* in *A. oligospora*, which was putatively involved in the production of 6-methylsalicylic acid. High-performance liquid chromatography analysis showed that the disruption of the PKS gene not only led to the total loss of the arthrosporol A but also resulted in significant reduction in the production of secondary metabolites in the cultural broth of the mutant Δ*AOL_s00215g283* strain. Interestingly, the mutant strain displayed significant increases in the trap formation and the nematicidal activity by 10 and 2 times, respectively, higher than the wild-type strain. These findings revealed a pathogenicity-related biosynthetic gene of this agriculturally important biological agent and have implications for establishment of efficient fungal biocontrol agents.

KEYWORDS: *nematode-trapping fungus, Arthrobotrys oligospora, 6-methylsalicylic acid, biosynthesis, arthrosporol*

INTRODUCTION

Plant-parasitic nematodes are one of the most devastating pest groups responsible for insidious disease symptoms in different crops and cause significant global crop loss.[1,2] Because of the banning of the most important chemical nematicides, the need for alternative management systems for the control of plant-parasitic nematodes has increased dramatically over the past decade. Therefore, biological control of phytonematodes has received an enhanced impetus.[3]

Nematode-trapping fungi show a distinguishing feature, predatism, which is the ability to capture nematodes with developing specific trapping devices, such as adhesive networks, adhesive knobs, and constricting rings to capture nematodes and then extract nutrients from their nematode prey.[4] The formation of trapping devices by nematode-trapping fungi is an important indicator of their switch from the saprophytic to the predacious lifestyles. They play important roles in controlling nematode population density in diverse natural environments and are of great importance for improving agricultural production. The behavior and ecology of nematode-trapping fungi have been studied in the past in attempts to make them better biological control agents.[5]

Arthrobotrys oligospora Fres., the first recognized nematode-trapping fungus,[6] is the most commonly isolated and by far the most abundant nematode-trapping fungus in the environment.[7] Strains of *A. oligospora* have been found in diverse soil environments, including heavy-metal-polluted soils and decaying wood, where they live mainly as saprophytes.[7,8] In the presence of nematodes, *A. oligospora* enters the predatory stage by forming complex three-dimensional networks to trap nematodes.[9] It has been assumed to be among the biggest contributors in controlling the population of nematodes and extensively studied as a model system for identifying and characterizing the ecology and biology of nematode-trapping fungi.[3−10]

A unique class of the oligosporon metabolites have been reported from the strains of *A. oligospora* from the Netherlands,[11] Australia,[12] and China.[13] The skeletal feature of these secondary metabolites is a polyketide-derived epoxy nucleus combined with a terpenoid-derived linear farnesyl unit. These compounds exhibited interesting biological activities, including nematicidal and antibacterial properties. Recently, we characterized a new group of oligosporon metabolites, arthrosporols (Figure 1), with a novel carbon scaffold from *A. oligospora*.[14] The arthrosporol metabolites possessed the similar polyketide-derived epoxy nucleus combined with a six-membered monocyclic farnesyl unit and displayed significant autoregulatory effects on the formation of conidiophores and the transition of hypha to a two-dimensional network in *A. oligospora*, which correlate with fungal reproductive and defensive capabilities, respectively.[14]

Up to now, the arthrosporol metabolites represented the most complex structural types of bioactive metabolites to be isolated and characterized from nematophagous fungi. Hybrid natural metabolites derived through mixed biosynthetic pathways, coupled with their interesting bioactivity profile, have

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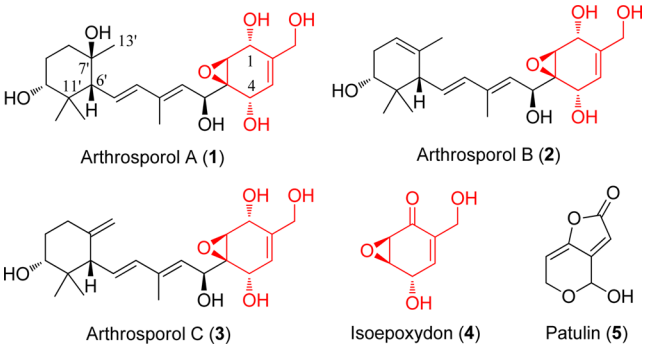

Figure 1. Arthrosporols A–C (**1**–**3**) from nematode-trapping fungus *A. oligospora*, isoepoxydon (**4**), and patulin (**5**).

been evoking our great interest in the biosynthesis of this class of naturally occurring metabolites from *A. oligospora*.

We now report the identification of the polyketide synthase (PKS) gene encoding 6-methylsalicylic acid synthase involved in the biosynthesis of the arthrosporol metabolites in *A. oligospora*. The effect of disruption of the PKS gene on the production of arthrosporol metabolites was investigated. The formation of adhesive trapping networks and capture of nematodes by the mutant strain were also studied. Our results suggested that the PKS gene might play an important role in regulating the trap formation and the lifestyle switching of this fungus.

MATERIALS AND METHODS

Sequence and Phylogenetic Analysis of *AOL_s00215g283* in *A. oligospora*. Predicted *AOL_s00215g283* in *A. oligospora* was annotated by BLAST searches against protein databases and InterProScan searches against protein domain databases. The amino acid sequence of AOL_s00215g283 in *A. oligospora* was downloaded from GenBank, and the sequences from different fungi were downloaded from GenBank. The amino acid sequences from different fungi were analyzed using the DNAman software package, version 5.2.2 (Lynnon Biosoft, Vaudreuil, Quebec, Canada). A neighbor-joining tree was constructed using the Mega 5.1 software package. A web-based analysis platform antiSMASH 2.0[15] was applied to perform genome mining of the biosynthetic gene clusters. The conserved functional domains were analyzed using InterProScan 4.8 with default parameter settings.

Fungal Material. The strain *A. oligospora* YMF 1.3170 was isolated from Jiuquan, Gansu, People's Republic of China,[14] and identified as *A. oligospora* by morphological features. The isolate was deposited in the strain collection of the Laboratory for Conservation and Utilization of Bio-Resources and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University. After the conidia of *A. oligospora* YMF 1.3170 had developed on potato dextrose agar (PDA) slants in test tubes at 25 °C, the strain was kept at −30 °C as stock cultures. DNA extraction of *A. oligospora* YMF 1.3170 and polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) rRNA gene were carried out according to the literature. A characteristic fragment (GenBank accession number JX244893) was amplified by PCR using the species-specific primers ITS4 and ITS5 and the genome DNA of *A. oligospora* YMF 1.3170 as the template. The BLAST searches in the GenBank showed that the DNA fragment was most similar to the published ITS sequences of *A. oligospora*, consistent with morphological identifications.

Disruption Vector Construction. The plasmid pAg1-H3,[16] which was used for gene disruption vector construction and transformation, was maintained in *Escherichia coli* strain DH5a (Takara, Shiga, Japan). The strain *A. oligospora* YMF 1.3170 used in this study was maintained on PDA at 28 °C. Genomic DNA of *A. oligospora* YMF 1.3170 was extracted as previously described.[17] Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs (Beverly, MA). In-Fusion HD Cloning Kits were from Clontech Laboratories (Mountain View, CA). The left and right DNA fragments flanking the hygromycin-resistant gene (hygR) in pAg1-H3 vector were PCR-amplified from the genomic DNA of *A. oligospora* YMF 1.3170 by GXL high-fidelity DNA polymerase (Takara, Shiga, Japan), using primer sets 283-5f, GAGCTCGGTACCAAGGCCCGGGGGAGCCGCAGAGGACAACA; 283-5r, AGGCCTGATCATCGATGGGCCCCGACATGCCATGCCGATT; 283-3f, TCTAGAGGATCCCCCGACTAGTATTATCGGTAGCCTTTGTC; and 283-3r, CACGAAGCTTGCATGCCTGCAGGAGGATTCGGTATTGTTGTT. The DNA fragments (5′ flank and 3′ flank) were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). The purified 5′ flank DNA fragment was inserted into the *Sma*I- and *Apa*I-digested pAg1-H3 vector by the in-fusion method to produce pAg1-H3-283-5′. Similarly, the 3′ flank DNA fragment was inserted into the *Spe*I and *Sbf*I sites of pAg1-H3-283-5′ to generate the completed disruption vector pAg1-H3-283-5′-3′.

The homologous fragment amplifications were carried out as follows. A total of 1 μL of the prepared genomic DNA template from *A. oligospora* YMF 1.3170 was added to a 50 μL PCR amplification system using GXL high-fidelity DNA polymerase following the instructions of the manufacturer (Takara). All PCRs were carried out in a Veriti 96-well thermal cycler (PE Applied Biosystems). The amplification program consisted of predenaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 57 °C for 15 s, and elongation at 68 °C for 2 min, with a final extension step at 68 °C for 10 min.

Preparation of Fungal Protoplasts. Regeneration medium PDASS (PDA supplied with 0.6 M sucrose, 0.3 g/L yeast extract, 0.3 g/L tryptone, and 0.3 g/L peptone) was used for protoplast regeneration,[18] and PDASS supplemented with 200 μg/mL hygromycin B (Amresco, Solon, OH) was used for selecting transformants. In the initial transformation for the disruption of the PKS gene, four 1 cm diameter mycelia plugs from YMA medium (2 g/L yeast extract, 10 g/L malt extract, and 18 g/L agar) for 7 days at 28 °C were inoculated into 100 mL of TG medium consisting of 1% tryptone (Oxoid, Basingstoke, U.K.) and 1% glucose and cultures at 30 °C and 180 rpm for 36 h. The mycelia were harvested and resuspended in 20 mL of a filter-sterilized enzyme solution that contained 120 mg of lysing enzymes (Sigma, St. Louis, MO), 0.4 mL of cellulase (Sigma, St. Louis, MO), and 100 mg of snailase (Solarbio, Beijing, China) in 0.6 M $MgSO_4$ at pH 6.0. Snailase, extracted from the crop and digestive tract of snails, consists of more than 30 enzymes, including cellulase, hemicellulase, β-glucuronidase, etc. The suspension was incubated for 4 h at 28 °C on a rotary shaker at 180 rpm. Protoplasts were collected by filtering through six layers of sterile lens-cleaning tissue and centrifuged at 1000*g*. The protoplasts were washed twice with KTC (1.2 M KCl, 10 mM Tris-HCl, and 50 mM $CaCl_2$) solution and finally resuspended in the same solution.

Transformation of Fungus *A. oligospora*. The protoplast-based protocol for the gene disruption in *A. oligospora* YMF 1.3170 was performed as described in a previous report,[18] with modifications. A total of 100 μL of protoplasts (ca. 8.0×10^7 mL^{-1}) were mixed with 10 μg of linear DNA in a 1.5 mL centrifuge tube. After 30 min of incubation on ice, 600 μL of PTC (50% polyethylene glycol 6000, 20 mM Tris–HCl at pH 7.5, and 50 mM $CaCl_2$) was added to the mixture and mixed gently. After incubation at 28 °C for 1 h and regeneration for 12 h, the putatively transformed protoplasts were plated onto PDAS medium (PDA supplemented with 5 g/L molasses, 0.6 M saccharose, 0.3 g/L yeast extract, 0.3 g/L tryptone, and 0.3 g/L casein peptone) containing 200 μg/mL hygromycin B (Roche Corporation, Germany). The transformation colonies were selected after incubation at 28 °C for 6–7 days. After single-spore isolation, the putative transformants were transferred onto TYGA medium alone (10 g/L tryptone, 10 g/L glucose, 5 g/L yeast extract, 5 g/L molasses, and 18 g/L agar) containing 200 μg/mL hygromycin B and incubated for 5 days at 28 °C. Genomic DNA of putative transformants were extracted and verified by PCR to check for the integration of genes in

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the genome. The positive transformants were further confirmed by Southern blot analyses. Southern analysis was carried out according to the instructions provided by the North2South chemiluminescent hybridization and detection kit (Pierce, Rockford, IL). The primer pair 283-S1 (TTGGAGAAACCGAACAACCT) and 283-S2 (TTGGTGAGGAGTGAATAGCG) were used as Southern hybridization probes, and restriction enzyme *Ssp*I was used to digest the genomic DNA of the wild-type *A. oligospora* and the mutant strains for Southern analysis.

Vegetative Growth and Conidia Yield Comparisons. The 9 mm diameter mycelia plugs from 7-day-old fungal colonies of the wild-type and mutant strains were inoculated onto 9 cm plates with different media (6 replicates/strain). The mycelia plugs of the same size and age were incubated at 28 °C at both light and dark conditions for 6−10 days, respectively. Radial colony growth rates and colony morphology were recorded at a 24 h interval for 1 week, and the growth rate was calculated as millimeters per hour each day. The growth rates and colony morphology of the wild-type and mutant strains were compared in PDA, YMA (2 g/L yeast extract, 10 g/L malt extract, and 18 g/L agar), and TYGA (10 g/L tryptone, 10 g/L glucose, 5 g/L yeast extract, 5 g/L molasses, and 18 g/L agar) media. Similarly, to test the adaptive abilities, the growth rates were compared in TGA medium supplemented with stress-related compounds, including 0.1−0.3 mol/L NaCl, 5−15 mmol/L H_2O_2, and 0.01−0.03% sodium dodecyl sulfate (SDS).

To assess sporulation capacities of the wild-type and mutant strains, 100 μL aliquots of conidial suspension were spread on 9 cm plates with YMA medium and incubated for 10 days at 28 °C and the conidia were washed in 10 mL of sterile water, followed by filtration through four layers of lens tissues to remove mycelial debris. The concentrations of the conidial suspension were determined with microscopic counts on a hemocytometer.[18]

Induction of Trap Formation and Virulence Assays. *Caenorhabditis elegans* (strain N2) was cultured in oatmeal medium at 22 °C for 6−7 days. The wild-type and mutant strains were cultivated on agar plates by spreading approximately 10^6 conidia per plate. After 3 days of incubation at 28 °C, 300 adult nematodes were added to the middle of the plate. Fungal mycelia and traps were observed, and the numbers of captured nematodes were recorded after 12 and 24 h, respectively, under light microscopy (Olympus, Tokyo, Japan). The experiments were performed in triplicate.

Fermentation and Extraction Procedures. The wild-type *A. oligospora* and the Δ*AOL_s00215g283* mutant strains cultured on PDA medium at 28 °C for 7 days were inoculated into 500 mL flasks containing 250 mL of production PD medium (200 g/L potato and 20 g/L dextrose). The culture broths of the 14-day-old liquid fermentations (1.5 L each strain) were filtered to separate the mycelia from the cultures. The culture filtrates were concentrated *in vacuo* to 50 mL and were exhaustively extracted overnight with ethyl acetate (1:1, v/v). The organic fractions were evaporated to dryness to give residues. The dried organic residues were then dissolved in 500 μL of methanol, filtered through 0.22 μm membranes, and further analyzed using high-performance liquid chromatography with photodiode array detection (HPLC−DAD) and gas chromatography−mass spectrometry (GC−MS).

HPLC Analysis. HPLC analysis was carried out using a HPLC-1200 series (Agilent, Waldbronn, Germany) system equipped with a G1311A binary pump and G1315A photodiode array ultraviolet/visible (UV/vis) detector, employing the following instrumental conditions: column, CAPCELL PAK C18, 5 μm, 4.6 × 250 mm (Shiseido). The total flow rate was 1 mL/min; mobile phase A was 0.1% formic acid in water; and mobile phase B was 0.1% formic acid in acetonitrile. The column temperature was maintained at 40 °C. The injection volume for the extracts was 25 μL. The liquid chromatography (LC) conditions were manually optimized on the basis of separation patterns with the following gradient: 0−2 min, 10% B; 10 min, 25% B; 30 min, 35% B; 35 min, 50% B; 45 min, 90% B; 47 min, 10% B; and 49 min, 10% B. UV spectra were recorded at 220−400 nm. The standard sample arthrosporol A was isolated from wild-type *A. oligospora* according to the modified method described in the literature.[14] A 14-day-old fermentation broth of strain *A. oligospora* YMF 1.3170 cultured on PDA medium was filtered to separate the mycelia from the culture. The culture filtrate was concentrated *in vacuo* and partitioned with acetyl acetate, and the organic part was evaporated to dryness to give an oily residue. The residue was loaded onto a macroporous resin column (7 × 80 cm) and eluted with H_2O−MeOH (0, 25, 50, and 90%, each in 3 L) to yield four fractions (A−D) based on thin-layer chromatography (TLC) behavior. Fraction C obtained on elution with 50−90% MeOH−H_2O was further subjected to a Sephadex LH-20 gel column (4 × 150 cm) eluting with MeOH (2.5 L) to yield six subfractions. Subfraction 3 was repeatedly subjected to a Sephadex LH-20 gel column (3 × 100 cm) eluting with acetone (2.3 L), a C18 column (3 × 50 cm) eluting with H_2O−MeOH (35, 37, and 39%, each in 1.8 L), and a silica gel column (2 × 40 cm) eluting with $CHCl_3$−MeOH (35:1 and 20:1, each in 1.5 L) to obtain arthrosporol A. The purified sample arthrosporol A was elucidated and confirmed with nuclear magnetic resonance (NMR) data.[14]

GC−MS Analysis, Metabolite Identification, and Semi-quantitation. The GC−MS analysis was performed with a 5890 series II Plus gas chromatograph interfaced with a model 5972 mass spectrometric detector (Hewlett-Packard, Avondale, PA) equipped with a 30 m long, 0.25 mm inner diameter, and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 100 to 300 °C at a rate of 5 °C/min. Helium was used as a carrier gas at a flow rate of 0.7 mL/min. The split ratio was 1:20; the injector temperature was 280 °C; the interface temperature was 300 °C; and the ionization voltage was 70 eV.

Identification of these peaks was performed through retention time index and mass spectrum.[19] Compounds were designated as metabolites if they were identified with a match 900 on a scale of 0−1000 and retention index (RI) deviation of 3.0. The semi-quantitative analysis of the main compounds was carried out by internal normalization with the area of each compound. The addition of each area of the compounds corresponds to 100% area.[20]

Statistical Analysis. Statistical analysis were performed using the SPSS package, version 17.0 for Windows (Chicago, IL). Differences with $p < 0.05$ were regarded as statistically significant, and differences with $p < 0.01$ were regarded as highly statistically significant.

RESULTS AND DISCUSSION

The arthrosporol metabolites possess a novel hybrid carbon skeleton consisting of a polyketide-derived epoxycyclohexenol combined with a terpenoid-derived monocyclic sesquiterpenol substructure. Arthrosporols B (**2**) and C (**3**) were assumed to be two dehydration derivatives of arthrosporol A (**1**).[14] All of the arthrosporols shared the same epoxycyclohexenol moiety, and the difference among them only occurred in the monocyclic sesquiterpenol substructure. From the biosynthetic point of view, a PKS gene should be involved in the production of arthrosporols in nematode-trapping fungus *A. oligospora*.

A search for the epoxycyclohexenol moiety in the SciFinder database found that the epoxycyclohexenol moiety of arthrosporols was quite similar to a known compound isoepoxydon (**4**) (Figure 1).[21] They even shared the same stereochemistry for the epoxy ring and the hydroxyl group at C-4. The only difference between them was that the hydroxyl group at C-1 in the epoxycyclohexenol moiety of arthrosporols was replaced by a ketone at C-1 in isoepoxydon.

Isoepoxydon is an important intermediate metabolite for the biosynthesis of a well-studied antibiotic and mycotoxin, patulin (**5**). Patulin a toxic chemical contaminant produced by *Aspergillus*, *Penicillium*, and *Byssochlamys* spp.[22] It is the most common mycotoxin found in apples and apple-derived products, such as juice, cider, compotes, and other food, intended for young children. The fact that exposure to this mycotoxin is associated with immunological, neurological, and gastrointestinal outcomes has led to patulin being included

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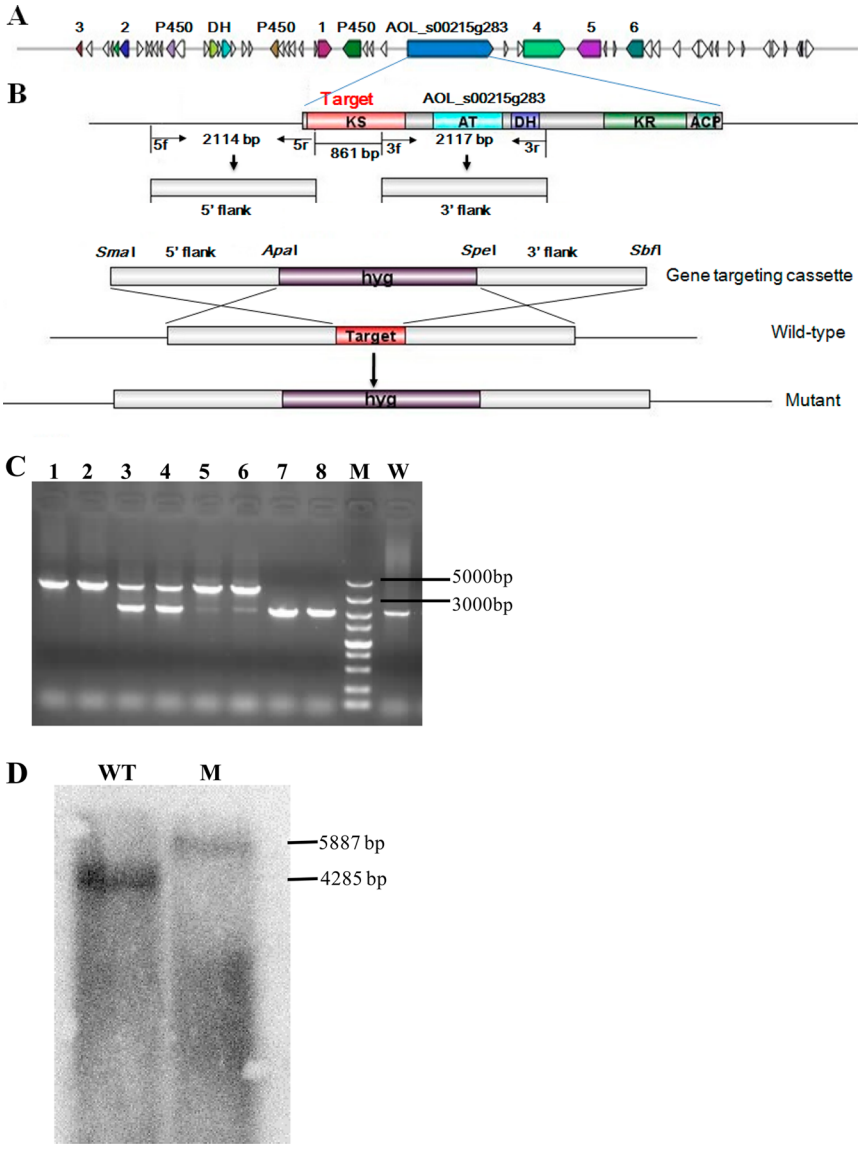

Figure 2. *AOL_s00215g283* gene cluster and schematic diagram of gene disruption. (A) *AOL_s00215g283* gene cluster spanning 51.843 kb and consisting of 11 genes with their predicted functions color-coded: 1, amidohydrolase (AOL_s00215g281); 2, mannose-6-phosphate isomerase (AOL_s00215g277); 3, polyprenyltransferase (AOL_s00215g276); 4, DNA translocase (AOL_s00215g284); 5, Gcd10p family (AOL_s00215g285); 6, single-stranded nucleic-acid-binding domain (AOL_s00215g286); P450, cytochrome P450s (AOL_s00215g282, AOL_s00215g280, and AOL_s00215g278); and DH, dehydrogenase (AOL_s00215g279). (B) Sketch map of the replacement of the targeting gene using a homologous recombination method. (C) Transformants confirmed by the PCR method: W, wild-type strain; lanes 1 and 2, two Δ*AOL_s00215g283* mutants; M, marker (DL5000). (D) Southern analysis of the wild-type strain (WT) and the Δ*AOL_s00215g283* mutant (M). The genomic DNA was digested using *Ssp*I.

among mycotoxins whose level in food is regulated in many countries around the world.[23] Similar to several other major mycotoxins, including aflatoxins, fumonisins, zearalenone, and ochratoxins, patulin is a polyketide metabolite.[24] The first step in the biosynthesis of patulin needs the involvement of a PKS, identified as 6-methylsalicylic acid synthase.[25] The most recent study revealed that 6-methylsalicylic acid synthase is also involved in the production of a meroterpenoid antibiotic yanuthone D in *Aspergillus niger*.[26]

Bioinformatic analysis of the *A. oligospora* genome identified five putative PKS genes. Among these genes, the gene *AOL_s00215g283* was annotated for type I PKS synthase.[17] Sequence analysis and a database search using the BLASTP program revealed that the amino acid sequence of AOL_s00215g283 shared 53% similarity to 6-methylsalicylic acid synthases from *Endocarpon pusillum* (XP_007785679.1), *Byssochlamys nivea* (AAK48943.1), and *Aspergillus terreus* (BAA20102.2 and XP_001215453.1). A total of 42 homologues of AOL_s00215g283 were retrieved, and a neighbor-joining phylogenetic tree was built using these amino acid sequences of these proteins. From this tree, we found that AOL_s00215g283 was clustered within the fungal polyketide synthases and formed a distinct monophyletic branch with no other closely related PKS as of yet. Strangely, no homologues identified from other nematode-trapping fungi, such as *Dactylellina haptotyla* and *Drechslerella stenobrocha*,[17] clustered with the amino acid sequence of AOL_s00215g283.

A previous study revealed that the genes involved in the patulin biosynthesis pathway have been found to be located in a cluster on one chromosome as is the case for a large number of

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mycotoxin pathways.[24] Five genes in the early portion of the patulin biosynthesis pathway up to the production of isoepoxydon have been identified as *PatK*, *PatG*, *PatH*, *PatI*, and *PatN*, encoding 6-methylsalicylic acid synthase, 6-methylsalicylic acid decarboxylase, and three cytochrome P450s, including *m*-cresol hydroxylase (P450), *m*-hydroxybenzyl alcohol hydroxylase (P450), and isoepoxydon dehydrogenase (P450), respectively.[22]

The analysis of the similar cluster involved in the biosynthesis of isoepoxydon in the *A. oligospora* genome was carried out. Through antiSMASH 2.0, a cluster containing the predicted PKS gene *AOL_s00215g283* was identified (Figure 2A). Most notably, the upstream flanking region of the gene *AOL_s00215g283* encodes an amidohydrolase (AOL_s00215g281), a dehydrogenase (AOL_s00215g279), a mannose-6-phosphate isomerase (AOL_s00215g277), a polyprenyltransferase (AOL_s00215g276), and three cytochrome P450s (AOL_s00215g278, AOL_s00215g280, and AOL_s00215g282). Interestingly, the downstream flank of the cluster encodes a DNA translocase (AOL_s00215g284), a member of the Gcd10p family (AOL_s00215g285), and a single-stranded nucleic-acid-binding domain (AOL_s00215g286). The amino acid sequence of AOL_s00215g281 shares 55% similarity to the 6-methylsalicylic acid decarboxylase (PatG). The amino acid sequence of AOL_s00215g282 shares 48 and 44% similarity to cytochrome P450s, PatH and PatI, respectively.

All of the above indicated that the gene cluster with the gene *AOL_s00215g283* was likely involved in the production of 6-methylsalicylic acid, which was predicted to be the first precursor for the biosynthesis of the polyketide-derived epoxycyclohexenol in arthrosporols.

To investigate the biological function of the gene *AOL_s00215g283*, the target fragment of this gene was knocked out by homologous recombination. Conserved domain analysis demonstrated that the gene *AOL_s00215g283* encoded five typical catalytic domains, including ketide synthase (KS), acyltransferase (AT), dehydratase (DH), *β*-ketoacylreductase domain (KR), and acyl carrier protein (ACP) for the PKS (Figure 2A). The fragment in the sequence encoding the KS domain was chosen to be the target. A modified method for genetic disruption of the gene *AOL_s00215g283* was developed using double-crossover recombination with the hygromycin-resistance gene (hyg) as a selection marker. The two homologous regions were amplified from *A. oligospora* genomic DNA using primers containing overlapping regions with the vector and the hyg-resistance cassette (Figure 2B). The disruption vector was transformed into the protoplasts of *A. oligospora* and yielded 20 transformants. Genomic DNAs of these transformants were isolated, and the site-specific insertion were confirmed using diagnostic PCR. In comparison to a 2035 bp fragment from the wild-type strain of *A. oligospora*, a 3637 bp fragment was amplified from the transformants using the primers 283yz-5f/283yz-3r (Figure 2C). In addition, these transformants were further confirmed using Southern blot analysis; the single band hybridizing the Δ*AOL_s00215g283* gene probe was observed in the wild-type strains and transformants (Figure 2D); and the sizes of the hybridized bands were consistent with expectation, indicating that the sequence encoding for the KS domain in the gene *AOL_s00215g283* in the mutant Δ*AOL_s00215g283* strain was disrupted. In comparison to the wild-type strain, the mutant Δ*AOL_s00215g283* strain showed similar growth rates and conditions on YMA, PDA, and TYGA media (Figure 3) and displayed a slight reduction in conidiation on the YMA

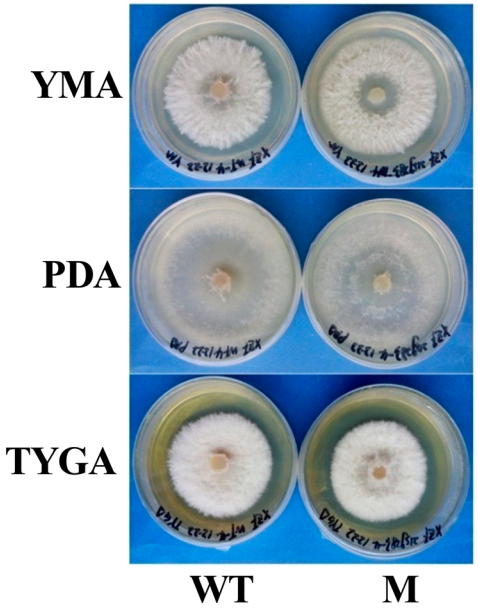

Figure 3. Comparison of the mycelia growths of the wild-type and mutant Δ*AOL_s00215g283* strains on YMA, PDA, or TYGA for 6 days at 28 °C.

medium. Similarly, both the mutant Δ*AOL_s00215g283* and wild-type strains exhibited the same growths in the presence of stress-related compounds. However, the mutant Δ*AOL_s00215g283* strain formed more adhesive networks than the wild-type strain after the nematodes were added on the 4 day cultures (Figure 4). After 12 h, the number of adhesive networks formed by the mutant Δ*AOL_s00215g283* was 318 cm^{-2} (Figure 4B), 10 times more than that of the wild-type strain (27 cm^{-2}). At the same time, the number of captured nematodes by the mutant Δ*AOL_s00215g283* was more than twice that of the wild-type strain (Figure 4C).

It was obvious that the mutant Δ*AOL_s00215g283* strain cultivated in liquid PDB for 14 days yielded light-colored culture compared to the wild-type strain. Both cultures were extracted, and the extracting organic portions were dried under *vacuo* to give a 276 mg extract for the wild-type strain and 54 mg for the mutant Δ*AOL_s00215g283*. The weight of the extract from the mutant Δ*AOL_s00215g283* strain was less than one-fifth of that from the wild-type strain. To investigate the involvement of the gene *AOL_s00215g283* in the biosynthesis of arthrosporols, both the extracts were then analyzed with the HPLC−DAD method (Figure 5). It is noteworthy that the HPLC metabolite profile of the mutant Δ*AOL_s00215g283* strain showed a significant difference from that of the wild-type strain. In comparison to the wild-type strain, the mutant Δ*AOL_s00215g283* strain lacked almost most of the peaks during the retention times ranging between 21 and 40 min in the HPLC chromatogram (Figure 5A). This was consistent with the above result that much less extract was obtained from the mutant Δ*AOL_s00215g283* strain. Further detailed analysis showed that no arthrosporol A was detected in the cultural broth of the mutant Δ*AOL_s00215g283* strain (Figure 5B), indicating that the disruption of the *AOL_s00215g283* gene led to a total loss of arthrosporol A

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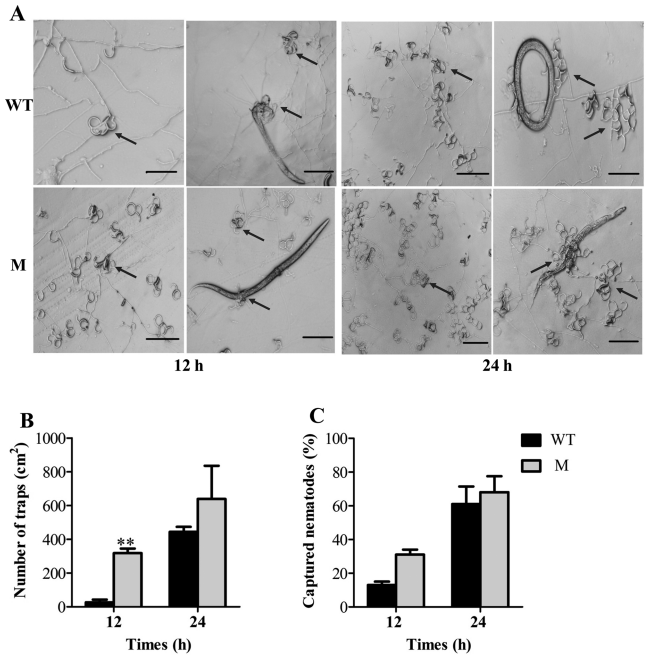

Figure 4. Comparison of trap formations and captured nematodes between the wild-type and mutant Δ*AOL_s00215g283* strains. (A) Formation of traps of the wild-type and mutant Δ*AOL_s00215g283* strains at 12 and 24 h: WT, wild-type strain; M, mutant Δ*AOL_s00215g283*. Scale bar = 50 μm. (B) Comparison of the trap formations. (**) $p < 0.01$ versus the wild-type strain. (C) Comparison of the fungal nematicidal activity against *C. elegans*.

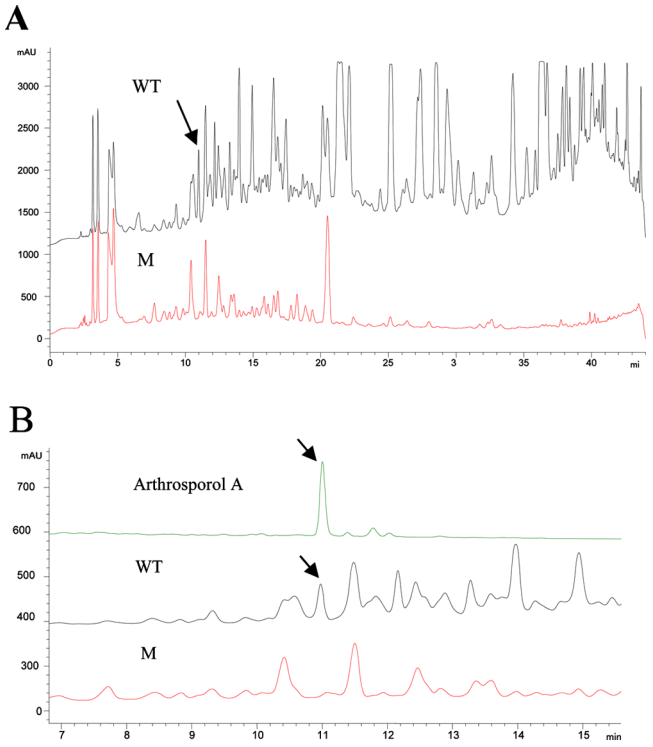

Figure 5. (A) HPLC analysis of the methanol extracts of the cultural broths from the wild-type strain (black line) and Δ*AOL_s00215g283* mutant (red line). (B) Amplified portion of the HPLC spectrum of the methanol extracts of the cultural broths from the wild-type strain (black line), mutant Δ*AOL_s00215g283* (red line), and arthrosporol A (green line). Black arrows indicate the peak of arthrosporol A at around 11 min.

and the gene *AOL_s00215g283* played a key role in the biosynthetic pathway for arthrosporol A.

Considering the possibility that the lack of the epoxycyclohexenol moiety caused by the disruption of the *AOL_s00215g283* gene might lead to the accumulation of the terpenoid-derived sesquiterpenol substructure, together with the fact that most sesquiterpenoids are volatile compounds, the culture broth of the wild-type and mutant Δ*AOL_s00215g283* strains were further analyzed by the GC−MS method. Surprisingly, two linear sesquiterpenoids, sesquirosefuran and 3,7,11-trimethyl-2,6,10-dodecatrienal, were found only in the wild-type strain. It is likely that the disruption of the *AOL_s00215g283* gene might also affect the production of the farnesol derivatives in *A. oligospora*.

A previous study displayed that farnesol secreted by *Candida albicans* could block the transition from yeast to hypha.[27] Reports of farnesol production by ascomycetes other than yeasts are rare, although some studies reported that farnesol blocked conidiation of *A. niger*,[28] inhibited cell wall integrity signaling and caused mislocalization of regulatory Rho proteins in *Aspergillus fumigatus*,[29] and also inhibited growth of other ascomycetes, for example, that of *Fusarium graminearum*.[30] In addition, farnesol displayed an intriguing effect on the *Neurospora crassa* circadian clock, which coordinated rhythmic asexual reproduction during vegetative growth.[31] Whether the lack of farnesol derivatives or terpenoid-derived arthrosporols in nematode-trapping fungus *A. oligospora* induced higher trap formation and less metabolite production need further detailed study.

In conclusion, by gene engineering and chemical metabolite profiling, we have identified the PKS gene *AOL_s00215g283* in nematode-trapping fungus *A. oligospora*. Our result revealed that the PKS gene played significant roles in not only the biosynthesis for the arthrosporols but also the fungal ability for predatory trap formation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04244.

Unrooted neighbor-joining tree of fungal PKS synthase homologues (Figure S1), comparison of the growths and sporulations of the wild-type and mutant Δ*AOL_s00215g283* strains (Figure S2), 10 day culture broths of the Δ*AOL_s00215g283* mutant and wild-type strains (Figure S3), list of predicted functions of genes in the *AOL_s00215g283* gene cluster (Table S1), compounds detected by GC−MS analysis in the wild-type strain (Table S2), and compounds detected by GC−MS analysis only in the mutant Δ*AOL_s00215g283* (Table S3) (PDF)

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Notes

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