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Identification of the oosporein biosynthesis gene cluster in an entomopathogenic fungus *Blackwellomyces cardinalis*

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ABSTRACT

Blackwellomyces cardinalis (≡ *Cordyceps cardinalis*) is an entomopathogenic fungus that hosts lepidopteran insect larvae. Oosporein, produced by *Bl. cardinalis*, is a red secondary metabolite that is also produced by other entomopathogens and is known to contribute to entomopathogenic activity. In this study, a homologous region of the oosporein biosynthesis gene cluster (*BcOpS* cluster) was found from the genome sequence of *Bl. cardinalis* strain NBRC 103832. Within the cluster, a putative transcription factor gene *BcOpS3* was deleted by homologous recombination. The deletion strain (*ΔBcOpS3*) did not produce oosporein. Real–time qPCR analysis showed that the expression of all genes was either lost or greatly reduced compared to the wild type strain (WT). Infection assay using silkworms showed that the virulence of the *ΔBcOpS3* strain was not different from that of the WT strain. We compared the expression levels of antimicrobial peptide genes in silkworm infected with these strains, and found that the increased expression of the *cecA* gene in WT was not observed in the Δ*BcOpS3* strain, suggesting that the immune response of the silkworm was altered.

Keywords: antimicrobial peptide, *Bombyx mori*, secondary metabolite

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1. Introduction

Entomopathogenic fungi are group of fungi which infect and kill insects. Some of them infect specific host, but others invade a wide variety of insect species (Zhao et al., 2016). For example, *Metarhizium acridum* (Driver & Milner) J.F. Bisch., Rehner & Humber infects locusts in a specific manner (Bateman et al., 1996), while *Beauveria bassiana* (Bals.-Criv.) Vuill. can infect more than 200 insect species including Lepidopteran larvae, mosquitoes, and beetles (Clark et al., 1968; Feng et al., 1994; Masuda, 2000). There are some fungal species that are used as pest control agents. They are considered to have the lowest environmental impact and can be used near agricultural fields and conservation areas, satisfying the demand for more environmentally sustainable technologies. On the other hand, the utility of insecticides is being undermined by problems of insecticide resistance, environmental contamination, and risks to human health (Federici et al., 2008; Thomas & Read, 2007).

Blackwellomyces cardinalis (G.H. Sung & Spatafora) Spatafora & Luangsa-ard is a member of Cordycipitaceae, described in 2004 in the southern Appalachians and southeastern Japan that hosts small moth larvae (Sung & Spatafora, 2004). Originally known as *Cordyceps cardinalis*, a new genus, *Blackwellomyces*, was proposed due to its morphogenetic novelty (Kepler, et al., 2017). Although there have been reports of transformation of *Bl. cardinalis* strain C033 by introducing foreign genes, there have been no reports of manipulation of specific genes of *Bl. cardinalis* itself by homologous recombination (Guo et al., 2013). If the genes of *Bl. cardinalis* itself can be disrupted and examined, it will be easier to understand the molecular mechanism of pathogenicity of this fungus and to improve its function as a microbial control agent (Zhao et al., 2016).

It is also known that fungi produce an abundance of low–molecular–weight organic compounds called secondary metabolites (SMs), which are not indispensable for the maintenance of life (Lim et al., 2012). Some of these compounds are harmful to humans, known as mycotoxins, and some are beneficial to humans, such as antibiotics and substances relevant to human health, and

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the regulation of their biosynthesis is of great interest (Calvo et al., 2002). Many of the genes involved in SM biosynthesis are located in the genome sequence in close together in a cluster structure (Chiang et al., 2008; Feng et al., 2015; Lo et al., 2012). Entomopathogenic fungi produce SMs as well, but several have been identified that are not produced by other fungi and are expected to be beneficial to humans or involved in pathogenicity against insects. For example, beauvericin produced by *Beauveria bassiana* is known to exhibit insecticidal and antimicrobial activities (Hamill et al., 1969), and destruxin produced by *Metharizium* spp. shows toxicity against insects and is expected to be used for drug therapy for osteoporosis and cancer (Chen et al., 1997; Itoh et al., 2009; Kershaw et al., 1999; Nakagawa et al., 2003).

The same is true for the *Bl. cardinalis* NBRC 103832 strain used in this study, which has been reported to produce cardinalisamides A–C (Umeyama et al., 2014). These compounds were first discovered in *Bl. cardinalis* cultures and are active against *Trypanosoma brucei*, the protozoan parasite that causes African trypanosomiasis. In *Bl. cardinalis* C033 strain was shown to produce, 5–Methyl–1,3–benzenediol, some ergosterol, uridine, and oosporein (Lu et al., 2018). Among these, oosporein is a red pigment that is known to be a compound with immunosuppressive and antifungal activity in insects and has been reported to contribute to the entomopathogenicity of *Be. bassiana* (Feng et al., 2015). The oosporein biosynthetic pathway has also been elucidated in *Be. bassiana*, which involves seven genes, *BbOpS1–7*, on the oosporein biosynthetic cluster (*BbOpS* cluster) (Feng et al., 2015). Within the cluster, BbOpS3 has been shown to encode a Gal4-like Zn(II)Cys6 transcription factor (Feng et al., 2015). Since *Bl. cardinalis* is pathogenic to insects, oosporein may be involved in pathogenicity, but there are no reports on *Bl. cardinalis* and its pathogenicity. In this study, we generated an *BcOpS3*–deficient strain of *Bl. cardinalis* and tested its pathogenicity against silkworm moth (*Bombyx mori*) larvae to investigate the relationship between the entomopathogenicity of *Bl. cardinalis* and oosporein.

2. Materials and methods

2.1. Strains and media

Blackwellomyces cardinalis NBRC 103832 was used as a wild type strain (WT) throughout this study. The strain was isolated from a single spore of a fruiting body that developed in lepidopteran larvae in Takatsuki, Osaka, Japan (Umeyama et al., 2014). *Beauveria bassiana* NBRC 103790 and *Be. brongniartii* NBRC 5299 were used for comparison of oosporein production. Fungal strains were grown on YM agar medium (yeast extract 2 g/L, malt extract 20 g/L, agar 20 g/L) at 25 °C, unless otherwise stated. *Aspergillus oryzae* NBRC 100959 was used for virulence assay as a low pathogenic control (Fitriana et al., 2021), and was grown on potato dextrose agar (Difco, Detroit, MI). For preparation of spore suspensions, distilled water containing 0.05% (v/v) Tween 80 was applied to the 7 d-old culture and spores were harvested using rubber policeman. For plasmid construction, *Escherichia coli* DH5α was used. *Escherichia coli* was grown in LB medium with appropriate antibiotics. *Agrobacterium tumefaciens* EHA105 was used for *Agrobacterium tumefaciens*–mediated transformation (AtMT). Inducing medium (IM; Guo et al., 2013) was used for activation of *A. tumefaciens*. Glucose minimal medium (GMM; Shimizu & Keller, 2001) containing 10 μg/mL tetracycline and 600 μg/mL phosphinothricin was used for selection of transformants.

2.2. Purification and identification of SM

Fungal spores were inoculated into SDB liquid medium (peptone from meat, bacteriological 5.0 g/L, tryptone 5.0 g/L, glucose 20 g/L) and incubated at 25 °C with shake at 140 rpm. After 7 d of incubation, the mycelia were removed by filtration and the culture filtrate was adjusted to pH 2.0 with HCl and extracted with an equal volume of ethyl acetate. The extract was dried in a rotary evaporator and dissolved in a mixture of DMSO/methanol (50:50, v/v). The extract was analyzed and fractionated by HPLC equipped with a reversed–phase column (250 mm × 10 mm i.d., 5 μm, Inert-Sustain C18 column, GL Sciences, Tokyo, Japan). A mixture of water/acetonitrile (75:25, v/v) containing 0.1% TFA was used as a mobile phase, and purification was performed under an isocratic condition.

The purified samples were analyzed by NMR and mass spectrometry to determine the structure; for NMR, the purified samples were dissolved in DMSO–d6 or DMSO–d6 containing triethylamine (10%, v/v) and analyzed by a JEOL ECZ 800 spectrometer (JEOL Ltd., Tokyo, Japan) at 800 MHz for ^{1}H NMR and 200 MHz for ^{13}C NMR. A high-resolution mass spectrum was obtained by MALDI–TOF MS analysis using an MS–S3000 SpiralTOF–plus spectrometer (JEOL) in the spiral negative mode. Sinapinic acid was used as a matrix, and its mass peaks at *m/z* 223.0612 $[M-H]^-$ and *m/z* 447.1297 $[2M-H]^-$ were used as internal standards for mass calibration.

2.3. Deletion of BcOpS3 by genetic manipulation

The oosporein biosynthetic gene cluster of *Bl. cardinalis* was predicted from the whole genome sequence (Nguyen et al., unpublished) using the Anti–SMASH fungal version (Blin et al., 2023). In order to delete the putative transcription factor gene *BcOpS3*, coding for a Gal4-like zinc finger domain protein as reported in *Be. bassiana BbOpS3*, the upstream and downstream 1 kb of *BcOpS3* were amplified with primers UPOpS3–F/UPOpS3–R and DWOpS3–F/DWOpS3–R, respectively, from the genomic DNA of *Bl. cardinalis*. The amplified fragments and a marker gene, *bar*, were cloned into pPZP vector by seamless cloning using SLiCE reaction mixture (Motohashi, 2015) to create pNKM6. The plasmid pNKM6 was then introduced into *A. tumefaciens* EHA105.

Agrobacterium tumefaciens–mediated transformation was performed with some modifications to the protocol of previous study (Guo et al., 2013). Briefly, *A. tumefaciens* harboring pNKM6 was cultured in a 3 mL LB liquid medium containing 50 μg/mL rifampicin and 50 μg/mL kanamycin for 24 h at 140 rpm with shake. One milliliter of the culture was then transferred to a flask containing 10 mL fresh liquid IM until the culture reached to $OD_{600} = 0.6$. The *A. tumefaciens* culture was then mixed with an equal volume of *Bl. cardinalis* spore suspension (1.0×10^6 spores/mL). The mixture was inoculated onto IM agar plate (IM with 2% agar) and incubated at 28 °C for 48 h. The cells on the plates were collected with sterile water and transferred to GMM agar plate containing 10 μg/mL tetracycline and 600 μg/mL phosphinothricin. The colonies that emerged after 1 to 2 wk-incubation were transferred to a fresh YM plate containing 600 μg/mL phosphinothricin and incubated for additional 2 d. The introduction of the marker gene, *bar*, was confirmed by PCR. The loss of oosporein production was confirmed by HPLC. The extracts prepared as above were analyzed by HPLC equipped with a reversed–phase column (150 mm × 4.6 mm i.d., 5 μm, Symphonia C18 column, JASCO Engineering, Tokyo, Japan). The LC conditions were as follows: mobile phase, water/acetoni-

trile containing 0.1% TFA; liner gradient of 5–100% acetonitrile for 9 min, isocratic elution of 100% acetonitrile for 3 min, followed by equilibration at 5% acetonitrile for 10 min before next injection; wavelength 287 nm.

2.4. Gene expression analysis

RNA was extracted using Sepasol®–RNA I Super G (Nacalei tesque, Kyoto, Japan) according to the manufacture's protocol. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. Real–time qPCR was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) according to the manufacturer's instructions with ABI StepOne (Thermo Fisher Scientific, Waltham, MA, USA). The obtained data was calculated by the StepOne software, and gene expression was normalized to actin.

2.5. Silkworm experiments

For virulence assay, fifth instar *Bombyx mori* larvae were inoculated with a 50 μL spore suspension (in 0.9 % NaCl, 0.05% Tween 80) at 1.0×10^3 to 1.0×10^5 spores/mL. Inoculated larvae were placed in plastic containers and kept at 25 °C without feeding. The number of dead individuals was recorded daily for 7 d. The experiments were triplicated, and the obtained mortality data were statistically analyzed by Log–rank (Mantel–Cox) test and Gehan–Breslow–Wilcoxon test using Prism8 (GraphPad Software, La Jolla, CA). For gene expression analysis, spores were inoculated into silkworm larvae as described above, and three days later, fat bodies were collected using dissecting scissors. RNA extraction, cDNA synthesis, real–time qPCR were performed as described above.

3. Results

3.1. Search for clusters

The genome sequence data of *Bl. cardinalis* NBRC 103832 strain (Nguyen et al., unpublished) was analyzed using Anti–SMASH fungal version to search for secondary metabolite biosynthetic gene clusters. Among the PKS–related gene clusters, a sequence with 55% homology to the oosporein biosynthetic gene cluster (*OpS* cluster) of *Be. bassiana* was found. In addition, seven genes, *BcOpS1–BcOpS7*, were found to be present in the *BcOpS* cluster as in *Be. bassiana* (Fig. 1). The identities of the deduced amino acid sequences to the corresponding homologs of those in *Be. bassiana* are summarized in Table 1.

3.2. Chemical structure of the red pigment produced by Blackwellomyces cardinalis

In order to determine the chemical structure of the red compound produced by *Bl. cardinalis* NBRC 103832, the crude extract from the culture was separated by HPLC, and the peak with a retention time of 7 min was collected. In total, 11 mg of red compound was obtained from 47 mg of the crude extract. The purified

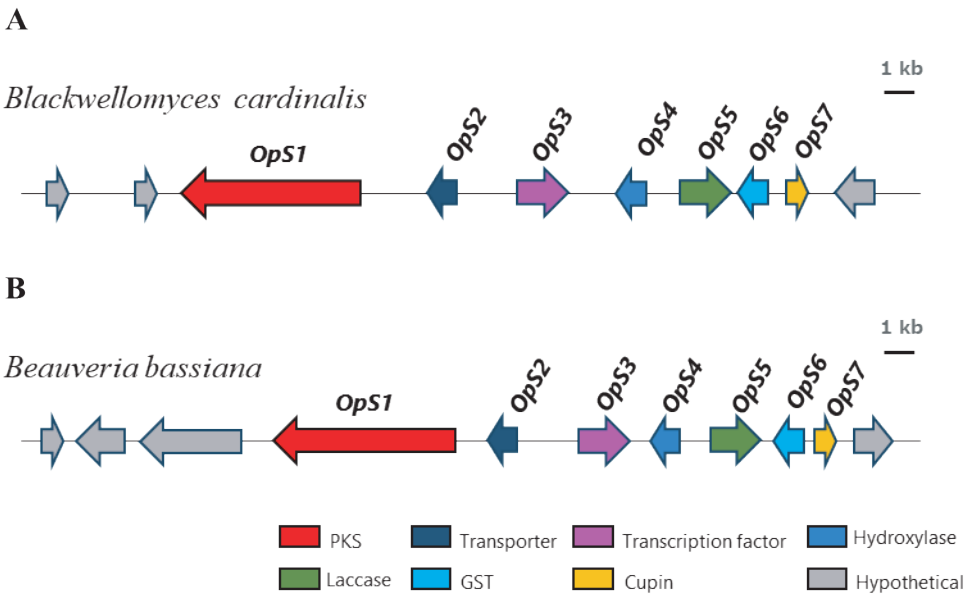

Fig. 1. Schematic structure of the *OpS* clusters involved in oosporein biosynthesis.
The Schematic structure of the *OpS* cluster in *Blackwellomyces cardinalis* (A) and in *Beauveria bassiana* (B, reproduced from Feng et al., 2015). Homologous genes are shown in the same color and their predicted function is stated at the bottom.

Table 1. Putative *BcOpS* cluster gene function and identity of protein.

Gene	Putative function	Accession No.	% Identity of protein
BcOpS1	Polyketide synthase	XP_008601498	55.51 %
BcOpS2	MFS transporter	KAH8713102	80.31 %
BcOpS3	Fungal specific transcription factor	XP_008601500	50.71 %
BcOpS4	FAD- dependent hydroxylase	KAH8713100	71.53 %
BcOpS5	Laccase2 (Oxidoreductase)	KAH8713099	72.34 %
BcOpS6	Glutathione-s-transferase 2	PMB67240	77.61 %
BcOpS7	Cupin, RmlC-type	OAA52237	72.97 %

compound was used for MALDI–TOF MS analysis showed a peak at *m/z* 305.0321 [M-H]⁻; calculated for $C_{14}H_{10}O_8$, 306.0376 (Supplementary Fig. S2A).^{1}H NMR analysis showed a peak due to a methyl group at 1.86 ppm (Supplementary Fig. S2B).^{13}C NMR analysis showed a peak at 8.12 ppm, 107.8 ppm, and 113.3 ppm, and analysis under basic conditions revealed two new peaks due to carbonyl groups at 172.8 ppm and 174.5 ppm (Supplementary Fig. S2C, D). Since these results are consistent with the analysis of oosporein in previous studies, the red compound was determined to be oosporein (Love et al., 2009).

3.3. Functional analysis of BcOpS3 by gene deletion

It has been reported that the *BbOpS3* is a transcription factor gene for the *OpS* cluster in *Be. bassiana* (Feng et al., 2015). Therefore, to elucidate the function of *OpS3* in *Bl. cardinalis*, we deleted the *BcOpS3* gene by AtMT (Fig. 2A). After several attempts, we obtained a total of 270 colonies that could grow on selective medi-

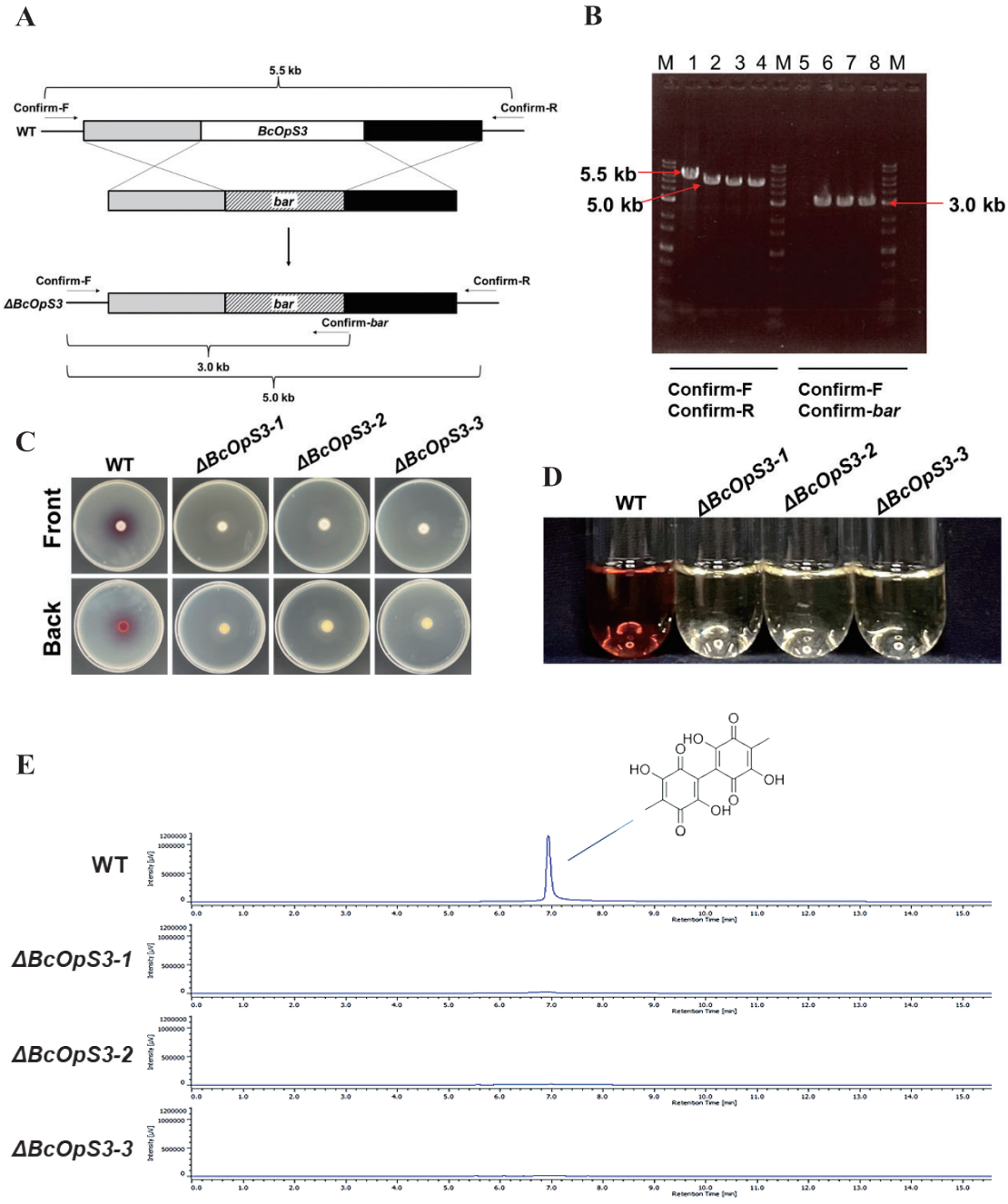

Fig. 2. Chemical characterization of the *BcOpS3* deletion strains.
A: The gene deletion strategy to knock out *BcOpS3* gene of *Blackwellomyces cardinalis* wild type strain NBRC103832. Upstream (grey box) and downstream (closed box) flanking sequences of the *BcOps3* gene (open box) were fused with the *bar* marker gene (striped box) and used for gene targeting. *BcOpS3* gene was eliminated from the genome by the *Agrobacterium tumefaciens*–mediated transformation (see text). B: Diagnostic PCR to confirm the deletion of *OpS3* gene in *Bl. Cardinalis*. M, 1 kb molecular marker; 1,5, wild type (WT); 2–4, 6–8 candidates of *BcOpS3* deletion strains (*ΔBcOpS3-1*, *2*, and *3*). Solid (C) and liquid (D) cultures of the wild type (WT) and *ΔBcOpS3* strains. E: HPLC analysis. Ethyl acetate extract from the wild type and the gene deletion strains were monitored and detected by UV at 287 nm.

um containing phosphinothricin. Out of them, 15 were identified to be deficient in *BcOpS3* by homologous recombination confirmed by the diagnostic PCR (Fig. 2B). As transformation of *Bl. cardinalis* was technically difficult because of the limited choice of the marker genes and of the low transformation efficiency, the *BcOpS3* complementary strain was not generated. Thus, the experiment was conducted using three independent *ΔBcOpS3* strains named *ΔBcOpS3-1*, *ΔBcOpS3-2*, and *ΔBcOpS3-3*. These knockout strains failed to produce red compound either on solid media or liquid culture (Fig. 2C, D), and oosporein was no longer detected by HPLC analysis (Fig. 2E). These results suggest that the *BcOpS3* gene plays an important role in the production of oosporein. To confirm whether *BcOpS3* gene product is a transcription factor, we analyzed *BcOpS1–BcOpS7* gene expression in WT and *ΔBcOpS3* strains (Fig.3A–G). Gene expression of *BcOpS1–7* were detected in WT, but were not expressed or greatly reduced in the *ΔBcOpS3* strains in both day 2 and day 4. These results suggest that the *BcOpS* cluster is a biosynthetic gene cluster for oosporein, and that *BcOpS3* is a transcription factor gene for the *OpS* cluster in *Bl. cardinalis* as in *Be. bassiana*.

3.4. Virulence against silkworm

Oosporein has been reported to have activity related to entomopathogenicity. In a previous study, inoculation of *Galleria mellonella* larvae with oosporein caused inhibition of activation of prophenol oxidase (PO), which is required for melanin formation, one of the insects humoral immunity, and inhibition of expression of the antimicrobial peptide galerimycin (Feng et al., 2015). In addition, an experiment in which *Be. bassiana* oosporein non-producing strain (*ΔBbOpS1*) was inoculated against *G. mellonera* larvae showed that the loss of oosporein production reduced virulence (Feng et al., 2015). Therefore, to confirm whether oosporein production is also involved in the virulence of *Bl. cardinalis*, we inoculated WT and *ΔBcOpS3* strains against silkworm. However, no significant difference was detected between WT and *ΔBcOpS3* strains even when inoculated with spores ranging from 1.0×10^3 spores/mL to 1.0×10^5 spores/mL (Fig. 4A–C). Most of the dead larvae killed by the WT spore inoculation turned purple within 1 day after death, while those killed by the *ΔBcOpS3* strain stayed pale yellow (Fig. 4D). To further evaluate the virulence of the *ΔBcOpS3* strain, we analyzed the expression of antimicrobial peptide genes in silkworms after infection. Antimicrobial peptides are known to be one of the innate immunities of insects, and their expression has been shown to increase by *Be. bassiana* infection but repressed by oosporein (Feng et al., 2015; Lu et al., 2016). We analyzed gene expression of *cecA*, *gloverin*, and *attacin*, which are known to be expressed by silkworms during fungal infection (Geng et al., 2021; Lu et al., 2016). The results showed that the expression of *cecA* gene was elevated only when inoculated with WT, and not significantly increased when inoculated with the *ΔBcOpS3* strain (Fig. 5A). Neither *gloverin* nor *attacin* showed significant differences in gene expression level in this experiment (Fig. 5B, C).

4. Discussion

Entomopathogenic fungi are expected to be used as pest control agents because of their ability to infect and kill only certain insects. *Be. bassiana* and *Metharizium* spp., which have been well studied, have already been applied as pest control agents, but many species have not been studied enough to reach commercial use (Andreadis et al., 2016; Shirotsuka et al., 2015). In this study, we aimed to elucidate the virulence mechanisms of *Bl. cardinalis*, which has rarely been reported as a research target.

4.1. Structural analysis of red compounds

In a previous study, *Bl. cardinalis* strain C033 was reported to produce oosporein (Lu et al., 2018). Oosporein is also known to be synthesized by a number of entomopathogenic fungal species including *Be. bassiana* and *Be. brongniartii* (Seger et al., 2005). It has been shown to contribute to the pathogenicity by causing immunosuppression of insects and competition avoidance on insect cadaver (Fan et al., 2017). In this study, we confirmed that *Bl. cardinalis* NBRC 103832 also produces oosporein, suggesting that oosporein is a major secondary metabolite of *Bl. cardinalis* in general.

4.2. Expression analysis of BcOpS cluster

As oosporein is biosynthesized by the function of *BbOpS* gene cluster in *Be. bassiana* (Feng et al., 2015), and we also found a homologous gene cluster in *Bl. cardinalis*, designated as *BcOpS* cluster, we further explored the involvement of the cluster in oosporein biosynthesis by knocking out a putative transcription factor gene *BcOpS3*. Deletion of *BcOpS3* in *Bl. cardinalis* resulted in no oosporein production (Fig. 2C–E). In addition, the loss of *BcOpS3* completely blocked or greatly reduced the expression level of the entire *BcOpS* cluster genes in *Bl. cardinalis* (Fig.3A–G). These results indicate that *BcOpS3* encodes a transcription factor of the *BcOpS* cluster in *Bl. cardinalis* and regulates oosporein biosynthesis by positively controlling transcription of *BcOpS* cluster genes. It has been reported that *BbOpS3* gene codes for a transcription factor that regulates gene expression of the *BbOpS* cluster in *Be. bassiana* (Fan et al., 2017; Feng et al., 2015), indicating that the transcriptional regulation mechanism is conserved in both fungal species.

4.3. Involvement of oosporein in entomopathogenicity

Oosporein is a compound that plays a crucial role in the pathogenicity of entomopathogenic fungi. In *Be. bassiana*, virulence assays using larvae of waxmoth *Galleria mellonera* have shown that the oosporein–nonproducing strain was less virulent than the wild–type strain, while the *OpS3*–overexpressing strain was more virulent than the wild–type strain (Feng et al., 2015). It was also shown by Fan et al. (2017), an oosporein overproduction strain lacking *Bbsmr1* gene was more virulent whereas another oosporein overproduction strain with overexpression of the transcription factor gene *BbOpS3* resulted in decreased virulence. They have concluded that the oosporein is unlikely entomotoxic at least in early to mid-infection processes. In our study, no significant difference in silkworm mortality was observed between the wild type and the *ΔBcOpS3* strains of *Bl. cardinalis* (Fig.4A–C). Further study using different insect host besides silkworm should be carried out that the *ΔBcOpS3* strains might demonstrate different virulence capability.

In response to fungal infection, insects are known to produce antimicrobial peptides which is released into the hemolymph to induce cell lysis and destruction of foreign enemy microorganisms (Faye et al., 1975). Upon infection, *Be. bassiana* induced the expression level of *cecA*, one of the antimicrobial peptide genes in silkworm (Lu et al., 2016). In another report, oosporein inoculation of *G. mellonera* larvae repressed gene expression of the antimicrobial peptide gallerimycin, suggesting that oosporein evades insect's immune system (Feng et al., 2015). In our study, *cecA* gene expression increased only when the wild type strain capable of producing oosporein was inoculated, and but not when *ΔBcOpS3* was chal-

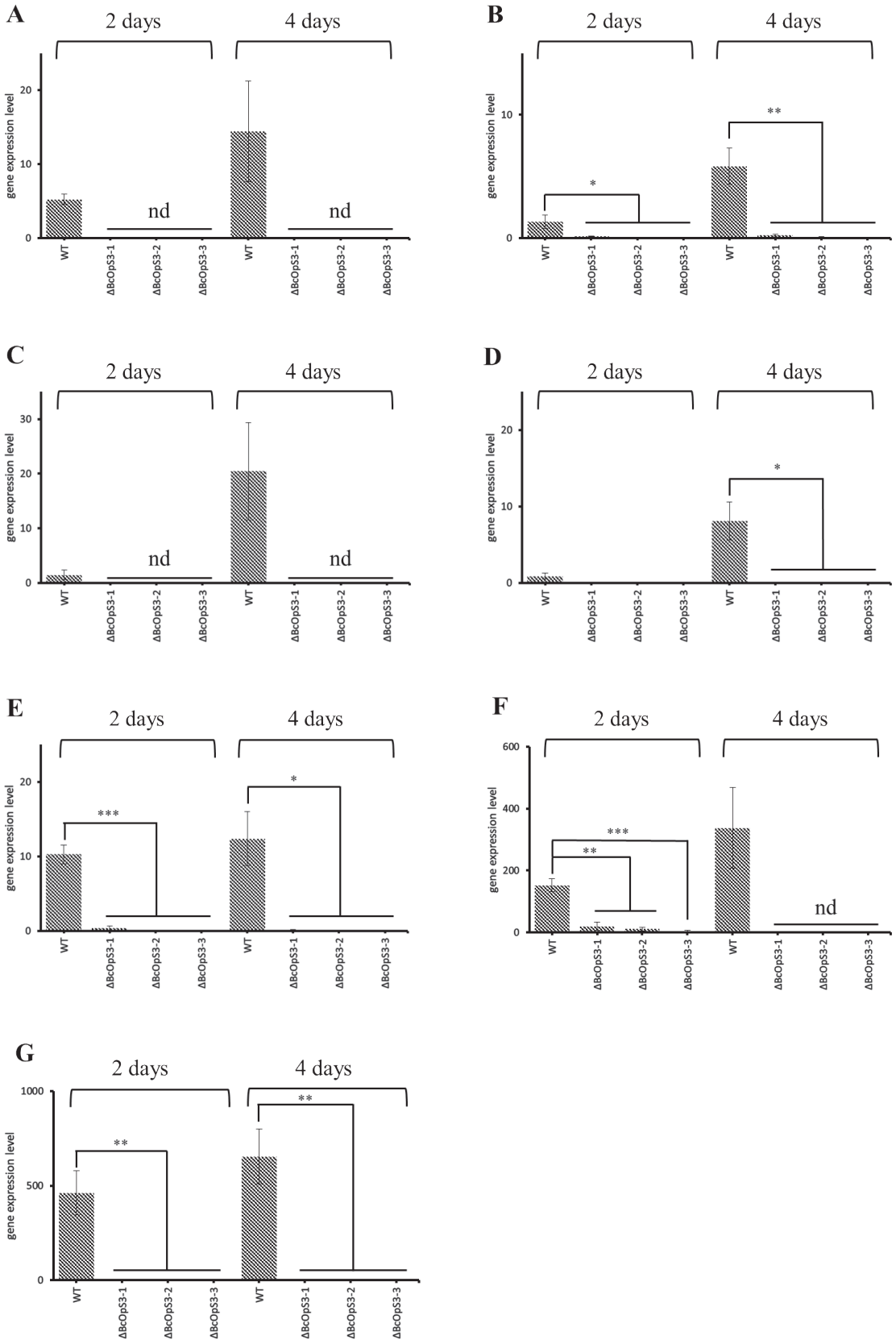

Fig. 3. Expression of the *BcOpS* cluster genes.
Expression of the *OpS* cluster genes in *Blackwellomyces cardinalis* wild type (WT) and *BcOpS3* deletion (*ΔBcOpS3*) strains at 2 or 4 day–old cultures by real-time-qPCR. The expression levels were normalized to those of the *actin* gene. Error bars represent standard errors of three independent experiments. nd, not detected. The asterisks represent significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by Dunnett's test with WT as a control for each day. *BcOpS1* (A), *BcOpS2* (B), *BcOpS3* (C), *BcOpS4* (D), *BcOpS5* (E), *BcOpS6* (F), and *BcOpS7* (G).

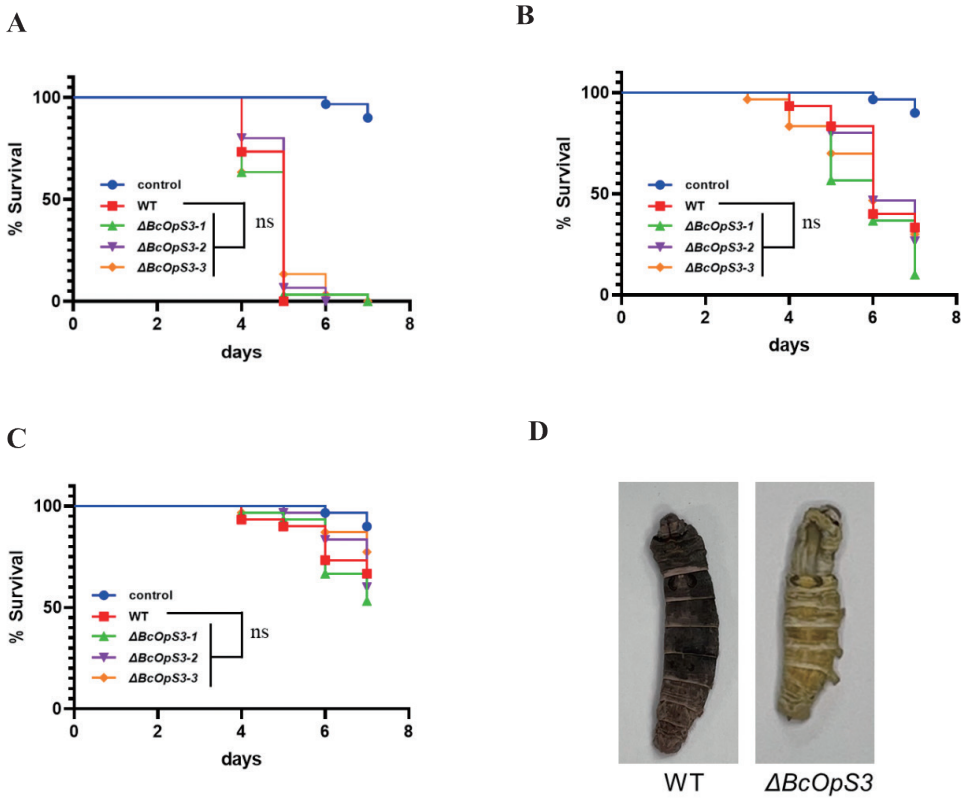

Fig. 4. Virulence of the *BcOpS3* deletion strains.
The wild type (WT) and *BcOpS3* deletion (*ΔBcOpS3*) strains of *Blackwellomyces cardinalis* were injected to the haemolymph of the silkworm *Bombyx mori* at different concentrations; A, 50×10^3 spores/insect; B, 5.0×10^2 spores/insect; C, 5.0×10^1 spores/insect. The silkworms were observed twice daily, and the survival curves were drawn. D: Cadavers of the silkworms killed by the WT (left) or the *ΔBcOpS3* strain (right) of *Bl. cardinalis*. Note that the *ΔBcOpS3*–injected silkworm remained whitish while the WT–injected turned purplish in color. ns, not significant by Log–rank (Mantel–Cox) test and Gehan–Breslow–Wilcoxon test.

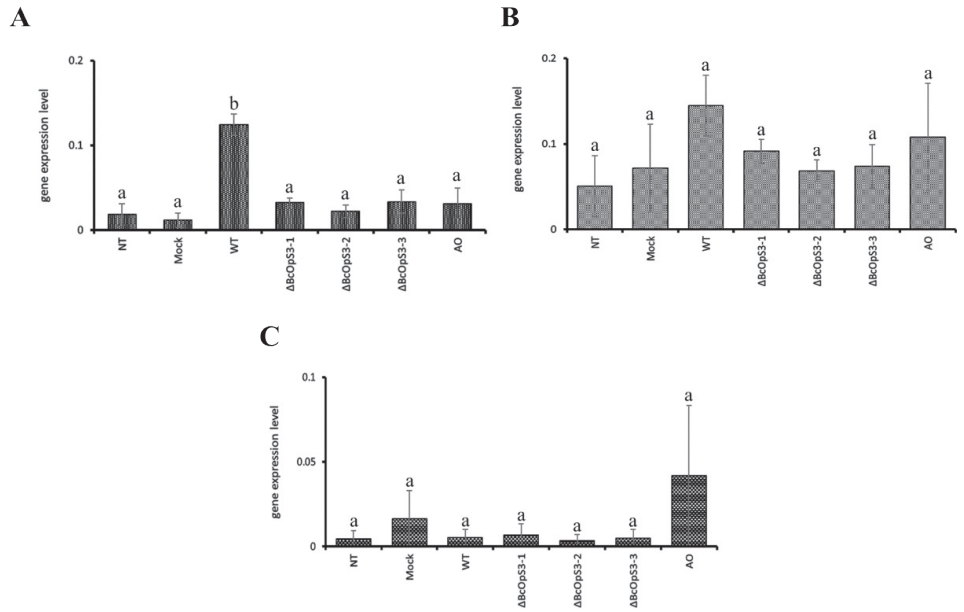

Fig. 5. Antimicrobial peptide gene expression in silkworms after inoculation by *Blackwellomyces cardinalis*.
The expression of antimicrobial peptide genes, *cecA* (A), *gloverin* (B), and *attacin* (C) in silkworms was analyzed by real-time qPCR. *Bl. cardinalis* spore suspension adjusted to 1.0×10^5 spores/mL was inoculated to the silkworms and the RNA was extracted from their fat bodies 3 days after inoculation. The gene expression levels were normalized to the silkworm *actin* gene. NT, not treated; Mock, saline with 0.1% (v/v) Tween 80 was inoculated; AO, *Aspergillus oryzae* spores were inoculated. Error bars indicate standard errors for three independent experiments. Different letters indicate significant differences after one-way ANOVA method and Tukey test ($P < 0.05$).

lenged. The gene expression level of other antimicrobial peptide genes, gloverin and attacin, was not significantly more highly induced by the infection of the wild type strain of *Bl. cardinalis*. Fan et al. (2017) noted that oosporein produced by *Be. bassiana* is not produced most likely after death of the host to thwart bacterial competition on a host cadaver. We also observed that the silkworms killed by the wild type *Bl. cardinalis* turned purplish which occurred 1 d after death, suggesting that *Bl. cardinalis* does not synthesize oosporein before silkworms are killed by the fungus (Fig. 4D). Oosporein itself may have an immunosuppressive activity to the insect hosts, but oosporein may not be produced to the level which is sufficient to induce antimicrobial peptide gene expression by the hosts.

We also observed that *Bl. cardinalis* produced oosporein more abundantly than *Be. bassiana* and *Be. brongniartii* (Supplementary Fig. S1; Supplementary Table S2). In *Be. bassiana*, *BbOpS3* has been shown to be regulated by the upstream regulatory cascade. For instance, it has been shown that oosporein production in *Be. bassiana* is regulated by BbPacC, a pH dependent transcription factor, and that it only produces oosporein under high pH conditions (Chen et al., 2022). A zinc finger transcription factor, BbSmr1 (*Be. bassiana* secondary metabolite regulator 1) has also been shown to control production of oosporein in *Be. bassiana* (Fan et al., 2017). Identifying the transcription factors upstream of *BcOpS3* may help elucidate the mechanism by which oosporein is produced in large amount in *Bl. cardinalis*, which in turn help us to further control fungal secondary metabolisms.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the countries where they were performed.

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