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Abstract

Insects fight against invading microbial pathogens through various immune-related measures that comprise ‘internal’, ‘external’ as well as ‘social’ immunities. The defenses by external immunity associated with the cuticular integument are supposed to be of particular importance in repelling entomopathogenic fungi that infect host insects transcutaneously. Among such integument-related defenses, external secretions of benzoquinone derivatives typical of tenebrionid beetles have been suggested to play important roles in the antimicrobial defenses. In the present study, by utilizing the experimental infection system composed of the red flour beetle Tribolium castaneum and generalist ascomycete entomopathogens Beauveria bassiana and Metarhizium anisopliae, we performed the functional assays of the three T. castaneum genes whose involvement in benzoquinone synthesis in the adults has been reported, namely GT39, GT62 and GT63. Observations by scanning electron microcopy (SEM) revealed that the conidia of the two fungal species did not germinate on the wild-type adult body surface but did on the pupae. The expression analyses demonstrated that the levels of GT39 and GT62 mRNA increased from middle pupae and reached high in early adults while GT63 did not show a clear adult-biased expression pattern. The RNA interference-based knockdown of any of the three genes in pupae resulted in the adults compromised to the infection of the both fungal species. SEM observations revealed that the gene silencing allowed the conidial germination on the body surface of the knockdown beetles, thereby impairing the robust antifungal defense of adult beetles. Thus, we have provided direct experimental evidence for the functional importance in vivo of these benzoquinone synthesis-related genes that support the antifungal defense of tenebrionid beetles.

Keywords	Tribolium castaneum; immunity; entomopathogenic fungus; benzoquinone; scanning electron microscopy.
Taxonomy	Host Defense, Entomopathogens

Corresponding Author	Ken Miura
Corresponding Author's Institution	Nagoya University
Order of Authors	Mitsuki Sawada, Takuma Sano, Kento Hanakawa, Patchara Sirasoonthorn, Takao Oi, Ken Miura
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Highlights

·Functions of *Tribolium* benzoquinone synthesis-related genes were examined in vivo.

·SEM observation revealed that germination of fungal conidia did not occur on adults.

·Knockdown of these genes disrupted robust antifungal host defense of adults.

·SEM observation demonstrated conidial germination on the knockdown beetles.

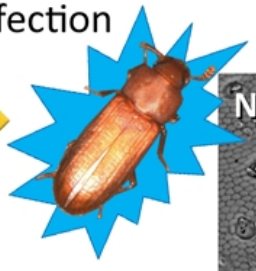
Antifungal functions of *Tribolium* benzoquinone synthesis-related genes

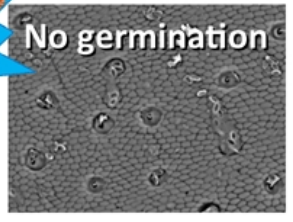
Naïve animals

Adults

Fungal challenge

Refractory to fungal infection





Pupae

Fungal challenge

Succumb to fungal infection

Knockdown adults of benzoquinone synthesis-related genes

KD Adults

Fungal challenge

Total disruption of antifungal defense

Benzoquinone synthesis-related genes of *Tribolium castaneum* confer the robust antifungal host defense to the adult beetles through the inhibition of conidial germination on the body surface.

Mitsuki Sawada[1]*, Takuma Sano[1]*, Kento Hayakawa[1], Patchara Sirasoonthorn[1], Takao Oi[2] and Ken Miura[1]**

1, Applied Entomology Laboratory, Department of Animal Sciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan.

2. Laboratory of Plant Physiology and Morphology, Department of Plant Production Sciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan.

*, These two authors contributed equally to this work.

**, Corresponding author

Telephone: +81-52-789-4033

Fax: +81-52-789-4032

Email: k-miura@agr.nagoya-u.ac.jp

Abbreviations used: RNAi, RNA interference; CHS1, chitin synthase 1; Lac2, laccase2; SEM, scanning electron microscopy; qRT-PCR, real-time quantitative RT-PCR; RPL32, ribosomal protein L32; dsRNA, double strand RNA; malE, maltose binding protein E.

ABSTRACT

Insects fight against invading microbial pathogens through various immune-related measures that comprise ‘internal’, ‘external’ as well as ‘social’ immunities. The defenses by external immunity associated with the cuticular integument are supposed to be of particular importance in repelling entomopathogenic fungi that infect host insects transcutaneously. Among such integument-related defenses, external secretions of benzoquinone derivatives typical of tenebrionid beetles have been suggested to play important roles in the antimicrobial defenses. In the present study, by utilizing the experimental infection system composed of the red flour beetle *Tribolium castaneum* and generalist ascomycete entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae*, we performed the functional assays of the three *T. castaneum* genes whose involvement in benzoquinone synthesis in the adults has been reported, namely *GT39*, *GT62* and *GT63*. Observations by scanning electron microcopy (SEM) revealed that the conidia of the two fungal species did not germinate on the wild-type adult body surface but did on the pupae. The expression analyses demonstrated that the levels of *GT39* and *GT62* mRNA increased from middle pupae and reached high in early adults while *GT63* did not show a clear adult-biased expression pattern. The RNA interference-based knockdown of any of the three genes in pupae resulted in the adults compromised to the infection of the both fungal species. SEM observations revealed that the gene silencing allowed the conidial germination on the body surface of the knockdown beetles, thereby impairing the robust antifungal defense of adult beetles. Thus, we have provided direct experimental evidence for the functional importance in vivo of these benzoquinone synthesis-related genes that support the antifungal defense of tenebrionid beetles.

Key words: *Tribolium castaneum*; immunity; entomopathogenic fungus; benzoquinone; scanning electron microscopy.

1. Introduction

Insects are threatened by many stresses from the surrounding environment, which involve physical, chemical as well as biological ones (Cloudsley-Thompson, 1988; Lacey et al., 2015). The biological stresses include, in addition to predators or parasitoids, a wide variety of microbial pathogens, thus the well-suited immune defense system is of particular importance (Butt et al., 2016; Lemaitre and Hoffmann, 2007). Insects solely rely on the innate immune system that utilizes germline-encoded receptors for invading foreign body recognition (Akira et al., 2006; Ferrandon et al., 2007).

Insect innate immune system is conveniently divided into humoral and cellular immunity, both of which are supposed to function in a coordinated fashion to exclude infecting pathogens (Hultmark, 2003; Lavine and Strand, 2002; Strand, 2008). Beside those immune responses exerted inside of the insect body, the insect cuticle provides a first-line response defense to block the entry of life-threatening microbial pathogens (Butt et al., 2016; Lu and St Leger, 2016; Ortiz-Urquiza and Keyhani, 2013; Pedrini et al., 2015; St Leger et al., 1994; St Leger et al., 1991). Entomopathogenic fungi are considered to be one of the major causes of insect death among such pathogens, and thus are utilized commonly as bio-pesticides (Lacey et al., 2015). Entomopathogenic fungi are unique in that they infect host insects transcutaneously through the cuticular integument (Thomas and Read, 2007). The insect cuticle is an extracellular matrix secreted outwardly by the monolayer of epithelia (Moussian, 2010). It covers whole insect outer body surface and lines as well the lumens of both hindgut and foregut that are also of ectodermal origin. The cuticle possesses a multilayered structure, a major portion of which is occupied by the thickest procuticle that is further divided into the endocuticle and exocuticle. The procuticle comprises as major structural components the lattice of chitin microfibrils and cuticular proteins filling the lattice interspace. These major components are

connected by covalent and non-covalent bonds formed during the sclerotization/pigmentation process. Oxidative enzymes that are also secreted by the epithelia into the cuticle catalyze the production of cross-linking agents (Arakane et al., 2005). The outermost cuticular layer, the epicuticle lacks the chitin fibrils but is rich in lipids and cuticular proteins. The epicuticle is generally covered with an envelope or a wax layer that faces directly to environment. This layer can retain microbiostatic or antimicrobial compounds that are secreted externally, and these compounds endow insects 'external immunity' in conjunction with the physical barriers provided by the cuticular integument. Indeed, defenses supported by such chemical secretion confer some insect species, such as *Tribolium castaneum*, robust refractoriness to microbial pathogens (Li et al., 2013; Pedrini et al., 2015). Tenebrionid beetles are known to secrete a variety of substituted benzoquinone compounds. Among these beetles, *Tribolium* spp. are known to possess two pairs of secretory glands specialized for this purpose, one in the prothorax and the other in the posterior abdomen, and these are referred to as odoriferous glands or stink glands (Roth, 1943). Major quinone derivatives found are reported to be ethyl-1,4-benzoquinone, methyl-1,4-benzoquinone and 2-methoxybenzoquinone. Besides benzoquinone derivatives, some species belonging to genus *Tribolium* are reported to secrete hydrocarbons (alkenes) externally, such as 1-pentadecene (Markarian et al., 1978; Pedrini et al., 2015).

By using the red flour beetle *T. castaneum* as a research model, we previously reported the details of its immune signaling pathways that invoke antimicrobial peptide gene induction as well as prophenoloxidase activation in response to the intrahemocoelic injection of opportunistic bacterial pathogens or budding yeast while utilizing *T. castaneum* genome information and RNA-interference (RNAi)-based approaches (Koyama et al., 2015; Yokoi et al., 2015; Yokoi et al., 2012a; Yokoi et al., 2012b). This was followed by the introduction of generalist ascomycete fungal entomopathogens into our experimental system, namely *Beauveria bassiana* and

Metarhizium anisopliae. We noticed during the course of studies with the model beetle and the fungal pathogens the greatest importance of the cuticular integument in fighting against the fungal infection. For example, while c.a. 70% pupae can survive when exposed to *B. bassiana* conidial suspension of a density of 1 x 10^5 cell/ml, the intrahemocoelic microinjection of as little as five cultured yeast-like hyphal body cells can effectively kill more than 80% of test pupae (Hayakawa et al., 2017). In addition, the adult beetles were found much more refractory to the fungal infection compared to the pupae. Since the activity of humoral immunity estimated by the microbial induction of antimicrobial peptide genes does not differ largely between adults and pupae (our unpublished observation), the robust antifungal defense phenotype of adult beetles was likely to be accounted for by the difference in defenses before the entry of the fungus into the haemocoel, i.e., cuticular defenses. Based on these observations, we started to investigate the antifungal functions provided by major components of the cuticular integument. So far, we have reported that the moderate knockdown of *chitin synthase 1* (*CHS1*) or *laccase2* (*Lac2*) genes in pupae renders the resulting adults susceptible to the transcutaneous infection of the two fungal species, *B. bassiana* and *M. anisopliae* (Hayakawa et al., 2017; Hayakawa et al., 2018). Interestingly, the knockdown effect of *Lac2* gene that encodes an oxidative enzyme responsible for cuticular sclerotization/pigmentation is clearer in adults than in pupae. Given these facts, we became more interested in defining precisely specific defense components responsible for the robust antifungal defense phenotype exhibited by the adult beetles in addition to the *Lac2* gene product. One such adult-specific component could be the external secretion of benzoquinone derivatives. As mentioned above, tenebrionid beetles like *T. castaneum* are especially known to secrete such chemicals and have been providing research models for ‘external immunity’ as well as ‘social immunity’ (Joop et al., 2014; Lu and St Leger, 2016; Rafaluk-Mohr et al., 2018).

In this paper, we first examined the differences of conidial behavior on the body surface of pupae or adults by utilizing the observation with scanning electron microscopy (SEM). Then, we investigated mRNA abundance for the three genes involved in the production of benzoquinone derivatives (Li et al., 2013) through the beetle development (i.e., from the onset of pupation to adulthood). Finally, we knocked down the three genes and compared the resulting antifungal defense phenotypes to control beetles in terms of survival upon infection as well as conidial behaviors on the body surface.

2. Materials and Methods

2.1. Insect and fungal species

T. castaneum was reared at 30 °C in the dark as described previously (Yokoi et al., 2012a; Yokoi et al., 2012b). Prepupae were collected from the culture each day, and the newly pupated ones on the following day defined as day 0 pupae. Pupae were reared in a 6-well plastic plate, and the newly emerged adult beetles (day 0 adult) were collected and transferred to a new container. The average pupal period was c.a. 5.5 days under our experimental conditions.

The *B. bassiana* strain and the *M. anisopliae* strain used in this study as well as in our previous studies (Hayakawa et al. 2017; Hayakawa et al., 2018) were isolated by K. Kamiya in the field of Fukui Prefecture and Saitama Prefecture in Japan, respectively. These fungal strains were the generous gift from K. Kamiya and used for the pupae and adult bioassays in this study. After the experimental fungal infection pupae or adults were kept at 25 °C under humid conditions.

2.2. Quantitative real-time RT-PCR (RT-PCR)

The extraction of total RNA from whole insect bodies, cDNA synthesis and RT-PCR-based mRNA determination were done as reported previously (Koyama et al., 2015). In brief, total

142 RNA was extracted using TRIZOL Reagent (Life Technologies), and the RNA samples with the
143 A260/A280 and A260/A230 ratios greater than 1.7 and 2.0 respectively were used as cDNA
144 synthesis templates using a PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA). The
145 resulting 1st strand cDNA preparations were subjected to qRT-PCR analyses in a Thermal Cycler
146 Dice Real Time System (Model TP-800, TAKARA) using a SYBR Premix Ex Taq Perfect Real
147 Time Kit Tli RNaseH Plus (TAKARA). Threshold cycle numbers of respective mRNA species in
148 cDNA samples were determined based on the second derivatives of the primary amplification
149 curves, and the relative mRNA abundances calculated by adopting *ribosomal protein L32* (*RPL32*)
150 mRNA in the same sample as a normalizer. The primer pairs used for qRT-PCR analyses are listed
151 in Table 1, and the accession numbers shown therein.
152
153 *2.3. RNAi*
154 RNAi-based gene silencing was performed based on the procedure described in our
155 previous papers (Yokoi et al., 2012a). cDNA templates for double strand RNA (dsRNA) synthesis
156 were prepared by a conventional PCR procedure using a gene-specific primer pairs that possessed
157 T7 RNA polymerase promoter sequences on the 5' termini (Table 2). The T7 RNA polymerase
158 promoter-double tagged cDNA fragments were purified and used as templates for dsRNA synthesis
159 using a MEGAscript RNAi Kit (Ambion). The homogeneity of dsRNA preparation was confirmed
160 by gel electrophoresis, and the amount determined spectrophotometrically. The dsRNA was
161 ethanol-precipitated, dried under vacuum, redissolved in 10 mM Tris-HCl, pH 8.0 at a concentration
162 of 3 μg/μl and stored at -20 ^{o}C until use.
163 Day 1 pupae were injected with 100 ng of dsRNA by using a Nanoject II (Drummond
164 Scientific Company) and allowed to develop at 30 ^{o}C. Control animals were treated similarly but
165 with dsRNA of *Escherichia coli maltose binding protein E* (*malE*) sequence, which derives from the

166 pmal-c2x plasmid (New England Biolabs, accession # AX377531.1). 1-day old adults, subjected to
167 RNAi-based gene knockdown during the pupal stage, were assessed for gene knockdown efficiency.
168
169 *2.4. Fungal infection and survival assay*
170 Experimental transcutaneous infection with either *B. bassiana* or *M. anisopliae* was
171 performed as described previously (Hayakawa et al., 2017). Test animals were immersed in
172 suspension of conidia (asexual spores) for 1 min and subsequently transferred to 12-well culture
173 plates that contained filter paper moistened with distilled water. The plates were kept in a moist
174 container at 25 ^{o}C, and the survival of animals were thereafter monitored and recorded every 24 h.
175 Life and death decisions were done by observing the responses of test animals when stimulated by a
176 thin and flexible plastic rod under a stereoscopic microscope (Olympus, models SZX12 and SZX16)
177 as described previously (Hayakawa et al., 2017). Data were drawn in Kaplan-Meier plots, and *P*-
178 values calculated by Gehan-Breslow-Wilcoxson test using a commercial software (Ekuseru-Toukei
179 2010, Social Survey Research Information Co., Ltd.). Conidial suspensions were prepared at the
180 density of 1 x 10^7 or 1 x 10^8 cell/ml in 0.02% of Tween-80 for both fungal species.
181
182 *2.5. SEM observation*
183 The appearances of conidia on the surface of cuticular integument of either pupae or adults
184 were observed by SEM. Day 3 wild-type pupa, day 0 or day 10 wild-type adults as well as day 6
185 knockdown mutant beetles (targeted knockdown and control knockdown) were infected with the two
186 fungal species. Ten insects underwent fungal infection for each category, and among them three to
187 four representative individuals subjected to SEM observation. They were observed directly without
188 sputter deposition while under a low-vacuum status in a table-top SEM (Model TM3030 Miniscope,
189 Hitachi).

3. Results

3.1. SEM observation of fungal conidia on the body surface of pupae or adults

We have previously reported that the *T. castaneum* pupae are much more vulnerable to the transcutaneous infection of entomopathogenic fungi than the adult beetles (Hayakawa et al., 2017). Considering the possibility that some components of the cuticle may influence the subsequent behavior of conidia that attached to the body surface of either pupae or adults, we observed directly the body surface of test animals by SEM.

Pupae were subjected to fungal infection via the immersion in conidial suspension of *B. bassiana* or *M. anisopliae*, and the body surface were thereafter observed and photographed by SEM periodically (Fig. 1). The germination of *B. bassiana* conidia on the pupal body surface was first recognized in a portion of conidia at 12 h post infection. The germination of the other portion of conidia as well as the hyphal growth of germinated conidia proceeded in a relatively slow fashion by 36 h, and the pupal body surface was covered locally by the network of hyphae by 48 h. As for *M. anisopliae*, conidial germination was found slower than the case of *B. bassiana*. Indeed, the germination did not become recognizable by 24 h post infection. The germination was first recorded at 30 h (data not shown), and this was followed by more rapid hyphal elongation and the formation of network compared to *B. bassiana*. Thus, it was clearly demonstrated that the pupal body surface allowed the conidia of both *B. bassiana* and *M. anisopliae* to germinate.

Importantly, the similar experiment using adult beetles instead of pupae brought about a contrasting result. Although we found appreciable numbers of conidia of both fungal species attached on the adult body surface, we could not detect any conidial germination of *B. bassiana* or *M. anisopliae* by 48 h post infection as shown in Fig. 2, where both dorsal and ventral views are

214 presented. We have employed the same conidial density of 1 x 10^8 cell/ml for both pupal and adult
215 infection, and this density is lethal to pupae for the two fungal species while the adults are shown
216 totally refractory to the same dosages of conidia (Hayakawa et al., 2017; our unpublished results).
217
218 *3.2. Changes of mRNA levels of benzoquinone synthesis-related genes, GT39, GT62 and T63 during*
219 *pupal and adult development*
220 Given that the body surface of adult beetles did not allow the conidial germination of both
221 *B. bassiana* and *M. anisopliae*, which was consistent with our previous survival assays, we
222 hypothesized that defensive chemical compounds secreted and spread over the adult body surface
223 was responsible to this particular phenotype. Such compounds could be benzoquinones (Pedrini et
224 al., 2015), and thus we first investigated the expression profiles of genes, the intimate involvement
225 of which in benzoquinone synthesis has been suggested, namely *GT39*, *GT62* and GT63 (Li et al.,
226 2013). The total RNA fractions were prepared from the whole body of unsexed animals from day 0
227 pupae to day 21 adults, and the mRNA levels of the three genes were determined by using qRT-PCR
228 (Fig. 3).
229 *GT39* and *GT62* exhibited similar developmental profiles. Their expression was almost
230 negligible during early and middle phases of pupal life and began to increase in late pupae. The
231 expression levels increased more after adult emergence by about 5-times in comparison with the late
232 pupae, and the elevated levels were maintained by day 21 of adult life with some fluctuations.
233 Meanwhile, *GT63* showed a transcription profile distinct from the two genes: The expression level
234 was found greater than that of *GT39* or *GT62* by about one order of magnitude; appreciable amount
235 of the mRNA existed in early and middle pupae; while it went up on day 0 and day 1 adults, the
236 abundance of *GT63* transcript on day 3 to day 7 pupae seemed to be comparable to that in pupae.

We considered that these results were consistent with the proposed function of the three genes, the involvement in adult-specific benzoquinone synthesis/secretion (Li et al., 2013), while the apparently distinct changing pattern of *GT63* mRNA might reflect its more versatile function in diverse tissues and in non-adult developmental stages.

3.3. Gene knockdown of GT39, GT62 and T63

In order to silence the expression of *GT39*, *GT62* and *GT63* genes during late pupal to early adult stages, day 1 pupa were treated with the corresponding dsRNA through the microinjection into the hemocoel. 1-day old knockdown adults were examined in terms of mRNA abundance in comparison with *malE* dsRNA-injected controls (Fig. 4). As for *GT39* and *GT62*, more than 98% of knockdown efficiencies were achieved. As regards *GT63*, while the knockdown was also statistically significant, the efficiency was less than 80%. The less effective knockdown of *GT63* might be related to its possible expression in more diverse tissues/organs, some of which could be RNAi-resistant.

3.4. Survival of GT39, GT62 and GT63 knockdown adults upon B. bassiana or M. anisopliae infection

The adult beetles that had been treated with dsRNA on day 1 of pupal life were examined in terms of antifungal defense phenotype. Day 6 adults were subjected to fungal infection through the immersion in the conidial suspension of either *B. bassiana* or *M. anisopliae* at the conidial density of 1 x 10^7 cell/ml, and the survival was recorded thereafter (Fig. 5). As is evident here, all the *GT39*, *GT62* and *GT63* knockdown had a devastating impact on the host defense against the two fungal species while this dosage of conidial treatment did not affect at all the survival of *malE* dsRNA-treated control beetles. The *GT63* knockdown beetle mutants display the most dramatic

outcome, with all beetles dying after 96 hours post-infection by both fungal species. The combination of *GT62* knockdown beetles and *M. anisopliae* was found equally devastating. The knockdown of *GT39* seemingly resulted in a less severe defensive phenotype, with most of the *B. bassiana*- or *M. anisopliae*-challenged beetles dying before 120 hours post-infection (Fig. 5). Based on these results, we concluded that all the functions of *GT39*, *GT62* or *GT63* genes were essential to the effective and robust cuticular antifungal host defense of adult beetles.

3.5. SEM observation of conidia on the body surface of GT39, GT62 and GT63 knockdown beetles

In order to link the impaired survival of the knockdown adults to the behavior of the two fungal pathogens on the host cuticle, SEM observation was conducted using the knockdown beetles that had been exposed to the conidial suspensions of the two fungal species on day 6 of adult life. Fig. 6 shows the results of *GT39*, *GT62* and *GT63* knockdown adults upon fungal infection. While on the body surface of the negative control, *malE*-treated beetles the conidia of the two species remained ungerminated, all the experimental gene knockdown obviously allowed some of attached conidia of both species to germinate and develop elongated hyphae. Together with the aforementioned survival assays, we concluded that these genes play crucial roles for robust defense of adult beetles against entomopathogenic fungi by inhibiting conidial germination on the body surface.

4. Discussion

Entomopathogenic fungi, especially generalist species such as *B. bassiana* and *M. anisopliae* have gained attention as an environmentally-friendly component of IPM program (Lacey et al., 2015). In this context, it is of particular importance to identify insect host genes that contribute potentially to the resistance to fungal infection in preparation for future problems of resistance

development. Resistant factors of insect against fungal pathogens can vary and include internal, external and social immunity as well as behavioral avoidance seen in some insect species (Lu and St Leger, 2016; Ortiz-Urquiza and Keyhani, 2013). We believe *T. castaneum*, while in fact this model beetle is not a crop-damaging pest but damages stored products, provides a good model experimental system for this purpose, considering its genomic information as well as its RNAi-amenable nature (Richards et al., 2008; Tomoyasu and Denell, 2004; Tomoyasu et al., 2008; Zou et al., 2007). In addition, this species combines well-defined internal and external immunity. In this study we focused on one of the constituents of its antifungal external immunity.

Genes associated with the production of benzoquinone derivatives were first identified by Wilmmer and co-workers in 2013 (Li et al., 2013). They picked up candidate genes that were highly and specifically expressed in *T. castaneum* odoriferous gland transcriptomes by adopting RNA sequencing approaches. This was followed by phenotypic assays of the knockdown beetles of the candidate genes in terms of the chemical determination of the gland contents and gland tissue morphology, leading to the identification of three particular genes *GT39*, *GT62* and *GT63*, the knockdown of which resulted in remarkable quinone-less phenotypes. They also performed the phylogenetic analyses on these three genes to infer their functions. The present study by us was performed based on the framework established by this preceding study (Li et al., 2013). However, these authors did not assess the antimicrobial phenotypes of the knockdown beetles. Instead, they conducted the knockdown of one gene, *GT63* among the three genes that they had identified, and tested the knockdown effect by placing the dissected stink glands on microbial culture plates of either a common food contaminant fungus *Aspergillus niger* or a Gram-positive soil bacterium *Arthrobacter globiformis* to estimate the growth inhibition ability of the gland contents. These authors demonstrated successfully that the growth inhibition ability ex vivo of the wild-type gland to these non-entomopathogenic microbes disappeared by knocking down the *GT63* gene. We employed

conventional survival assays of knockdown beetles in this study and demonstrated that the knockdown of one gene out of the three was sufficient to disrupt totally the robust defense of the adult beetles against two distinct fungal entomopathogens. The observed defense phenotypes are consistent with the previous results by Li et al. (2013) that the gland levels of major benzoquinone derivatives, methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone become negligible upon the single knockdown among the three genes, thereby proving the indispensable nature of these genes in host defense at an individual level.

As a next step, we addressed the question regarding which phase of fungal infection process is blocked by the adult beetles. To examine the appearances and behaviors of conidia on the host body surface, we employed the SEM observation. We first compared the status of conidia attached onto either pupae or adults, and this test clearly visualized the distinct behaviors of conidia depending on the developmental stages of *T. castaneum* that they infected. Whereas the appreciable numbers of conidia of *B. bassiana* and *M. anisopliae* were able to attach onto the body surfaces of both pupae and adult beetles, they were able to germinate and elongate hyphae only on the pupae. No conidial germination was detectable on the adult body surface, indicating that the inhibition of conidial germination rather than that of hyphal growth or penetration into the cuticle is the determining factor for the robust antifungal defense phenotype exhibited by the adult beetles. This in vivo observation is consistent with the previous result of in vitro germination assay of *B. bassiana* conidia exposed to benzoquinone (Pedrini et al., 2015). Then, in order to correlate the impaired antifungal defense phenotypes of the adults, which had undergone the knockdown of benzoquinone synthesis-related genes, to the conidial behavior on their body surface, a similar SEM observation was conducted. By using this approach, we successfully demonstrated that the knockdown tolerated the germination of the both fungal species on the adult beetles, which could account for a large fraction of the resulting impaired defense phenotype. As for the ungerminated status of *B. bassiana*

and *M. anisopliae* conidia on the naïve or control knockdown beetles, this is likely a fungistatic effect rather than fungicidal one, considering the in vitro germination result by Pedrini et al. (2015) that have shown that prolonged incubation in the presence of benzoquinone allows *B. bassiana* conidia to germinate presumably through the loss of benzoquinone by oxidation with time.

To counteract the action of benzoquinone derivatives by *T. castaneum* adult beetles *B. bassiana* is known to utilize a specific detoxifying enzyme, NAD(P)H: 1,4-benzoquinone oxidoreductase (BbBqrA) (Pedrini et al., 2015). Cognate enzymes that would be potentially able to reduce quinones or related substrates are encoded in the genomes of several fungal species. These authors searched genomic databases and performed phylogenetic analyses. Interestingly, whereas they found that *M. anisopliae* genome does encode an orthologous gene to *B. bassiana*'s *BbBqrA*, the two genes were clustered into closely related but distinct subgroups. Pedrini et al. (2015) prepared a genetically modified *B. bassiana* strain overexpressing *BbBqr* and tested its virulence in comparison with the parental strain. The mortality of *T. castaneum* adults increased compared to that of around 20% by the parental strain, but it was still less than 50%. This suggests that neither the wild-type level nor even the artificially elevated level of BbBqr protein is sufficient to overcome this specific defense measures by the adult beetles of *T. castaneum*. Conversely, in this study the disarmament of the host insects via the knockdown of benzoquinone synthesis-related genes disrupted almost completely the host defense against *M. anisopliae* in addition to *B. bassiana*. This may be indicative of the extreme usefulness of the benzoquinone-based defense not only to specified fungal pathogens but to a wider spectrum of microbial pathogens.

We have been studying over the past few years the defenses associated with cuticular integument by using the *T. castaneum*-fungal entomopathogen system. So far, we have been dealing with major and common cuticular components, and among them we have already reported the involvement of *CHS1* and *Lac2* in antifungal host defense. Indeed, the dsRNA treatment of these

two gene sequences in pupae resulted in the adult phenotypes, in which refractoriness to fungal infection are significantly impaired (Hayakawa et al., 2017; Hayakawa et al., 2018). However, the problem was that since these genes are also indispensable to successful molting/metamorphosis, a high degree of gene knockdown would bring about lethality via a failure of proper ecdysis. Therefore, we needed to employ a very mild method to obtain knockdown beetles by injecting 1 ng dsRNA per pupa, which is 1/100 of our regular dosage. Meanwhile, the knockdown of the three benzoquinone synthesis-related genes, which are tenebrionid-specific rather than common to all insects, did not lead to such lethality arisen from developmental defects. Thus, we were able to use our regular dosage of dsRNA, 100 ng per animal, to silence these genes in this study. The outcome was notable: In any cases of single knockdown out of the three genes through dsRNA treatment in early pupae, the resulting adults of day 6 totally succumbed to the two fungal species. While we have indeed obtained a similar drastic phenotypic shift in adults by moderate knockdown of *Lac2* in pupal stage, a head-to-head comparison between our previous (*Lac2*) and present results is difficult since the two experiments differ in the degree of knockdown as well as the density of conidial suspension used. Nonetheless, we consider that the three genes upholding the external secretion of benzoquinones clearly play a major role to establish the robust antifungal host defense of *T. castaneum* adults in conjunction with other critical genes, such as aforementioned *CHS1*, *Lac2*, as well as adult specific cuticular protein genes. As for *Lac2*, its possible involvement in oxidation steps of the benzoquinone synthetic pathway has been suggested (Hayakawa et al., 2018; Li et al., 2013). In order to further investigate the function of *Lac2* independently of the developmental context, analyses using adult beetles that are treated with the dsRNA after emergence are underway.

Tribolium beetles are known to secrete the stink gland contents not only onto their body surface but as well to the surrounding environment. This is often visualized by a pinkish color of flour infested by these beetles. In the present study, we showed that the expression levels of the three

benzoquinone synthesis-related gene were very low in early to middle pupae except the *GT63* and that the inhibition of conidial germination was negligible on the pupal surface compared to the adults. This indicates that *T. castaneum* pupae per se lack the defense supported by the external secretion of benzoquinone derivatives that would otherwise give them a more robust antifungal defense phenotype. The immature stages of *T. castaneum* may possibly achieve benefits from the benzoquinones secreted externally by the adult beetles in the same colony, which could sterilize the environment and give a prophylactic effect. It may be useful to investigate the defense phenotypes of pupae or larvae through quantitative as well as morphological studies when co-cultured with either control or knockdown beetles of benzoquinone synthesis-related genes to address the issues related to social immunity. Determining whether or not secreted benzoquinones can make a transition from the adult to the pupae or larvae by using chemical determination as well as SEM observation is of particular interest.

In summary, we performed the functional analyses of the three *T. castaneum* genes that have been reported to be involved in the synthesis of benzoquinone derivatives. We demonstrated that the single knockdown of any of the three genes resulted in a total disruption of host defense of the adult beetles against the two generalist fungal pathogens, *B. bassiana* and *M. anisopliae*. SEM observations revealed that the loss of antifungal phenotype in the knockdown adults arose from the loss of inhibition of conidial germination found in control or wild-type beetles. Thus, we have provided direct experimental evidence that proves the crucial nature in vivo of these genes to the robust antifungal defense of adult tenebrionid beetles.

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546 Legends to figures

547 **Fig. 1.** SEM observations of conidia on the body surface of pupae. Day 3 pupae were infected with
548 either *B. bassiana* (left) or *M. anisopliae* (right) by the immersion in conidial suspensions. The cell
549 density of the suspension was 1 x 10^8 cell/ml for both fungal species. The surface of test pupae was
550 thereafter observed and photographed by SEM every 12h post challenge. The magnification was x
551 3000 throughout, and the scale bars represent 10 μm.

552

553 **Fig. 2.** SEM observations of conidia on the body surface of adults. Day 1 adults were infected with
554 either *B. bassiana* (left) or *M. anisopliae* (right) by the immersion in conidial suspensions. The cell
555 density of the suspension was 1 x 10^8 cell/ml for both fungal species. The surface of test adults was
556 observed and photographed by SEM at 48h post challenge. The pictures of both dorsal and ventral
557 body surfaces are shown. The magnification was x 1000 throughout, and the scale bars represent 30
558 μm.

559

560 **Fig. 3.** Developmental expression profiles of *GT39*, *GT62* and *GT63* during pupal and adult stages.
561 The mRNA levels of the three genes were determined by qRT-PCR, and shown as relative
562 abundances to those of reference gene *RPL32*. The numerals that follow the symbol P (pupa) or A
563 (adult) are ages in days of respective developmental stages. Each vertical bar represents mean ± SD
564 from three biological replicates.

565

566 **Fig. 4.** Knockdown efficiencies of *GT39*, *GT62* and *GT63*. Day 1 pupae were injected with 100 ng
567 of *GT39*, *GT62* and *GT63* dsRNA, and the mRNA levels determined in resulting day 1 adults. Each
568 bar represents mean ± SD from three biological replicate. *MalE* dsRNA-treated animals served as
569 negative controls, and the significantly different values from the control marked by asterisks with *p*-

values.

Fig. 5. Survival of knockdown adults upon fungal infection. Day 1 pupae were treated with 100 ng dsRNA of *GT39*, *GT62* or *GT63*, and the resulting adults of age day 6 was examined in terms of survival against the infection of *B. bassiana* (left) or *M. anisopliae* (right). The survival rates were recorded every 24h up to 192 h post fungal challenge, and the results shown in Kaplan-Meier plots. The conidial density used for infection was 1 x 10^7 cell/ml for both fungal species. The *malE* dsRNA-treated animals were used as negative controls. The survival curves that are significantly different from the control are indicated by asterisks with *p*-values.

Fig. 6. Conidial germination and hyphal growth on adult body surface after knockdown of *GT39*, *GT62* and *GT63*. Day 1 pupae were injected with 100 ng dsRNA of *GT39*, *GT62*, *GT63* or negative control *malE*. The resulting adults of age day 6 were challenged by the two fungal species, and observed by SEM at 48 h post infection. Other details are the same as in Fig. 2.

Table 1. Primer pairs used for qRT-PCR.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product length	Accession #
GT39	TTGCTGAAGTCTACGAGAACAC	GAGCTCGATGGTGTCATTGTC	149 bp	JX569829
GT62	GCGACGATATGGGACATAACGA	GTACAAGCATTCTGGACGTAGTA	121 bp	JX569830
GT63	ACGAAGCGACCGCAAATGTTGA	ACCGTCCCAGCATCCATCAC	154 bp	JX569831
RPL32	ACCGTTATGGCAAACTCAAACG	TGTGCTTCGTTTTGGCATTGGA	183 bp	Glean_06106

Table 1. Sawada et al.

Table 2. Primer pairs used for cDNA template preparation for dsRNA synthesis.

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product length
GT39	*TAATACGACTCACTATAGGG*-GGAGGTCACCCAGAACAACT	*TAATACGACTCACTATAGGG*-TGACATCCCTTGGCACATATTC	458 bp
GT62	*TAATACGACTCACTATAGGG*-AAGGTGGCACATACGATGGATA	*TAATACGACTCACTATAGGG*-GCGGATTGGCATTCGGATCAAT	375 bp
GT63	*TAATACGACTCACTATAGGG*-TCAGTGGAACGTGTGGTCGAATA	*TAATACGACTCACTATAGGG*-TTGCGCCCAATTCGTCACCAT	462 bp
malE	*TAATACGACTCACTATAGGG*-ATTGCTGCTGACGGGGGTTAT	*TAATACGACTCACTATAGGG*-ATGTTCGGCATGATTTCACCTTT	518 bp

T7 RNA polymerase promoter sequences are shown in italic.

Table 2. Sawada et al.

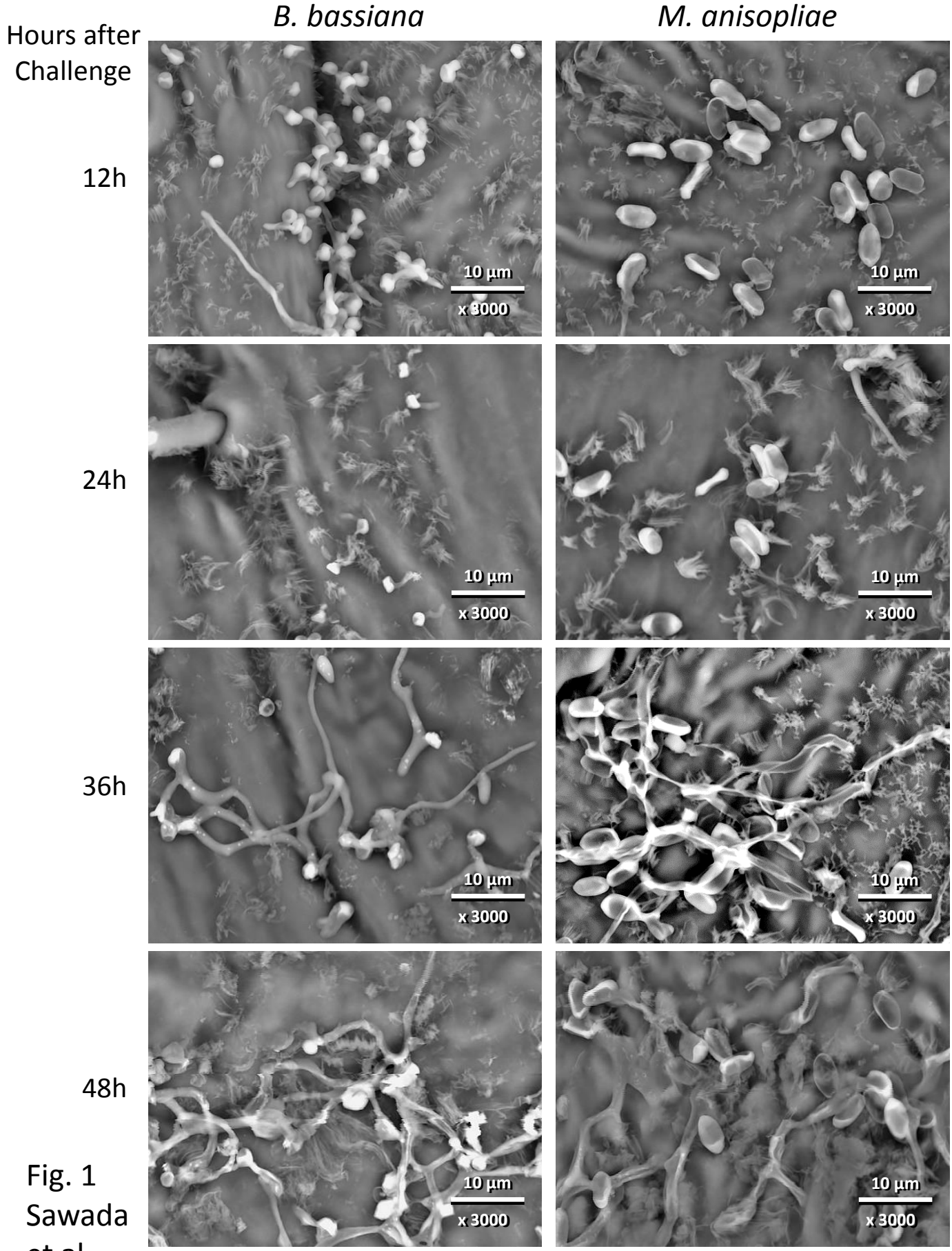


Fig. 1
Sawada et al.

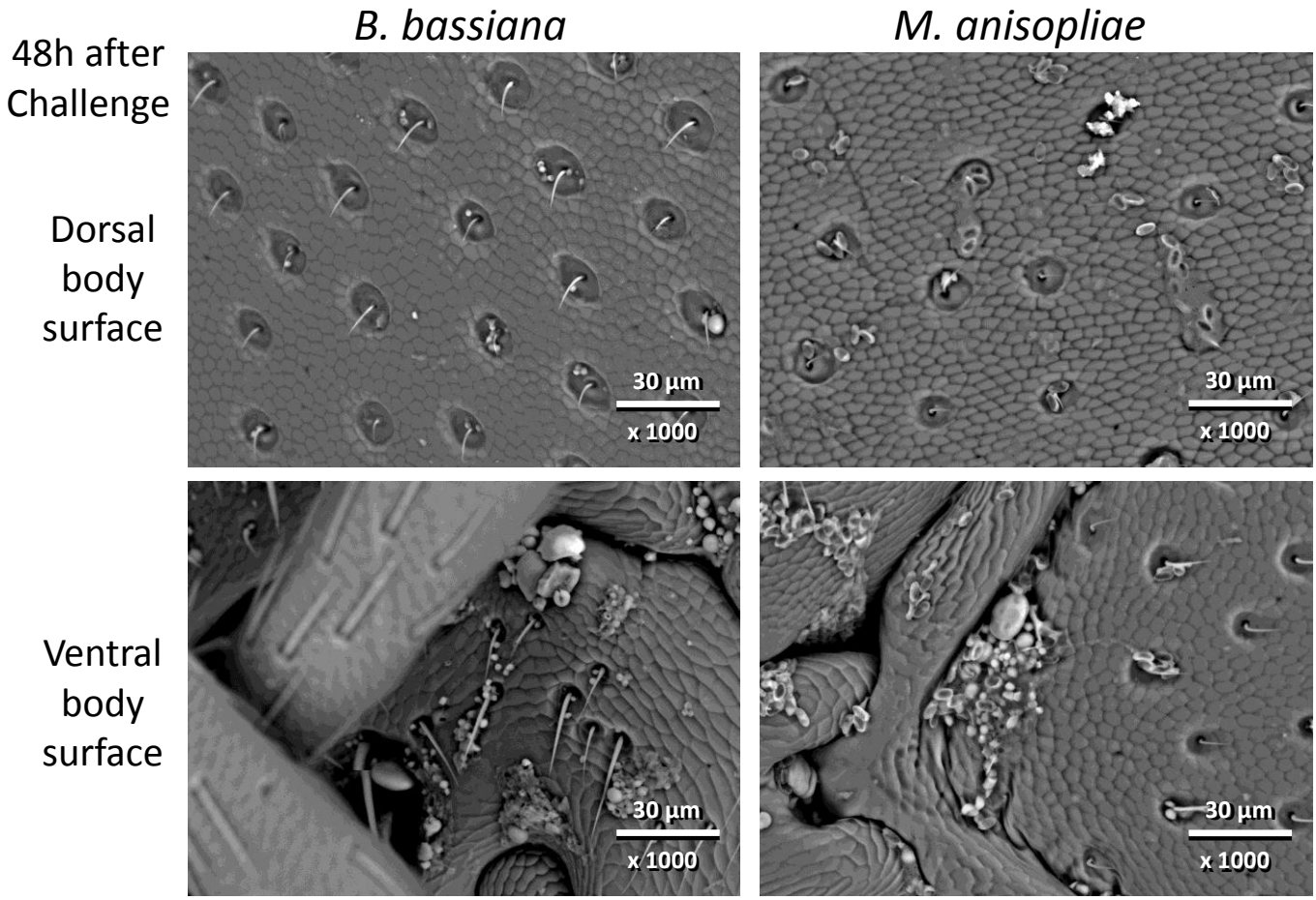


Fig. 2. Sawada et al.

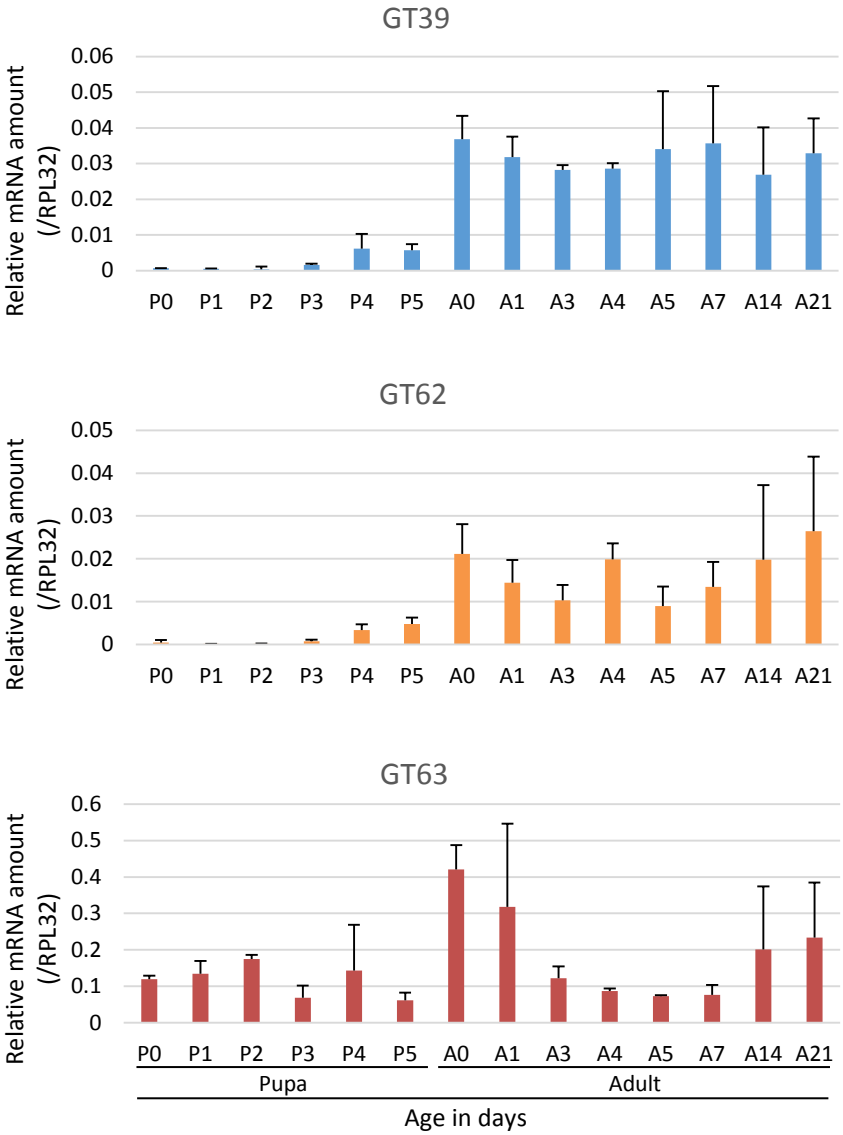
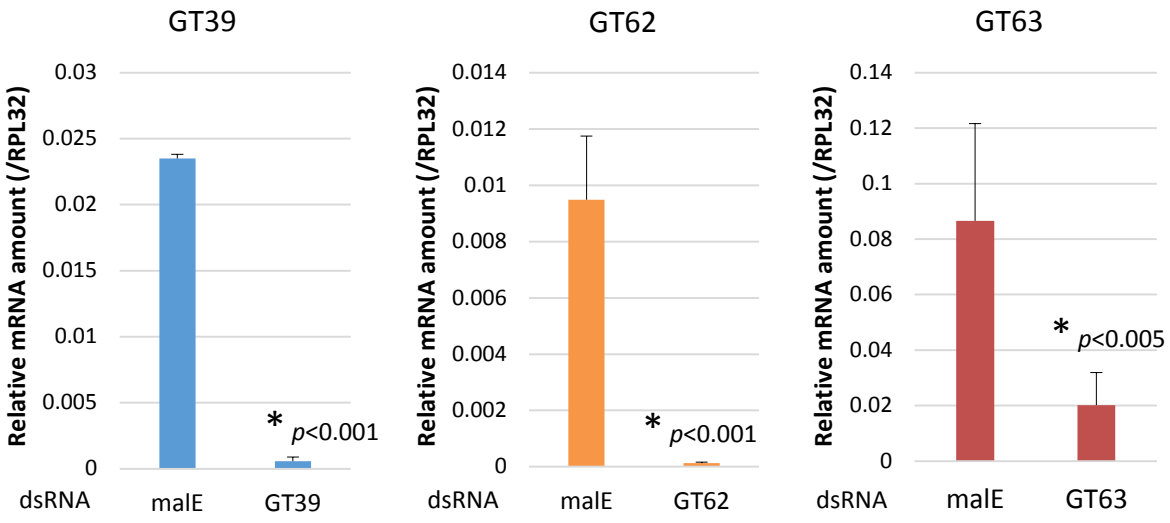


Fig. 3. Sawada et al.



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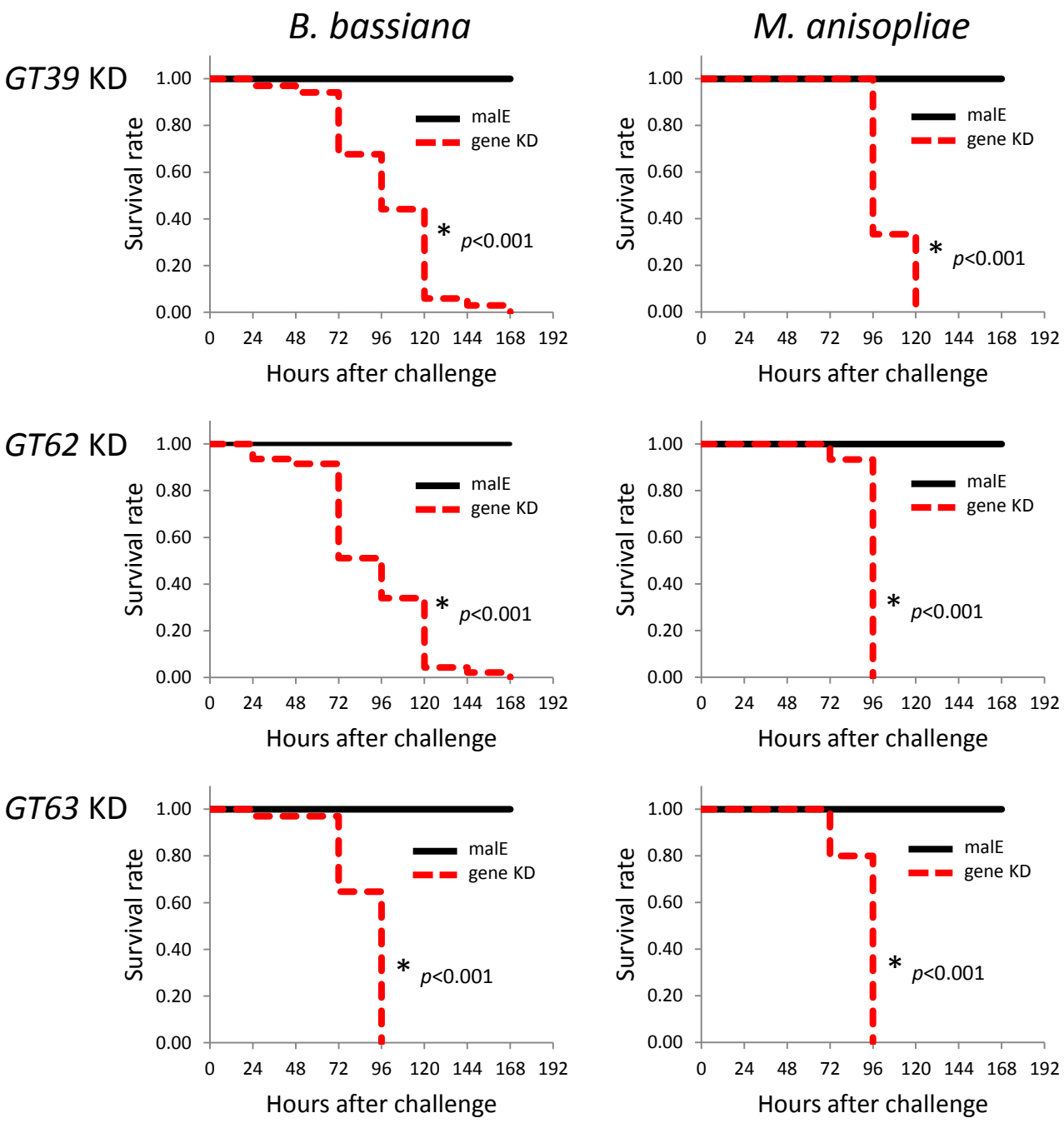

Fig. 5. Sawada et al.

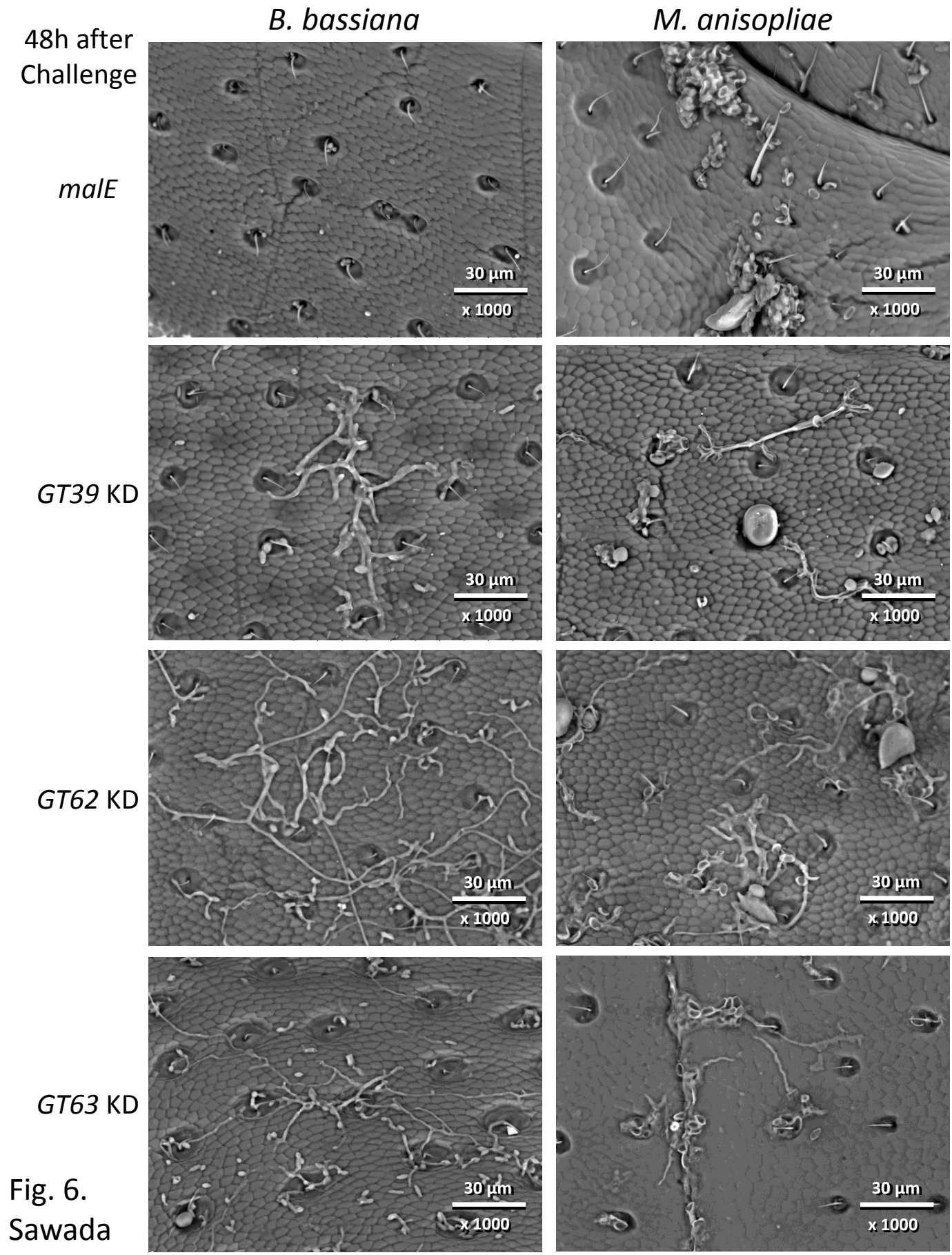

Fig. 6. Sawada et al.