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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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41 **HIGHLIGHTS**

42

43 ● Changes of the gene expression in the fat body of silkworm larvae were observed

44 during *Cordyceps militaris* infection.

45

46 ● Genes encoding oligopeptide transporters and involved in phenolic substrate

47 degradation were upregulated in *Cordyceps militaris*.

48

49

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, 1×10^6/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

168 two groups (Trapnell et al., 2010). Upregulated and downregulated genes were defined
169 with a log 2-fold change (log FC) ≥2.5 and ≤2.5, respectively, with a *q*-value cutoff of
170 0.05.
171
172 **2.4 RT-qPCR**
173 RNA extraction was described above in 2.3 cDNA was prepared using PrimeScript RT-
174 PCR Kit (TAKARA Bio, Kusatsu, Japan) and 500 ng of total RNA. Quantitative PCR
175 was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan).
176 Data were analyzed using comparative CT method ($2^{\Delta\Delta CT}$ method) using actin A3 gene
177 of *B. mori* (Genbank: NM_001126254.1) or the actin gene of *C. militaris* (Genbank:
178 XM_006670515.1). Sequences of primers are provided in Table 1.
179
180 **2.5 Growth of mycelia in silkworm larvae**
181 Twenty microliters of each concentration (1 and 10 mM) of catechol or 4,5-
182 dichlorocatechol dissolved with DMSO was injected at three sites in 5th instars of
183 silkworm larvae after they died with the injection of 25 μL conidia (1×10^6/mL) of
184 conidia) of each fungus (*C. militaris*, *B. bassiana*, *M. anisopliae*). Silkworm larvae died
185 at 72–96 h after the injection of conidia of *M. anisopliae* and *B. bassiana*, and silkworm
186 larvae died at 144–168 h after the injection of conidia of *C. militaris* (Kato et al., 2021).
187 These larvae injected with catechol or 4,5-dichlorocatechol were incubated at 23−24°C
188 for 144 h until the mycelia appeared on the larvae surface. As a negative control, the
189 same volume of DMSO as catechol or 4,5-dichlorocatechol was injected. In one
190 experiment, 5 silkworm larvae were used. Experiments were performed in duplicate.
191

192 **3 RESULTS**

193

194 **3.1 Sequencing of both *B. mori* and *C. militaris* reads**

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

204

205 **3.2 Differential gene expression analysis in *B. mori***

206 First, to identify the genes involved in the response of silkworm larvae to *C. militaris*
207 mycosis, differential gene expression (DEG) in the fat body of silkworm larvae at 144 h
208 after the conidia and PBS injection was analyzed. In this DEG analysis, 168 and 187
209 genes were significantly up and downregulated by greater than log Fold-Change (FC)
210 2.5 ($q < 0.05$), respectively (Supplementary Table 1) in the fat body of silkworm larvae.
211 Changes of gene expression in *B. mori* are explained below.

212

213 *Proteases and protease inhibitors*

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

216 is an inhibitor of proPO activating enzyme and KWMTBOMO15016 encoding protease
217 inhibitor were greatly up-regulated. On the other hand, the expression of
218 KWMTBOMO01542 encoding fungal protease inhibitor annotated as BmSPI38,
219 KWMTBOMO11319 encoding chymotrypsin inhibitor CI-8A and KWMTBOMO13850
220 encoding kazal-type proteinase inhibitor were expressed in 5th instar of larvae
221 substantially, but these genes were greatly downregulated by *C. militaris* mycosis (Table
222 2).
223
224 *Cuticular proteins*
225 The increased expression of some genes encoding cuticular proteins
226 (KWMTBOMO02607, KWMTBOMO04384, KWMTBOMO13153,
227 KWMTBOMO12563 and KWMTBOMO13142) in this study (Table 3) also
228 corresponded to the previous paper, which reported that cuticular protein genes are
229 upregulated in silkworms by *B. bassiana* conidia injection (Xing et al., 2017). However,
230 KWMTBOMO09333 encoding cuticular protein glycine-rich 16 precursor was
231 substantially down-regulated by the infection of *C. militaris*.
232
233 *Hemolymph*
234 In contrast to the upregulated genes by the fungal mycosis, some hemolymph protein
235 genes encoding several hemolymph proteins, including storage proteins,
236 microvitellogenins and lipoproteins were downregulated (Table 4). Especially,
237 KWMTBOMO11903 encoding low molecular 30 kDa lipoprotein PBMHPC-19-like
238 precursor and KWMTBOMO11904 encoding low molecular mass 30 kDa lipoprotein
239 19G1-like precursor were expressed in 5th instar of larvae abundantly, but greatly

240 downregulated by the fungal mycosis. Upregulation of genes encoding hemolymph
241 proteins including storage proteins, microvitellogenins and lipoproteins were not
242 observed.
243
244 **3.3 Differential gene expression analysis in *C. militaris* before the death by mycosis**
245 To identify the genes of *C. militaris* involved in the in vivo growth, DEG in *C. militaris*
246 grown in silkworm larvae at between 144 h and 168 h after the conidia injection was
247 performed, following raw reads were aligned to the reference genome of *C. militaris*.
248 Supplementary Table 2 indicates the DEG in *C. militaris* between 144 h (the survival
249 rate of silkworm larvae was around 50%) and 168 h (the survival rate of silkworm
250 larvae was 0%). During this, 91 genes were significantly upregulated, showing that they
251 work greatly for its growth *in vivo*. Changes of gene expression in *C. militaris* are
252 explained below.
253
254 *Transporters*
255 Genes encoding transporters and permeases were upregulated in *C. militaris* (Table 5).
256 Especially, genes encoding oligopeptide transporters (A9K55_003014, A9K55_003410,
257 A9K55_003881, A9K55_006730, A9K55_006784 and A9K55_008881) and amino acid
258 permeases (A9K55_000740 and A9K55_009238) were up-regulated.
259
260 *Gene clusters*
261 Interestingly, 4 upregulated gene clusters were also found (Table 6, Fig. 1). In
262 chromosome 7 (Chr. 7), two gene clusters were found, but, in one cluster, genes
263 encoding *A9K55_008710, A9K55_008711, and AK955_008712* were not annotated and

264 encode hypothetical proteins. In another cluster, genes encoding putative glutathione
265 metabolism were found. In Chr. 3, one gene cluster comprising genes encoding
266 *A9K55_001226, A9K55_001227, A9K55_001228, and A9K55_001229* were
267 upregulated. A9K55_001226 is putative non-hemolytic phospholipase C and
268 A9K55_001229 has a putative caleosin domain.
269 Additionally, one cluster in Chr. 5 composed of genes encoding *A9K55_005209*,
270 *A9K55_005210*, *A9K55_005211*, *A9K55_005212*, and *A9K55_005213* was upregulated.
271 Especially, the expression of *A9K55_005210*, *A9K55_005211*, and A9K55_005213 was
272 increased significantly (Log_2 FC: 7.22, 6.63 and 5.97) in silkworm larvae at 168 h after
273 conidia injection. Interestingly, *A9K55_005210*, *A9K55*_005211, and *A9K55_005213*
274 genes encode putative catechol 1,2-dioxygenase, salicylate hydrolase, and 2,3-
275 dihydroxybenzoate decarboxylase. Additionally, the expression of one gene encoding
276 putative phenol 2-monooxygenase in Chr. 4 was also increased significantly (Log_2 FC:
277 7.14). To validate the expression data of genes in the cluster of Chr. 5, RT-qPCR was
278 conducted (Fig. 2). the *C. militaris* genes (*A9K55_005210*, *A9K55_005211*,
279 *A9K55_005212,* and *A9K55_005213*) in the gene cluster of Chr. 5 (Fig. 1) was
280 increased at 168 h after conidia injection than at 144 h (Fig. 2). Especially, the gene
281 (A9K55_005210) encoding putative catechol 1,2-dioxygenase was expressed by
282 approximately 180-fold at 168 h after conidia injection compared to at 144 h,
283 corresponding to Table 6, even though the significant difference was undetected.
284
285 **3.4 Effects of a catechol 1,2-dioxygenase inhibitor on the *in vivo* growth of**
286 **entomopathogenic fungi**
287 The upregulation of one gene cluster, including genes encoding putative catechol 1,2-

288 dioxygenase, salicylate hydrolase, and 2,3-dihydroxybenzoate decarboxylase during the
289 *in vivo* growth of *C. militaris* was found. These enzymes are involved in the degradation
290 of phenolic substrates through catechol formation. Especially, catechol 1,2-dioxygenase
291 catalyzes the ring cleavage of catechol to form *cis*,*cis*-muconic acid. In other
292 entomopathogenic fungi, *B. bassiana* and *M. anisopliae*, the gene cluster similar to that
293 of *C. militaris* is found (Fig. 3A). It was supposed that the degradation pathway of
294 catechol, salicylate, and 2,3-dihydroxybenzoate may be activated in *C. militaris* to grow
295 *in vivo* (Fig. 3B). Insect cuticles have many kinds of phenolic substrates, including
296 catechol and salicylate as substrates of proPO for cuticular sclerotization (Andersen,
297 2010; Atkinson et al., 1973; Hopkins et al., 1999). Additionally, these phenolic
298 substrates work in the host immunity together with PO to produce melanin (Lu et al.,
299 2014; Butt et al. 2016; Sugumaran & Barek, 2016). To survive in silkworm larvae (*in*
300 *vivo*), this cluster may be upregulated for the degradation of phenolic substrates. To
301 confirm the need of the gene cluster for *in vivo* growth, the effect of 4,5-
302 dichlorocatechol, an inhibitor of catechol 1,2-dioxygenase, on *in vivo* growth in
303 silkworm larvae was investigated (Matera et al., 2010; Potrawfke et al., 2001) (Fig. 4A
304 and B). In this experiment, as a control, DMSO and catechol were also investigated in
305 parallel with 4,5-dichlorocatechol. The growth of *C. militaris* was slower than *B.*
306 *bassiana* and *M. anisopliae,* and the larvae infected with *C. militaris* also looked more
307 bacteria-infected. This result is consistent with the previous report (Kryukov et al.,
308 2020). The growth of *C. militaris* was slightly inhibited by 4,5-dichlorocatechol
309 compared to catechol and DMSO and the growth of *B. bassiana* and *M. anisopliae* was
310 also inhibited by 4,5-dichlorocatechol.
311

312 **4 DISCUSSION**

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

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

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

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

336 upregulated in silkworms by *B. bassiana* conidia injection (Xing et al., 2017). However,
337 some genes encoding cuticular proteins were also down-regulated by the infection of *C.*
338 *militaris*. Insect cuticles are barriers for entomopathogenic fungi to penetrate to both
339 inside and outside of larvae. These results suggest that *C. militaris* may rearrange the
340 composition of cuticular proteins in cuticles for the penetration of cuticles and the
341 growth by the control of the expression of genes encoding cuticular proteins of
342 silkworms. It was reported that some cuticular proteins in *Tribolium castaneum* have
343 anti-fungal roles (Sirasoonthorn et al., 2021) and some cuticular proteins in insects may
344 be controlled for the penetration of cuticles by entomopathogenic fungi.
345 Some hemolymph protein genes encoding several hemolymph proteins, including
346 storage proteins, microvitellogenins and lipoproteins were downregulated (Table 4). The
347 gene encoding apolipophorin III is downregulated in the 6th instar of *Thitarodes pui*
348 after the expression is increased once (Sun et al., 2012). In silkworm larvae, some
349 lipoproteins in hemolymph have anti-fungus activities and are functional in the immune
350 system (Li et al., 2019; Ye et al., 2021). It suggests that *C. militaris* may inhibit the
351 expression of silkworm lipoprotein genes to evade the immune system in the silkworm
352 for its growth *in vivo*.
353 In addition to the change of gene expression in silkworm larvae, in *C. militaris*, the
354 remarkable changes of gene expression were also observed by the RNA-seq.
355 Genes encoding oligopeptides and amino acid transporters and other transporters,
356 including ABC multidrug transporters, were upregulated (Table 5). Additionally, some
357 protease genes were also upregulated. These results suggest that *C. militaris* may
358 retrieve peptides and amino acids from silkworm larvae as a nutrient to grow *in vivo*. In
359 fact, Gao *et al*. propose that *Metarhizium* species may access products of proteins

360 degradation in insects because *Metarhizium* species have more amino acid and
361 oligopeptide transporters than other fungi (Gao et al., 2011). In *B. bassiana*, ABC
362 multidrug transporters contribute to the antioxidation and pathogenicity (Song et al.,
363 2013). The upregulation of ABC multidrug transporter genes in entomopathogenic fungi
364 may be an important factor for the *in vivo* growth to cope with oxidative stresses.
365 In Chr. 3, one gene cluster including genes encoding *A9K55_001226,*
366 *A9K55_001227, A9K55_001228, and A9K55_001229* were upregulated (Table 6).
367 A9K55_001226 is putative non-hemolytic phospholipase C and A9K55_001229 has a
368 putative caleosin domain. Caleosin is a lipid droplet-associated protein and, in *B.*
369 *bassiana*, the loss of the caleosin gene causes abnormal multi-lamellar formation and
370 the decrease in its virulence (Fan et al., 2015). It was reported that phospholipase is
371 involved in the virulence in human pathogenic fungi (Ghannoum, 2000; Keyhani,
372 2018). In *M. anisopliae*, phospholipase C activity is observed on the conidial surface
373 (Santi et al., 2010). These results suggest that lipid metabolism may also be an
374 important factor for the colonization of *C. militaris*.
375 One cluster in Chr. 5 composed of genes encoding *A9K55_005209*, *A9K55_005210*,
376 *A9K55_005211*, *A9K55_005212*, and *A9K55_005213* was also upregulated.
377 *A9K55_005210*, *A9K55*_005211, and *A9K55_005213* genes encode putative catechol
378 1,2-dioxygenase, salicylate hydrolase, and 2,3-dihydroxybenzoate decarboxylase,
379 respectively. These enzymes are involved in the degradation of phenolic substrates
380 through catechol formation. In Fig. 4, 4,5-dichlorocatechol, which is an inhibitor of
381 catechol 1,2-dioxygenase, inhibited the growth of entomopathogenic fungi in silkworm
382 larvae. These results suggest that the degradation of phenolic substrates may be crucial
383 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

632 **TABLE 2** List of genes encoding proteases and protease inhibitors expressed differentially in the fat body of silkworms injected with *C.*

633 *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

635 **TABLE 3** List of genes encoding cuticle proteins expressed differentially in the fat body of silkworms injected with *C. militaris* conidia

636 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

637

638

639 **TABLE 4** List of genes encoding hemolymph proteins which were downregulated in the fat body of silkworms injected with *C. militaris*

640 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

641

642

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

646

647 **Figure legends**

648

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

653

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

658

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

664

665 **FIGURE 4** Effects of catechol and 4,5-dichlorocatechol on the growth of *C. militaris*,
666 *B. bassiana,* and *M. anisopliae* in silkworm larvae. **(A)** Injection of each concentration
667 (0.1 and 1-mM) of catechol or 4,5-dichlorocatechol into each entomopathogenic fungus-
668 infected silkworm larva. As a negative control, the same volume of DMSO was
669 injected. These solutions were injected into infected silkworm larvae after they died but
670 before the mycelia did not appear on the surface. **(B)** Morphologies of each

entomopathogenic fungus-infected silkworm larvae in the presence of DMSO, catechol, or 4,5-dichlorocatechol. Three independent experiments were performed and typical results were shown.

FIGURE 5 Infection mechanism of *C. militaris* to silkworm larvae proposed in this study.

Figure 1. Kato et al.

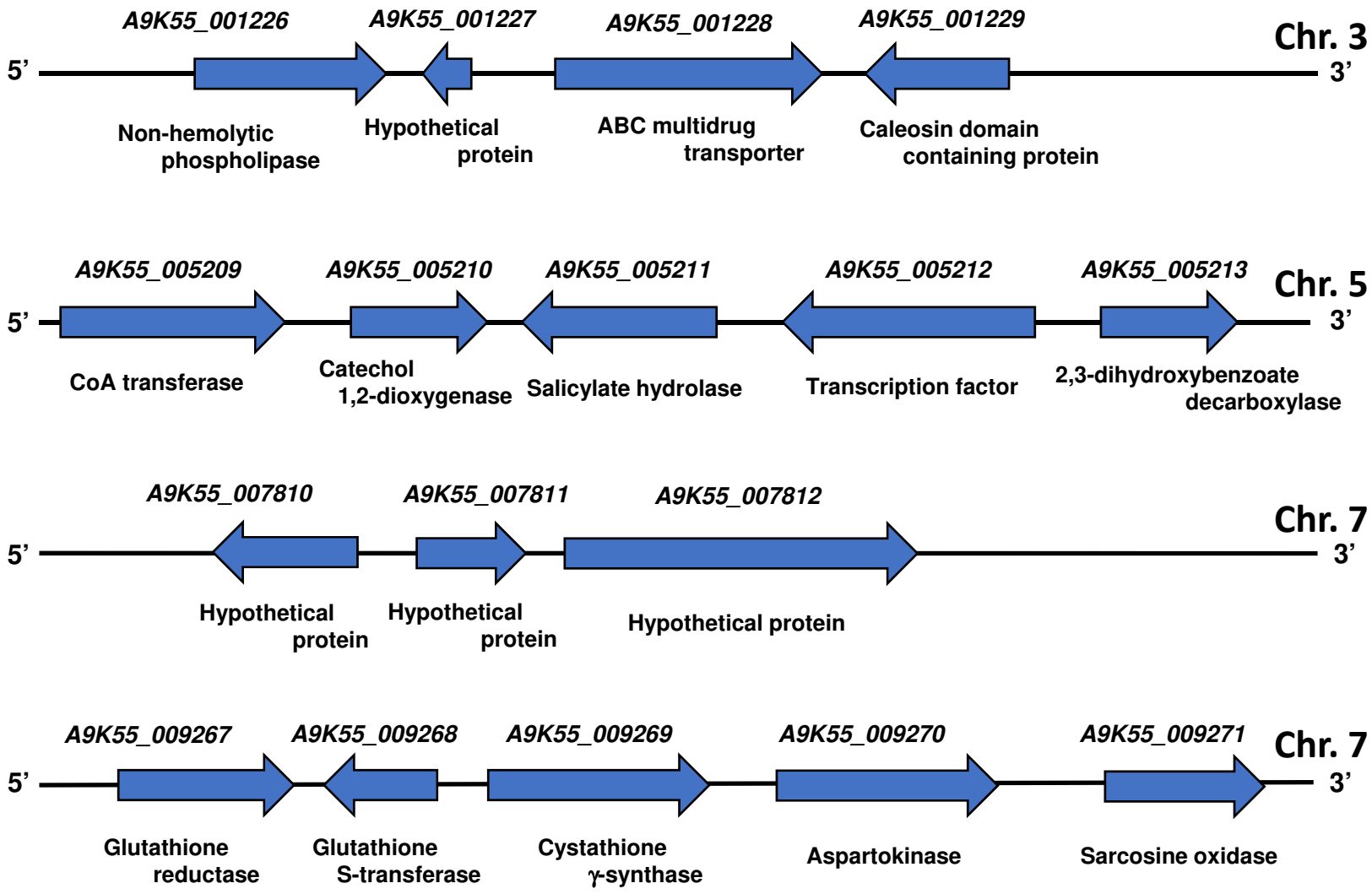

Figure 2. Kato et al.

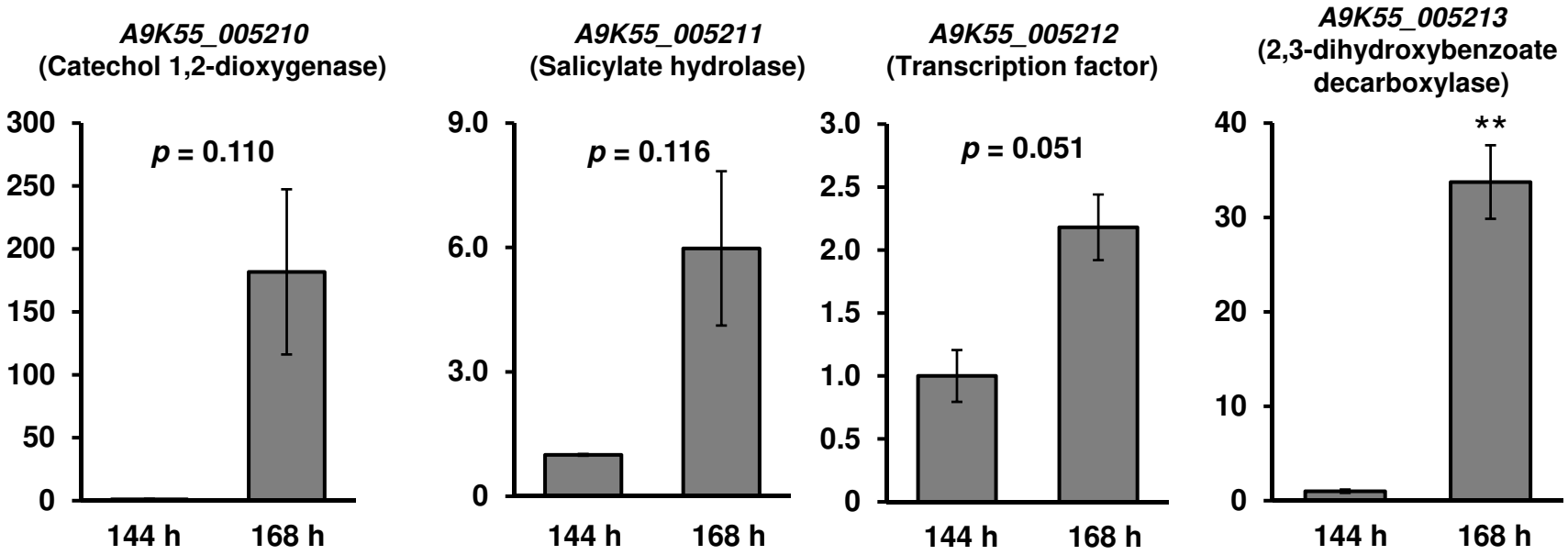

Figure 3. Kato et al.

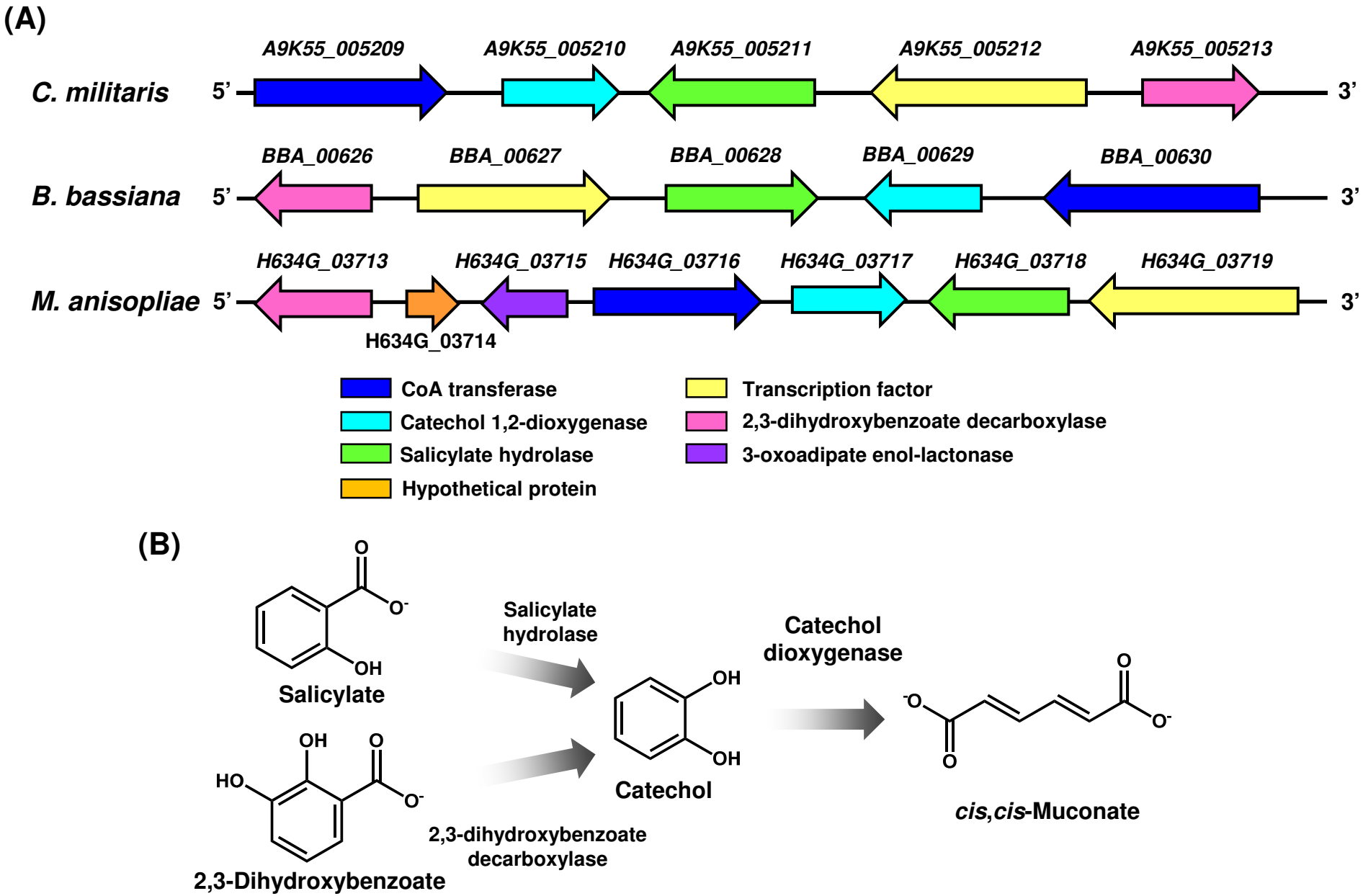

Figure 4. Kato et al.

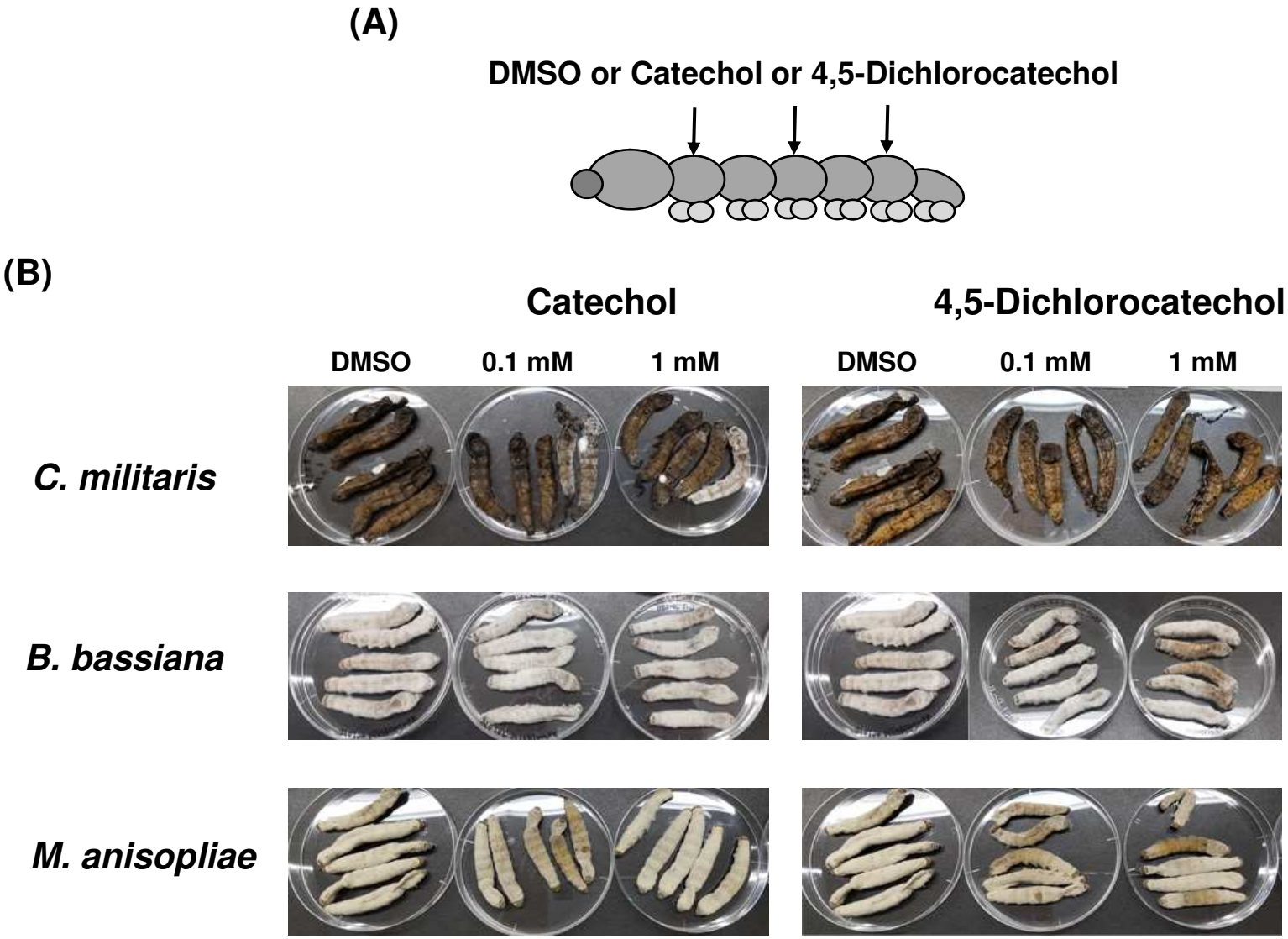
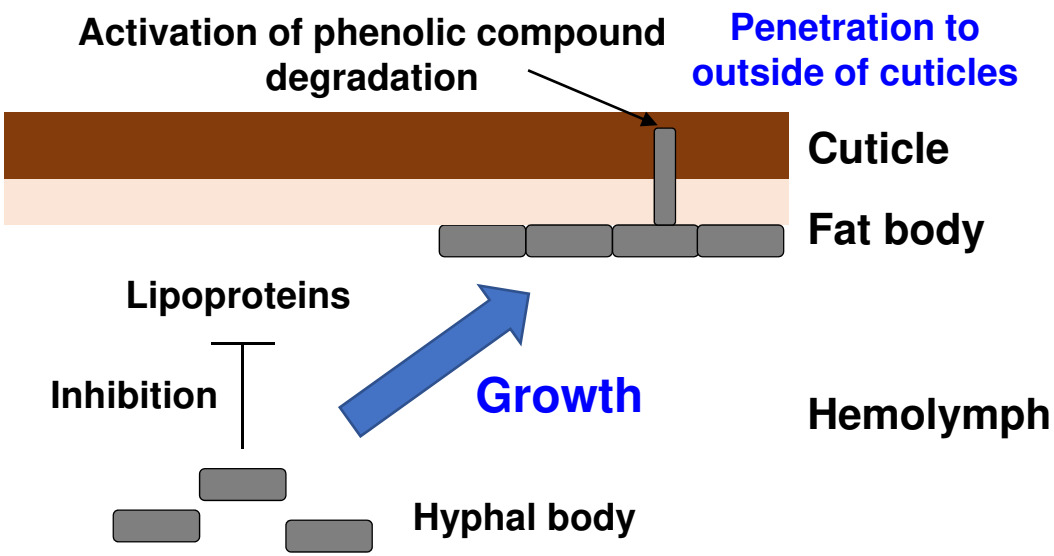
Figure 5. Kato et al.