Highlights

·Antifungal functions of *Tribolium laccase2* (*Lac2*) and *Yellow-e* (*Y-e*) were investigated.

·Moderate knockdown of *Lac2* totally disrupted antifungal host defense only in adults.

·*Y-e* knockdown had no or limited effects on the host defense of both pupae and adults.

·*Lac2* knockdown adults developed refractoriness to *Beauveria bassiana* by age 10 days.

Antifungal functions of *Tribolium Laccase2* gene

***Laccase2* knockdown**

Day 1 adult

Fungal challenge

Almost totally succumb to infection of both *Beauveria bassiana* and *Metarhizium anisopliae*

Development

Day 10 adult

Fungal challenge

Total recovery of defense only against *B. bassiana*

Day 3 pupa

Fungal challenge

No effect of gene knockdown

Knockdown of *Yellow-e* has no or limited effects

Involvement of *laccase2* and *yellow-e* genes in antifungal host defense of the model beetle, *Tribolium castaneum*.

Yuuki HAYAKAWA, Mitsuki SAWADA, Masaki SEKI, Patchara SIRASOONTHORN, Shotaro SHIGA, Katsumi KAMIYA, Chieka MINAKUCHI and Ken MIURA*

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

*Corresponding author

Telephone: +81-52-789-4033

Fax: +81-52-789-4032

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

Abbreviations used: CHS1, chitin synthase 1; RNAi, RNA interference; Lac2, laccase2; NADA, N-acetyldopamine; NBAD, N-β-alanyldopamine; DHI, 5, 6-dihydroxyindole; DHICA, 5, 6-dihydroxyindole-2-carboxylic acid; DCE, dopachrome conversion enzyme; Y-e, yellow-e; qRT-PCR, real-time quantitative RT-PCR; RPL32, ribosomal protein L32; dsRNA, double strand RNA; malE, maltose binding protein E.

Abstract

We previously reported that the moderate knockdown of *chitin synthase 1* gene of the model beetle *Tribolium castaneum* impairs the host defense against entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, which infect host insects via the direct penetration of cuticular integuments (Hayakawa et al, 2017). In this study, we focused on the antifungal roles of *laccase2* (*Lac2*) as well as *yellow-e* (*Y-e*) genes, both of which are shown to be important to the establishment of stable cuticular structures in this beetle species. The expression profiles of the two genes somewhat resembled each other, peaking in late prepupae and mid to late pupae, while the transcript levels of *Lac2* were higher than *Y-e* throughout. The knockdown of *Lac2* gene at the prepupal and pupal peaks with relatively small amounts of dsRNA resulted in pupae with a lighter color and adults with a lighter color and dimpled/wrinkled elytra, respectively. Meanwhile, similar gene knockdown of *Y-e* but with 10 times more dsRNA compared to *Lac2* resulted in pupae having a normal appearance and adults with a darker color. We conducted fungal infection assays with *B. bassiana* and *M. anisopliae* using these knockdown animals. The knockdown of *Y-e* gene had no or limited effects in both pupae and adults in terms of the antifungal host defense. Similarly, the knockdown of *Lac2* gene did not change significantly the defense phenotypes of the resulting pupae. By sharp contrast, the host defense of the adult beetles against the two fungal species was almost totally destroyed by the moderate knockdown of *Lac2* gene, suggesting its indispensable role in antifungal host defense presumably through the construction of sound cuticles of the adults. Finally, we investigated the maturation of host defense against fungal infection in the *Lac2* knockdown adults and found that while the day 10 adults were still susceptible to *M. anisopliae* infection with some delay of death in comparison with day 1 adults, they exhibited complete refractoriness to *B. bassiana*.

Key words: *Tribolium castaneum*; immunity; entomopathogenic fungus; insect cuticle; laccase2.

1. Introduction

Insects are threatened by a wide variety of pathogens or parasites, such as viruses, bacteria, fungi, nematodes and parasitoids (Butt et al., 2016; Lemaitre and Hoffmann, 2007). The insect innate immune system that solely relies on germline-encoded receptors for non-self recognition is conveniently divided into humoral and cellular immune reactions, and these two processes work in a coordinated manner to eliminate invading pathogens (Hultmark, 2003; Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007; Strand, 2008). Meanwhile, the insect cuticular integument provides a first line of defense against life-threatening pathogenic invaders as well as harsh environmental stresses such as droughts or predators (Butt et al., 2016; Gillespie et al., 2000; Moussian, 2010). Entomopathogenic fungi are uncharacteristic among insect pathogens in that they can penetrate directly the insect integument and invade into the nutrient-rich hemocoel (an insect body cavity filled with blood) underneath this barrier layer (Thomas and Read, 2007). Indeed, entomopathogenic fungi represent a major cause of insect diseases, and thus have been utilized as bio-insecticides complementary or alternative to chemical ones (Butt et al., 2016; Lu and St Leger, 2016; Zhao et al., 2016). *Beauveria bassiana* and *Metarhizium anisopliae*, which have relatively wide host ranges, are the most widely-used fungal species for both field applications and insect pathology laboratory studies (Butt et al., 2016; de Faria and Wraight, 2007; Ortiz-Urquiza and Keyhani, 2016). Their conidia (asexual spores) germinate once they attach onto the appropriate surface of insect cuticle through hydrophobic interactions, develop a structure for invasion, and the hyphae penetrate into the cuticle by using both physical forces and hydrolytic enzymes for cuticular constituents. Once the elongating hyphae reach

the hemocoel, they transform into yeast-like unicellular hyphal bodies. The hyphal bodies proliferate there while excreting toxic secondary metabolites and eventually colonize and kill the host insect. After the vegetative growth and the depletion of nutritional resources, the fungal hyphae penetrate the cuticle of dead insect outward. The hyphae then cover the cadaver, sporulate and produce a massive amount of conidia. On the other hand, host insects counteract the fungal infection during these infection processes (Lu and St Leger, 2016). Some lipophilic constituents of the outermost insect cuticle layer, the envelope, inhibit the germination of conidia (Gołebiowski et al., 2008). The chitinous procuticle as well as the non-chitinous epicuticle and envelope provide stabilized physical barriers through the covalent and non-covalent associations of the major constituents, cuticular proteins, as well as through those between chitin microfibrils and cuticular proteins in the case of procuticle. These composite cuticular layers not only represent a physical barrier but also a chemical barrier by the occurrence of prophenoloxidase and its activating system (Asano and Ashida, 2001; Ashida and Brey, 1995) and antimicrobial peptides secreted by epithelial cells (Brey et al., 1993; Ferrandon et al., 2007) as reported in some insect species. The fungi in the hemocoel are considered to be attacked by the host's humoral and cellular immune reactions, although the information to date on the involvement of the latter in antifungal immunity is very scarce (Lu and St Leger, 2016). Among the major humoral immune responses, the melanization reaction catalyzed by the activated form of phenoloxidase has been reported to play a role in host defense against fungal infection (Binggeli et al., 2014; Yokoi et al., 2015) while antimicrobial peptides that can counteract the experimental, opportunistic infection of human pathogens, such as *Candida albicans* and *Aspergillus fumigatus*, seem to have a limited effectiveness against naturally occurring entomopathogens like *B. bassiana* and *M. anisopliae* (Ekengren and Hultmark, 1999).

Our research group has been studying insect immunity in recent years using the red

flour beetle, *Tribolium castaneum* as a model. We first performed the molecular dissection of the beetle's immune signaling Toll and IMD pathways while referring to the well-established *Drosophila* counterparts (Yokoi et al., 2012a; Yokoi et al., 2012b). We used model microbes, such as *Escherichia coli*, *Micrococcus luteus* and *Saccharomyces cerevisiae* in these studies and employed the microinjection of the microbial suspension to elicit the host's immune reactions while knocking down systemically *Tribolium* immune-related genes (Koyama et al., 2015; Yokoi et al., 2012a; Yokoi et al., 2012b). We subsequently introduced natural fungal entomopathogens into our experimental system (Yokoi et al., 2015). Recently, we have been interested in the function of insect cuticular integument as a barrier against fungal infection, and have been examining the phenotypic changes in antifungal host defense upon systematic knockdown of the genes that are thought to be involved in the construction of the sound cuticular integument. Most recently, we have demonstrated that the moderate knockdown of *chitin synthase 1* (*CHS1*) gene effectively impairs the host defense of resulting animals against *B. bassiana* and *M. anisopliae* (Hayakawa et al., 2017). As mentioned above, chitin and cuticular proteins are the major constituents of insect cuticles, and they are cross-liked by quinones generated by oxidative enzymes and phenolic substrates, resulting in the sclerotization and pigmentation of the cuticle (Andersen, 2010). The *T. castaneum laccase2* (*Lac2*) gene is responsible for the cuticle sclerotization and pigmentation while other candidate genes coding for other oxidative enzymes, such as *prophenoloxidases* and *laccase1* genes, are not involved in this particular process (Arakane et al., 2005). This specific role of *Lac2* gene is also confirmed in other insect species (Futahashi et al., 2011; Niu et al., 2008). The Lac2 enzyme oxidizes mainly two acyldopamines, N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD), to generate NADA-quinone and NBAD-quinone respectively, and these compounds are thought to be functioning as cross-liking agents between protein-protein as well as protein-chitin to

stabilize cuticular structure (Andersen, 2010). In addition, during the sclerotization by these quinones brownish or yellowish cuticle coloration is thought to be generated. On the other hand, the blackish coloration associated with insect cuticle is considered to be generated by using other substrates, such as L-dopa and dopamine. These compounds are oxidized as well by Lac2, generating dopaquinone and dopaminequinone, respectively. They are converted respectively to dopachrome and dopaminechrome through non-enzymatic reactions, then further converted to 5, 6-dihydroxyindole (DHI) and 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) by the action of dopachrome conversion enzyme (DCE) and in part by non-enzymatic reactions. DHI and DHICA are oxidized to melanochromes by Lac2, eventually forming melanin (Andersen, 2010; Lomakin et al., 2011). DCE and related enzymes are known to be encoded by the *yellow* locus in *Drosophila* (Nash and Yarkin, 1974). The *Drosophila yellow* gene family comprises 14 members, among which *yellow-f1* and *yellow-f2* encode proteins possessing DCE activities (Han et al., 2002). Similarly, the involvement of *yellow* gene family members in proper body pigmentation is demonstrated in two lepidopteran species (Futahashi et al., 2008; Ito et al., 2010). The counterpart of *Drosophila yellow* gene family in *T. castaneum* is also composed of 14 members. While the gene(s) encoding protein(s) bearing DCE activities is yet to be determined in *T. castaneum*, the knockdown of *yellow-e* (*Y-e*) gene render the adults darker in color and susceptible to dry conditions, suggesting its function to protect the adult beetles from dehydration (Noh et al., 2015a). In the present study, we investigated the functions of *Lac2* and *Y-e* gene in antifungal host defense.

2. Materials and Methods

2.1. Insect

T. castaneum was reared at 30 °C as described previously (Yokoi et al., 2012a; Yokoi et al., 2012b). For fungal infection, animals were transferred to and kept at 25 °C on the day of pupation or emergence, and the infection and consecutive experiments were conducted as indicated respectively.

2.2. Fungal species used

The *B. bassiana* and *M. anisopliae* strains used in this study were respectively isolated by K. K. in Fukui and Saitama prefectures, Japan (Hayakawa et al., 2017). See section 2.5 for detail of experimental infection procedures.

2.3. RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA extraction, 1st strand cDNA synthesis and qRT-PCR were performed basically as described in our previous papers (Koyama et al., 2015; Yokoi et al., 2012b). The developmental stages of animals used for total RNA extraction were last instar (7th) larvae including the prepupal stage, pupae and adults, and the whole body was subjected to the extraction using TRIZOL reagent (Invitrogen). RNA preparations with the A260/A280 and A260/A230 ratios higher than 1.7 and 2.0 respectively were used for 1st strand cDNA synthesis with a PrimeScript RT reagent Kit with gDNA Eraser (TAKARA), which digests and removes contaminating genomic DNA prior to reverse transcription reaction. The resulting 1st strand cDNA pools were examined in terms of transcript abundance of genes to be analyzed, using a SYBR Premix Ex Taq Perfect Real Time Kit Tli RNaseH Plus (TAKARA) and a Thermal Cycler Dice Real Time System (Model TP800, TAKARA). Threshold cycle number for each

transcript in the sample was determined based on the second derivative of its primary amplification curve and normalized to that of *ribosomal protein L32* (*RPL32*) in the same sample, giving relative transcript abundance. Sequences of the primers used for qRT-PCR analyses are shown in Table 1 along with the accession numbers of respective mRNA sequences. RNA extraction and the following cDNA synthesis and qRT-PCR analysis were performed using a pool of three animals each time, and these experiments were independently repeated three times.

2.4. RNA interference (RNAi)

Gene knockdown via RNAi was performed as described previously (Hayakawa et al., 2017). We routinely treat *T. castaneum* with 100 ng of double strand RNA (dsRNA) in our regular experiments (Koyama et al., 2015; Yokoi et al., 2015), but this dosage causes lethality of resulting adults always in the case of *Lac2* dsRNA and with a much less frequency for *Y-e* dsRNA. Therefore, we injected smaller amounts of dsRNA depending on both the developmental stages of animals and target genes in order to avoid severe phenotypes. dsRNA was synthesized using a MEGAscript RNAi Kit (Ambion), purified, checked for uniformity by agarose gel electrophoresis, and determined spectrophotometrically. The dsRNA preparation was diluted in 10 mM Tris-HCl, pH 8.0 and injected into day 0 prepupae or day 1 pupae by using a Nanoject II (Drummond Scientific Company). The dsRNA-injected animals were used to estimate RNAi efficiencies and defense phenotypes upon fungal infection as well as to photograph the appearances of animals under a microscope (Olympus, model SZX12). The sequences of primer pairs used for the preparation of cDNA templates for dsRNA synthesis are shown in Table 2 along with those for a negative control *maltose binding protein E* (*malE*).

2.5. Survival assay upon fungal infection

The knockdown pupae or adults of *T. castaneum* were subjected to *B. bassiana* and *M. anisopliae* infection along with *malE* dsRNA-treated control knockdown animals, and the survival afterward was recorded every 24 h. The experimental infection procedures for *B. bassiana* and *M. anisopliae* were basically as described in our previous paper (Hayakawa et al., 2017; Yokoi et al., 2015). Briefly, *B. bassiana* and *M. anisopliae* conidia were collected from fully-grown, dried culture plates of SDYA and PDA, respectively, and stored at 4 ^{o}C. We usually renew these conidial stocks every two months whereas the *B. bassiana* stock usually has a longer shelf life. The stored conidia were suspended in 0.02% Tween-80 just prior to use, filtered through absorbent cotton, counted on a hemocytometer and diluted. To prepare *M. anisopliae* conidial suspension at its highest concentration of 3.0 x 10^8 cell/ml, 0.1% Tween-80 was used to obtain a uniformly dispersed suspension. Animals were immersed in the conidial suspensions for 1 min, put on filter paper to remove excessive liquid, and placed in 12-well culture plates. The experimental infection procedure mimics the natural way of fungal infection, and is hereafter referred to as 'the immersion method'. The 12-well culture plates were kept in a moist container at 25 ^{o}C, and the survival of animals observed and recorded every 24 h. Life and death decisions of test animals were performed by observing the responses when they were stimulated by a thin and flexible plastic rod under a stereoscopic microscope (Olympus, models SZX12 and SZX16). Data were drawn in Kaplan-Meier plots, and *P*-values calculated by Gehan-Breslow-Wilcoxon test by utilizing a commercial software package (Ekuseru-Toukei 2010, Social Survey Research Information Co., Ltd.).

3. Results

3.1. Developmental changes of Lac2 and Y-e transcript levels from last instar larvae to adults.

We first examined the expression profiles of *Lac2* and *Y-e* from the late phase of *T. castaneum* development, namely from last (7th) instar larvae (only a small portion of larvae undergoes an additional larval-to-larval molt) to day 14 after adult emergence (Fig. 1A). The *Lac2* transcript levels did not show a clear tendency during last larval instar. It peaked in day 1 prepupae, then declined and exhibited low levels in early pupae. This was followed by a seemingly broad peak in mid and late pupae, the levels of which varied widely in each day. The level was still high on the day of emergence and then decreased gradually. The developmental profile of *Lac2* obtained in this study by qRT-PCR analyses was consistent with that estimated by RT-PCR and gel analyses during prepupal to adult development in the pioneering work (Arakane et al., 2005). Similarly, the developmental expression profile of *Y-e* was examined (Fig. 1B). While the overall expression levels were lower compared to *Lac2*, *Y-e* showed a similar changing profile. It showed a small peak in day 1 prepupae and a larger and broader one in the late half of pupal development, which was similar to the profile presented by a previous work (Noh et al., 2015a). Given these results, we considered that the expression of these two genes in day 1 prepupae was responsible for the sclerotization and pigmentation of pupal cuticle and that the greater expression in mid to late pupae accounted for those of adult cuticle, although we did not recognize clear pupal peaks for both genes because of large variations of transcript levels.

3.2. RNAi-based moderate gene knockdown of Lac2 and Y-e and appearances of resulting animals.

We targeted the two peaks of *Lac2* and *Y-e* genes, one is on day 1 of prepupal stage and the other is during mid to late pupal development, by injecting dsRNA. To target the prepupal expression peak, dsRNA was injected into day 0 prepupae while for the mid to late pupal peak dsRNA injection was performed using day 1 pupae, and the degree of gene silencing was estimated by qRT-PCR of targeted genes in day 1 prepupae and day 4 pupae, respectively. Since the injection of these dsRNA at high doses leads to lethality arisen mainly from molting defects and deformed body structures especially in the case of *Lac2* (Arakane et al., 2005), we used smaller amounts of dsRNA than our regular dose 100 ng per animal. As for *Lac2*, 10 ng dsRNA per animal was employed to moderately silence the prepupal peak, while a dosage of 1 ng per animal was used for the moderate knockdown of mid to late pupal peak. The dsRNA-treated animals were examined by qRT-PCR in terms of transcript abundance of targeted genes. The *Lac2* gene knockdown results of day 1 prepupa and of day 4 pupae are shown in Fig. 2A and 2B. In both cases of prepupal and pupal peaks, the *Lac2* transcript declined to 20 to 30% levels of *malE* dsRNA-treated controls. The appearances of resulting *Lac2*-knockdown pupae and adults are shown in Fig. 3. The day 3 knockdown pupae (five days post dsRNA injection into day 0 prepupae) looked lighter in color compared to the *malE* controls, and no other differences were recognizable under binocular viewing conditions (Fig. 3A, left and center panels). The day 1 knockdown adults (six days post dsRNA injection into day 1 pupae) as well exhibited a lighter color than the controls as was shown in the *Lac2* knockdown pupae. In addition, the elytra of *Lac2*-knockdown adults had a slightly wrinkled/dimpled appearance, as we have reported in the adults that underwent moderate *CHS1* gene knockdown (Hayakawa et al., 2017). The elytra opened a little in this picture, but this was also observed for some control adults. The adults that underwent this level of moderate *Lac2* knockdown were similarly viable to control animals (data not shown). .

For *Y-e* knockdown, we employed 10 times larger amounts of dsRNA, 100 ng for prepupal injection and 10 ng for pupal injection, than the case of *Lac2*, since the lethal effect caused by *Y-e* dsRNA injection was less severe in comparison with *Lac2* dsRNA. The knockdown efficiencies of the prepupal and pupal *Y-e* expression peaks were around 90% and higher than the cases of *Lac2*, possibly because of 10 times higher administered doses of dsRNA (Fig. 2C and 2D). The appearances of *Y-e*-knockdown pupae and adults are presented in Fig. 3. The day 3 *Y-e* knockdown pupae did not show a distinctive outward difference from controls, while the day 1 knockdown adults exhibited a slightly darker body color than the controls (Fig. 2A and 2B, left and right panels), which was initially reported by Arakane and coworkers (Noh et al., 2015a). We observed that while the prepupa administered with 100 ng of *Y-e* dsRNA pupated normally, but a small portion of resulting pupae failed to emerge. The pupae administered with 10 ng of *Y-e* dsRNA emerged normally, and the resulting adults were as viable as control animals (data not shown). The control transcript levels of *Lac2* and *Y-e* genes in day 4 pupae presented in Fig. 2 seemed lower than those shown in Fig. 1. We considered that this could partly be attributed to large variations of the transcript levels of day 4 pupae in Fig 1, and we did not investigate this point further.

3.3. Antifungal defense phenotypes of Y-e knockdown animals

The day 3 pupae that had been treated with *Y-e* or *malE* on day 0 of the prepupal stage were examined for the defense phenotypes against *B. bassiana* or *M. anisopliae*. The pupae underwent the fungal infection by the immersion method, and afterward the survival of the pupae was recorded (Fig. 4A and 4B). The control pupae were basically resistant to the fungal infection at the defined conidial concentrations employed, and 80 to 90% survived by 168 h post infection of both *B. bassiana* and *M. anisopliae*. Meanwhile, the *Y-e* knockdown did not

weaken the antifungal host defense greatly. The knockdown pupae tended to show slightly lower survival rates compared to the controls, but the differences were not statistically significant for either *B. bassiana* or *M. anisopliae* infection. The results of adults exhibited a similar tendency, but the modestly reduced survival upon *B. bassiana* infection found in *Y-e* knockdown adults was statistically significant (Fig. 4C and 4D).

3.4. Antifungal defense phenotypes of Lac2 knockdown animals

As regards fungal infection in pupae, the *Lac2* knockdown pupae and control *malE* dsRNA-treated pupae were found similarly refractory to the infection of both *B. bassiana* and *M. anisopliae* (Fig. 5A and 5B). The *Lac2* knockdown seemingly caused a little lower survival of the pupae, which was not statistically significant as in the case of *Y-e* knockdown pupae. Contrastingly, the moderate knockdown of *Lac2* brought about a striking phenotype change in terms of antifungal host defense (Fig. 5C and 5D). While the control, *malE* dsRNA-treated adults were almost totally refractory to the infections of both *B. bassiana* and *M. anisopliae*, only 1 ng *Lac2* dsRNA administration on day 1 of the pupal life impaired very effectively the host defense of resulting day 1 adults. More than 60% of the knockdown adults succumbed to *B. bassiana* infection by 96 h post infection, and all died by 120 h (Fig. 5C). Similarly, *M. anisopliae* infection killed over 60% of the knockdown adults by 96 h post infection, and this was followed by 90% mortality by 168 h (Fig. 5D). Although the survival curves seem to follow somewhat different kinetics depending on the fungal species infected, these results indicate that the function of *Lac2* gene is indispensable to the host defense of *T. castaneum* adults against at least the two fungal species tested, possibly through its contribution to constructing consolidated adult cuticles.

3.5. Antifungal defense maturation of Lac2 knockdown adults

As shown in section 3.4, moderate *Lac2* knockdown in the pupae considerably weakened the defense against both *B. bassiana* and *M. anisopliae* of the resulting adults on the next day of emergence (Fig. 5C and 5D). The lighter body color associated with *Lac2* knockdown adults thereafter got darkened and became indistinguishable from that of control beetles by day 4 (data not shown), probably because of the remaining Lac2 enzymatic activity. Therefore, we hypothesized that the maturation of cuticular barrier function occurred gradually even in *Lac2* knockdown adults and that the barrier function might eventually become comparable to that of control beetles. We tested this hypothesis by performing the fungal infection of day 10 *Lac2* knockdown adults along with control ones (Fig. 6). To our surprise, the day 10 knockdown adults exhibited entirely different defense phenotypes against the two fungal species. The day 10 *Lac2* knockdown adults were revealed to be totally refractory to *B. bassiana* infection as the control ones (Fig. 6A). By contrast, the knockdown adults started to succumb to the *M. anisopliae* infection at 144 h post infection, and more than 70% died by 168 h.

4. Discussion

Previously, we have shown experimentally that the *CHS1* gene, which is responsible for the synthesis of one of the major constituents of insect cuticle, chitin, is crucial to the antifungal host defense of the model beetle, *Tribolium castaneum* (Hayakawa et al., 2017). The administration of as little as 1.0 ng of dsRNA bearing *CHS1* sequence on day 1 of the pupal stage significantly weakened the host defense against both *B. bassiana* and *M. anisopliae* infection by the immersion method without affecting viability of the resulting adults. The impairment in host defense by the RNAi was also observed in pupae that had been injected with

0.5 ng of the dsRNA on day 0 of prepupal stage. But, the impairment was only found for *B. bassiana*, and the degree was lesser compared to that in adults. We also reported that the pupae are more susceptible to these fungal species than the adults but some refractoriness develops with pupal age in days. On the other hand, when we injected cultured hyphal body cells directly into the hemocoel, only a few dozen of the cells were sufficient to kill the naïve pupae (Hayakawa et al., 2017) or adults (our unpublished observation). These observations collectively suggest the followings: (i) in this experimental infection system composed of the host *T. castaneum* and the fungal species *B. bassiana* and *M. anisopliae*, defenses provided by the cuticular integument is of first importance over those functioning in the hemocoel; (ii) the host defense by the cuticle matures with age as well as with developmental stage progression; (iii) more robust defense of the adults beetles compared to the pupae depends on adult-specific properties of the cuticle. In the present study, we further focused on genes potentially important to the establishment of immunologically sound cuticles, and investigated the loss-of-function phenotypes of *Lac2* and *Y-e* genes in pupae and adults as well as the developmental maturation of host defense in *Lac2* knockdown adults.

We chose the *Lac2* as a knockdown target since its intimate involvement of insect cuticle formation is intensively documented (Arakane et al., 2005) and since it has been attracting attention as a target of pest control strategies because of its indispensability to insect normal molting/metamorphosis (Christiaens et al., 2016). We were interested in its antifungal role through the establishment of sound cuticular structures, so we knocked down this gene with a small amount of dsRNA to avoid severe phenotypes concerning detects in molting/metamorphosis. The *Y-e* gene, the dehydration resistance role of which in the adult cuticle is reported (Noh et al., 2015a), was also adopted as a knockdown target in this study, since we considered that its knockdown might influence as well the antifungal properties of the

cuticle. The examination of developmental profiles of the two genes revealed that the both of them had a smaller expression peak at the late prepupal stage and a larger one during mid to late phases of the pupal stages. We targeted these two peaks by intrahemocoelic dsRNA injection, gaining 70 to 90% reduction of transcript levels. The resulting knockdown pupae or adults were viable while they exhibited some visible changes in outward appearances. Then, we conducted experimental fungal infections via the immersion method using the knockdown animals of either *Lac2* or *Y-e*. Interestingly, the knockdown of the two genes did not impair significantly the host defense of resulting pupae against *B. bassiana* or *M. anisopliae* while the knockdown pupae seemingly succumbed to the fungal infections a little earlier than the control *malE* dsRNA-treated ones. Given these results, we consider that although the two genes are expressed to some extent prior to pupation, their contribution seems to be low in terms of rendering pupal cuticles refractory to the fungal infection. Similarly, *Y-e* did not seem to play a major role in antifungal defense in adults: the knockdown slightly reduced the survival of the adults only upon *B. bassiana* infection.

In sharp contrast are the results of the *Lac2* knockdown adults. The pupal administration of only 1 ng *Lac2* dsRNA per animal brought about almost complete destruction of host defense against both *B. bassiana* and *M. anisopliae* in the resulting adults. This unequivocally indicates the indispensability of *Lac2* in conferring protection from fungal infection to the adults probably through constructing the appropriate adult cuticular structures. Given that *Lac2* is expressed as well in larvae and prepupae, it seems reasonable to consider that its adult-specific function regarding the antifungal host defense could be supported by some adult-specific factors. We infer that such potential factors could be adults-specific cuticular proteins. Arakane and coworkers have investigated the expression of several cuticular protein genes and have found three cuticular proteins that are enriched in highly sclerotized, rigid adult

cuticle portions (Arakane et al., 2012; Noh et al., 2015b). When we assume that the marked refractoriness of the naïve adult beetles to entomopathogenic fungi is upheld by the consolidated cuticular structure through the cross-linking involving these adult-specific cuticular proteins, our results that the *Lac2* knockdown brought about a devastating outcome only in adults might be reasonable.

When we tested in the previous study the effect of moderate *CHS1* knockdown on antifungal host defense in adults, the reductions of the survival rate by 168 h post infection were c.a. 50% for *B. bassiana* and c.a. 30% for *M. anisopliae*, respectively (Hayakawa et al., 2017). On the other hand, those by *Lac2* knockdown in this study were 100% for *B. bassiana* and c.a. 90% for *M. anisopliae*, respectively. Taken together, these results are likely to indicate that the antifungal function of *Lac2* in the adults is more important than that of *CHS1*. These two sets of experiments were taken place under somewhat different conditions: for example, we injected 1 ng of either *CHS1* or *Lac2* dsRNA into day 1 pupae, but the peak level reduction of the targeted transcripts in pupae were less than 50% for *CHS1* (Hayakawa et al., 2017) and around 70% for *Lac2* in this study. Therefore, it may be difficult to perform a direct comparison between the results of the two distinct experiments. However, considering that the adults that underwent moderate *Lac2* knockdown suffered a much greater impairment in antifungal host defense, we prefer the idea that *Lac2* is more critical than *CHS1* to the formation of rigid cuticles in the adults with the help of some sort of adult-specific cuticular proteins such as above-mentioned ones.

Finally, we discuss here the maturation of antifungal host defense in the *Lac2* knockdown adults. The day 1 knockdown adults exhibited a slightly lighter body coloration compared to *malE*-dsRNA-injected control adults. The knockdown adults possessed dimpled/wrinkled elytra, and this phenotype is also associated with the adults subjected to

moderate *CHS1* knockdown (Hayakawa et al., 2017). The *Lac2* knockdown adults thereafter developed darker body coloration and became indistinguishable from the control ones by day 4 of the adult stage while the dimpled/wrinkled elytra did not show significant changes. We performed fungal infection assays using day 10 knockdown adults along with control ones of the same age, expecting that some refractoriness to the infection might develop with age since the body coloration comparable to control ones could be supported by residual or restored Lac2 enzymatic activities, which might as well provide an improved defense against fungi. Indeed, as for *M. anisopliae* infection, the day 10 *Lac2* knockdown adults seemed somewhat to develop refractoriness and succumbed to the infection more slowly than the day 1 knockdown adults, with the survival being higher by 168 h post infection. We had expected that the day 10 *Lac2* knockdown adults would show as well a modestly improved refractoriness to *B. bassiana* infection. However, the results were far beyond our expectation: the day 10 *Lac2* knockdown adults were revealed to be totally refractory to *B. bassiana* infection as the control ones. Given these results, it is reasonable to deduce that the *Lac2* knockdown adults develop some defense mechanisms more specific to *B. bassiana* infection with age. One such mechanism could be defensive secretions of quinone-containing compounds associated with the tenebrionid beetles including *T. castaneum*, (Li et al., 2013), while we do not rule out the involvement of remaining Lac2 activity in the delayed cuticular maturation. The major effective constituents of these secretions are reported to be methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone. These compounds are secreted by the prothoracic and abdominal glands of the *T. castaneum* adults, cover the body surface, and are acting as antifungal agents against *B. bassiana*. Meanwhile, this fungal species is known to counteract these host insect-derived defensive compounds to some extent through the activity of its NAD(P)H:1,4-benzoquinone oxidoreductase (Pedrini et al., 2015). Since *T. castaneum* develops these secreting glands well by day 10 of the adult

stage (Li et al., 2013), we consider at present that the recovered refractoriness to *B. bassiana* observed in the day 10 *Lac2* knockdown adults can at least in part be ascribed to the secretion of these defensive compounds and that *M. anisopliae* may have higher resistance to these compounds. In addition, it is interesting to consider the possible involvement of Lac2 enzyme as well in the defensive secretions while the enzymes responsible for the quinone production in the odoriferous glands have yet to be determined. Indeed, Li et al. (2013) have found sereral oxydative enzyme transcripts enriched in the glands, which include *Lac2*. If this is the case, the quinone production in the glands of the adults that underwent moderate *Lac2* knockdown might be restored to some extent by day 10.

In summary, we investigated the antifungal functions of *Lac2* and *Y-e* genes in this study and demonstrated that the severe impairment of host defense in adults were arisen from moderate knockdown of *Lac2*. We do not exclude the possibility that some structural defects/changes in the cuticles of the *Lac2* knockdown adults may also alter the transport or proper distribution of immunity- or defense-related effectors, such as oxidative enzymes or antimicrobial agents in this extracellular matrix, leading to the quite susceptible antifungal defense phenotype together with physically weakened cuticular structures by the knockdown. The severe knockdown phenotype was found only in the adults, and was totally recovered with times in the case of *B. bassiana* infection. We hypothesize the involvement of adults-specific factors such as some cuticular proteins for the severe defense phenotype observed only in the knockdown adults, and the involvement of defensive glandular secretions for the maturation/improvement of defense against *B. bassiana* infection in the knockdown adults. Our current and future studies are focusing on these points for better understanding of antifungal functions of insect cuticles.

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Legends to figures

Fig. 1. Developmental expression profiles of *Lac2* and *Y-e* genes. The transcript abundance was determined by qRT-PCR for either *Lac2* (A) or *Y-e* (B) gene. Developmental stages examined were last (7th) instar larvae (LL), prepupae (Pp), pupae (P) and adults (A). Numerals that follow the developmental symbols indicate age in days of respective stages: e.g., P3 represents day 3 pupae. Determination was performed using a pool of three animals each time, and values normalized to the levels of *RpL32* transcript. Experiments were independently repeated three times for respective developmental points, and bars represent mean ± SD.

Fig. 2. Gene knockdown efficiencies in animals treated with sublethal doses of *Lac2* or *Y-e* dsRNA. A, 10 ng of *Lac2* dsRNA was injected into day 0 prepupae, and the transcript levels were determined 24 h later at the stage of day 1 prepupae. B, 1 ng of *Lac2* dsRNA was injected into day 1 pupae, and the transcript levels were determined 72 h later in day 4 pupae. C, the same as in A except that 100 ng of *Y-e* dsRNA was used. D, the same as in B except that 10 ng of *Y-e* dsRNA was used. Control animals were treated with the equal dosage of *malE* dsRNA. Determination was done using a pool of three animals each time. Experiments were repeated independently three times, and bars represent mean ± SD. Significantly different values are marked with asterisks and *P*-values (Student's *t*-test).

Fig. 3. Appearances of dsRNA-treated animals. Day 0 prepupae or day 1 pupae were treated with *Lac2* or *Y-e* dsRNA as described in Fig. 2, and the resulting day 3 pupae (A) or day 1 adults (B) photographed. Amounts of dsRNA used are shown along with photographs. *MalE* RNA-treated animals serve as controls. Horizontal bars represent 1 mm.

Fig. 4. Survival of animals that underwent *Y-e* knockdown upon fungal infection. Day 0 prepupae were injected with 100 ng dsRNA of either *Y-e* or *malE*, and reared at 30 ^{o}C until pupation. The animals were transferred to 25 ^{o}C at pupation, and the resulting day 3 pupae subjected to fungal infection by the immersion method. The conidial concentrations used were 1.0 x 10^5 cell/ml for *B. bassiana* and 1.0 x 10^3 cell/ml for *M. anisopliae*. The survival of animals were thereafter recorded every 24 h, and the results by 168 h post fungal infection are shown in Kaplan-Meier plots for *B. bassiana* (A) and *M. anisopliae* (B). Similarly, day 1 pupae were injected with 10 ng dsRNA of either *Y-e* or *malE*, and then reared at 30 ^{o}C until emergence. Newly emerged day 0 unsexed adults were transferred to 25 ^{o}C, and the day 1 adults examined in terms of antifungal phenotypes as in pupae, but with conidial suspensions of much higher concentrations: 1.0 x 10^9 cell/ml for *B. bassiana* and 3.0 x 10^8 cell/ml for *M. anisopliae*. Kaplan-Meier plots of *B. bassiana*- (C) or *M. anisopliae*-challenged adults (D) are shown. Animals treated with the same amount of *malE* dsRNA served as controls, and a statistically different survival curve is marked with an asterisk and a calculated *P*-value. N. S., not significant. Experiments of respective knockdown or control categories were performed each time using c.a. 10 animals and independently repeated three times. The sum of the three independent experiments is shown for each plot.

Fig. 5. Survival of animals that underwent moderate *Lac2* knockdown upon fungal infection. The detailed experimental conditions are the same as in Fig. 4 except that 10 and 1 ng of *Lac2* dsRNA were used for prepupal and pupal gene knockdown respectively. A, survival of *Lac2* knockdown pupae upon *B. bassiana* infection. B, survival of *Lac2* knockdown pupae upon *M. anisopliae* infection. C, survival of *Lac2* knockdown adults upon *B. bassiana* infection. D,

survival of *Lac2* knockdown adults upon *M. anisopliae* infection.

Fig. 6. Survival of day 10 *Lac2* knockdown adults upon fungal infection. Day 10 adults that had received 1 ng dsRNA of *Lac2* on day 1 of the pupal stage were subjected to fungal infection and survival experiments. The other details are the same as in Figs. 4 and 5.

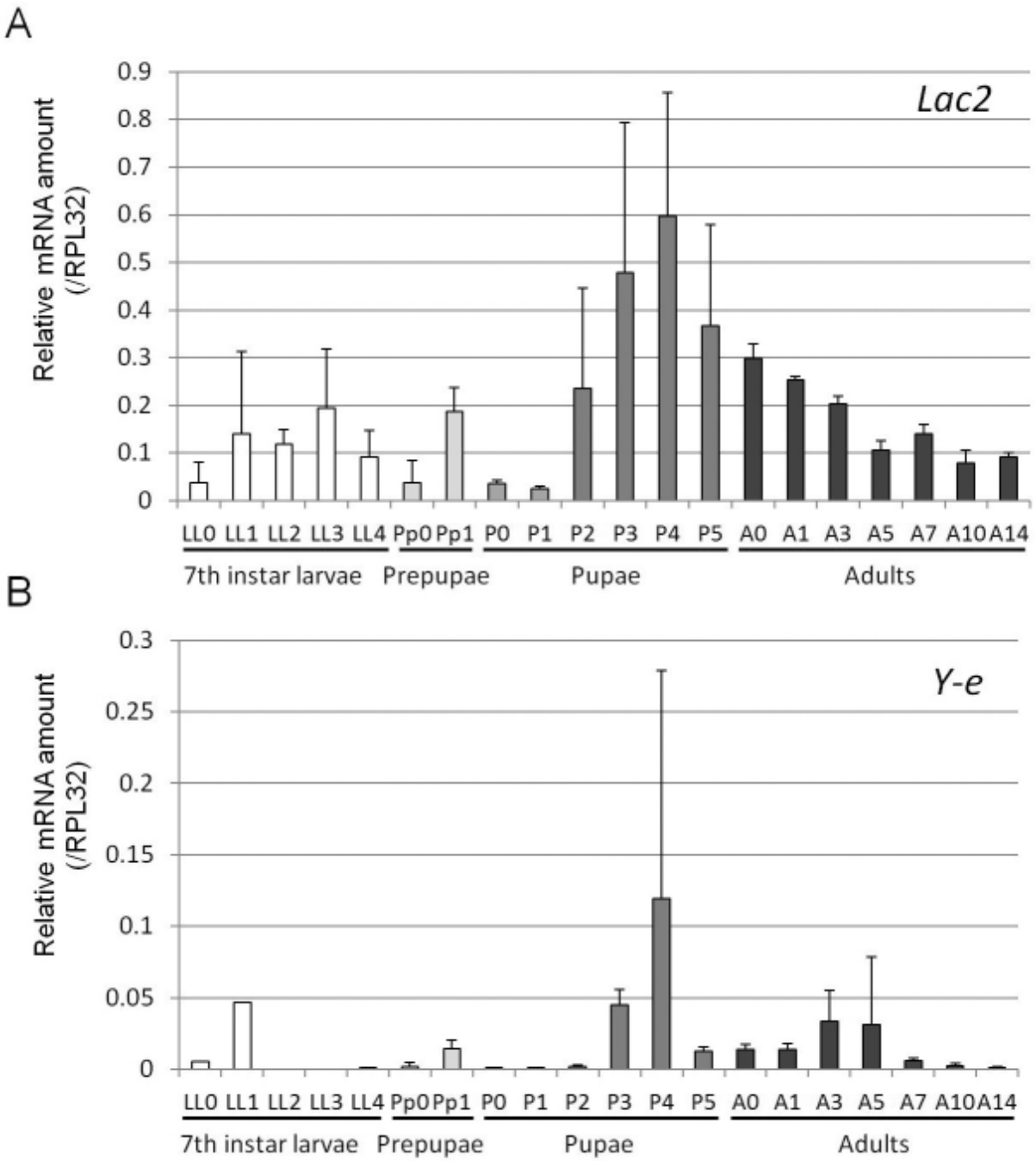


Fig. 1. Hayakawa et al.

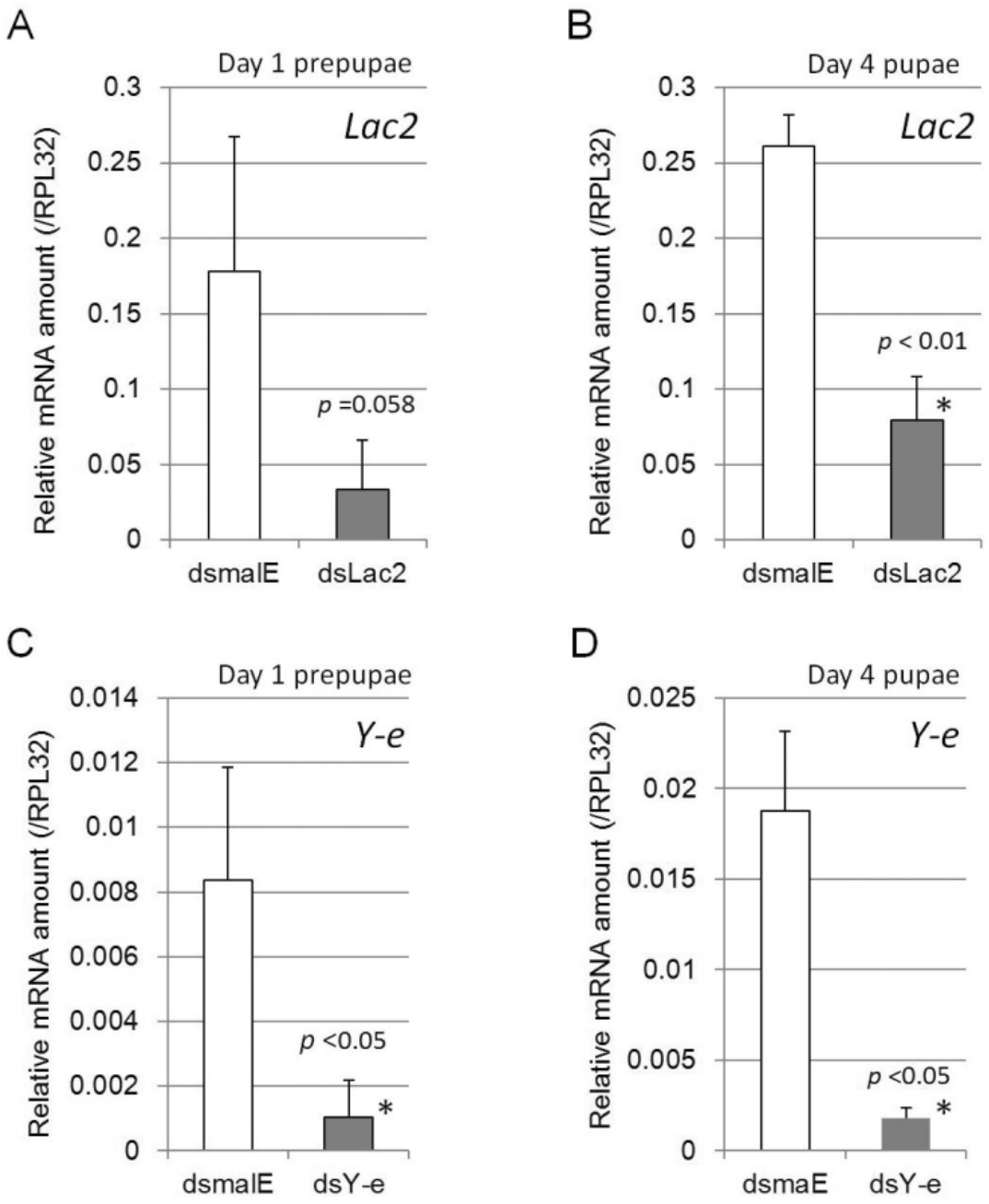
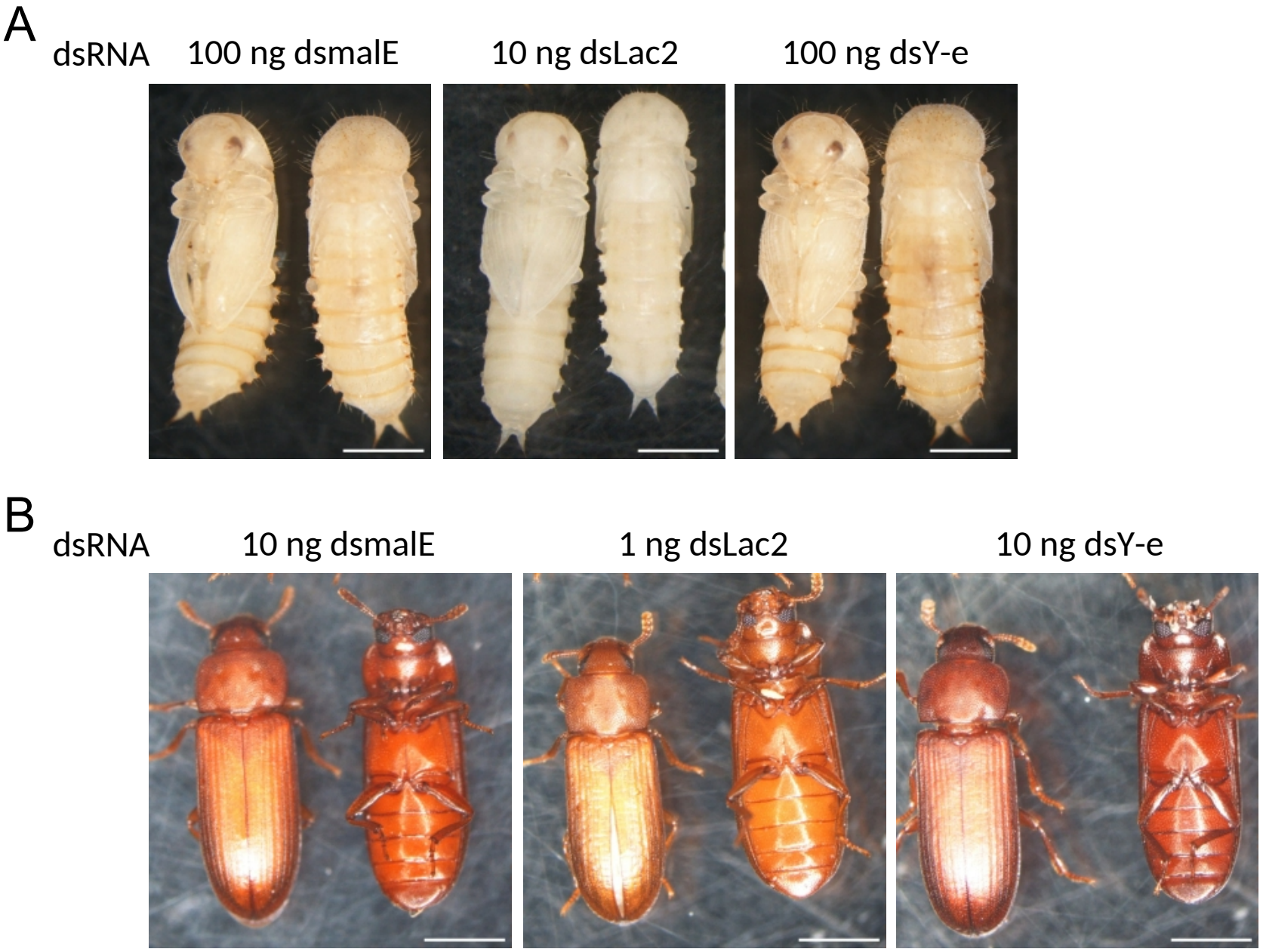


Fig. 2. Hayakawa et al.



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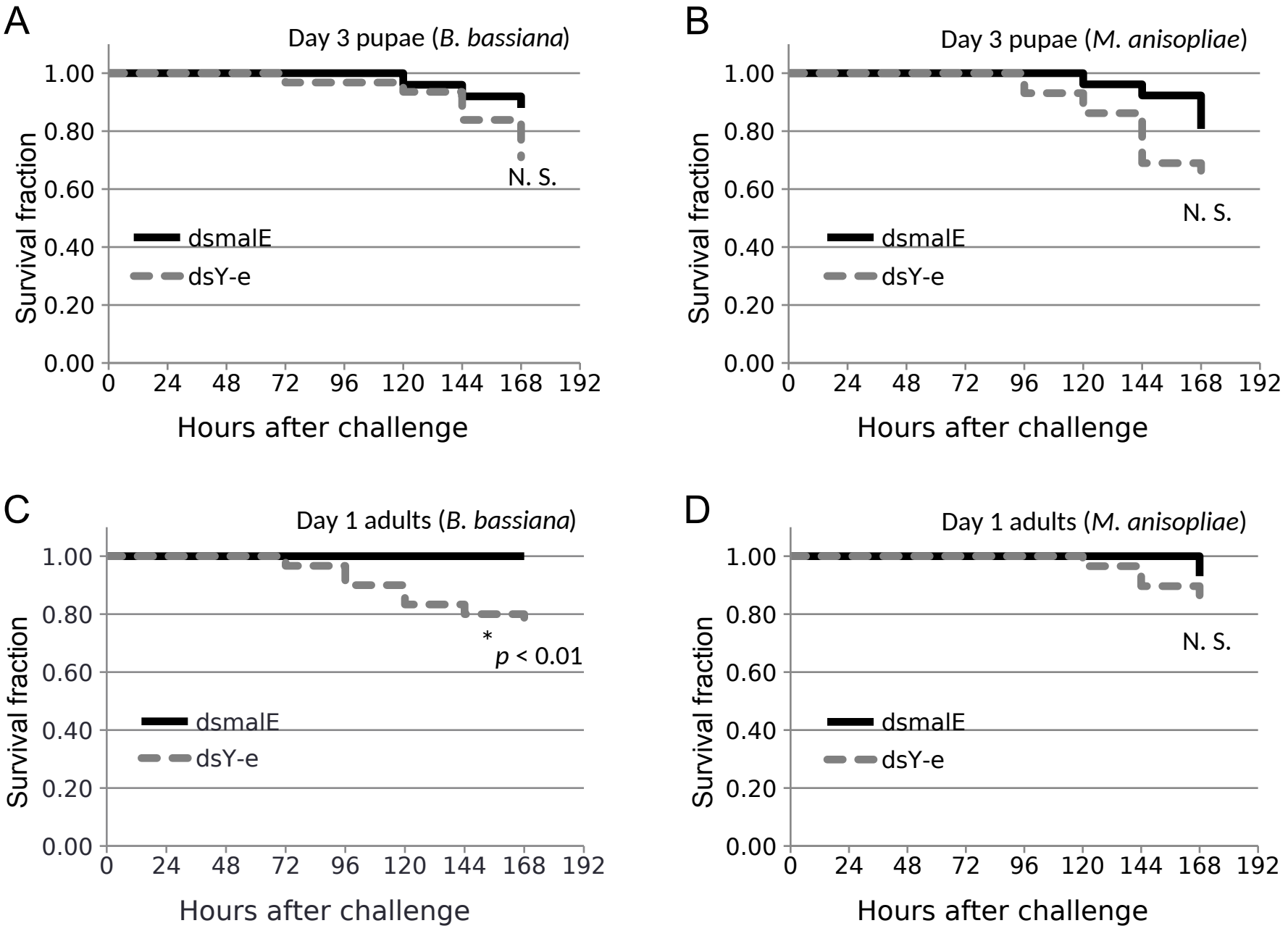

Fig. 4. Hayakawa et al.

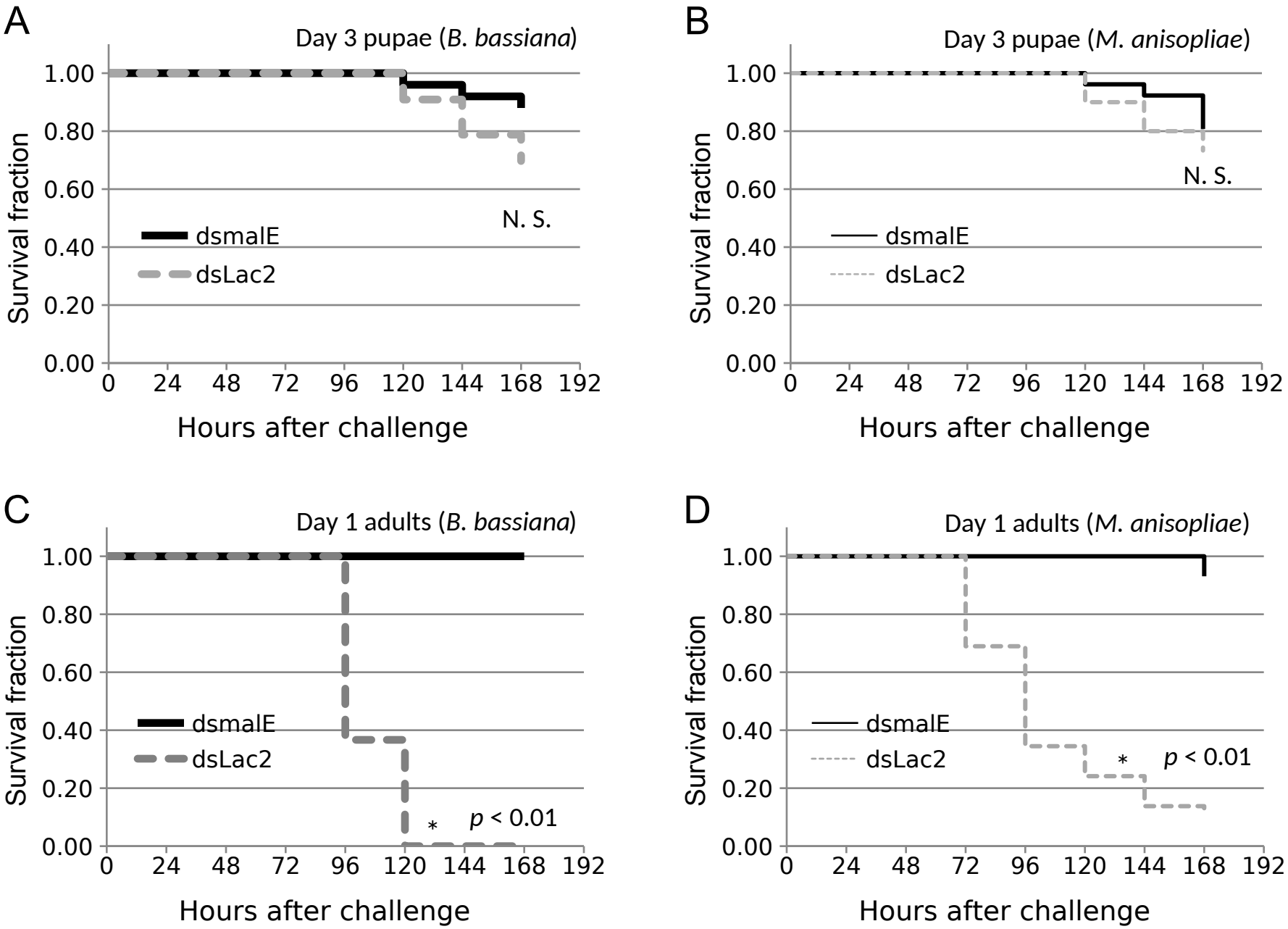

Fig. 5. Hayakawa et al.

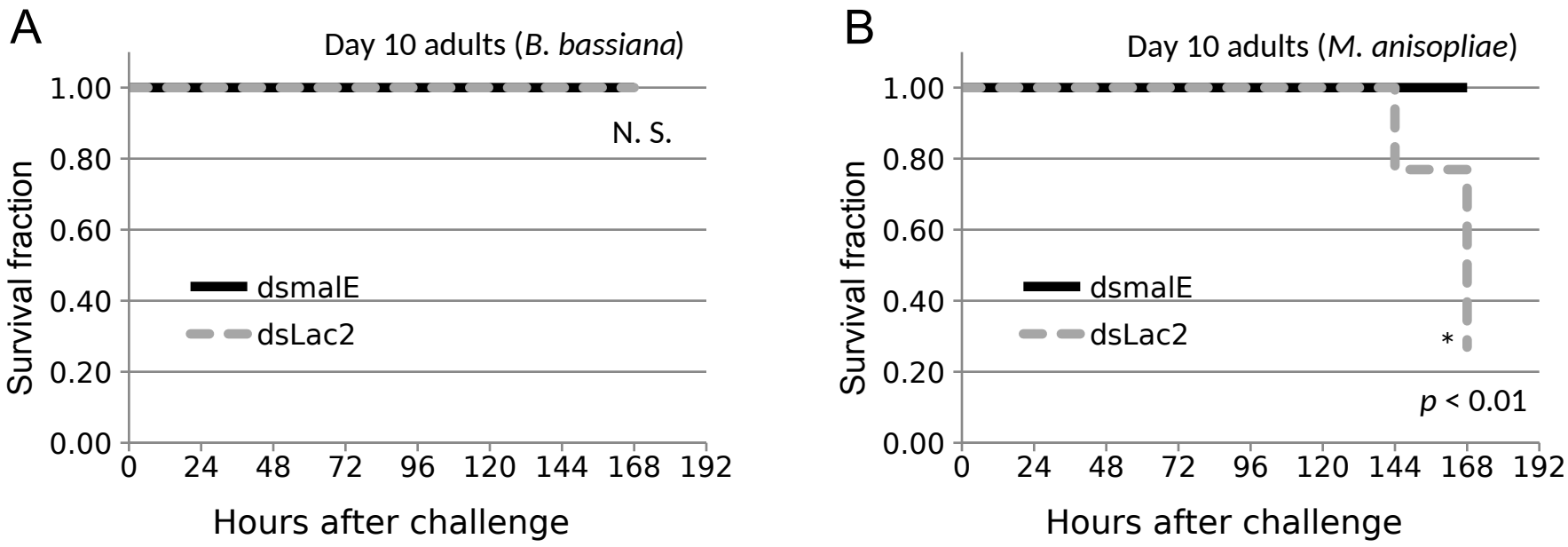

Fig. 6. Hayakawa et al.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession #
Y-e	TAGCCCTTGACCCAAAAGACG	CGATTGGTCCTCATCCTTCCA	AX377531
Lac2	CGTTTTCAGGTGAACGATACGA	GTTGGTATGGCCCTTTGGCATA	NM_001039398
RPL32	ACCGTTATGGCAAACTCAAACG	TGTGCTTCGTTTTGGCATTGGA	GU111765

Table. 1. The sequences of primer pairs used for qRT-PCR analyses.

Table. 1. Hayakawa et al.

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
Y-e	*TAATACGACTCACTATAGGG*- -TCGACTTTCAATTCCCCTTCGA	*TAATACGACTCACTATAGGG*- -AGGACACACACGTTGGAAATC
Lac2	*TAATACGACTCACTATAGGG*- -GCACCAGGCGCCAAGAAGAA	*TAATACGACTCACTATAGGG*- -CGACGTCGATCACAACCTTGT
malE	*TAATACGACTCACTATAGGG* - -ATTGCTGCTGACGGGGGTTAT	*TAATACGACTCACTATAGGG* - -ATGTTCGGCATGATTTCACCTTT

Table. 2. The sequences of primer pairs used for the preparation of dsRNA synthesis templates.
T7 RNA promoter sequences are in italic.

Table. 2. Hayakawa et al.