# Changes of the gene expression in silkworm larvae and Cordyceps militaris at late stages of the pathogenesis

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## CONFLICT OF INTEREST

The authors have declared no conflict of interest.


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## HIGHLIGHTS

- Changes of the gene expression in the fat body of silkworm larvae were observed during *Cordyceps militaris* infection.

- Genes encoding oligopeptide transporters and involved in phenolic substrate degradation were upregulated in *Cordyceps militaris*.

**Abstract**

*Cordyceps militaris* is an entomopathogenic fungus that forms its fruiting body. The gene expression change in *C. militaris* and silkworm larvae were analyzed using RNA-seq to investigate the relationship of *C. militaris* with the host, silkworm larvae before the death by mycosis. At 144 h after the injection of *C. militaris* conidia, genes encoding proteases, protease inhibitors, and cuticle proteins in the fat body of silkworm larvae were upregulated, but genes encoding lipoproteins and other proteins in hemolymph were downregulated. On the other hand, at 168 h after the injection of *C. militaris* conidia, genes encoding amino acid and oligopeptide transporters and permeases in *C. militaris* were upregulated, suggesting that *C. militaris* may use peptides and amino acids in silkworm larvae as a nutrient to grow *in vivo*.

Additionally, one gene cluster composed of genes putatively involved in the degradation of phenolic substrates was also upregulated. The addition of 4,5-dichlorocatechol, an inhibitor of catechol 1,2-dioxygenase, inhibited the *in vivo* growth of *C. militaris*, *Beauveria bassiana* and *Metarhizium anisopliae*. These results also suggest that the expression of the gene cluster may be crucial for the *in vivo* growth of *C. militaris* and entomopathogenic fungi. This study will clarify how *C. militaris* grows in insect hosts by avoiding host's immune systems.

KEYWORDS

*Cordyceps militaris*, silkworm, RNA-seq, entomopathogenic fungi, catechol.

## 1 INTRODUCTION

*Cordyceps militaris* is a pathogenic insect fungus and produces its fruiting body on the insect (Shrestha et al., 2012). The fruiting body has various types of bioactive compounds and has been used for Chinese medicine and supplementary diet. Cordycepin, a well-known bioactive compound in *C. militaris*, possesses anti-inflammatory, anti-tumor, and immunomodulatory activities (Ashraf et al., 2020; Tan et al., 2020; Yang et al., 2020). Therefore, the demand for the fruiting body for human health has risen annually.

Entomopathogenic fungi grow as blastospores and hyphal bodies in the hemolymph of insects upon entering the body of insects (Butt et al., 2016). During the entomopathogenic fungus infection, these fungi attack the host immune system and adapt to osmotic stress of the hemolymph. Entomopathogenic fungi produce some proteins, bioactive metabolites, and peptides to adapt to the environment in the hemolymph. *Metarhizium anisopliae* produces collagenous protective coat protein, MCL1, on the surface to avoid the host's immune system by shielding own β-glucans (Wang & St. Leger, 2006). *Beauveria* species produce beauvericin, bassianolide, and oosporein, which are involved in the pathogenicity (Feng et al., 2015; Xu et al., 2008; Xu et al., 2009). *Metarhizium* species also have various genes for their secondary metabolite synthesis (Donzelli & Krasnoff, 2016).

In insect hosts, during the entomopathogenic fungus infection, cellular and humoral immunities are induced to adapt against the infection (Butt et al., 2016). The humoral responses in hosts during the entomopathogenic fungi infection include the expression of anti-microbial peptides (AMPs) and protease inhibitors (Bulet & Stöcklin, 2005),

activation of phenol-oxidase (PO) (Eleftherianos & Revenis, 2011), and reactive oxygen species (ROS) production (Troha & Buchon, 2019). The entomopathogenic fungi have several methods to adapt against these immunities in hosts to survive and grow in hemolymph.

*Metarhizium and Beauveria* have been used as biopesticides, given their pathogenicity to insects (Lovett & St Leger, 2018). Numerous papers describe the gene expression of these entomopathogenic fungi during infection to insects (Iwanicki et al., 2020). Transcriptome analysis is a tool for showing the change in global gene expression in the fungi and hosts. Additionally, proteome and metabolome analyses also provide indicative information on fungal and host's responses to infection and host's immunity (Bhadani et al., 2021; Brancini et al., 2019).

Unlike *Metarhizium and Beauveria*, *C. militaris* causes epizootics in populations of Lepidoptera. However, the mechanisms of this epizootics in addition to the ways of infection and the development of pathogenesis in hosts are still poorly understood (Kryukov et al., 2020; Xiao et al., 2012). Compared to *Metarhizium and Beauveria*, how *C. militaris* infects the host has been not investigated fully (Aw & Hue, 2017; Ortiz-Urquiza & Keyhani, 2016). Until now, it was reported that cordycepin, which is one of bioactive compounds produced by *C. militaris*, inhibits the immune response of hemocytes and the expression of genes encoding anti-microbial peptides and lysozyme in the host, and the growth of *C. militaris* in *Galleria mellonella* larvae at 25°C is inhibited by the expression of genes encoding anti-fungal peptides, which is decreased at 15°C (Kryukov et al., 2020; Wellham et al., 2021; Woolley et al., 2020). In this study, the *C. militaris* gene expression and the host before its death by mycosis using RNA-seq were investigated to show the infection mechanism of the fungus.

## 2 MATERIALS AND METHODS

### 2.1 Strain, cultivation, and conidia isolation

*C. miliaris* NBRC103752, *B. bassiana* NBRC4848 and *M. anisopliae* NBRC 8556 purchased from Biological Resource Center, National Institute of Technology and Evaluation (NBRC, Japan), was used in this study. This fungus was cultivated on potato dextrose agar medium at 28°C. Its conidia were isolated according to the method described in a previous paper (Zheng et al., 2011).

### 2.2 Infection to silkworm larvae

As a host of *C. militaris*, the 5th instar of larvae, purchased from Ehimesansyu (Ehime, Japan), was used. For *C. militaris* infection of the silkworm larvae, $1 \times 10^6$/mL (25 μL) of conidia suspended in phosphate-buffered saline (PBS, pH 7.4) was injected into silkworm larvae. As a negative control, PBS was injected with the same volume of conidia. The larvae were raised on an artificial diet, Silkmate S2 (Nosan, Yokohama, Japan) at 23−24°C. Five or ten silkworm larvae were put into a plastic container (15 × 20 × 5.5 cm).

### 2.3 RNA extraction and RNA-seq analysis

After 144 and 168 h, total RNA (each sample n = 2) was extracted from the fat body of 3 silkworm larvae in a group with Trizol (Thermo Fisher Scientific K. K., Tokyo, Japan). Fat body (100 mg) was placed into liquid nitrogen and incubated for one minute. Frozen fat body was crashed, and 1 mL of Trizol was added to the sample. After

144 incubation for 5 min at room temperature, 200 μL of chloroform was added to the
145 mixture, followed by incubation for 3 min. The mixture was centrifuged at 12,000 × g
146 for 15 min after which the supernatant was collected. Then, 2-propanol precipitation
147 was conducted. Precipitated RNA was dissolved with RNA-free water, followed by
148 DNase treatment and its purification.
149 To investigate the gene expression of silkworm larvae to respond to *C. militaris*
150 mycosis until they died, RNA-seq analysis was conducted using total RNA (including
151 RNAs of silkworm larvae and *C. militaris*) extracted from the fat body of silkworm
152 larvae, which has influences of intrinsic proteins in hemolymph and cuticles, at 144 h
153 after the conidia and PBS injection. Alternatively, to investigate the gene expression of
154 *C. militaris* in silkworm larvae *in vivo*, RNA-seq analysis was conducted using total
155 RNA (including RNAs of silkworm larvae and *C. militaris*) extracted from the fat body
156 of silkworm larvae at 144 h (survival rate of silkworm larvae: 50%) and 168 h (survival
157 rate of silkworm larvae: 0%) after conidia injection. Extracted total RNA was used for
158 strand-specific RNA-seq library construction using an Illumina HiSeq 4000 with the
159 sequence mode 2 × 100 bp (Eurofins Genomics K. K., Tokyo, Japan). Raw reads were
160 aligned to the reference genome of *Bombyx mori* (Kawamoto et al., 2019) and *C.*
161 *militaris* ATCC34164 (GenBank assembly accession: GCA_008080495.1). In each,
162 about 80–90 million raw reads were yielded, and Q30 values of each sample were
163 around 95%. Gene expression data were calculated based on FPKM (Fragments Per
164 Kilobase of exon per Million mapped reads) and compared between samples using NGS
165 data analysis system MASER (Kinjo et al., 2018). In brief, mapping was performed by
166 TopHat2 (Kim et al., 2013), followed by the estimation of expression levels of genes
167 using CuffLinks2. CuffDiff was used for the comparison of gene expression levels in

two groups (Trapnell et al., 2010). Upregulated and downregulated genes were defined with a log 2-fold change (log FC) ≥2.5 and ≤2.5, respectively, with a *q*-value cutoff of 0.05.

**2.4 RT-qPCR**

RNA extraction was described above in 2.3 cDNA was prepared using PrimeScript RT-PCR Kit (TAKARA Bio, Kusatsu, Japan) and 500 ng of total RNA. Quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). Data were analyzed using comparative CT method ($2^{\Delta\Delta CT}$ method) using actin A3 gene of *B. mori* (Genbank: NM_001126254.1) or the actin gene of *C. militaris* (Genbank: XM_006670515.1). Sequences of primers are provided in Table 1.

**2.5 Growth of mycelia in silkworm larvae**

Twenty microliters of each concentration (1 and 10 mM) of catechol or 4,5-dichlorocatechol dissolved with DMSO was injected at three sites in 5th instars of silkworm larvae after they died with the injection of 25 μL conidia ($1 \times 10^6$/mL) of conidia) of each fungus (*C. militaris*, *B. bassiana*, *M. anisopliae*). Silkworm larvae died at 72–96 h after the injection of conidia of *M. anisopliae* and *B. bassiana*, and silkworm larvae died at 144–168 h after the injection of conidia of *C. militaris* (Kato et al., 2021). These larvae injected with catechol or 4,5-dichlorocatechol were incubated at 23−24°C for 144 h until the mycelia appeared on the larvae surface. As a negative control, the same volume of DMSO as catechol or 4,5-dichlorocatechol was injected. In one experiment, 5 silkworm larvae were used. Experiments were performed in duplicate.

## 3 RESULTS

### 3.1 Sequencing of both *B. mori* and *C. militaris* reads

Our previous paper indicates that the survival rate of silkworm larvae drops at 122 h after the injection of *C. militaris* NBRC103752 conidia ($1 \times 10^6$/mL, 25 μL) and reaches 0% at 168 h (Kato et al., 2021). After 168 h, any mycelia and stromata were not observed on the surface of cadavers. Therefore, to investigate the gene expression of silkworm larvae to respond to *C. militaris* mycosis until they died, RNA-seq analysis was conducted at 144 h after the conidia and PBS injection. Alternatively, to investigate the gene expression of *C. militaris* in silkworm larvae *in vivo*, RNA-seq analysis was conducted at 144 h (survival rate of silkworm larvae: 50%) and 168 h (survival rate of silkworm larvae: 0%) after conidia injection.

### 3.2 Differential gene expression analysis in *B. mori*

First, to identify the genes involved in the response of silkworm larvae to *C. militaris* mycosis, differential gene expression (DEG) in the fat body of silkworm larvae at 144 h after the conidia and PBS injection was analyzed. In this DEG analysis, 168 and 187 genes were significantly up and downregulated by greater than log Fold-Change (FC) 2.5 ($q < 0.05$), respectively (Supplementary Table 1) in the fat body of silkworm larvae. Changes of gene expression in *B. mori* are explained below.

*Proteases and protease inhibitors*

Genes encoding several proteases and protease inhibitors (Table 2) were upregulated. KWMTBOMO03899 encoding serine protease inhibitor (Serpin 3) which

is an inhibitor of proPO activating enzyme and KWMTBOMO15016 encoding protease inhibitor were greatly up-regulated. On the other hand, the expression of KWMTBOMO01542 encoding fungal protease inhibitor annotated as BmSPI38, KWMTBOMO11319 encoding chymotrypsin inhibitor CI-8A and KWMTBOMO13850 encoding kazal-type proteinase inhibitor were expressed in 5th instar of larvae substantially, but these genes were greatly downregulated by *C. militaris* mycosis (Table 2).

*Cuticular proteins*

The increased expression of some genes encoding cuticular proteins (KWMTBOMO02607, KWMTBOMO04384, KWMTBOMO13153, KWMTBOMO12563 and KWMTBOMO13142) in this study (Table 3) also corresponded to the previous paper, which reported that cuticular protein genes are upregulated in silkworms by *B. bassiana* conidia injection (Xing et al., 2017). However, KWMTBOMO09333 encoding cuticular protein glycine-rich 16 precursor was substantially down-regulated by the infection of *C. militaris*.

*Hemolymph*

In contrast to the upregulated genes by the fungal mycosis, some hemolymph protein genes encoding several hemolymph proteins, including storage proteins, microvitellogenins and lipoproteins were downregulated (Table 4). Especially, KWMTBOMO11903 encoding low molecular 30 kDa lipoprotein PBMHPC-19-like precursor and KWMTBOMO11904 encoding low molecular mass 30 kDa lipoprotein 19G1-like precursor were expressed in 5th instar of larvae abundantly, but greatly

downregulated by the fungal mycosis. Upregulation of genes encoding hemolymph proteins including storage proteins, microvitellogenins and lipoproteins were not observed.

### 3.3 Differential gene expression analysis in *C. militaris* before the death by mycosis

To identify the genes of *C. militaris* involved in the in vivo growth, DEG in *C. militaris* grown in silkworm larvae at between 144 h and 168 h after the conidia injection was performed, following raw reads were aligned to the reference genome of *C. militaris*. Supplementary Table 2 indicates the DEG in *C. militaris* between 144 h (the survival rate of silkworm larvae was around 50%) and 168 h (the survival rate of silkworm larvae was 0%). During this, 91 genes were significantly upregulated, showing that they work greatly for its growth *in vivo*. Changes of gene expression in *C. militaris* are explained below.

*Transporters*

Genes encoding transporters and permeases were upregulated in *C. militaris* (Table 5). Especially, genes encoding oligopeptide transporters (A9K55_003014, A9K55_003410, A9K55_003881, A9K55_006730, A9K55_006784 and A9K55_008881) and amino acid permeases (A9K55_000740 and A9K55_009238) were up-regulated.

*Gene clusters*

Interestingly, 4 upregulated gene clusters were also found (Table 6, Fig. 1). In chromosome 7 (Chr. 7), two gene clusters were found, but, in one cluster, genes encoding *A9K55_008710, A9K55_008711, and AK955_008712* were not annotated and

encode hypothetical proteins. In another cluster, genes encoding putative glutathione metabolism were found. In Chr. 3, one gene cluster comprising genes encoding *A9K55_001226, A9K55_001227, A9K55_001228, and A9K55_001229* were upregulated. A9K55_001226 is putative non-hemolytic phospholipase C and A9K55_001229 has a putative caleosin domain.

Additionally, one cluster in Chr. 5 composed of genes encoding *A9K55_005209*, *A9K55_005210*, *A9K55_005211*, *A9K55_005212*, and *A9K55_005213* was upregulated. Especially, the expression of *A9K55_005210*, *A9K55_005211*, and A9K55_005213 was increased significantly ($Log_2$ FC: 7.22, 6.63 and 5.97) in silkworm larvae at 168 h after conidia injection. Interestingly, *A9K55_005210*, *A9K55*_005211, and *A9K55_005213* genes encode putative catechol 1,2-dioxygenase, salicylate hydrolase, and 2,3-dihydroxybenzoate decarboxylase. Additionally, the expression of one gene encoding putative phenol 2-monooxygenase in Chr. 4 was also increased significantly ($Log_2$ FC: 7.14). To validate the expression data of genes in the cluster of Chr. 5, RT-qPCR was conducted (Fig. 2). the *C. militaris* genes (*A9K55_005210*, *A9K55_005211*, *A9K55_005212,* and *A9K55_005213*) in the gene cluster of Chr. 5 (Fig. 1) was increased at 168 h after conidia injection than at 144 h (Fig. 2). Especially, the gene (A9K55_005210) encoding putative catechol 1,2-dioxygenase was expressed by approximately 180-fold at 168 h after conidia injection compared to at 144 h, corresponding to Table 6, even though the significant difference was undetected.

### 3.4 Effects of a catechol 1,2-dioxygenase inhibitor on the *in vivo* growth of entomopathogenic fungi

The upregulation of one gene cluster, including genes encoding putative catechol 1,2-

dioxygenase, salicylate hydrolase, and 2,3-dihydroxybenzoate decarboxylase during the *in vivo* growth of *C. militaris* was found. These enzymes are involved in the degradation of phenolic substrates through catechol formation. Especially, catechol 1,2-dioxygenase catalyzes the ring cleavage of catechol to form *cis*,*cis*-muconic acid. In other entomopathogenic fungi, *B. bassiana* and *M. anisopliae*, the gene cluster similar to that of *C. militaris* is found (Fig. 3A). It was supposed that the degradation pathway of catechol, salicylate, and 2,3-dihydroxybenzoate may be activated in *C. militaris* to grow *in vivo* (Fig. 3B). Insect cuticles have many kinds of phenolic substrates, including catechol and salicylate as substrates of proPO for cuticular sclerotization (Andersen, 2010; Atkinson et al., 1973; Hopkins et al., 1999). Additionally, these phenolic substrates work in the host immunity together with PO to produce melanin (Lu et al., 2014; Butt et al. 2016; Sugumaran & Barek, 2016). To survive in silkworm larvae (*in vivo*), this cluster may be upregulated for the degradation of phenolic substrates. To confirm the need of the gene cluster for *in vivo* growth, the effect of 4,5-dichlorocatechol, an inhibitor of catechol 1,2-dioxygenase, on *in vivo* growth in silkworm larvae was investigated (Matera et al., 2010; Potrawfke et al., 2001) (Fig. 4A and B). In this experiment, as a control, DMSO and catechol were also investigated in parallel with 4,5-dichlorocatechol. The growth of *C. militaris* was slower than *B. bassiana* and *M. anisopliae,* and the larvae infected with *C. militaris* also looked more bacteria-infected. This result is consistent with the previous report (Kryukov et al., 2020). The growth of *C. militaris* was slightly inhibited by 4,5-dichlorocatechol compared to catechol and DMSO and the growth of *B. bassiana* and *M. anisopliae* was also inhibited by 4,5-dichlorocatechol.

## 4 DISCUSSION

In this study, some characteristic gene expression in the fat body of silkworm larvae during the *C. militaris* mycosis of the larvae was found using the RNA-seq.

Some proteases and protease inhibitors are involved in insect immunity, including PO activity, the expression of AMPs, and Toll immune pathway (Felföldi et al., 2011; Kanost & Jiang, 2015; Wang et al., 2017). Especially, KWMTBOMO03899 encoding serine protease inhibitor (Serpin 3) is also expressed more in silkworm hemocytes and fat body by the injection of bacteria and in this study (Zou et al., 2009). The increase in the expression of KWMTBOMO3899 in this study (Table 2) corresponds to the decrease in the PO activity in hemolymph of silkworm larvae injected with *C. militaris* conidia in the previous papers, which report that the injection of *C. militaris* conidia decreases PO activity in *Galleria mellonella* and silkworms (Kryukov et al., 2020; Kato et al., 2021). Additionally, *Manduca sexta* Serpin 3, an ortholog to *B. mori* Serpin 3, inhibits the activation of pro-PO activity in hemolymph by inhibiting PO-activating proteinases (Zhu et al., 2003).

The expression of KWMTBOMO01542 encoding fungal protease inhibitor annotated as BmSPI38 and KWMTBOMO13850 encoding kazal-type proteinase inhibitor was reduced by *C. militaris* mycosis (Table 2). These genes are overexpressed in *B. bassiana*-resistant silkworm larvae K8 strain (Xing et al., 2017). BmSPI38 is also involved in fungal resistance in silkworms (Li et al., 2012; Li et al., 2015). These results suggest that *C. militaris* may suppress the expression of these genes encoding protease inhibitors to survive and grow in silkworm larvae.

The increased expression of some cuticular proteins in this study (Table 3) also corresponded to the previous paper, which reported that cuticular protein genes are

upregulated in silkworms by *B. bassiana* conidia injection (Xing et al., 2017). However, some genes encoding cuticular proteins were also down-regulated by the infection of *C. militaris*. Insect cuticles are barriers for entomopathogenic fungi to penetrate to both inside and outside of larvae. These results suggest that *C. militaris* may rearrange the composition of cuticular proteins in cuticles for the penetration of cuticles and the growth by the control of the expression of genes encoding cuticular proteins of silkworms. It was reported that some cuticular proteins in *Tribolium castaneum* have anti-fungal roles (Sirasoonthorn et al., 2021) and some cuticular proteins in insects may be controlled for the penetration of cuticles by entomopathogenic fungi.

Some hemolymph protein genes encoding several hemolymph proteins, including storage proteins, microvitellogenins and lipoproteins were downregulated (Table 4). The gene encoding apolipophorin III is downregulated in the 6th instar of *Thitarodes pui* after the expression is increased once (Sun et al., 2012). In silkworm larvae, some lipoproteins in hemolymph have anti-fungus activities and are functional in the immune system (Li et al., 2019; Ye et al., 2021). It suggests that *C. militaris* may inhibit the expression of silkworm lipoprotein genes to evade the immune system in the silkworm for its growth *in vivo*.

In addition to the change of gene expression in silkworm larvae, in *C. militaris*, the remarkable changes of gene expression were also observed by the RNA-seq. Genes encoding oligopeptides and amino acid transporters and other transporters, including ABC multidrug transporters, were upregulated (Table 5). Additionally, some protease genes were also upregulated. These results suggest that *C. militaris* may retrieve peptides and amino acids from silkworm larvae as a nutrient to grow *in vivo*. In fact, Gao *et al*. propose that *Metarhizium* species may access products of proteins

degradation in insects because *Metarhizium* species have more amino acid and oligopeptide transporters than other fungi (Gao et al., 2011). In *B. bassiana*, ABC multidrug transporters contribute to the antioxidation and pathogenicity (Song et al., 2013). The upregulation of ABC multidrug transporter genes in entomopathogenic fungi may be an important factor for the *in vivo* growth to cope with oxidative stresses.

In Chr. 3, one gene cluster including genes encoding *A9K55_001226, A9K55_001227, A9K55_001228, and A9K55_001229* were upregulated (Table 6). A9K55_001226 is putative non-hemolytic phospholipase C and A9K55_001229 has a putative caleosin domain. Caleosin is a lipid droplet-associated protein and, in *B. bassiana*, the loss of the caleosin gene causes abnormal multi-lamellar formation and the decrease in its virulence (Fan et al., 2015). It was reported that phospholipase is involved in the virulence in human pathogenic fungi (Ghannoum, 2000; Keyhani, 2018). In *M. anisopliae*, phospholipase C activity is observed on the conidial surface (Santi et al., 2010). These results suggest that lipid metabolism may also be an important factor for the colonization of *C. militaris*.

One cluster in Chr. 5 composed of genes encoding *A9K55_005209*, *A9K55_005210*, *A9K55_005211*, *A9K55_005212*, and *A9K55_005213* was also upregulated. *A9K55_005210*, *A9K55*_005211, and *A9K55_005213* genes encode putative catechol 1,2-dioxygenase, salicylate hydrolase, and 2,3-dihydroxybenzoate decarboxylase, respectively. These enzymes are involved in the degradation of phenolic substrates through catechol formation. In Fig. 4, 4,5-dichlorocatechol, which is an inhibitor of catechol 1,2-dioxygenase, inhibited the growth of entomopathogenic fungi in silkworm larvae. These results suggest that the degradation of phenolic substrates may be crucial for the *in vivo* growth of entomopathogenic fungi. Therefore, it is supposed that the

increased expression of the gene cluster in Chr. 5 indicated in Fig. 3 was detected for the growth of *C. militaris* mycelia from 144−168 h after the injection of *C. militaris* conidia (Table 6). Cuticles of silkworm larvae have a large number of phenolic substrates for the ecdysis and the response (melanization) to microbial attack (Ortiz-Urquiza & Keyhani, 2013; Yatsu & Asano, 2009). To survive, grow and spread in silkworm larvae, entomopathogenic fungi may cope with the large number of phenolic compounds (Fig. 5). However, we need to investigate the activity of the catechol 1,2-dioxygenase and the phenolic substrate degradation at the stage.

## 5 Conclusion

In this study, RNA-seq analyses showed the changes of genes encoding proteases, protease inhibitors, cuticular proteins and hemolymph proteins in *B. mori* by *C. militaris* growth just before the larvae died. In addition, the analyses also revealed that the degradation pathway of phenolic substrates, including catechol in *C. militaris* was activated during its *in vivo* growth. From these results, we propose the mechanism of *in vivo* growth of *C. militaris* in silkworm larvae (Fig. 5). However, during the mycosis of insects by *C. militaris* at 23 °C, the growth of bacteria is also predominant in insects (Fig. 4B) because of the dysfunction of host's immune systems (Kryukov et al., 2020). Therefore, changes of the gene expression in the fat body of silkworm larvae in this study is also caused by the growth of bacteria. It is important to consider the interplay between insect hosts, entomopathogenic fungi and symbiotic bacterial community.

## AUTHORS CONTRIBUTIONS

**Tatsuya Kato:** Conceptualization (lead), Data curation (lead), Formal analysis

(supporting), Funding acquisition (lead), Investigation (supporting), Methodology (lead), Project administration (lead), Resources (supporting), Supervision (lead), Validation (lead), Visualization (lead), Writing-Original Draft Preparation (lead), Writing-Review & Editing (lead). **Konomi Nishimura:** Conceptualization (supporting), Data curation (supporting), Formal analysis (supporting), Investigation (lead), Methodology (supporting), Project administration (supporting), Validation (supporting). **Sadahiko Misu:** Formal analysis (supporting), Investigation (supporting), Software (Supporting). **Kazuho Ikeo:** Formal analysis (lead), Investigation (supporting), Software (lead), Writing-Review & Editing (supporting). **Enoch Y. Park:** Conceptualization (supporting), Funding acquisition (lead), Investigation (supporting), Resources (lead), Supervision (supporting), Writing-Review & Editing (supporting)..

## CONFLICT OF INTEREST

The authors have declared no conflict of interest.


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## SUPPLEMENTARY INFORMATION

**Table S1** Profile of differentially gene expression in silkworm larvae at 144 h after the injection of *C. militaris* conidia and PBS.

**Table S2** Profile of differentially gene expression in *C. militaris* between 144 h and 166

h after the injection of *C. militaris* conidia.

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630 **TABLE 1** Primers used in this study

| Name | 5′-3′ |
|---|---|
| Cmactin-F | GTCCCCGTCATCATGGTATC |
| Cmactin-R | GGTGTGGTGCCAAATCTTCT |
| Cm_amidohydrolase_F | CGTGCCATTTTACCTGCACC |
| Cm_amidohydrolase_R | TTGACGTCCTCGAACCAGTG |
| Cm_transcript fact_F | CAGTCGGCTGTTTCCGTTTG |
| Cm_transcript fact_R | CAGGTCCAAGACGTCCCATC |
| Cm_salicylate hydr_F | TGGAGAGCGGAACAATCACG |
| Cm_salicylate hydr_R | ATCTTGTCCCACTTGCCCTC |
| Cm_catechol diox_F | AAAACGGCACGACAATCACG |
| Cm_catechol diox_R | ACCTGCTTCTCATCCTGCTG |

631

**TABLE 2** List of genes encoding proteases and protease inhibitors expressed differentially in the fat body of silkworms injected with *C. militaris* conidia and PBS (q-value < 0.05).

| Gene | Annotation | Cm 144h | PBS 144h | $Log_2$ FC | q-value |
|---|---|---|---|---|---|
| KWMTBOMO04136 | uncharacterized protein LOC105841582 | 2.26939 | 64.9877 | -4.83979 | 3.05E-05 |
| KWMTBOMO10720 | chymotrypsin-2 | 2.25524 | 46.9419 | -4.37952 | 0.000150673 |
| KWMTBOMO04985 | serine protease gd | 4.43512 | 87.4091 | -4.30074 | 0.00248281 |
| KWMTBOMO01542 | fungal protease inhibitor F-like | 91.9173 | 1399.5 | -3.92843 | 0.000210305 |
| KWMTBOMO11319 | chymotrypsin inhibitor CI-8A, partial | 85.0038 | 796.036 | -3.22723 | 0.00759012 |
| KWMTBOMO13850 | kazal-type proteinase inhibitor precursor | 128.131 | 1199.68 | -3.22696 | 0.0213181 |
| KWMTBOMO13450 | inducible metalloproteinase inhibitor protein-like | 6.48719 | 0.557108 | 3.54157 | 3.82E-05 |
| KWMTBOMO13852 | protease inhibitor 1 precursor | 77.6846 | 6.55854 | 3.56618 | 6.40E-06 |
| KWMTBOMO08941 | matrix metalloproteinase 1 isoform 1 | 84.7479 | 4.17022 | 4.34498 | 0.000427735 |
| KWMTBOMO03899 | serine protease inhibitor 3 precursor | 550.862 | 21.3358 | 4.69035 | 3.63E-06 |
| KWMTBOMO06700 | uncharacterized protein LOC101746589 isoform X2 | 0.759282 | 0.0261449 | 4.86003 | 0.000682077 |
| KWMTBOMO12098 | salivary cysteine-rich peptide precursor | 398.034 | 10.7372 | 5.21220 | 1.01E-06 |
| KWMTBOMO15016 | protease inhibitor protein | 2695.72 | 69.8156 | 5.27098 | 2.52E-05 |
| KWMTBOMO02640 | A disintegrin and metalloproteinase with thrombospondin motifs 14 | 3.48149 | 0.0866836 | 5.32780 | 7.18E-05 |
| KWMTBOMO07132 | serine protease 7 | 104.418 | 2.55292 | 5.35408 | 1.00E-08 |

| | | | | | |
|---|---|---|---|---|---|
| KWMTBOMO15225 | melanization protease 1 | 51.2343 | 0.863814 | 5.89025 | 8.58E-07 |
| KWMTBOMO03772 | trypsin-like serine protease isoform X1 | 8.22752 | 0.112625 | 6.19086 | 0.000137206 |
| KWMTBOMO16361 | serine protease easter | 11.7144 | 0.114992 | 6.6706 | 2.20E-05 |
| KWMTBOMO14032 | uncharacterized protein LOC114246586 isoform X3 | 41.9292 | 0.265015 | 7.30574 | 1.72E-09 |
| KWMTBOMO04597 | carboxypeptidase D-like isoform X2 | 16.3792 | 0.0608962 | 8.07129 | 1.90E-06 |

634

**TABLE 3** List of genes encoding cuticle proteins expressed differentially in the fat body of silkworms injected with *C. militaris* conidia and PBS (q-value < 0.05).

| Gene | Annotation | Cm 144h | PBS 144h | $Log_2$ FC | q-value |
|---|---|---|---|---|---|
| KWMTBOMO09333 | cuticular protein glycine-rich 16 precursor | 28.864 | 3670.53 | -6.99057 | 4.93E-10 |
| KWMTBOMO06667 | cuticle protein 6.4 | 2.23838 | 57.1307 | -4.67374 | 0.00186685 |
| KWMTBOMO14312 | cuticular protein RR-2 motif 143 | 0.854633 | 13.6172 | -3.99398 | 0.00842974 |
| KWMTBOMO04385 | cuticular protein RR-1 motif 3 precursor | 23.1891 | 0.355374 | 6.02797 | 0.000295781 |
| KWMTBOMO13337 | cuticular protein RR-3 motif 148 precursor | 119.808 | 1.78459 | 6.06899 | 1.12E-05 |
| KWMTBOMO02607 | TPA: putative cuticle protein | 331.141 | 3.64879 | 6.50388 | 7.49E-05 |
| KWMTBOMO04384 | TPA: putative cuticle protein | 51.7868 | 0.295935 | 7.45116 | 1.86E-06 |
| KWMTBOMO13153 | cuticular protein RR-1 motif 32 precursor | 12.1662 | 0.0436811 | 8.12165 | 0.00589931 |
| KWMTBOMO12563 | cuticular protein RR-1 motif 21 isoform X1 | 378.405 | 0.873731 | 8.75853 | 2.69E-08 |
| KWMTBOMO13142 | cuticular protein glycine-rich 21 isoform X1 | 5.36725 | 0.00629397 | 9.73600 | 0.00684964 |

**TABLE 4** List of genes encoding hemolymph proteins which were downregulated in the fat body of silkworms injected with *C. militaris* conidia (q-value < 0.05).

| Gene | Annotation | Cm 144h | PBS 144h | $Log_2$ FC | q-value |
|---|---|---|---|---|---|
| KWMTBOMO11955 | microvitellogenin | 0.846819 | 264.043 | -8.28451 | 4.08E-12 |
| KWMTBOMO01262 | sex-specific storage-protein 2 precursor | 0.0264395 | 6.61221 | -7.96630 | 0.0255479 |
| KWMTBOMO04913 | carbonic anhydrase 7 | 0.176093 | 26.9208 | -7.25624 | 5.48E-06 |
| KWMTBOMO11903 | low molecular 30 kDa lipoprotein PBMHPC-19-like precursor | 33.0201 | 4507.03 | -7.09269 | 3.55E-09 |
| KWMTBOMO11904 | low molecular mass 30 kDa lipoprotein 19G1-like precursor | 11.945 | 1148.22 | -6.58685 | 1.45E-05 |
| KWMTBOMO08267 | hemicentin-1 | 0.0285761 | 1.43683 | -5.65194 | 0.033124 |
| KWMTBOMO01263 | silkworm storage protein | 14.9467 | 409.94 | -4.77751 | 0.00148665 |
| KWMTBOMO11946 | microvitellogenin isoform X1, microvitellogenin isoform X2 | 4.17965 | 111.437 | -4.73671 | 6.67E-05 |
| KWMTBOMO11903 | low molecular 30 kDa lipoprotein PBMHPC-19-like precursor | 62.4695 | 1420.82 | -4.50742 | 0.000560136 |
| KWMTBOMO08264 | roundabout homolog 3 | 0.359146 | 6.95044 | -4.27446 | 0.00513965 |
| KWMTBOMO08515 | leucine-rich repeat-containing protein 24 | 12.488 | 228.127 | -4.19122 | 0.00124637 |
| KWMTBOMO11944 | microvitellogenin isoform X1 | 0.937786 | 16.2211 | -4.11247 | 0.0112246 |
| KWMTBOMO13038 | yellow-c | 1.18018 | 16.8939 | -3.83942 | 0.000840765 |

643 **TABLE 5** List of genes encoding transporters and permeases which were upregulated in *C. militaris* at 168 h (q-value < 0.05).

| | Gene | Annotation | Cm 144h | Cm 168h | $Log_2$ FC | q-value |
|---|---|---|---|---|---|---|
| Chr. 2 | *A9K55_000740* | high affinity methionine permease | 0.239955 | 22.7181 | 6.56494 | 0.00137337 |
| | *A9K55_001034* | ABC multidrug transporter | 0.23899 | 9.47377 | 5.30891 | 0.0100232 |
| Chr. 3 | *A9K55_001228* | ABC multidrug transporter | 0.0263955 | 3.40255 | 7.01018 | 2.73E-05 |
| Chr. 4 | *A9K55_002395* | ABC multidrug transporter | 0.0531925 | 11.3527 | 7.7376 | 0.000547884 |
| | *A9K55_003014* | small oligopeptide OPT family | 0.0977206 | 29.4449 | 8.23514 | 2.59E-05 |
| | *A9K55_003410* | Tetrapeptide OPT1 isp4 | 1.1095 | 32.1653 | 4.85753 | 0.0217291 |
| Chr. 5 | *A9K55_003881* | OPT peptide transporter Mtd1 | 0.0639788 | 4.32712 | 6.07967 | 0.000115189 |
| | *A9K55_003920* | General substrate transporter | 0.14568 | 15.4402 | 6.72775 | 0.00652256 |
| | *A9K55_004408* | bicyclomycin resistance | 1.37565 | 26.6367 | 4.27524 | 0.028083 |
| Chr. 6 | *A9K55_005984* | MFS multidrug transporter | 0.239429 | 8.31024 | 5.11722 | 0.0237589 |
| | *A9K55_006730* | Oligopeptide transporter | 1.06794 | 35.6944 | 5.0628 | 0.0237589 |
| | *A9K55_006784* | Oligopeptide transporter OPT superfamily | 1.05646 | 30.2642 | 4.8403 | 0.00866372 |
| Chr. 7 | *A9K55_007089* | major facilitator superfamily transporter | 1.0676 | 87.3402 | 6.35421 | 0.000405537 |
| | *A9K55_007381* | protein CCC1, putative | 1.102 | 49.1297 | 5.47841 | 0.0016399 |
| | *A9K55_008881* | oligopeptide transporter | 0.0127827 | 19.739 | 10.5926 | 5.56E-06 |
| | *A9K55_009238* | amino-acid permease inda1 | 0.00637409 | 5.07179 | 9.63606 | 0.0405053 |

644

645 **TABLE 6** List of genes in a cluster which were upregulated in *C. militaris* at 168 h (q-value < 0.05).

| | Gene | Annotation | Cm 144h | Cm 168h | $Log_2$ FC | q-value |
|---|---|---|---|---|---|---|
| Chr. 3 | *A9K55_001226* | non-hemolytic phospholipase C | 0.0845705 | 4.06736 | 5.5878 | 0.000220309 |
| | *A9K55_001227* | hypothetical protein A9K55_001227 | 1.35619 | 180.899 | 7.05948 | 4.09E-08 |
| | *A9K55_001228* | ABC multidrug transporter | 0.0263955 | 3.40255 | 7.01018 | 2.73E-05 |
| | *A9K55_001229* | caleosin domain containing | 1.87461 | 104.745 | 5.80414 | 7.76E-06 |
| Chr. 5 | *A9K55_005210* | catechol dioxygenase | 0.113638 | 16.9733 | 7.22267 | 0.0154719 |
| | *A9K55_005211* | Salicylate hydrolase | 0.105612 | 10.5049 | 6.63616 | 0.0100232 |
| | *A9K55_005213* | amidohydrolase family | 0.374856 | 23.5821 | 5.97521 | 0.0326857 |
| Chr. 7 | *A9K55_007810* | hypothetical protein A9K55_007810 | 0.70273 | 47.8577 | 6.08964 | 0.0100232 |
| | *A9K55_007811* | hypothetical protein A9K55_007811 | 17.7859 | 1537.38 | 6.4336 | 0.00137337 |
| | *A9K55_007812* | hypothetical protein A9K55_007812 | 0.330197 | 7.31883 | 4.47021 | 0.0437199 |
| | *A9K55_009268* | glutathione-S-transferase GST | 0.133532 | 15.5788 | 6.86626 | 0.0133208 |
| | *A9K55_009269* | Cys Met pyridoxal phosphate-dependent enzyme | 0.224183 | 7.5145 | 5.06693 | 0.0297471 |
| | *A9K55_009271* | sarcosine oxidase | 0.549478 | 15.1118 | 4.78147 | 0.0139504 |

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## Figure legends

**FIGURE 1** Upregulated gene clusters in *C. militaris* during the *in vivo* growth. DEG of genes, which are composed of gene clusters, in *C. militaris* between 144 h and 168 h was indicated in Table 6 (q-value < 0.05). Here the construction of 4 upregulated gene clusters was indicated.

**FIGURE 2** RT-qPCR analysis to validate the RNA-seq analysis Expression of *C. militaris* genes (*A9K55_005210, A9K55_005211, A9K55_005212, A9K55_005213*) was expressed in the gene cluster of Chr. 5 in silkworm larvae at 144 h and 168 h after the injection of *C. militaris* conidia. Three biological replicates were performed (n = 3).

**FIGURE 3** The gene cluster composed of genes encoding putative phenolic substrate degrading enzymes in *C. militaris*, *B. bassiana* and *M. anisopliae*. **(A)** Construction of the gene cluster composed of genes encoding putative phenolic substrate degrading enzymes in these entomopathogenic fungi **(B)** Proposed degrading reaction of salicylate, catechol and 2,3-dihydroxybenzoate.

**FIGURE 4** Effects of catechol and 4,5-dichlorocatechol on the growth of *C. militaris*, *B. bassiana,* and *M. anisopliae* in silkworm larvae. **(A)** Injection of each concentration (0.1 and 1-mM) of catechol or 4,5-dichlorocatechol into each entomopathogenic fungus-infected silkworm larva. As a negative control, the same volume of DMSO was injected. These solutions were injected into infected silkworm larvae after they died but before the mycelia did not appear on the surface. **(B)** Morphologies of each

671 entomopathogenic fungus-infected silkworm larvae in the presence of DMSO, catechol,
672 or 4,5-dichlorocatechol. Three independent experiments were performed and typical
673 results were shown.
674
675 **FIGURE 5** Infection mechanism of *C. militaris* to silkworm larvae proposed in this
676 study.

**Figure 1. Kato et al.**

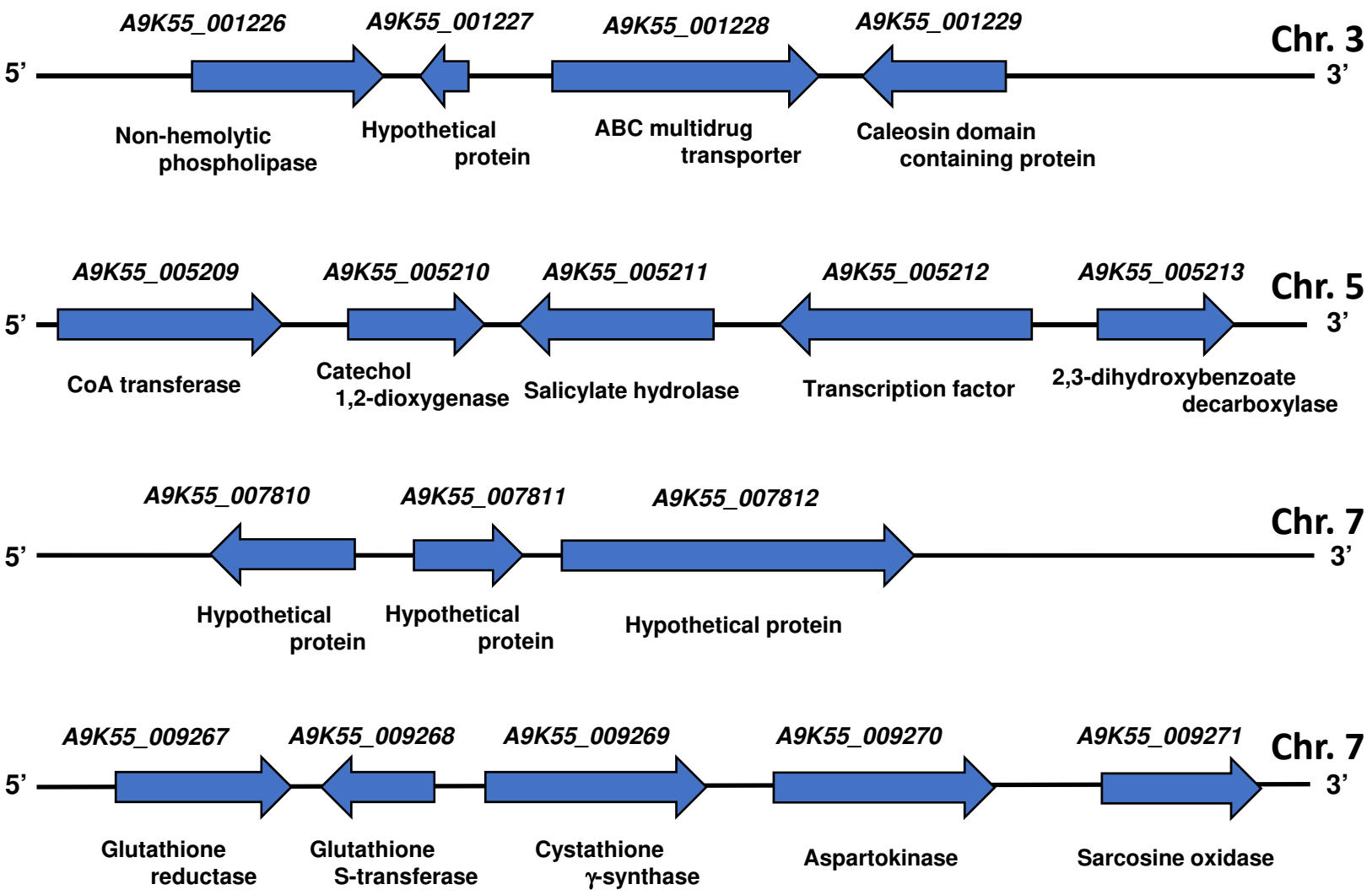


**Figure 2. Kato et al.**

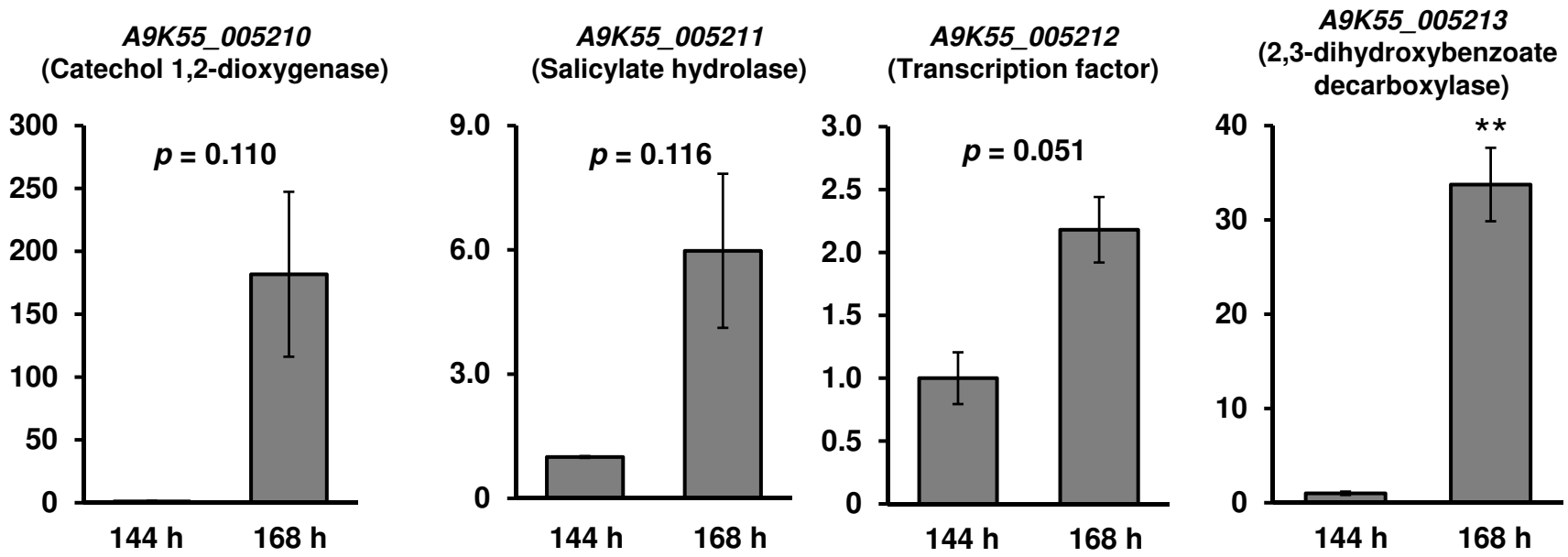
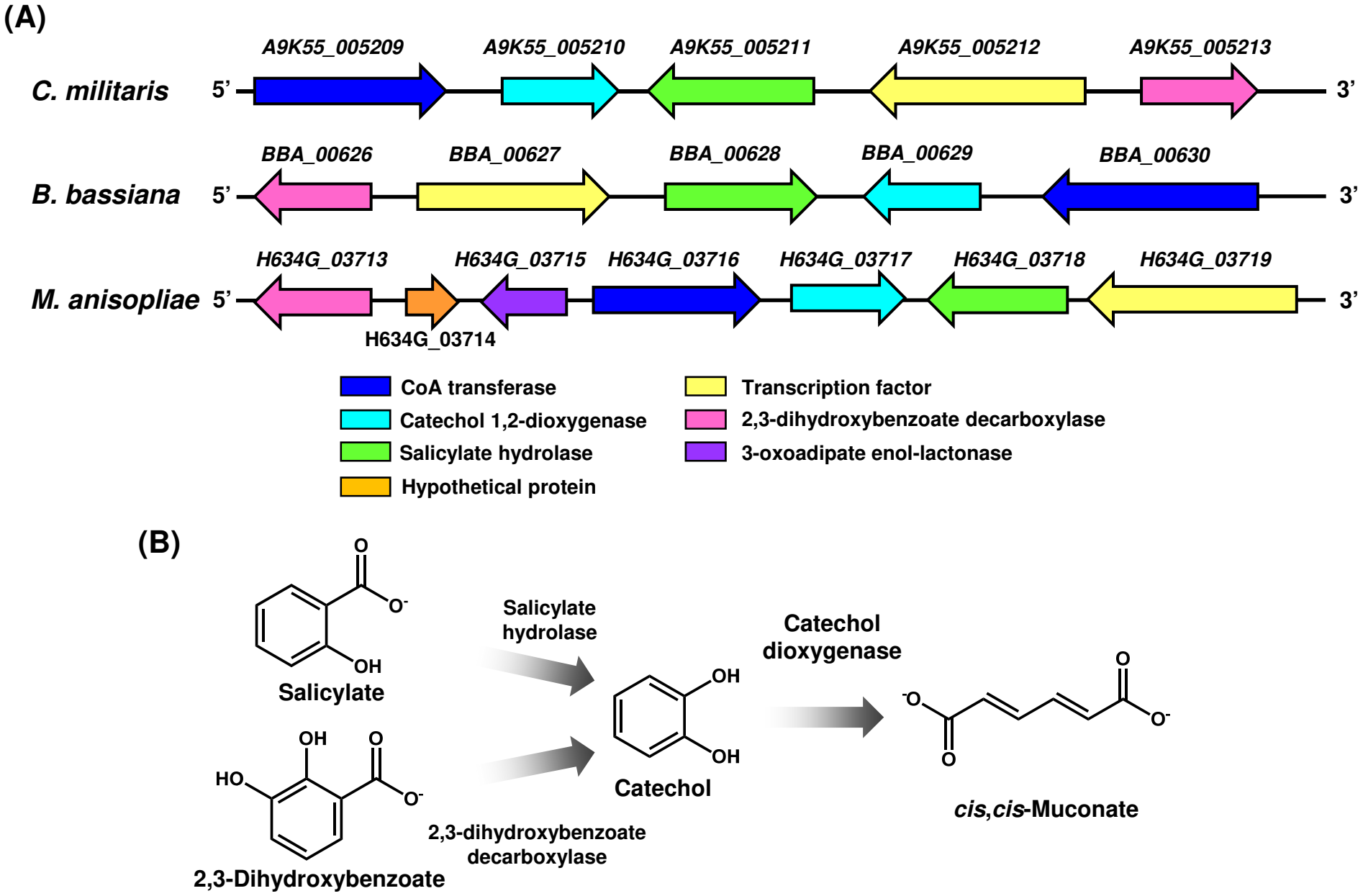
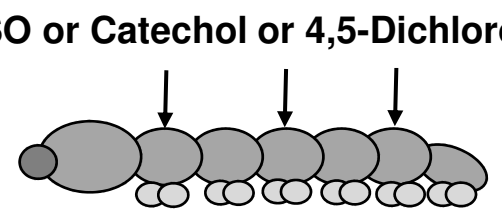


Figure 3. Kato et al.



**(A)**


DMSO or Catechol or 4,5-Dichlorocatechol

**(B)**

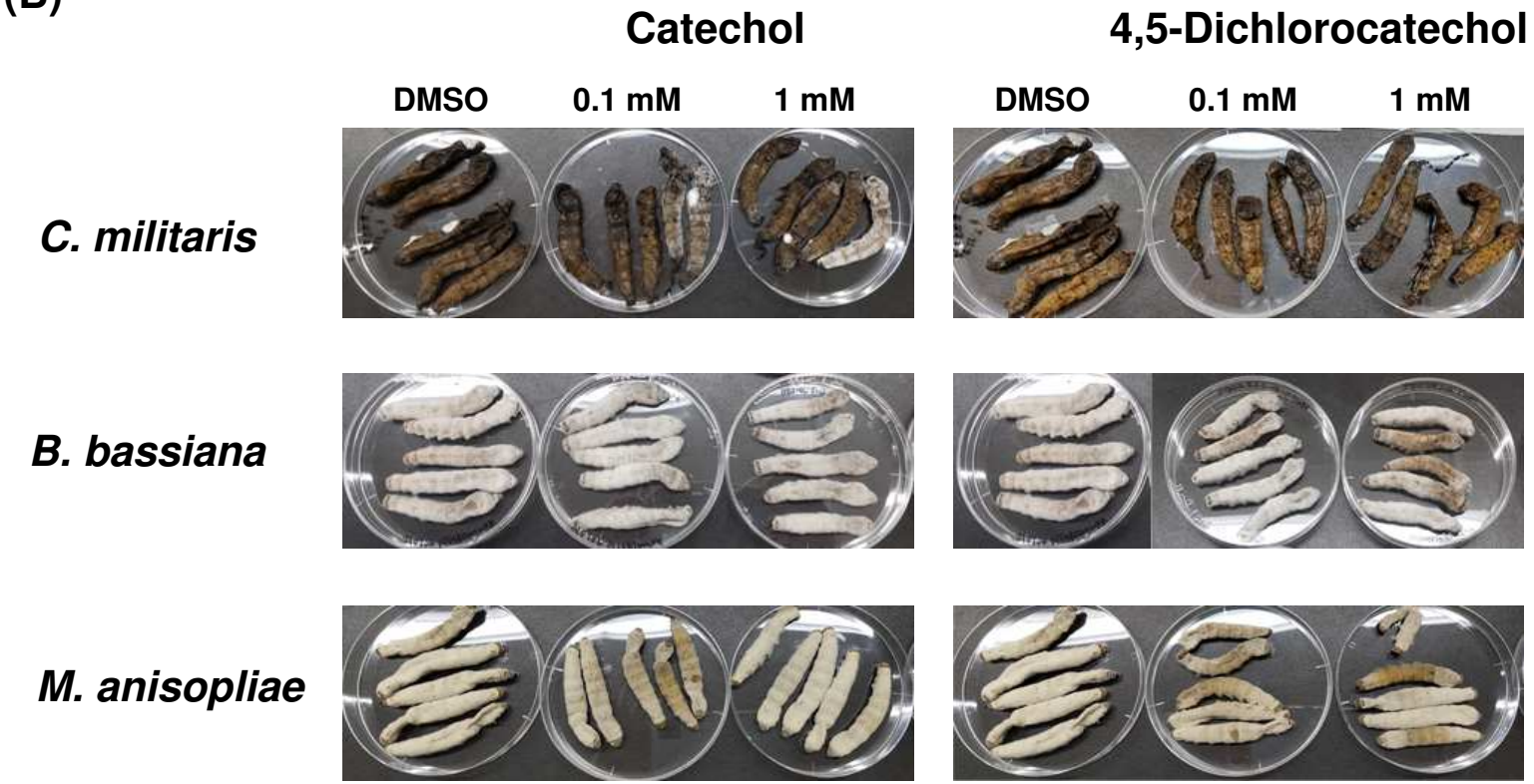

Catechol
4,5-Dichlorocatechol
DMSO
0.1 mM
1 mM
DMSO
0.1 mM
1 mM
C. militaris
B. bassiana
M. anisopliae

Figure 5. Kato et al.