SI Appendix

Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity

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SI Materials and Methods

Gene Deletion and Overexpression. Targeted gene deletion was performed by homologous recombination (1). Briefly, the 5' and 3' flanking sequences of *OpS1-OpS7* genes were amplified using different primer pairs (Table S1) with the Phanta™ Super-Fidelity DNA Polymerase (Vazyme, Piscataway, NJ). PCR products were digested with respective restriction enzymes (Table S1) and then inserted into the corresponding sites of the binary vector pDHt-bar to generate the disruption plasmids for *Agrobacterium*-mediated transformation (AMT) of *B. bassiana* (2). Deletion of the *OpS1* orthologous *PKS* gene was also conducted in *B. brongniartii*. Drug-resistant colonies were subcultured on PDA and verified for successful gene deletions by PCR. To verify gene functions, substrate feeding assays were performed including the addition of OA into the *ΔOpS1* culture and **6** into the *ΔOpS7* culture. To examine the function of the putative transcription factor gene *OpS3*, an overexpression vector of *OpS*3 was constructed using the vector pDHt-Ben containing a benomyl-resistant gene (2). The promoter of *gpdA* (glyeraldehyde-3-phosphate dehydrogenase) from *B. bassiana* was used to control the *OpS*3 gene to generate the plasmid pDHt-OpS3 for AMT transformation of the WT and *ΔOpS4-ΔOpS7* mutants of *B. bassiana*. The obtained genetically stable transformants were maintained on PDA plates for two weeks, and the conidia of the WT, *ΔOpS3* and WT*::OpS3* strains were inoculated in SDB for five days before harvesting for RNA extraction. The cDNA samples were prepared using the First Strand cDNA Synthesis Kit (TOYOBO) and used for RT-PCR analysis of gene expression.

Pigment Induction and Component Analyses. Conidia of the WT and different mutants were harvested and suspended in sterile aqueous Tween-20 (0.05% v/v) at 1×10^8 conidia/ml. Spore suspensions (50 μl each) were inoculated into 100 ml Erlenmeyer flasks containing 30 ml SDB medium and incubated at 25℃ on a rotatory shaker for 3 days (3). These seed cultures (1 ml each) were then transferred into 250 ml flasks containing 50 ml SDB medium

and incubated statically at 25℃ with controlled light (12 h/day) for 5 days. The culture filtrates were either extracted with ethyl acetate or directly used for chromatographic analysis with a LC-20AD HPLC system (Shimadzu Scientific Instruments) equipped with a C18 reverse phase column (Athena C18; particle size: 5 μm; length: 4.6×250 mm) and an SPD-M20A UV detector. The mobile phase consisted of water (with 0.1% TFA)/acetonitrile (88:12) for 35 min, at a flow rate of 1 ml/min. All samples were monitored at a wavelength of 254 nm. The molecular weights of different components were determined using a Q-TOF LC-MS system (Agilent G6520A). The oosporein was analyzed as $[M+H]^+$ ion and all other compounds were analyzed as $[M-H]^-$ ions. Collision-induced dissociation MS analyses were also conducted using the Q-TOF system to negatively ionize the compounds for fragmentation at the collision energy of 10 eV. To verify the structure of different compounds, H and C spectra of compounds **3**, **5** and **6** were also obtained using a Bruker Advanced III-500 MHz NMR spectrometer. For proper detection of the 13C signals of compound **6**, triethylamine was added in solvent dimethyl sulfoxide for NMR analysis (4).

Yeast Expression of Different Target Genes. To verify gene functions, heterologous expressions of the *OpS1* and *OpS4* genes were conducted in *P. pastoris* GS115. The *OpS1* and *OpS4* genes were amplified by PCR using cDNA as the template (with 2× Phanta Master Mix from Vazyme) and were inserted into the commercial vector pPICZ B under the control of the methanol-inducible *Aox1* gene promoter (Invitrogen). The plasmids were transformed into *P. pastoris* GS115 through electroporation as described in the manufacturer's protocol (*Pichia* Expression Kit, Invitrogen). To activate the heterologously expressed *OpS1*, a phosphor-pantetheinyl transferase gene BBA_06793 of *B. bassiana* was cloned into a commercial vector pPIC3.5k (Invitrogen) and transformed into the *OpS1*-expressing mutant (GS115*::OpS1*). Orsellinic acid (OA, Analytic grade 97%, Alfa Aesar) was added into the culture of the *OpS4*-expressing transformant (i.e., GS115*:: OpS4*) for bioconversion analysis.

Insect Bioassays. Insect bioassays were conducted using the newly emerged last instar larvae of the wax moth (*Galleria mellonella*). Each individual insect was injected in the second proleg with 10 μl of the spore suspension containing 1×10^6 conidia/ml of the WT, *ΔOpS1* and WT::*OpS3* strains suspended in 0.05% (v/v) Tween-20. Each strain had three replicates with 15 insects each, and the experiments were repeated twice. Additional insects were injected for bleeding to observe and compare fungal developments within insect hemocoels after treatment with different strains for 24, 36 and 48 h. Mortality was recorded every 12 h and the comparison of survival rates was determined by Kaplan-Meier analysis (5).

Insect Immune Interference Assays. To determine the effect of oosporein on inhibition of insect immune responses, the wax moth larvae were injected (10 μl each) with the

heat-killed spores (1×10^7 conidia/ml suspended in 0.05% Tween-20) with oosporein at the dosages of 10, 20 and 40 μg per insect. The untreated insects and insects injected with 10 μl of Tween were used as controls. The hemolymph was collected 12 hrs post treatment for PPO activity assay with the substrate dopamine (Sigma) using a Microplate Reader (Expert 96, Biochrom) (6). The fat bodies were dissected and used for RNA extraction and cDNA conversion. Western-blotting was also performed to verify the inhibition of PPO cleavage and activation. Semi-quantitative reverse transcription PCR was conducted to examine the expression of antifungal peptide gallerimycin gene (7).

Supporting references

1. Chen Y, Feng P, Shang Y, Xu YJ, Wang C (2015) Biosynthesis of non-melanin pigment by a divergent polyketide synthase in *Metarhizium robertsii*. *Fungal Genet Biol* 81:142-149.
2. Huang W, Shang Y, Chen P, Gao Q, Wang C (2015) MrpacC regulates sporulation, insect cuticle penetration and immune evasion in *Metarhizium robertsii*. *Environ Microbiol* 17(4): 994-1008.
3. Seger C, Erlebach D, Stuppner H, Griesser UJ, Strasser H (2005) Physicochemical properties of oosporein, the major secreted metabolite of the entomopathogenic fungus *Beauveria brongniartii*. *Helv Chim Acta* 88:802-810.
4. Love BE, Bonner SJ, Forrest LA (2009) An efficient synthesis of oosporein. *Tetrahedron Lett* 50, 5050-5052.
5. Gao Q, Shang Y, Huang W, Wang C (2013) Glycerol-3-phosphate Acyltransferase contributes to triacylglycerol biosynthesis, lipid droplet formation, and host invasion in *Metarhizium robertsii*. *Appl Environ Microbiol* 79(24):7646-7653.
6. Shao Q, et al. (2012) Hindgut innate immunity and regulation of fecal microbiota through melanization in insects. *J Biol Chem* 287(17):14270-14279.
7. Schuhmann B, Seitz V, Vilcinskas A, Podsiadlowski L (2003) Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella*. *Arch Insect Biochem Physiol* 53(3):125-133.

Supporting Table:

Table S1. Primers used in this study.

Gene	Primers	Primer sequences	Restriction enzymes	Length (bp)	Application
OpS1	*OpS*1-UF	CGGAATTCATGTAGCCGTTTCTCGTCAT	*EcoR*I	1590	Gene deletion
	*OpS*1-UR	CGGAATTCCCGATCAACATCGTGAATTA	*EcoR*I		
	*OpS*1-DF	GCTCTAGATGTATCATTGCTCTCCCGTA	*Xba*I	1561	
	*OpS*1-DR	GCTCTAGAAATCTCGCGATTCAAAGAAC	*Xba*I		
	OpS1-YF	AGAGCCTTTGCTGATTTTTG		1251	Verification
	OpS1-YR	TACGGGAGAGCAATGATACA			
OpS2	*OpS*2-UF	CCCTCGAGCAGATGACACCAGAACTTGC	*Xho*I	918	Gene deletion
	*OpS*2-UR	CGGGATCCGGTATCCTCACTCAGCGTTT	*Xho*I		
	*OpS*2-DF	GGACTAGTCCATTTCTCGCCATCTTTAT	*Spe*I	855	
	*OpS*2-DR	GGACTAGTGACTGTTGAGCATGTGAAGC	*Spe*I		
	OpS2-YF	CTTTGGATTTCCAGACTCGT		1377	Verification
	OpS2-YR	CTCATAGCGACCCTGTTCTT			
OpS3	*OpS*3-UF	CGGAATTCAACTAACTCGACGGCTTACG	*EcoR*I	878	Gene deletion
	*OpS*3-UR	CGGAATTCCTTCTCTTCCGTCCAGACAC	*EcoR*I		
	*OpS*3-DF	GGACTAGTGGAGCAGCATCGAGTACTTT	*Spe*I	902	
	*OpS*3-DR	GGACTAGTGATACTGTGGAGCATGGACA	*Spe*I		
	OpS3-YF	CGCAGTCGATAAGGTGAGTA		1312	Verification
	OpS3-YR	GCCTTTTCTAGCTGATTTGC			
OpS4	*OpS*4-UF	CGGGATCCAAAATCCCGATTCTGTCTTG	*BamH*I	855	Gene deletion
	*OpS*4-UR	CGGGATCCTGTAGAGAAGTGGCGACTCA	*BamH*I		
	*OpS*4-DF	GCTCTAGAGCCTACTTCGTTGAACTCG	*Xba*I	862	
	*OpS*4-DR	GCTCTAGAATATCTGTTGGGCTTGGATT	*Xba*I		
	OpS4-YF	TAACAGTCGACCCAACACTG			Verification
	OpS4-YR	CATTTTTCACCTCCACACAA			
OpS5	*OpS*5-UF	CGGGATCCATGCGTCCTTGGACAATACT	*BamH*I	952	Gene deletion
	*OpS*5-UR	CGGGATCCGATGTTTTTGCCATTGATGA	*BamH*I		
	*OpS*5-DF	GGACTAGTTCATGTGGACTCTGAACGAC	*Spe*I	907	
	*OpS*5-DR	GGACTAGTCTCGCTTAGATGTGCTTGAA	*Spe*I		
	OpS5-YF	GGTGGCTCAGCTAACAAAAT		1236	Verification
	OpS5-YR	GGCAAAGACCATGTATCCTC			
OpS6-7	*OpS*6-7-UF	CGGAATTCAATTGGGAAAGCAATGGTGG	*EcoR*I	787	Gene deletion
	*OpS*6-7-UF	CGGAATTCTTACCTCGAGTTTCCTGACG	*EcoR*I		
	*OpS*6-7-DF	GGACTAGTCGGCGTTTCAGATTATGCAA	*Spe*I	780	
	*OpS*6-7-DF	GGACTAGTTACGGCACCATTTCTCGTAA	*Spe*I		

	*OpS*6-7-YF	TCCTGGATTGCTGGAAGTTT		1146	Verification
	*OpS*6-7-YF	TCTTGGTCGTGTTTAGGGTC			
OpS3	*OpS*3-F	GCTCTAGAATGTTTTACACTTTCAATGC	*Xba*I	2226	Over expression
	*OpS*3-R	GCTCTAGATCAAAACGAATATAATTCGG	*Xba*I		
OpS1	*OpS*1-F	ACTAATTATTCGAAACGAGGATGCCGTCTTT CTTCCCAGTCTTCTCTGGA	PCR-fusion	6633	Yeast expression
	*OpS*1-R	CCGGCTGGGCCACGTGAATTCTTCAACTCAA ACTCCGAGCAGGCATC			
OpS4	*OpS*4-F	AGGAATTCATGGGCAGCATACGTGAACC	*EcoR*I	1284	Yeast expression
	*OpS*4-R	TGGCGGCCGCCTAGCTTGGTGTTTGCTAC	*Not*I		
OpS7	*OpS*7-F	AGGAATTCATGGGATTCGTACCGATTAC	*EcoR*I	918	Yeast expression
	*OpS*7-R	TGGCGGCCGCCAAATTCTTTTTATCCTCAC	*Not*I		
BBA_ 06793	6793-F	ATTCGAAGGATCCGACGTAGATGGCCACGGT CATACAATG	PCR-fusion	909	Yeast expression
	6793-R	CGCGGCCGCCCTAGGGAATTCTAATGATGAT GATGATGATGTCTTGTAGCTTTGAGCT			
Gallerimycin, AF453824	GalF	CTCGTAAAATACACATCCGGGG	/	150	Insect antifungal gene expression
	GalR	CTCGTAAAATACACATCCGGGG			
18 S gene, AF286298	18SF	CGCGCTACACTGAAGGAATC	/	165	RT-PCR gene reference
	18SR	TTGATTACGTCCCTGCCCTT			

Supporting Figures:

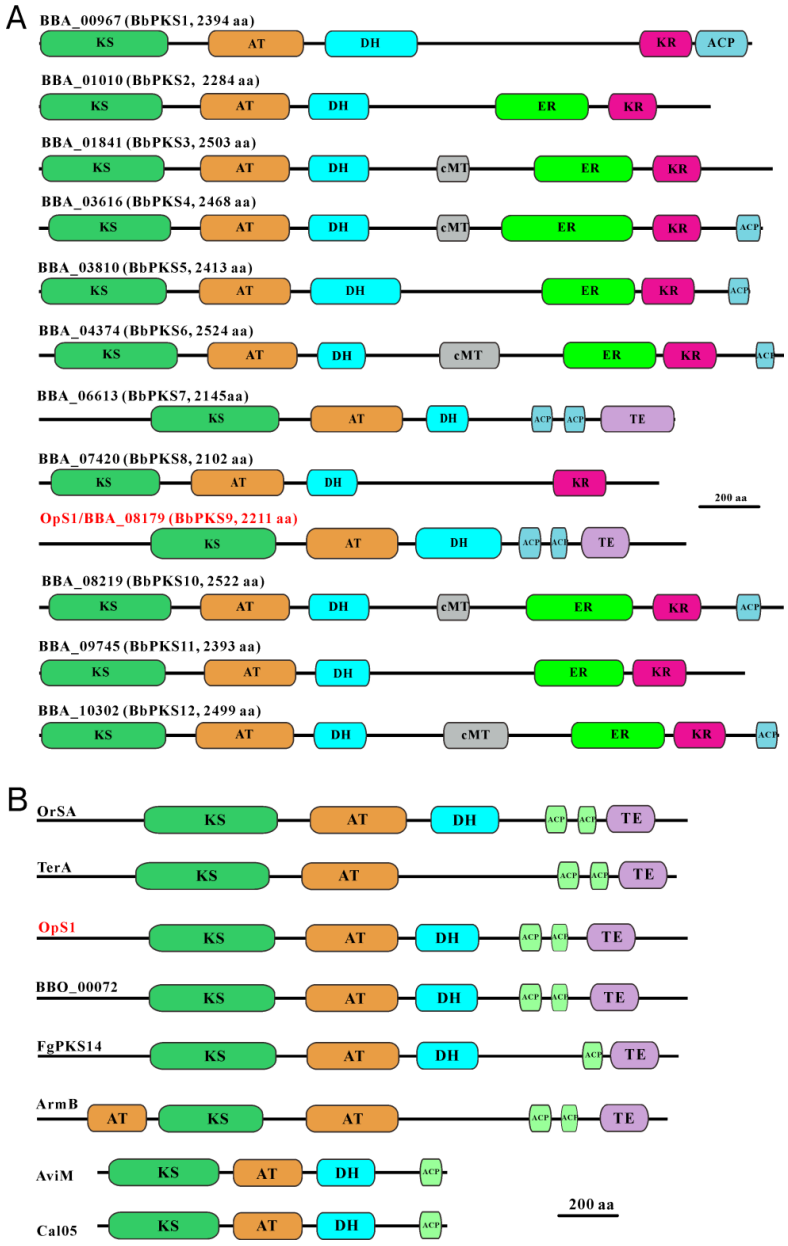

Fig. S1. Modular analysis of polyketide synthases (PKSs). (*A*) Modular analysis of 12 PKS enzymes, labeled in order as BbPKS1-BbPKS12, encoded in the genome of *Beauveria bassiana*. (*B*) Modulation and structural comparison of PKSs experimentally verified in biosynthesis of orsellinic acid in different fungi and bacteria. In addition to OpS1 and BBO_00072 from this study, OrsA (AN7909) is from the filamentous fungus *Aspergillus nidulans*; TerA (ATEG_00145) from *A. terreus*; FgPKS14 (FGSG_03964) from *Fusarium graminearum*; ArmB (AFL91703) from the mushroom *Armillaria mellea*; AviM (AAK83194) and Cal05 (AAM70355) are from the bacteria *Streptomyces viridochromogenes* and *Micromonospora echinospora*, respectively.

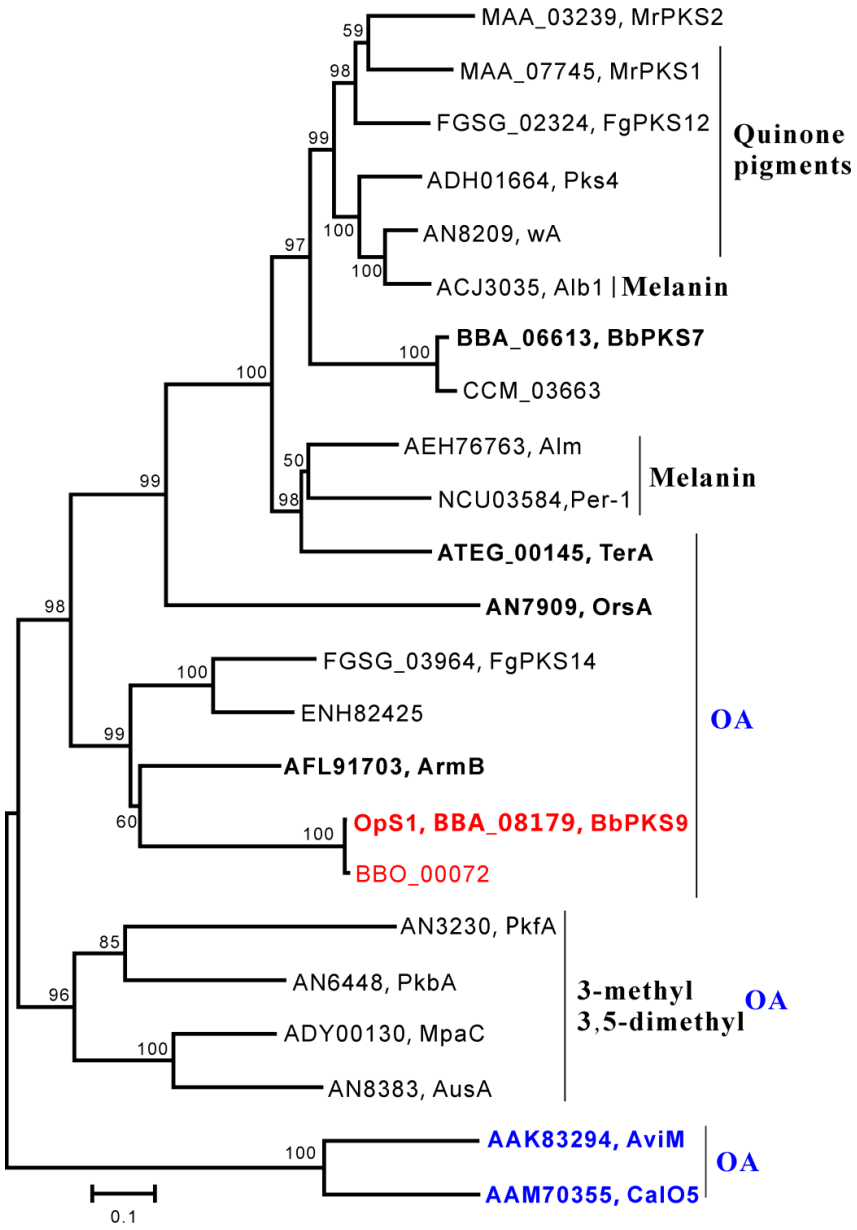

Fig. S2. Phylogeny analysis of different PKS enzymes involved in the biosynthesis of orsellinic acid (OA) and quinone pigments. The ketosynthase domain sequence from each PKS was retrieved and used for generation of this maximum-likelihood tree using a Dayhoff substitution model, with 500 bootstrap replications for the phylogeny test and a partial deletion for gaps/missing data treatment.

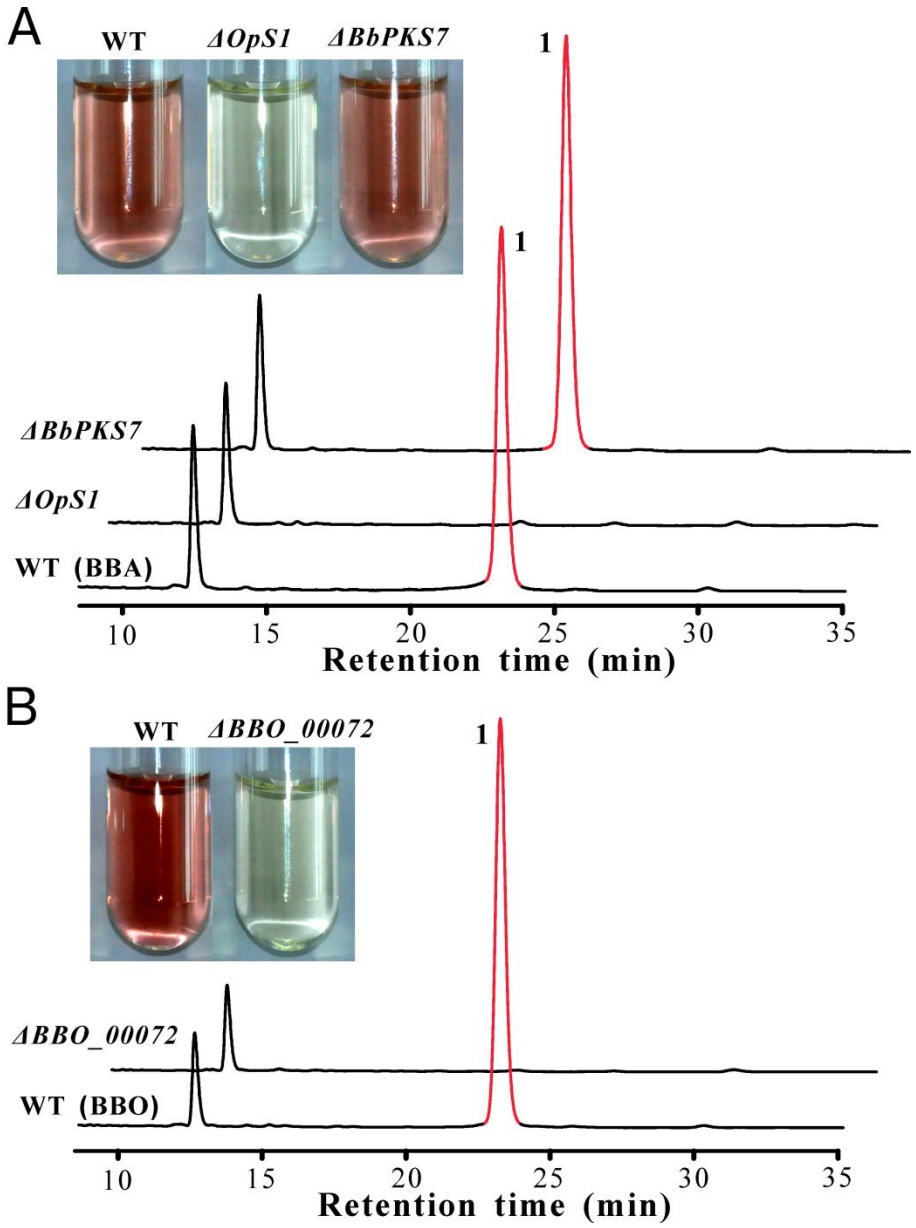

Fig. S3. Chromatographic examination for oosporein production in different strains. (*A*) Relative to the wild type (WT), deletion of *OpS1* (*BbPKS9*), but not *BbPKS7*, abolished oosporein (peak 1) production in *B. bassiana*. *Inset* shows the corresponding color of the culture filtrates of different strains. (*B*) Deletion of *OpS1* orthologous gene *BBO_00072* in *B. brongniartii* also led to the failure of oosporein production. *Inset* shows the corresponding color of the WT and null mutant culture filtrates.

A

Gene	Gene code	Putative function	Top hit with *A. nidulans* gene/name/protein identity
BBA_08179	***OpS1***	Type I iterative polyketide synthase	**AN7909/OrsA**/28.2%
BBA_08180	***OpS2***	MFS multidrug resistance transporter	**AN5656**/NA/46.2%
BBA_08181	***OpS3***	GAL4-like Zn2Cys6 transcription factor	**AN8441**/NA/22.7%
BBA_08182	***OpS4***	FAD binding domain-containing hydroxylase	**AN7684**/NA/44.7%
BBA_08183	***OpS5***	Laccase 2/Multicopper oxidase	**AN9170/LccB**/50.6%
BBA_08184	***OpS6***	Glutathione-S-transferase, theta	**AN4905/GstA**/34.4%
BBA_08185	***OpS7***	Cupin, Cupin_2 superfamily	NA

B

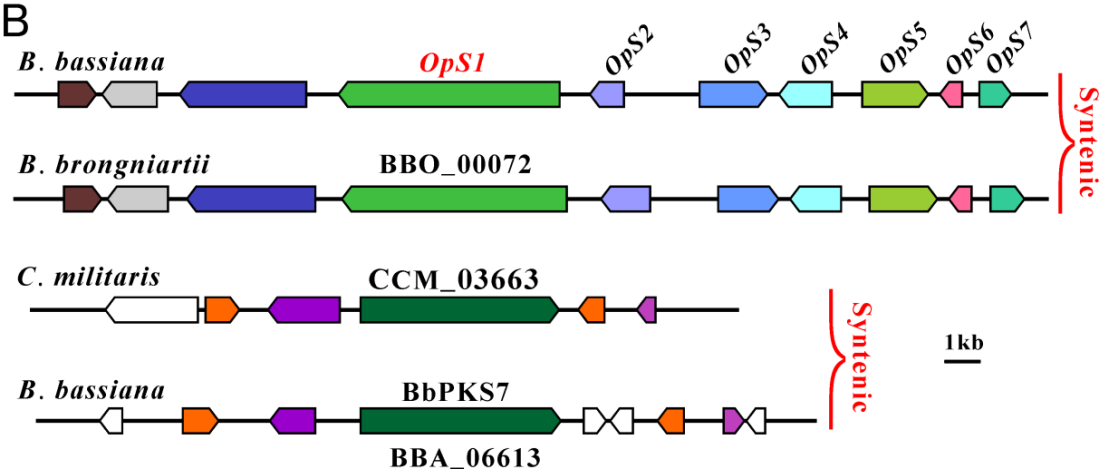

Fig. S4. Characterization of the *OpS1* gene cluster. (*A*) Annotation of the *OpS1* gene cluster. Except for putative cupin OpS7, homologous proteins of other genes are present in the *Aspergillus nidulans* genome. However, these genes are not clustered together in the *Aspergillus* genome, an indicator of non-oosporein production. (*B*) Synteny analysis of the *OpS1* (i.e., *BbPKS9*) and *BbPKS7* gene clusters. Consistent with the PKS gene function characterization, the cluster responsible for oosporein production is highly syntenically present in the genomes of *B. bassiana* and *B. brongiartii*. The non-producing *BbPKS7* cluster, however, has an orthologous counterpart in the *Cordyceps militaris* genome.

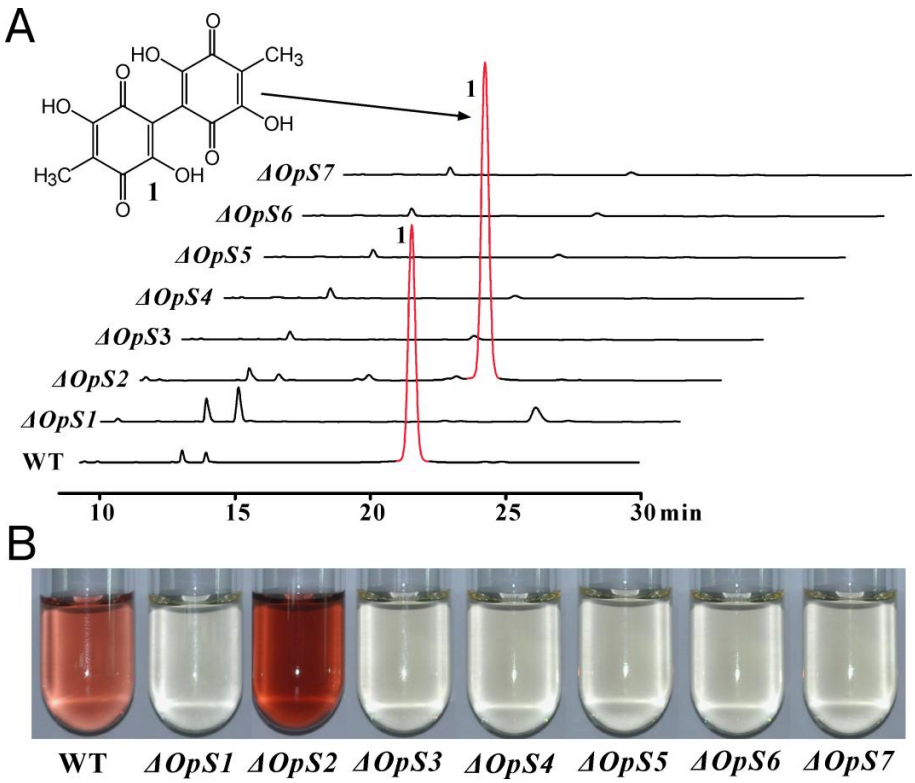

Fig. S5. (*A*) Chromatographic profiling of the WT and the gene deletion mutants. The red peak is for oosporein (**1**). (*B*) Culture filtrate color of the WT and the gene deletion mutants.

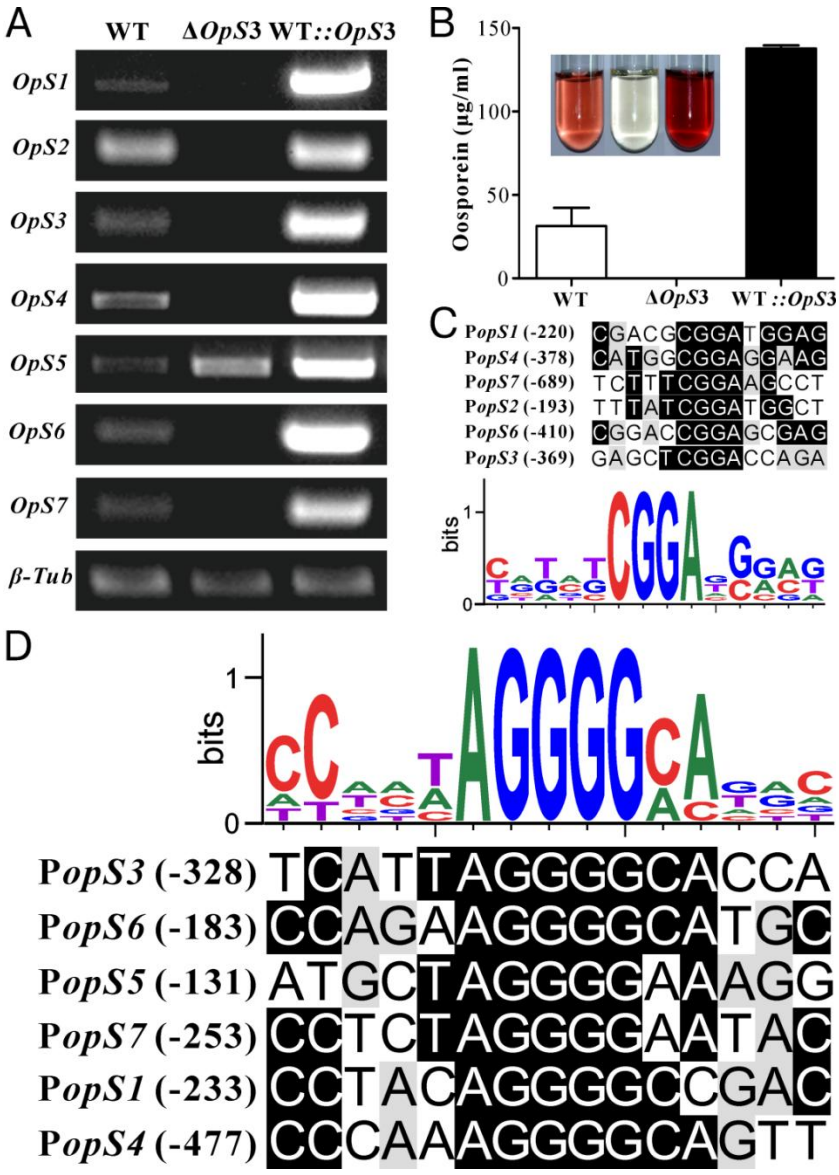

Fig. S6. Transcriptional regulation of the *OpS1* gene cluster by OpS3. (*A*) RT-PCR profiling of gene expression by the WT, *ΔOpS1* and WT*::OpS3* strains after growth in SDB for five days under the 12:12 hr of light:dark control. The β-tubulin (β-Tub) gene was used as a reference. (*B*) Quantification of oosporein production by different strains. *Inset* shows the culture filtrate color differences between the WT, *ΔOpS1* and WT*::OpS3* strains, in order. (*C*) Analysis of OpS3 putative binding motif in the promoter region of different genes. Consistent with the RT-PCR analysis showing in panel (*A*), the conserved motif, CGGA, is not present in the *OpS5* gene promoter. (*D*) Analysis of a putative Msn2-type transcription factor binding site in the promoter region of different genes. The putative binding motif is not present in the promoter region of *OpS2*.

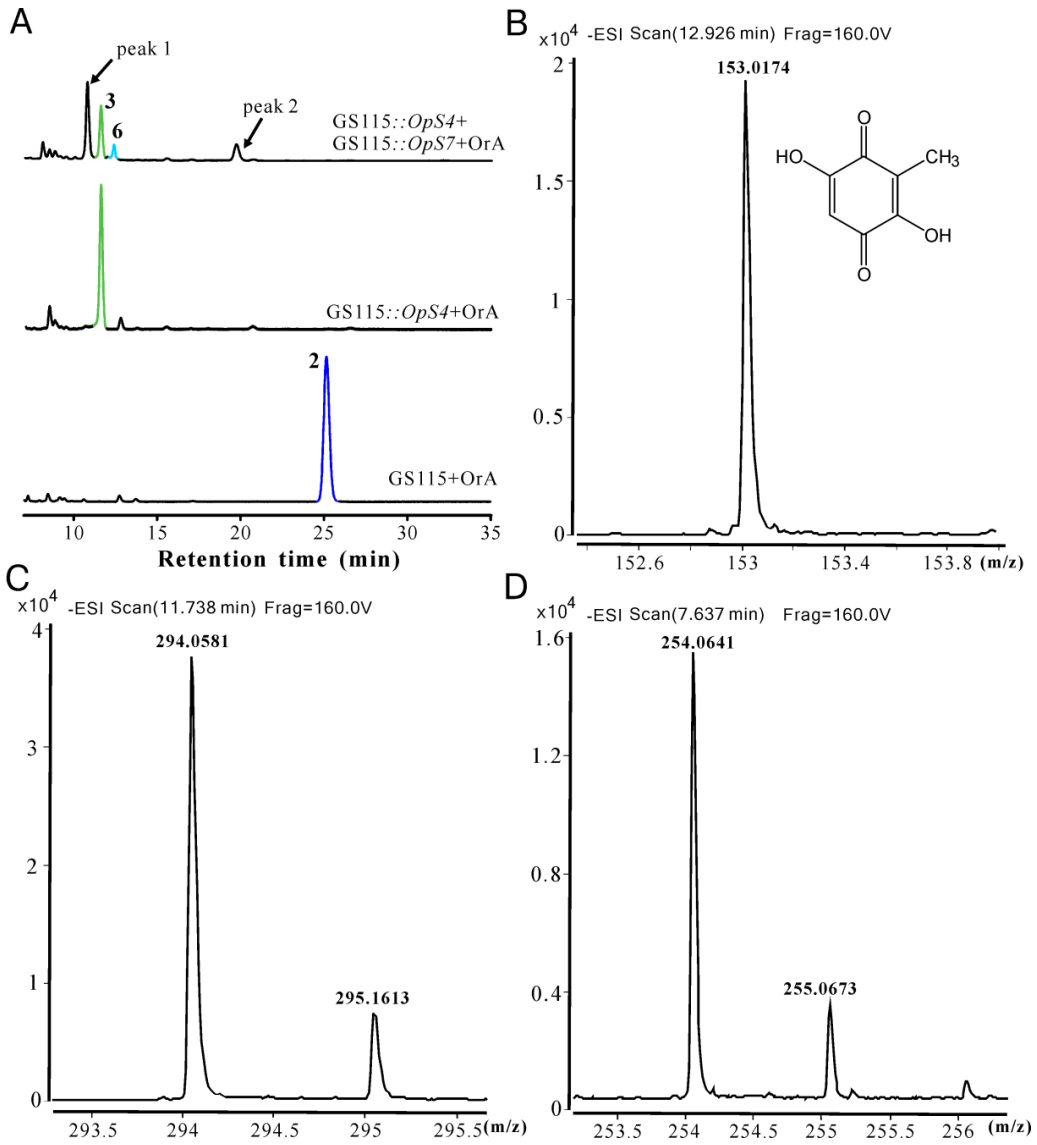

Fig. S7. Chromatographic and MS analyses of mixed fermented yeast mutant cells. (*A*) Mixed fermentation of yeast cells for HPLC analysis. Three groups of samples, i.e., GS115, GS115*::OpS4*, and GS115*::OpS4*+GS115*::OpS7*, were individually incubated in 100 ml of a minima medium containing methanol (0.5%) for 24 h before the addition of orsellinic acid (OA) (final concentration at 200 μg/ml). After fermentation for another 24 h, the yeast cells were harvested by centrifugation at 4000 g for 5 min. The cells were then extracted with methanol plus 1% trifluoroacetic acid. The crude extracts were subject to HPLC (254 nm) and LC-MS analysis, respectively. Compound: **2**, OA; **3**, for 6-methyl-1,2,4-benzenetriol; **6**, 2,5-dihydroxy-3-methyl- 2,5-cyclohexadiene-1,4-dione. (*B*) Mass spectrum of **6**. (*C*) Mass spectrum of the compound labeled as peak 1 in panel (*A*). (*D*) Mass spectrum of compound labeled as peak 2 in panel (*A*). The compounds corresponding to peaks 1 and 2 in panel (*A*) are otherwise uncharacterized.

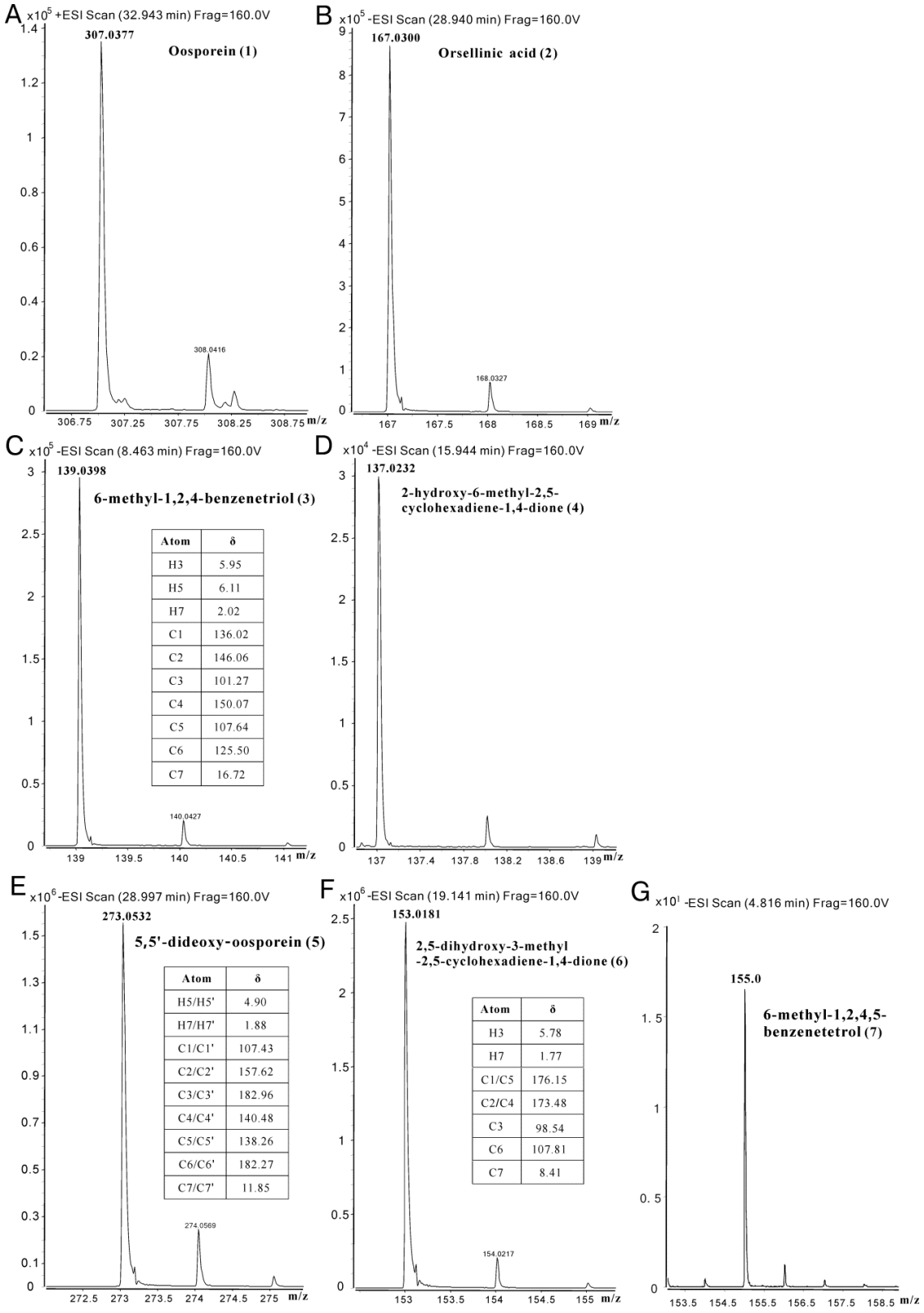

Fig. S8. MS and NMR analyses of different compounds detected in this study. (*A*) Mass spectrum of oosporein (**1**). (*B*) Mass spectrum of orsellinic acid (**2**). (*C*) Mass and NMR (*inset*) spectra of

6-methyl-1,2,4-benzenetriol (**3**). (*D*) Mass spectrum of 2-hydroxy-6-methyl-2,5-cyclohexadiene-1,4-dione (**4**). (*E*) Mass and NMR (*inset*) spectra of 5,5′-dideoxy-oosporein (**5**). (*F*) Mass and NMR (*inset*) spectra of 2,5-dihydroxy-3-methyl- 2,5-cyclohexadiene-1,4-dione (**6**). (*G*) Mass spectrum of 6-methyl-1,2,4,5-benzenetetrol (**7**).

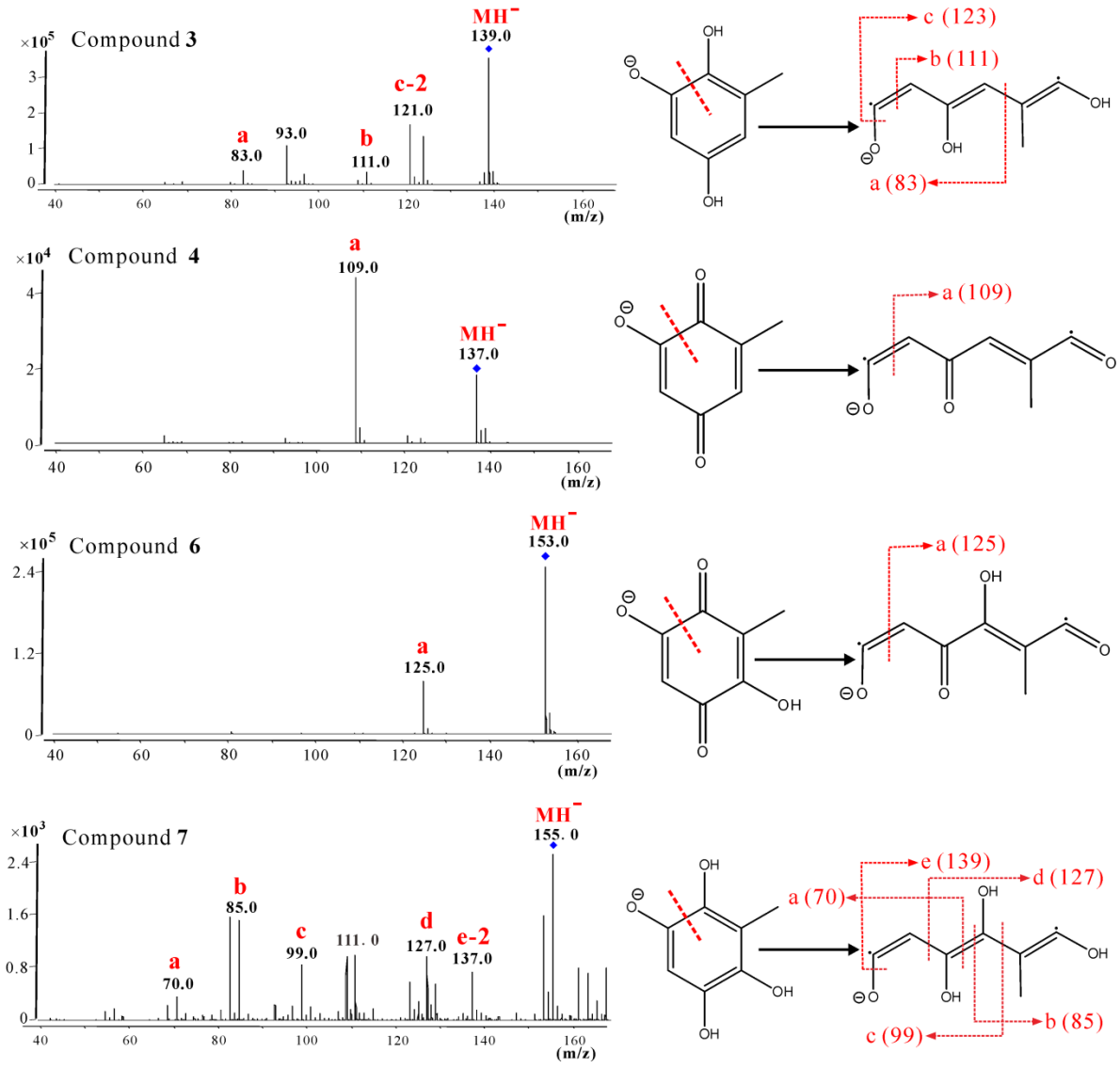

Fig. S9. Collision-induced dissociation (10 eV) MS/MS spectra of ionized compounds **3, 4, 6** and **7**. The mass peaks labeled with blue diamonds are for the respective parental compounds. Similar fragmentation patterns were detected between the compounds **3** and **7**, and between the compounds **4** and **6**, respectively. The masses of detected fragment(s) of each compound are illustrated in the right panels with the corresponding mass data in parenthesis.

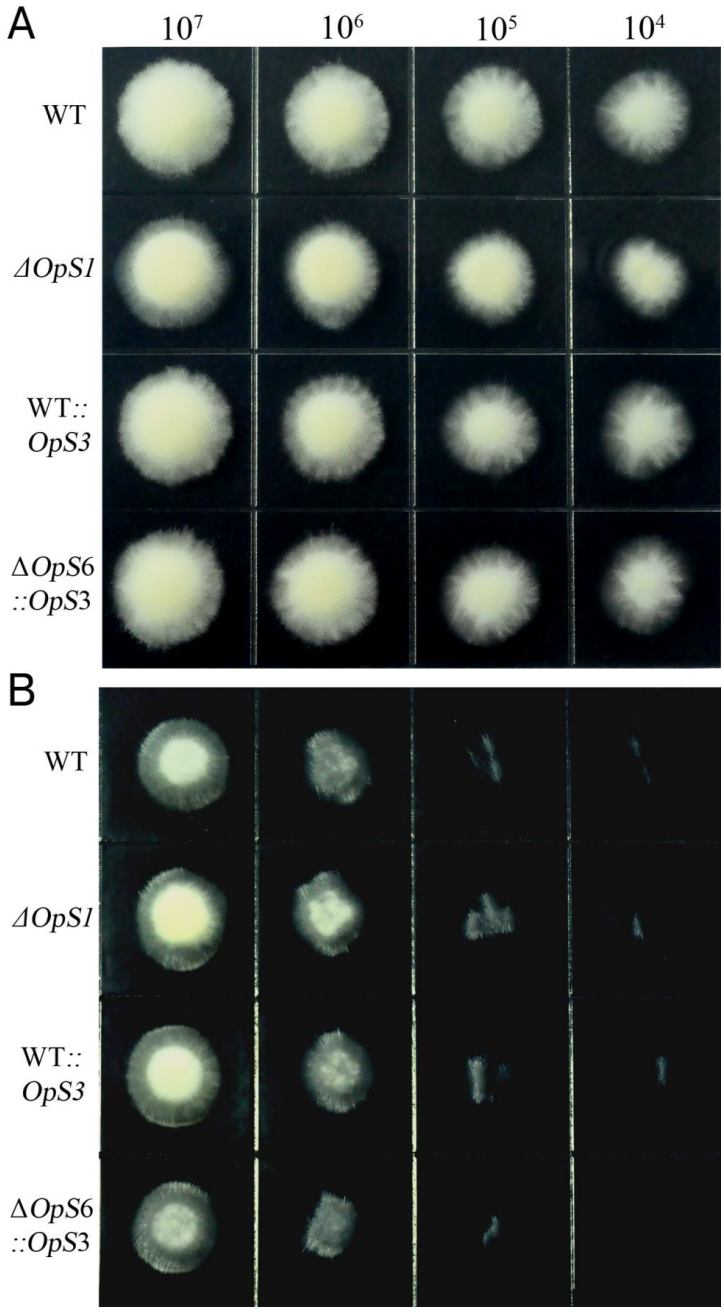

Fig. S10. Phenotypic characterization. (*A*) The phenotypes of different strains of *B. bassiana* inoculated (10 μl per spot of serial spore suspension; unit, spores per ml) on PDA for four days. (*B*) The phenotypes of different strains of *B. bassiana* inoculated on PDA amended with 2.5 mM H_2O_2 for four days.

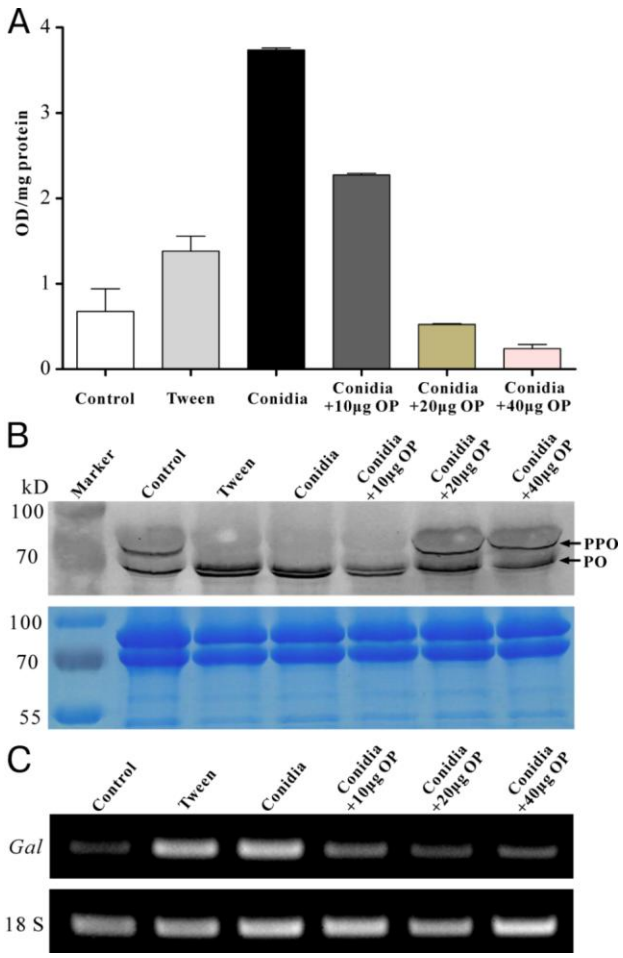

Fig. S11. Insect immune interference assays. (*A*) Inhibition of PPO activity by oosporein. The insects were injected with Tween (0.05%) or Tween-suspended heat-killed conidia (boiled for 5 min) containing different dosages of oosporein (OP). Control insects were not injected. The hemolymph was collected for PPO activity assay using the substrate dopamine. The absorbance values (OD) were measured using a Microplate Reader at the wave length of 492 nm. Relative to the PPO activation by *Beauveria* conidia, oosporein inhibition of PPO activity was found in a dose-dependent trend. (*B*) Verification of PPO cleavage and activation. The hemolymph samples (20 μg protein each) were separated for Western-blot analysis (upper panel). The parallel SDS-PAGE gel was included as a reference (lower panel). Cleavage of PPO to PO is the hallmark of PPO activation. Consistent the enzyme activity assay (*A*), oosporein was evident to be able to block PPO cleavage. (*C*) Down-regulation of antifungal peptide gallerimycin (*Gal*) gene by oosporein. A semi-quantitative RT-PCR analysis showed that, relative to fungal conidial injection, oosporein could reduce the expression of *Gal* gene. The 18 S rRNA gene (AF286298) of *Galleria* was used a reference. Consistent with PPO enzyme activity assay (*A*), the insects wounded by injection with Tween could also trigger immune responses.