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RESEARCH ARTICLE

Termite soldier mandibles are elongated by *dachshund* under hormonal and Hox gene controls

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ABSTRACT

In social insects, interactions among colony members trigger caste differentiation with morphological modifications. In termite soldier differentiation, the mandible size considerably increases through two moltings (via the presoldier stage) under the control of juvenile hormone (JH). Regulatory genes are predicted to provide patterning information that induces the mandible-specific cell proliferation. To identify factors responsible for the mandibular enlargement, expression analyses of 18 candidate genes were carried out in the termite *Hodotermopsis sjostedti*. Among those, *dachshund* (*dac*), which identifies the intermediate domain along the proximodistal appendage axis, showed mandible-specific upregulation prior to the molt into presoldiers, which can explain the pattern of cell proliferation for the mandibular elongation. Knockdown of *dac* by RNAi reduced the mandibular length and distorted its morphology. Furthermore, the epistatic relationships among *Methoprene tolerant*, *Insulin receptor*, *Deformed* (*Dfd*) and *dac* were revealed by combined RNAi and qRT-PCR analyses, suggesting that *dac* is regulated by *Dfd*, downstream of the JH and insulin signaling pathways. Thus, caste-specific morphogenesis is controlled by interactions between the factors that provide spatial information and physiological status.

KEY WORDS: *dachshund*, Mandible, Soldier differentiation, Epistasis, Hormones, Hox gene

INTRODUCTION

Although the morphological design of organisms is determined by genetic information, morphologies can also be altered by environmental stimuli, known as phenotypic plasticity or polyphenism (Nijhout, 2003). Insects are a major group of arthropods and share a basic body plan in serially homologous body segments; however, the allometry or proportions of body parts is diversified, particularly the size of appendages, including the mouthparts (Angelini et al., 2012a; Boxshall, 2004). As mouthpart modifications in insects played important evolutionary roles in adaptations to various environments, they exhibit considerable variation (Grimaldi and Engel, 2005; Koch, 2001; von Lieven, 2000). In some cases, however, the function of mouthparts is not related to feeding. Enlargement of mandibles is a striking example, as seen in male stag beetles fighting over mates (Emlen, 2008) and in termite soldiers defending their colonies against predators (Deligne et al., 1981).

In termites, the soldier caste has a unique morphology and function, and is a synapomorphy specific to the termite lineage, as it is present in all termite species with a few exceptions (Noirot, 1969). Soldiers can be differentiated from workers (or pseudergates, i.e. false workers or working larvae) through two molting events: the first molt leads to the presoldier stage; the second to the soldier stage (Fig. 1A; Roisin, 2000). During the differentiation, the anterior body parts are largely modified through solider-specific morphogenesis (Koshikawa et al., 2002; Miura and Matsumoto, 2000; Watanabe and Maekawa, 2008). Although soldier morphology is diversified among termite species, the biting type is thought to be the most primitive (Prestwich, 1984). In these species, soldiers possess elongated and heavily sclerotized mandibles (Koshikawa et al., 2002; Weesner, 1969).

The differentiation of termites into soldiers is regulated by a key endocrine factor: juvenile hormone (JH) (Cornette et al., 2008; Miura, 2019; Nijhout and Wheeler, 1982). In many termite species, the application of JH or its analogue (JHA) to workers induces differentiation into soldiers and morphological changes (Fig. 1C; Howard and Haverty, 1979). Although the mechanisms that control the effects of JH are unclear, knockdown of the JH receptor gene *Methoprene-tolerant* (*Met*) or hexamerin genes inhibit soldier differentiation (Masuoka et al., 2015; Zhou et al., 2006). Therefore, the downstream gene network upregulated by JH likely plays an important role in soldier-specific morphogenesis (Cornette et al., 2013; Miura, 2001, 2005; Miura and Scharf, 2011). The insulin/insulin-like growth factor signaling (IIS) pathway is also upregulated in mandibles before the presoldier molt and is required for soldier morphogenesis (Hattori et al., 2013).

The detailed morphogenetic processes of soldier differentiation have been studied in the damp-wood termite *Hodotermopsis sjostedti* (Archotermopsidae), a basal termite species (Koshikawa et al., 2002; Koshikawa et al., 2003; Fig. 1A). In soldier differentiation, the apical region of the mandible is more elongated than the proximal region (Fig. 1B; Koshikawa et al., 2002). Prior to the molt into presoldiers (PS), when mandibles are dramatically elongated, new folded cuticles and epidermal layers grow under the mandibular cuticle of pseudergate (PE) and expand at the presoldier molt (Koshikawa et al., 2003; Sugime et al., 2015). Therefore, epithelial cells of mandibles are suggested to proliferate before the PS molt. However, the mechanisms by which endocrine signals regulate mandible-specific allometric development are unclear, although several molecular studies on downstream factors have been reported (e.g. Ishikawa et al., 2010; Koshikawa et al., 2005, 2010; Miura et al., 1999; Toga et al., 2013). Thus, because the mandible grows

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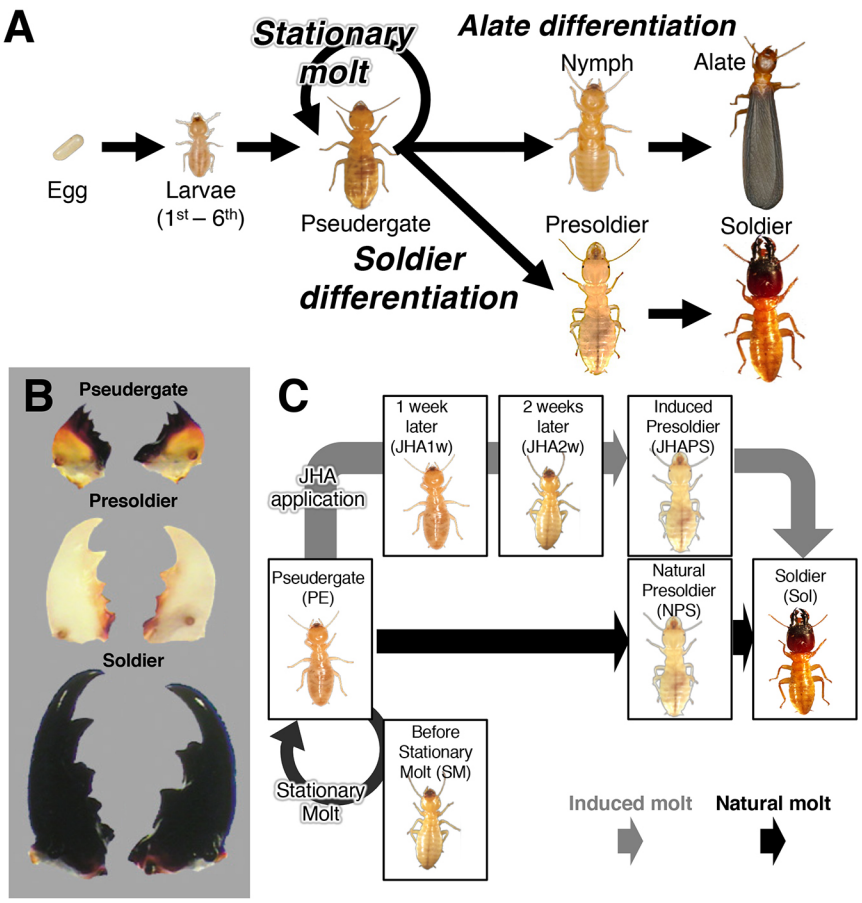

Fig. 1. Caste differentiation pathway of *H. sjostedti*, mandibular modification during soldier differentiation and the developmental stages compared. (A) Under laboratory-rearing conditions, most pseudergates (PEs) undergo stationary molt (SM, from pseudergate to pseudergate). The presoldier (PS) molt was induced in 2 weeks by the JHA pyriproxyfen. (B) Transition of mandibular morphology during the soldier differentiation. Apical parts of mandibles are elongated. (C) Gene expression levels were compared between seven developmental stages (boxes). The genes highly expressed at 1 or 2 weeks after JHA application (JHA1w and JHA2w) were analyzed, because epithelial proliferation for mandibular elongation occurs prior to the PS molt.

allometrically during soldier differentiation, we hypothesized that patterning factors provide spatial information to the mandibular epithelia.

Here, we have investigated the expression patterns of morphogenetic regulatory genes that are widely conserved and play important roles in body-part identity and the proximodistal patterning of insect appendages (Carroll, 1995; Kojima, 2004). In this study, using *H. sjostedti*, the expression patterns of 18 candidate genes were analyzed in mandibles during soldier differentiation. These included Hox genes, which provide segment identity [*labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*)]; segment polarity genes, which regulate the anteroposterior polarity of each segment [*decapentaplegic* (*dpp*), *wingless* (*wg*), *engrailed* (*en*) and *hedgehog* (*hh*)]; and appendage-patterning genes, which determine the proximodistal patterns and joint formation in appendages [*Distal-less* (*Dll*), *dachshund* (*dac*), *homothorax* (*hth*), *extradenticle* (*exd*), *aristaless* (*al*), *Serrate* (*Ser*), *spitz* (*spi*), *paired* (*prd*), and *cap'n'collar* (*cnc*)] (Angelini et al., 2012b; Coulcher and Telford, 2012; Kojima, 2004; Table S1). The temporal and spatial expression patterns were analyzed at seven developmental stages (Fig. 1C) and in five appendages. Results showed that *dachshund* (*dac*), which identifies the intermediate domain along the proximodistal appendage axis, was upregulated in the mandible-specific manner, and functional analyses using RNA interference (RNAi) showed that *dac* was required for the mandibular elongation. Furthermore, to clarify the epistatic relationships among differentially expressed toolkit genes (*dac*), hormone-receptor genes (*Met* and *InR*) and *Dfd*, the expression levels of these factors were analyzed after RNAi knockdown of each.

RESULTS

Expression profiles during the soldier differentiation

For expression analyses, sequences of toolkit genes were obtained by RNA sequencing of transcriptomes from eggs, alates, soldiers and neotenics in the focal termite species (see Materials and Methods for details). To screen for genes that were highly and specifically expressed in mandibles before the presoldier molt, the expression levels of target genes in mandibles were first compared at different developmental stages (Fig. 1C): pseudergate (PE), 1 week after JHA application (JHA1w), 2 weeks after JHA application (JHA2w), JHA-induced presoldier (JHAPS), natural presoldier (NPS), soldier (Sol) and stationary molt (SM). In the qPCR examinations (Fig. 2), genes that showed significantly higher expression during the course of the soldier differentiation (JHA1w and JHA2w) compared with pseudergate (PE) and stationary molt (SM) were chosen as 'primary candidates'. Among the Hox genes (*lab*, *pb*, *Dfd*, *Scr*, and *Antp*), *pb* and *Antp* were more highly expressed in JHA1w than in the other developmental stages (Fig. 2, Tukey's test; $P<0.05$). By contrast, *lab*, *Dfd* and *Scr* were not highly expressed in JHA1w and JHA2w. Among the segment polarity genes (*hh*, *dpp*, *wg* and *en*), *hh* was more highly expressed in JHA1w and JHA2w than in PE, but was not significantly different from that in SM. The expression of *dpp* showed no significant difference between JHA1w and PE, but was lower in JHA2w. Although the expression levels of *wg* and *en* were significantly higher in JHA1w than in PE, that of *en* did not significantly differ between JHA1w and NPS. Of the leg-patterning genes (*Dll*, *dac*, *hth*, *al*, *exd*, *prd*, *spi* and *Ser*), only *dac* showed significantly higher expression in JHA1w than at the other developmental stages. *Dll* was not upregulated during the differentiation. *hth* and *al* were more

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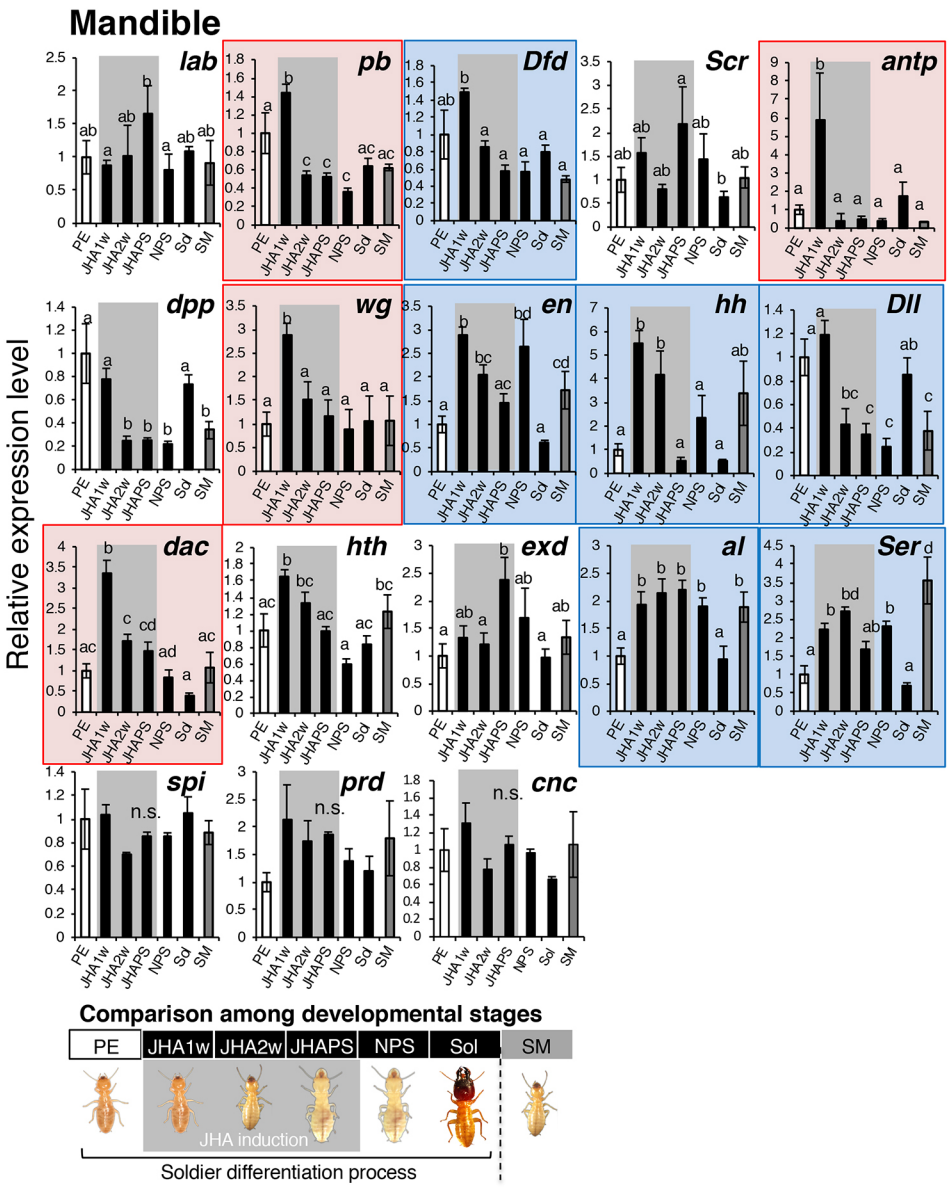

Fig. 2. Expression levels of 18 candidate toolkit genes at seven developmental stages in mandibles. Stages of artificial induction are highlighted in gray. Relative expression levels (data are mean±s.d., biological triplicates) were normalized to those in PE. Letters on bars denote significant differences between categories with different letters (Tukey's test, $P<0.05$). Genes that show significantly higher expression levels at JHA1/2w than PE and SM are highlighted in red (primary candidates). Genes showing relatively higher expression at JHA1/2w but not significantly different from PE and/or SM are highlighted in blue (secondary candidates). PE, pseudergate; JHA1w, 1 week after JHA treatment; JHA2w, 2 weeks after JHA treatment; JHAPS, presoldiers induced by JHA; NPS, natural presoldiers; Sol, soldier; SM, stationary molt.

highly expressed in JHA1w than in PE, but its expression was not significantly different from that in SM. *prd* and *spi* expression did not show significant differences between the different developmental stages, and *Ser* expression was significantly higher in JHA1w and JHA2w than in PE, and was highest in SM. Therefore, the four genes (*pb*, *Antp*, *dac* and *wg*) showing the highest upregulation in JHA1w were chosen as the 'primary candidates'.

In addition to the four genes, six genes (*Dfd*, *en*, *hh*, *Dll*, *Ser* and *al*) were chosen as 'secondary candidates', as these genes showed interesting expression patterns (upregulation at JHA1w/2w) and/or are known to possess important functions, but did not show significant differences from either PE or SM.

Expression profiles among appendages

The expression levels of all the 18 genes at JHA1w were then compared among antennae (Ant), mandibles (Md), maxillae (Mx), labium (Lab) and forelegs (FL) to determine whether they were upregulated in a mandible-specific manner (Fig. 3). Only the expression of *dac* was significantly (twofold) higher in mandibles than in the other appendages among the primary candidate genes (Fig. 3, Tukey's test; $P<0.05$). Among the secondary candidate genes, *Dfd* was expressed specifically in mandibles although at higher levels in maxillae. It was also shown that *en* was expressed at high levels in mandibles, but the difference was not as large as for *dac*. Furthermore, *prd* clearly showed a mandible-specific expression pattern, although significant upregulation was not detected during the soldier differentiation (Fig. 2). Therefore, only *dac* showed the most distinctive expression patterns (i.e. soldier differentiation-specific and mandible-specific patterns), so further functional analyses focused on *dac*.

Expression localization of *dachshund* in mandibles

To determine the detailed localization of *dac* inside a mandible, qRT-PCR and immunological staining were performed. Results of qRT-PCR comparing distal and proximal parts of right/left mandibles, by cutting a mandible into two parts, clearly showed that the expression levels in distal parts were higher (more than two folds) than those in proximal parts in both right and left mandibles

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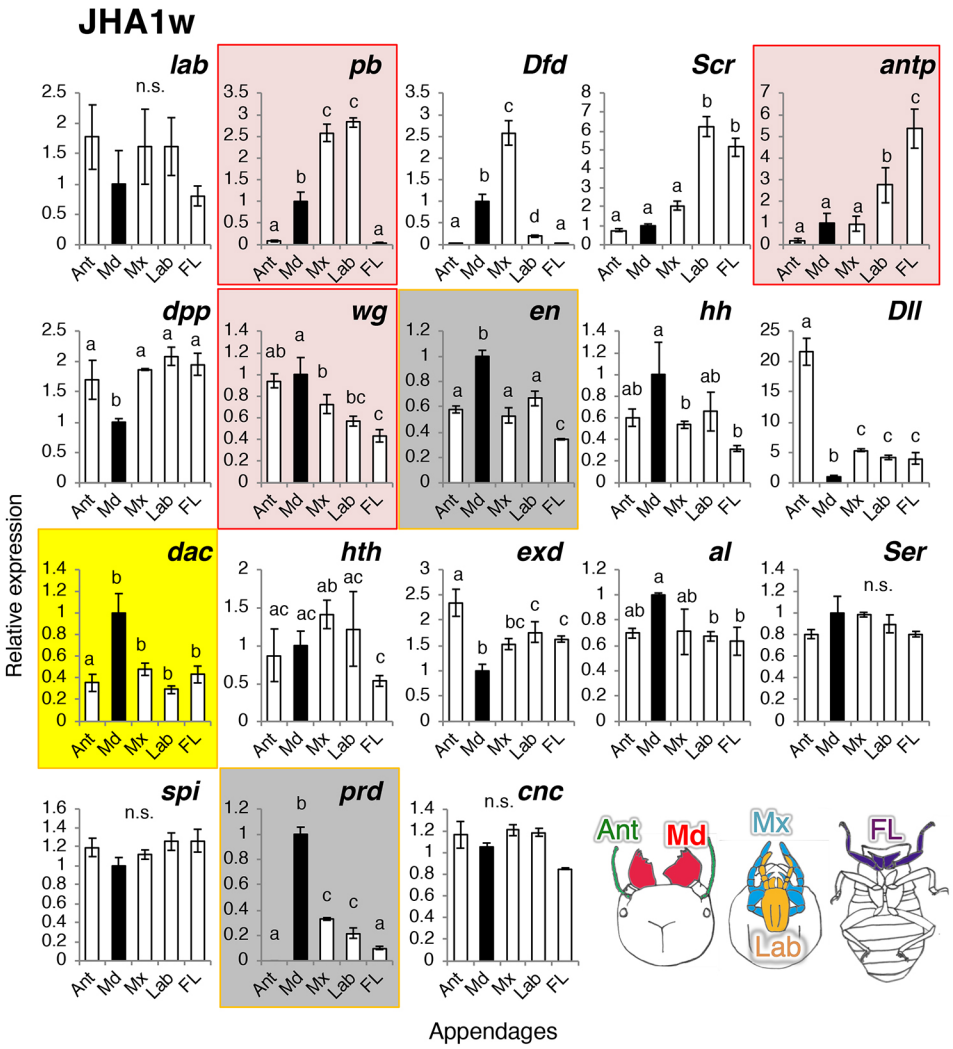

Fig. 3. Expression levels of 18 candidate toolkit genes 1 week after JHA application (JHA1w) in five different appendages. Relative expression levels (mean±s.d., biological triplicates) were to those in Md. Letters on bars denote significant differences between categories with different letters (Tukey's test, $P<0.05$). Among the primary candidates (*pb*, *Antp*, *wg* and *dac*; highlighted in red), only *dac* showed mandible-specific upregulation (highlighted in yellow). In addition, *en* and *prd* also showed mandible-specific upregulation (highlighted in gray). Md, mandible; Mx, maxilla; Lab, labium; Ant, antenna; FL, foreleg.

(Fig. 4A). Additionally, other genes that are known to provide axis information in insect appendages, i.e. *dpp*, *wg*, *hh*, *en*, *hth*, *al* and *Dll*, were also examined, of which only *en* showed significantly higher expression in the distal part (Fig. S2). On the other hand, *hth* showed higher expression levels in the proximal part, although a significant difference was detected only in left mandibles. The expression of *Dll* was undetectable.

In addition to the qPCR examinations, immunological staining was also carried out using two different monoclonal antibodies against *Drosophila* Dac protein. One of the two examined antibodies (mAbdac1-1) clearly showed the localization in the focal termite mandibles. Strong Dac signals were detected in epithelial tissues at the apical tip of mandibles and in the area between the first and second tooth in the distal half of the mandibles (Fig. 4B). In the proximal part, Dac signals were also detected in the marginal area of mandibles (basal teeth area), although the signals were weaker than the apical area. Dac signals were not detected at the outer margin of mandibles.

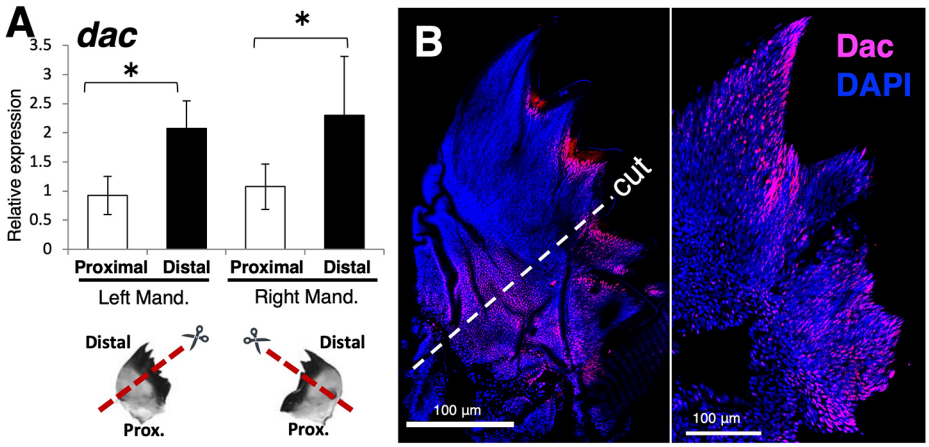

Fig. 4. Localization of *dac* expression in mandibles shown by qRT-PCR and immunostaining. (A) qRT-PCR comparisons between proximal and distal parts of mandibles obtained by cutting mandibles into two parts show that *dac* expression was higher (about twofold) in the distal region in both right and left mandibles (Welch's *t*-test, $*P<0.05$). (B) Confocal images of immunostaining for the Dac protein using anti-Dac antibody. The two panels are derived from different individuals. The Dac signals are detected in the apical tip and areas between the mandibular teeth. The signals are not detected at the outer margin of the mandible.

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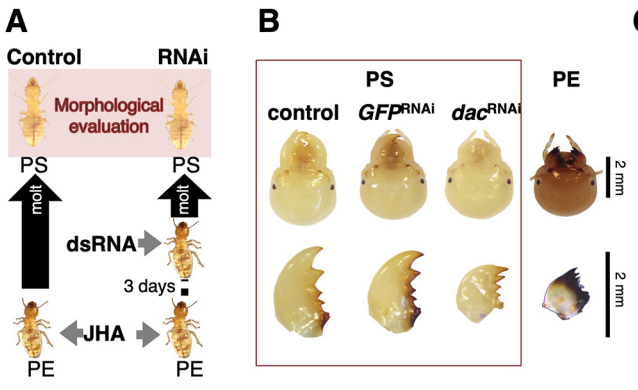

Fig. 5. Functional analyses of *dac* by RNAi. (A) Schematic diagram of RNAi. dsRNA (3 µg) was injected into an individual 3 days after JHA application. Effects of dac^{RNAi} on mandibular enlargement were evaluated in presoldiers. (B) Termite heads and left mandibles of a JHA-induced control presoldier, a JHA-induced GFP^{RNAi} presoldier, a JHA-induced dac^{RNAi} presoldier and an untreated pseudergate. dac^{RNAi} influences the mandibular elongation in PS. (C) Mandibular lengths (data are mean±s.d., PS, n=11; GFP^{RNAi}, n=28; dac^{RNAi}, n=26; PE, n=15). Letters on bars denote significant differences between categories with different letters (Tukey's test, P<0.05).

Functional analyses of *dachshund* by RNAi

To elucidate the function of *dac* in mandibular morphogenesis during soldier differentiation, RNAi was performed to JHA-treated pseudergate (PE) individuals (Fig. 5). *dac* RNAi resulted in presoldiers (PS) with markedly smaller mandibles than those of normal presoldiers and the *GFP* control (Fig. 5B,C, Fig. S3). The effects of *dac* RNAi on morphogenesis of 13 other body parts showed that the mandible length (ML) and the length between the apical and first teeth (AL) were smaller in dac^{RNAi} PSs than in normal PS (Fig. 5C, Fig. S3, Tukey's test; P<0.05). However, most of the other parameters did not significantly differ between normal and dac^{RNAi} PS. Moreover, the mesonotum width (MsW) and the metanotum width (MtW) of the dsRNA-injected PS (dac^{RNAi}, GFP^{RNAi}) were larger than those of normal PS. In principal component analysis (PCA) using the 13 parameters (Fig. S3), the distinction between PE and PS was represented by PC1; thus, PC1 reflects total body size as well as allometric differences. In addition, the distinction between dac^{RNAi} PS and control PS (RNAi untreated and GFP^{RNAi}) was represented by PC2. Thus, the mandibular morphology in dac^{RNAi} was intermediate between that of PE and PS (Fig. 5B,C, Fig. S3).

Furthermore, geometric morphometric analysis was conducted using 20 landmarks along the outline of the left mandibles in dac^{RNAi} individuals (Table S4, Fig. S4). In this analysis, Dll^{RNAi} was also added to confirm whether *Dll* did not affect the mandibular elongation in the focal termite species. Principal component analysis showed that the shape of dac^{RNAi} mandibles (PC1: −0.1) reduced the degree of elongation, particularly at the apical part, and showed a highly curved (i.e. round-shaped) outer edge (Fig. S4). Thus, *dac*-RNAi disrupted the soldier-specific mandibular morphogenesis during the soldier differentiation.

Epistatic analyses

To determine the epistatic relationships among hormonal factors, Hox genes and *dac*, RNAi knockdown of *Met*, *InR*, *Dfd* and *dac* was performed, followed by quantification of gene expression levels of those genes (Fig. 6). *dac* expression was reduced in Dfd^{RNAi}, Met^{RNAi} and InR^{RNAi} presoldiers (Fig. 6A, Tukey's test; P<0.05). *Dfd* expression was reduced in Met^{RNAi} and InR^{RNAi}, but not in dac^{RNAi} PS (Fig. 6B, Tukey's test). *Met* and *InR* expression was, respectively, reduced by InR^{RNAi} and Met^{RNAi}, which indicates reciprocal regulation (Fig. 6C,D, Tukey's test; P<0.05). By contrast,

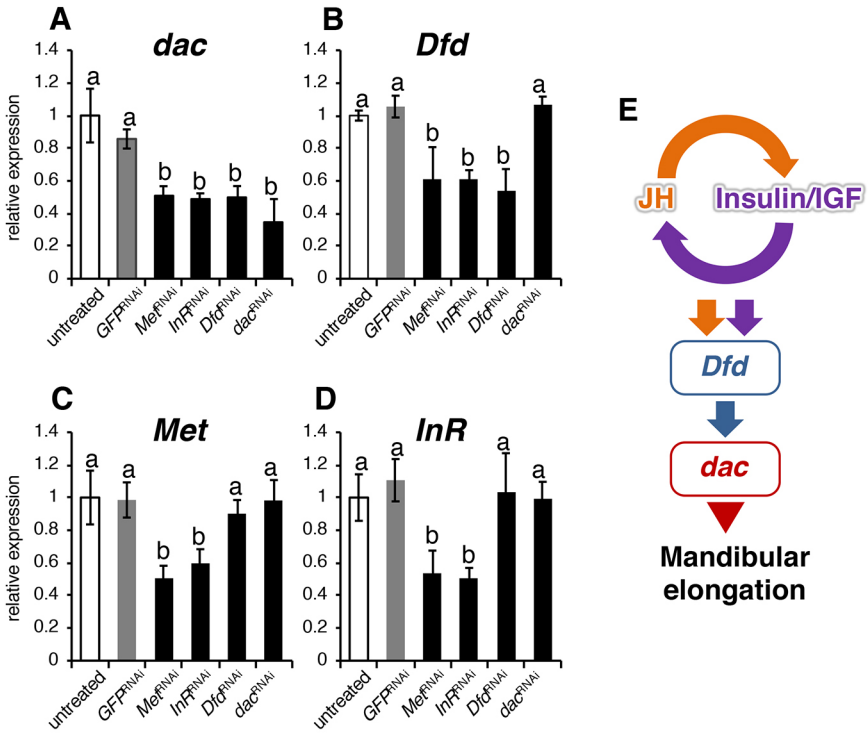

Fig. 6. Analyses by RNAi and qRT-PCR for the epistatic relationships among hormonal and patterning factors. Expression levels of (A) *dachshund* (*dac*), (B) *Deformed* (*Dfd*), (C) *Methoprene tolerant* (*Met*) and (D) *Insulin Receptor* (*InR*) in mandibles 1 week after JHA application. RNAi was performed 3 days after JHA application. The expression level of *dac* was reduced by Met^{RNAi}, InR^{RNAi} and Dfd^{RNAi}, and that of *Dfd* was reduced by Met^{RNAi} and InR^{RNAi}. *Met* and *InR* expression was affected by InR^{RNAi} and Met^{RNAi}, respectively. Letters on bars denote significant differences between categories with different letters (Tukey's test, P<0.05). (E) Schematic diagram of the epistatic relationships between pathways and factors that lead to the mandibular elongation during soldier differentiation.

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dac^{RNAi} did not affect the expression of *Dfd*, *Met* and *InR* (Fig. 6B-D, Tukey's test).

DISCUSSION

Our data strongly suggest that *dac* is a key factor in mandibular elongation during soldier differentiation, and that its expression is regulated by *Dfd* downstream of JH and insulin signaling. Four genes (i.e. *pb*, *Antp*, *wg* and *dac*) were significantly upregulated in mandibles at 1 week post-JHA application (JHA1w, Fig. 2). Because the folded mandibular tissue is prepared and almost ready to expand in JHA2w (Koshikawa et al., 2003), the patterning information must be provided by regulatory genes before JHA2w. Therefore, upregulation of the four regulatory genes in JHA1w is plausible if these genes are responsible for the mandibular morphogenesis.

Expression patterns of Hox genes

Among the Hox genes that determine the identity of body parts along the anterior-posterior axis, *Dfd* is known to provide the mandibular identity (Carroll, 1995; Hughes and Kaufman, 2002; Hughes and Kaufman, 2000). In addition, *Dfd* reportedly contributes to mandibular remodeling during the soldier differentiation (Toga et al., 2013). In *H. sjostedti*, *Dfd* expression was upregulated in JHA1w, although it was not significantly different from that in PE (Fig. 2). The comparison of *Dfd* expressions among appendages showed that *Dfd* was expressed not only in mandibles but also in maxillae (Fig. 3). This pattern of *Dfd* expression exactly corresponds, to our knowledge, to that revealed in other insects (Hughes and Kaufman, 2000; Rogers and Kaufman, 1997). Although the identity of maxillae also requires expression of *pb* and/or *Scr*, *Dfd* seems to be the only required factor for the mandibular identity, which is also the case in the termite soldier differentiation.

By contrast, *pb* and *Antp* were highly expressed in JHA1w, although expression was not mandible specific (Fig. 3). The spatial expression patterns of *pb* and *Antp*, i.e. upregulation in more-posterior appendages, also corresponded to those in other insects (Hughes and Kaufman, 2000; Rogers and Kaufman, 1997). Expression of these Hox genes could also be necessary, because the posterior appendages are mildly modified in the soldier differentiation (Koshikawa et al., 2002).

As the mandible-specific morphogenesis occurs in response to high JH titer, spatial information for the body-part identity should be provided by the upregulation of Hox genes downstream of the activity of JH, or by continuous expression throughout postembryonic development. The expression levels of some Hox genes were high even at the PE stage, and some were upregulated by JH, although the patterns differed among Hox genes (Fig. 2). This suggests that the Hox genes need to be expressed even during the postembryonic development, probably for postembryonic modifications.

Expression patterns of appendage-patterning genes

The antero-posterior appendage patterning genes, *en* and *hh* were upregulated in mandibles, respectively at JHA1w and JHA2w, but both were upregulated also in SM (Fig. 2). Hence, these genes are likely expressed at each molt, as are *hth*, *al* and *Ser* (Fig. 2). By contrast, *dpp* expression in mandibles was decreased by JHA (Fig. 2). This pattern was different from wing-disc formation in *Drosophila melanogaster*, in which *en* and *hh* expression induces *dpp* for antero-posterior axis formation (Estella et al., 2012; Kojima, 2004). *Ser* was slightly upregulated during soldier differentiation but the expression level was higher in SM. In *Drosophila* wing formation, *Ser* is expressed at the D/V boundary of dorsal cells (Kim et al., 1995). Such axis formation patterning would be also required for patterning at every molt. In insect embryos, *dpp* expression is known to localize at the distal tip (Angelini and Kaufman, 2005a). However, during the soldier differentiation in termites, *dpp* was downregulated in mandibles, suggesting that the mandibular modification in postembryonic development is different from the general appendage formation.

Mandibular enlargement during soldier differentiation occurs along the proximo-distal axis (Koshikawa et al., 2002, 2003); thus, the above result (Fig. 2) was likely due to the restriction of antero-posterior growth of mandibles (from teeth to the outer edge) during soldier differentiation. The antagonistic effects of *wg* and *dpp* within the imaginal disc provide patterning information along the proximo-distal axis of appendages (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Struhl and Basler, 1993). In the imaginal discs of *Drosophila*, high levels of *wg* and *dpp* signals reportedly induce *Dll* expression, leading to the formation of distal appendages (Lecuit and Cohen, 1997). During the mandibular elongation of termite soldiers, the expression pattern of *wg* corresponded to the pattern in *Drosophila*, but those of *dpp* and *Dll* did not (Fig. 2). Thus, *Dll* expression was reduced simultaneously with the downregulation of *dpp* in mandibles after JHA treatment. Upregulation of *wg* and *dac* at JHA1w suggests an interaction between the two factors (Fig. 2), but *wg* expression was not mandible specific (Fig. 3).

During the formation of insect appendages, *Dll* makes the major contribution to the patterning of the distal region (Panganiban and Rubenstein, 2002). The mandible is thought to be a gnathobasal appendage that lost the distal appendage region, where *Dll* is expressed (Angelini et al., 2012a; Boxshall, 2004; Panganiban et al., 1994; Popadić et al., 1998; Snodgrass, 1935). However, the apical region (from the first teeth to the tip) of the mandible is considerably enlarged during soldier differentiation (Koshikawa et al., 2003). Although some genes for the patterning of apical mandibular region were predicted, our data (Fig. 2, Fig. S4) suggest that *Dll* is not involved in or required for the mandibular enlargement during soldier differentiation, as reported in other insects (Angelini and Kaufman, 2004, 2005b; Beermann et al., 2001; Coulcher and Telford, 2013; Panganiban et al., 1994; Popadić et al., 1998; Simonnet and Moczek, 2011). This situation is very similar to the mandibular elongation in male stag beetles (Gotoh et al., 2017).

dachshund expression and function in mandibular elongation

Among 18 candidate genes, only *dac* showed characteristic expression patterns in the mandibles during soldier differentiation; i.e. mandible-specific upregulation prior to molt into presoldiers (Figs 2 and 3), and knockdown of *dac* expression restricted mandibular elongation during the molt into presoldiers (Fig. 5, Figs S3 and S4). The *dac* expression was higher in the distal part of the mandibles (Fig. 4A), which is similar to the of embryonic mandibles in *T. castaneum* (Coulcher and Telford, 2013). The detailed expression localization of *dac* (Fig. 4B) corresponded well to the areas of epithelial tissues where extensive cell proliferation takes place along the inner edges of mandibles before the molt into presoldiers (Koshikawa et al., 2002). However, the *dac* localization at the inner edge did not explain the cell proliferation at the outer (lateral) edges of mandibles. Interestingly, this situation is consistent with the phenotypes of *dac*-RNAi individuals.

Knockdown of *dac* affected enlargement of the mandibles, but not that of other appendages (Fig. 5, Fig. S3). The mandibular

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elongation in *dac*-RNAi individuals was significantly suppressed compared with those of *GFP*- and *Dll*-RNAi individuals (Fig. S4). The function of *dac* in mandibular development corresponds to that in other insects (Angelini and Kaufman, 2004; Coulcher and Telford, 2013; Giorgianni and Mann, 2011; Gotoh et al., 2017; Popadić et al., 1998; Simonnet and Moczek, 2011). In *H. sjostedti*, the number of mandibular teeth does not change during soldier differentiation, but the proportion changes owing to the increase in tooth distance (Koshikawa et al., 2003). dac^{RNAi} PS exhibited restricted enlargement of the apical region of the mandibles with highly curved or rounded outer edges (Fig. 5, Fig. S4). Considering that the *dac* expression was restricted to the inner edges (Fig. 4B), the RNAi knockdown of *dac* should inhibit the cell proliferation at the inner edge, so that the epithelial cell proliferation would only occur at the outer edge, resulting in the mandibular shape with curved outer edge. It is therefore suggested that there are other factors responsible for the cell proliferation at the outer edge.

In *Drosophila* leg discs, *dac* expression is directly regulated by *Dll* (Giorgianni and Mann, 2011). Because *Dll* was not upregulated in mandibles, this regulatory mechanism was not applicable to soldier differentiation. In *Tribolium*, however, *dac* is also expressed in mandibles and the proximal region of legs, which are not affected by *Dll* (Prpic et al., 2001). So this pattern of *dac* expression in *Tribolium* likely corresponds to that in termite mandibles (Angelini et al., 2012a). Unlike the mandibular development at metamorphosis in holometabolous insects, in the case of termite soldier differentiation, pre-existing mandibles are just modified, so that the loss of function of *dac* does not result in complete or partial loss of mandibles but in the allometric changes observed in this study.

Epistatic relationships between *dac*, *Dfd* and hormonal pathways

To assess morphogenesis during postembryonic development in insects, clarification of the links between hormonal signals that circulate throughout the body and patterning genes that mediate tissue-specific responsiveness is needed (Gotoh et al., 2015; Lavine et al., 2015; Truman and Riddiford, 2007; Villarreal et al., 2015). Therefore, we also examined the epistatic relationships of *dac* with putative upstream factors (i.e. JH and insulin), which provide temporal physiological information, and with Hox genes, which provide spatial information (Fig. 6). *dac* and *Dfd* expression in JHA1w was regulated by *Met* and *InR*, and *Dfd* expression was necessary for that of *dac* (Fig. 6E). These findings suggest that JH and IIS have their effects upstream of morphogenetic factors during soldier differentiation. In *T. cataneum* larvae, the expression of *hh*, which is important for the proliferation of imaginal cells, is inhibited by JH (Villarreal et al., 2015). In *H. sjostedti*, however, *hh* was upregulated in mandibles under the high-JH condition, although it was not significantly different from that during SM (Fig. 2). The difference in the response of *hh* expression to JH between *H. sjostedti* and *T. castaneum* was possibly due to alternation of the cis and/or trans regulatory elements. Some other factors, such as the Fat/Hippo signaling pathway, have been suggested to mediate between endocrine and morphogenetic factors (Gotoh et al., 2015; Lavine et al., 2015). Thus, tissue-specific cell proliferation leading to the mandible-specific elongation during termite soldier differentiation is coordinated by the interactions between endocrine signals and patterning genes (Fig. 6E). These interactions would also be applied to the differentiation of other castes, such as alates. The detailed link between endocrine signals and patterning genes is still unclear, so that further studies will enhance understanding of the regulatory mechanisms underlying morphological diversity.

MATERIALS AND METHODS

Insects

Colonies of *H. sjostedti* Holmgren (family Archotermopsidae) inhabiting rotten wood were collected on Yakushima Island (Kagoshima Prefecture, Japan) in May 2011 and May 2014. Colonies were maintained in plastic containers with nest logs at ∼25°C under constant darkness, and were occasionally fed moistened pinewood. Caste categories were identified based on previous studies of the focal species (Miura et al., 2000; 2004): larval instars, pseudergates (PE), nymphs, alates, neotenics, presoldiers (PS) and soldiers (Sol) (Fig. 1A).

Identification of toolkit gene orthologs

To identify major toolkit genes in the focal termite species, RNA-sequencing was performed (see supplementary Materials and Methods). Total RNAs were extracted from eggs, alates, soldiers and neotenics of *H. sjostedti*, yielding 181,084,900 paired-end reads. After filtering, 140,958,127 paired reads and 18,567,603 single reads, the paired-end read counterparts of which were filtered out, were retained for further analyses. *De novo* transcriptome assembly of the sequence reads generated 164,434 contigs with a total of 161,836,366 bases and a N50 of 2075 bp. The predicted amino acid sequences of *Tribolium castaneum* toolkit genes were used for BLASTp searches to identify the termite orthologs from transcriptome data of *H. sjostedti* (Boxshall, 2004; Richards et al., 2008). The top-hit sequences from the termite transcriptome database were defined as putative orthologs of the toolkit genes. We performed phylogenetic analyses that included genes from other animals to confirm the orthologs of putative toolkit genes (Table S1, Fig. S1A-J, see supplementary Materials and Methods for details).

JHA application and sampling stages

To generate individuals undergoing soldier differentiation, a JH analog, pyriproxyfen, was applied (Ogino et al., 1993). As previously described (Sugime et al., 2015), 10 pseudergates (PE) were placed in a Petri dish (ø 70 mm) lined with filter paper containing 10 µg pyriproxyfen (Sigma-Aldrich) and moistened with distilled water. Petri dishes were monitored daily and maintained at 25°C under constant darkness. The presoldier molt typically occurred ∼14 days after JHA application. During soldier differentiation, samples were obtained 1 week after JHA application (JHA1w), JHA2w and PS induced by JHA (JHAPS) stages. In addition to these JHA-treated termites, pseudergates (PE), natural PS (NPS), Sol and PE in the stages prior to the stationary molt (SM) were sampled from the stock colonies. NPS were included for comparison with JHAPS to evaluate artificial effects that might be induced by the application of an unnatural compound, i.e. JHA. To obtain the SM, PE that exhibited whitish abdomens due to gut purging were collected from the stock colonies (Koshikawa et al., 2005; Sugime et al., 2015).

Quantitative polymerase chain reaction

To quantify expression levels in mandibles during the soldier differentiation, total RNA was extracted from mandibles along a time course, i.e., PE, JHA1w, JHA2w, JHAPS, NPS, Sol and SM, and real-time qPCR was performed for the selected toolkit genes. Total RNAs were also extracted from different appendages, including mandibles (Md), maxillae (Mx), labium (Lab), antennae (Ant) and forelegs (Fl) dissected from 20 individuals 1 week after the JHA application (JHA1w) from three different colonies. The samples were frozen in liquid nitrogen and preserved at −80°C until RNA extraction. Methods for RNA extraction, reverse-transcription, real-time qPCR, selection of reference genes and statistical analysis were performed as previously described (Cornette et al., 2013; Hattori et al., 2013; Ishikawa et al., 2010; Koshikawa et al., 2005, 2010). Details are provided in the supplementary Materials and Methods.

Expression localization for *dac*

To analyze localization of the *dac* expression in mandibles, qRT-PCR and immunostaining were carried out. First, to roughly determine in which part of mandibles *dac* is expressed, qRT-PCR comparisons between distal and proximal mandibles were carried out. At 1 week after the JHA application (JHA1w), right and left mandibles were dissected, each of which was further dissected into two parts (distal and proximal parts) as shown in Fig. 3. RNA

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extraction and qPCR were performed as mentioned above. To further determine the detailed spatial patterns of *dac* expression, immunological staining was performed using the monoclonal antibodies against the *Drosophila* Dachshund protein (mAbdac1-1 and mAbdac1-2; Mardon et al., 1994; purchased from Developmental Studies Hybridoma Bank, University of Iowa) and a confocal laser microscope (OLYMPUS FV3000). See supplementary Materials and Methods for details.

RNA interference

To evaluate its function, *dac* was knocked down by RNAi using a method established to assess soldier differentiation of the focal species (Hattori et al., 2013; Koshikawa et al., 2010). *Dll*, which is responsible for the elongation of distal appendages, was also knocked down by RNAi (Panganiban and Rubenstein, 2002). Fragments of the target genes were amplified by PCR using specific primers and subcloned, and the sequences confirmed. dsRNA was then synthesized and diluted to a final concentration of 3 µg/µl (*dac*, *Dll* and *GFP*) or 1 µg/µl (*InR*), and injected to PE at day 3 after JHA application. See supplementary Materials and Methods for details. The termites were maintained in Petri dishes at 25°C for 2-3 weeks. Only individuals that molted within 3 weeks were used for the morphometric study. Phenotypes for RNAi-treated individuals were evaluated by morphometric analyses. To evaluate the effects of *dac* RNAi on soldier differentiation, we conducted principal component analysis based on the measurements of 13 body parts. To further investigate the effects of RNAi on detailed mandibular shapes (untreated, GFP^{RNAi}, Dll^{RNAi} and dac^{RNAi}), morphological changes were assessed, based on 20 landmarks along the outline of the mandibles, using geometric morphometric techniques (see supplementary Materials and Methods for details, Table S3).

Epistatic analyses of morphogenetic and endocrine factors

To evaluate the epistatic relationships among *dac*, JH signaling, insulin signaling and *Dfd* expression, qPCR was performed after reciprocal RNAi treatments. dsRNAs for *InR* and *dac*, and siRNAs for *Met* and *Dfd* were injected into termites. Detailed methods for the preparations of dsRNA and siRNA are described in the supplementary Materials and Methods. After RNAi of *dac*, *Met* (JH receptor), *InR* (insulin receptor) or *Dfd* (Hox), the transcript levels of these genes in mandibles were quantified 7 days after JHA application (i.e. 4 days after RNAi) by qPCR. RNA extraction, reverse transcription and qPCR were performed as described above.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.M.; Methodology: Y.S., K.O., H.G., Y.H., M.M., S.S., S.K., T.M.; Validation: Y.S., T.M.; Formal analysis: Y.S.; Investigation: Y.S., K.O., H.G., Y.H., S.K., T.M.; Resources: Y.H., S.S.; Data curation: K.O., Y.H., M.M., S.S., T.M.; Writing - original draft: Y.S., K.O., T.M.; Writing - review & editing: K.O., H.G., Y.H., S.K., T.M.; Visualization: T.M.; Supervision: T.M.; Project administration: T.M.; Funding acquisition: Y.S., K.O., T.M.

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Data availability

The dataset of transcriptome assembly is available in the DDBJ Sequence Read Archive under accession number DRA005483. Accession numbers of the candidate genes identified in *H. sjostedti* are listed in Table S2.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.171942.supplemental

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