

[REVIEW]

The Sawfly, *Athalia rosae ruficornis* (Hymenoptera) as a Model Insect for Developmental and Reproductive Biology: What Has Been Done and What Should Be Done?*

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1. Introduction

The general mode of reproduction of Hymenoptera is arrhenotokous parthenogenesis, also called haplodiploidy: fertilized eggs give rise to diploid females and unfertilized eggs give rise to haploid males (Suomalainen *et al.*, 1987). It is apparent in this group that egg activation and fertilization are independent phenomena, although they usually occur simultaneously. Events occurring at and around egg activation and fertilization have been extensively studied in many animals (Hardy, 2002). In insects, however, the studies in these fields have been sparsely done mainly due to the technical difficulties of artificial control of egg activation and fertilization, even in the model species, *Drosophila melanogaster*. On the other hand, it is possible to induce parthenogenetic development by merely immersing mature unfertilized eggs in distilled water in more than 200 sawfly species that belong to the primitive group of Hymenoptera, Symphyta (Naito, 1982). Thus, the hymenopteran species with the easy manipulation of egg activation serve as an experimental system for answering questions related to oocyte maturation, egg activation, fertilization and early embryonic development in insects.

Oishi *et al.* (1993, 1995, 1998) selected the sawfly, *Athalia rosae ruficornis* (Fig. 1), and developed this species as a model insect to unravel the mechanisms underlying egg maturation and the events leading to early embryonic development. Artificial activation of unfertilized eggs provides embryos with synchronized developmental stages (Sawa and Oishi, 1989a). This is an

advantage to investigate early embryonic development on morphology, biochemistry and molecular biology. One of the most remarkable technical achievements with this species is *in vitro* fertilization by microinjection of sperm into eggs (Sawa and Oishi, 1989b). The technique is the same as intracytoplasmic sperm injection (ICSI), and is commonly used in humans and experimental and domestic animals (Yanagimachi, 2005). ICSI in *A. rosae ruficornis* enables to evaluate the potential of immature male gametes on syngamy in insects.

Recently, gene functional analysis has been accelerated in insects other than the model species, *D. melanogaster*. Genome sequencing has been proceeding in many insects, and the accumulation of genomic information makes it easier to isolate specific genes of a certain species by using PCR-based cloning methods. Transgenesis is a prerequisite technology for analyzing gene function, and that has been established in more than 20 species to date spanning four insect orders. Molecular analyses of gene function have thus been facilitated in non-model insects. *A. rosae ruficornis* is one of such species with the success of germline transformation (Sumitani *et al.*, 2003).

In this review we describe findings on the potential of male gametes to participate in development, the regulatory pathway in meiotic cell cycle arrest during oocyte maturation, and the formation of abdominal appendages during embryonic development. Recent progress in the establishment of molecular tools for gene functional analysis, such as transgenesis, RNA interference (RNAi) and systems for regulating gene

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expression, are also mentioned.

2. Potential of male gametes to participate in development

A. rosae ruficornis is the only insect species in which artificial fertilization is feasible by means of intracytoplasmic sperm injection (ICSI). This technique is quite useful to examine the potential of male gamete to participate in development and interaction with cytoplasmic factors in recipient eggs. To achieve artificial fertilization by ICSI, we usually microinject mature sperm at the anterior end of the egg where the micropyle opens to mimic normal sperm penetration (Fig. 2A). The male gamete generally participates in development only when its nucleus unites with egg nucleus. ICSI, however, enables the unusual penetration of sperm into eggs in *A. rosae ruficornis*. Syngamy never takes place when sperm are injected into the posterior end of eggs, the opposite side of the micropyle opening, but independent participation of gamete nuclei occurs in a small fraction of the injected eggs, resulting in the production of haploid-haploid chimeras (Hatakeyama *et al.*, 1994a) (Fig.

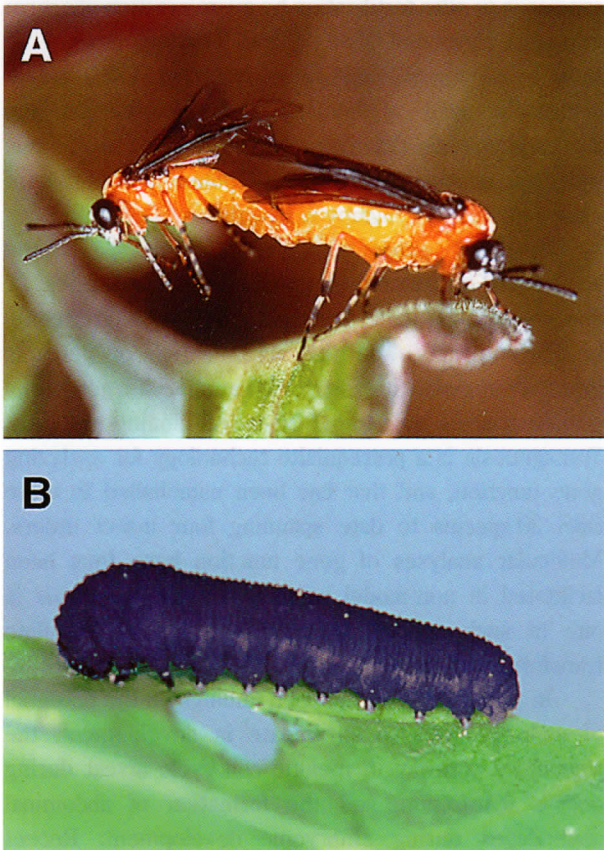


Fig. 1 An adult male (left) and female (right) of the sawfly, *Athalia rosae ruficornis* Jakovlev (Hymenoptera, Symphyta, Tenthredinidae) under copulation (A). A female fifth instar larva feeding on a leaf of the brassicaceous (cruciferous) plant, *Raphanus sativus* (B). General biology of *A. rosae ruficornis* is described in Sawa *et al.* (1989).

2B). Either diploid embryos or haploid-haploid chimeras are produced when sperm are injected into the lateral side (Fig. 2C). These findings indicate that mature sperm, as well as mature eggs have pluripotency, and that strict genomic imprinting seems unlikely. It is also suggested that egg cytoplasmic factor(s) responsible for the promotion of pronuclei formation and the prevention of supernumerary nuclei from development are involved. One of the candidate factors to promote pronuclei formation is the *Hira* gene product, which was first identified in *Xenopus laevis* to have critical function in nucleosome assembly (Ray-Gallet *et al.*, 2002). *Hira* protein is required for the reassembly of nucleosomes of sperm chromatin in *D. melanogaster*, and the male pronucleus is not formed without this protein (Loppin *et al.*, 2000, 2001, 2005). It is demonstrated that a maternal effect lethal mutation, *sesame (ssm)* of *D. melanogaster* is caused by a point mutation in the *Hira* gene (Loppin *et al.*, 2005). Possible factors to exclude supernumerary nuclei are the spindle checkpoint proteins, Bub1-related kinase (BubR1) and monopolar spindle1 (Mps1). Polar body nuclei are usually excluded during syncytial divisions in insect eggs and shown to form unique condensed chromosomes in *D. melanogaster* and *A. rosae ruficornis* (Campos-Ortega and Hartenstein, 1985; Page and Orr-Weaver, 1997; Yamamoto *et al.*, 2008). Both BubR1 and Mps1 accumulate at kinetochores of polar body chromosomes of *D. melanogaster* to maintain condensed conformation and to prevent them from participating in development (Perez-Mongiovi *et al.*, 2005; Fisher *et al.*, 2004). Some cytoplasmic factors are temperature-sensitive, since heat treatment of mature eggs upon activation disturbs the factors and allows polar body nuclei to participate in development of *A. rosae ruficornis* and the silkworm, *Bombyx mori* (Astaurov, 1967; Hatakeyama *et al.*, 1990).

ICSI in animals is developed primarily aiming at remedy of male-factor infertility in humans and preservation of endangered species. For this reason, immature male gametes as well as mature sperm have been used for ICSI. There are successful reports demonstrating production of offspring by using premature sperm, spermatids and even spermatocytes for ICSI in some mammalian species, including humans (Yanagimachi, 2005). The results reveal the capacity of premature male gametes to fertilize eggs in mammals. A question then arises as to whether this potential is general in premature male gametes of insects, and whether the presumed cytoplasmic factors have a similar effect. This is answered using ICSI in *A. rosae ruficornis* (Hatakeyama *et al.*, 2000).

Most spermatocytes complete meiosis before pupation, and spermiogenesis proceeds in testicular cysts in pupa of *A. rosae ruficornis* (Fig. 3A). Spermatids are round in the early pupal stage, and termed round

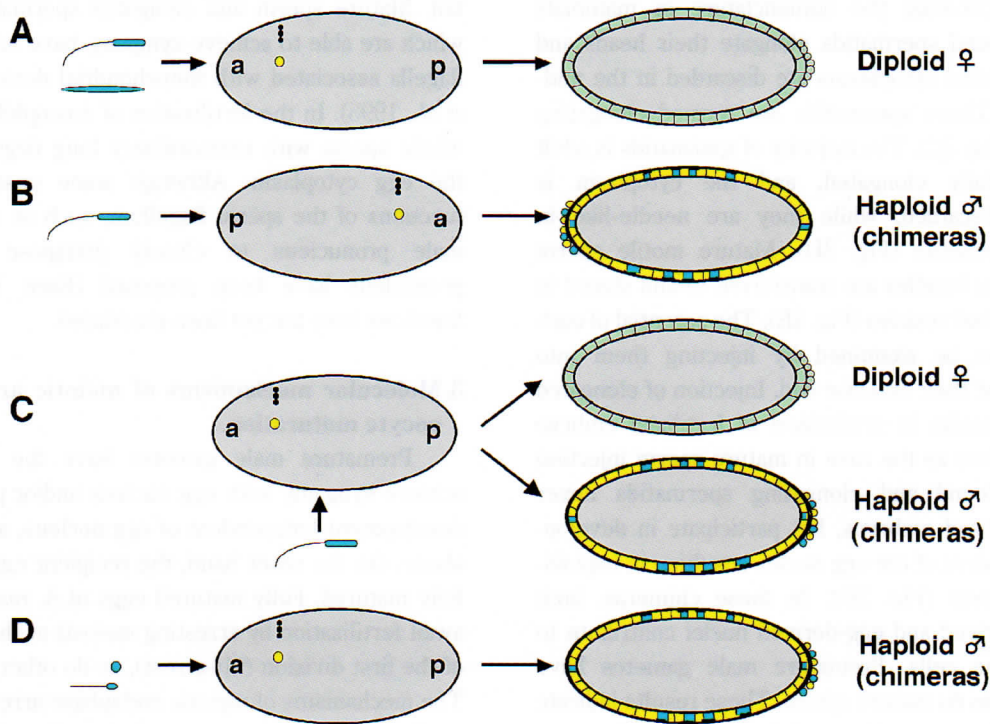


Fig. 2 Manner of participation of male gametes in development. Injection of mature sperm and elongated spermatid at the anterior end of eggs results in production of diploid female due to syngamy of male and female pronuclei (A). Mature sperm, when it is injected into an egg at the posterior end, participates in development independent of female pronucleus resulting in haploid-haploid chimera (B). Either diploid female or haploid-haploid chimera is produced when mature sperm is injected into the lateral side of an egg (C). Round spermatid and elongating spermatid participate in development independent of female pronuclei resulting in haploid-haploid chimeras when they are injected into eggs at the anterior end (D). Nuclei derived from male and female gametes are in blue and yellow, respectively. Green shows zygotic nuclei derived from both male and female gametes. Small black dots located at the anterior dorsal region of an egg represent polar body nuclei that are excluded from syncytial divisions. Orientation of an egg is marked with "a" and "p" for the anterior and the posterior, respectively.

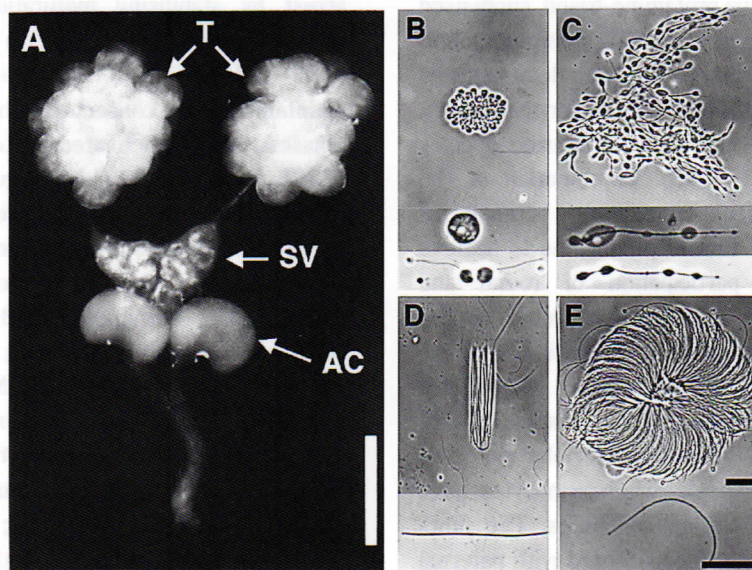


Fig. 3 A pair of testes and internal reproductive organs dissected from a one-day-old adult male (A). Morphology of male gametes in various developmental stages taken from testes or seminal vesicles (B-E). Spermatids in testicular cysts of early pupa are round in shape, the round spermatids (B). Elongating spermatids (C) of that heads and tails begin elongating are seen in testes at mid- to late-pupal stage. Most of spermatids in adult testes are elongated spermatids (D). They are fully elongated and residual cytoplasm is completely discarded, while they are immotile. Motile mature sperm form bundle (E) and are transferred to seminal vesicles. AC: accessory gland, SV: seminal vesicle, T: testis. Bars indicate 500 μm in (A) and 20 μm in (E).

spermatids following the nomenclature in mammals (Fig. 3B). Round spermatids elongate their heads and tails, and residual cytoplasm is discarded in the mid-pupal stage. These spermatids are termed elongating spermatids (Fig. 3C). The majority of spermatids in adult testes are fully elongated, and the cytoplasm is completely discarded, while they are needle-like in shape and immotile (Fig. 3D). Mature motile sperm forming sperm bundles are transferred to and stored in the male seminal vesicles (Fig. 3E). The potential of each spermatid can be examined by injecting them into mature eggs at their anterior end. Injection of elongated spermatids results in production of fertilized embryo (diploid females) as the case in mature sperm injection (Fig. 2A). Round and elongating spermatids never produce fertilized embryos, but participate in development independent of the egg nuclei, resulting in haploid-haploid chimeras (Fig. 2D). In these chimeras, both spermatid-derived and egg-derived nuclei contribute to their germline cells. Premature male gametes have pluripotency, as do mature sperm. These results indicate how injected spermatids participate in development (syngamy with egg nucleus or independent participation), depends on the stages of differentiation.

What are the differences between spermatids and mature sperm that affect the manner of participation in development? One of the marked differences is the condensation of chromatin. The chromosomal proteins, histones, are substituted by basic protamines during spermiogenesis. This substitution occurs soon after the completion of spermatid elongation in *D. melanogaster* (Jayaramaiah-Raja and Renkawitz-Pohl, 2005; Rathke *et al.*, 2007). Mature sperm and elongated spermatids of *A. rosae ruficornis* should have protamine-based, condensed chromatin, while round and elongating spermatids still have histone-based chromatin. It has been demonstrated that round spermatids form pronuclei faster than sperm when they are injected into eggs in murine species (Ogura and Yanagimachi, 1993). Coordination of the timing of male pronucleus formation is critical for synchronization with the female pronucleus to achieve syngamy. Independent participation of round and elongating spermatids in development would be the outcome of precocious pronucleus formation in these spermatids. Organization of centrosomes would also affect the timing of syngamy. Each mature sperm possesses only centrioles since pericentriolar materials are lost during spermiogenesis, whereas oocytes have pericentriolar materials without centrioles. The functional centrosome is usually reconstituted with paternal centrioles and maternal pericentriolar materials after sperm entry (Callaini *et al.*, 1999). On the other hand, spermatids might proceed to syncytial divisions without centrosome reconstitution since they retain functional centrosomes. Another obvious difference is the shape of

tail. Mature sperm and elongated spermatids, both of which are able to achieve syngamy, have fully expanded flagella associated with mitochondrial derivatives (Oda *et al.*, 1995). In the fertilization of drosophilid species, a whole sperm with extraordinary long flagellum enters the egg cytoplasm. Although some post-fertilization functions of the sperm flagellum, such as assisting the male pronucleus to closely juxtapose to female pronucleus have been proposed (Karr, 1996), these functions have not yet been elucidated.

3. Molecular mechanisms of meiotic arrest during oocyte maturation

Premature male gametes have the potential to achieve syngamy with egg nucleus and/or participate in development independent of egg nucleus, as mentioned above. On the other hand, the recipient eggs should be fully matured. Fully matured eggs of *A. rosae ruficornis* await fertilization by arresting meiosis at the metaphase of the first division (MI-arrest), so do other insect eggs. The mechanisms of meiotic metaphase arrest have been studied extensively in vertebrates, in which meiotic arrest generally occurs at the second metaphase (MII-arrest). In contrast, little is known about the molecular mechanisms of meiotic arrest at the first metaphase (MI-arrest) in most invertebrates, including insects. One reason is the scarcity of appropriate organisms in which fertilization or egg activation could be easily manipulated. *A. rosae ruficornis* with the easy manipulation of egg activation serves as a good model representing invertebrates to elucidate the molecular mechanisms of MI-arrest.

The cytoplasmic activity accomplishing metaphase arrest by stabilizing maturation-promoting factor/M-phase promoting factor (MPF) is termed cytostatic factor (CSF) (Masui, 2000). MII-arrest is established and maintained by the consecutive phosphorylation of protein kinases, mitogen-activated protein kinase (MAPK) via MAPK/extracellular signal-regulated protein kinase (MEK). The serine/threonine protein kinase, Mos that is the product of *c-mos* protooncogene has an essential role as an upstream regulator of the MEK-MAPK pathway (Sagata 1996; Kishimoto, 2003; Tunquist and Maller, 2003; Liu *et al.*, 2007). Recently, one of the downstream effectors of the Mos-MEK-MAPK pathway was identified. Emi-related protein1 (Erp1) inhibits the activity of anaphase-promoting complex/cyclosome (APC/C) to keep MPF active during MII-arrest (Shoji *et al.*, 2006; Nishiyama *et al.*, 2007; Inoue *et al.*, 2007) (Fig. 4). In invertebrates, most of the findings on meiotic metaphase arrest have been obtained using a few marine species, such as starfish and jellyfish, and the requirement of the Mos-MEK-MAPK pathway has been shown (Tachibana *et al.*, 2000; Mori *et al.*, 2006; Kondoh *et al.*, 2006).

MI-arrest of insect eggs has been examined in two species, *A. rosae ruficornis* and *D. melanogaster*. We demonstrated that the Mos-MEK-MAPK pathway is also conserved and plays a central role in MI-arrest in *A. rosae ruficornis* (Yamamoto *et al.*, 2008). Both MEK and MAPK are inactive (dephosphorylated) in immature oocytes. These are activated (phosphorylated) in mature (MI-arrested) eggs, and the activity is sustained until eggs are activated. MI-arrest is released upon egg activation, and meiosis proceeds to anaphase I (20 min), metaphase II (40 min), anaphase II (60 min) and is completed 90 min after egg activation (Fig. 5). One of the

four nuclei produced by meiotic divisions migrates inside the egg cytoplasm to become the female pronucleus, while the other three remain on the periphery to become the polar body nuclei that form unique condensed chromosomes. The activities of both MEK and MAPK decrease as meiosis proceeds and disappear within 40 min. When MI-arrested eggs are treated with the chemical inhibitor of MEK, MAPK is inactivated and MI-arrest is released. Thus, MEK activates MAPK as shown in other animals and establishes MI-arrest. Mos orthologue of *A. rosae ruficornis* functions as the upstream regulator of the MEK-MAPK pathway, as

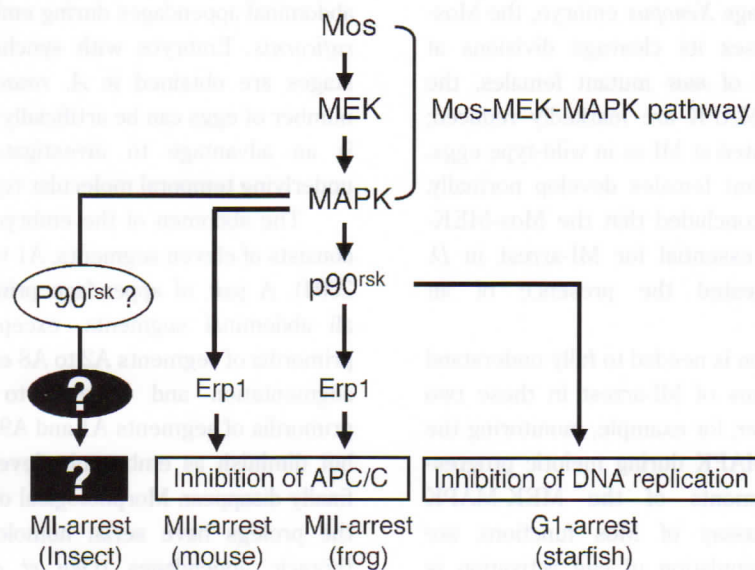


Fig. 4 Regulatory pathway of meiotic cell cycle arrest in animals. The Mos-MEK-MAPK pathway is a consecutive phosphorylation of protein kinases and plays a central role in meiotic metaphase arrest in animal eggs. The downstream effectors are identified in vertebrates and starfish. In vertebrates, MII-arrest is established by inhibition of APC/C with Erp1. p90 ribosomal S6 kinase (p90^{rsk}) is considered to be the factor connecting the Mos-MEK-MAPK pathway and its downstream effector, Erp1 in the frog (*X. laevis*). Although p90^{rsk} functions downstream of the Mos-MEK-MAPK pathway in starfish, it inhibits DNA replication resulting in G1-arrest. Involvement of the Mos-MEK-MAPK pathway in MI-arrest has been shown in two insects, *A. rosae ruficornis* and *D. melanogaster*; however, the downstream effector(s) have not yet been identified.

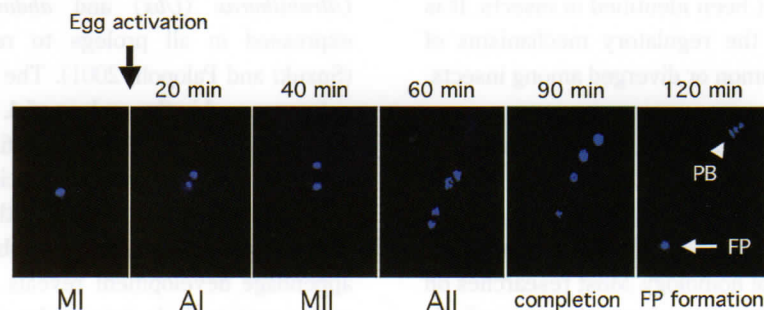


Fig. 5 Progression of meiosis upon artificial egg activation. Chromosomes are visualized with 4', 6-diamidino-2-phenylindole (DAPI) staining. Meiosis is arrested at the first metaphase in a fully matured egg (MI-arrest). Once an egg is activated, meiosis resumes and proceeds to anaphase I (after 20 min of egg activation), metaphase II (40 min), and anaphase II (60 min). Meiosis completes at 90 min after egg activation and four nuclei are produced in egg cytoplasm. One of the four nuclei migrates inside and becomes a female pronucleus and the other three become polar body nuclei remaining periphery. MI: metaphase I, AI: anaphase I, MII: metaphase II, AII: anaphase II. Female pronucleus (FP) and polar body nuclei (PB) are indicated by an arrow and arrowhead, respectively.

shown in the vertebrates and invertebrates examined to date. Injection of Mos protein into activated eggs in which both MEK and MAPK have lost their activities, MEK and MAPK are activated again. In these Mos-injected eggs syncytial divisions are arrested again at metaphase. These findings indicate unequivocally the requirement of the Mos-MEK-MAPK pathway for maintaining MI-arrest.

In contrast, interpretation of the results obtained in *D. melanogaster* using *mos* mutation is different (Ivanovska *et al.*, 2004). Both MEK and MAPK are active in immature oocytes and mature MI-arrested eggs. Cytostatic activity of Mos orthologue of *D. melanogaster* is demonstrated by a biological assay using embryos of *Xenopus laevis*. When *Drosophila* Mos is injected into one blastomere of two-cell-stage *Xenopus* embryo, the Mos-injected blastomere ceases its cleavage divisions at metaphase. In the eggs of *mos* mutant females, the activities of MEK and MAPK are markedly reduced; however, meiosis is arrested at MI as in wild-type eggs. Eggs laid by these mutant females develop normally. Ivanovska *et al.* (2004) concluded that the Mos-MEK-MAPK pathway is not essential for MI-arrest in *D. melanogaster* and suggested the presence of an independent pathway.

Additional information is needed to fully understand the molecular mechanisms of MI-arrest in these two species. In *D. melanogaster*, for example, monitoring the activities of MEK and MAPK during meiotic progression, inhibitory experiments of the MEK-MAPK pathway and *in vivo* assay of Mos functions are necessary. Artificial manipulation of egg activation is indispensable for these analyses. Activation of eggs is possible by artificial means in *D. melanogaster*, while up to 50% of eggs complete meiosis (Mahowald *et al.*, 1983; Horner and Wolfner, 2008). On the other hand, the loss-of-function analyses of Mos, such as *mos* mutant analysis in *D. melanogaster* are required to prove the necessity of the Mos-MEK-MAPK pathway for MI-arrest in *A. rosae ruficornis*. The downstream effectors of the Mos-MEK-MAPK pathway have not been identified in insects. It is still unknown whether the regulatory mechanisms of insect MI-arrest are common or diverged among insects.

4. Model for abdominal appendage development

Diversified larval forms are completed during embryonic development. In particular, appendages are extremely modified, reflecting the life style of species, although they share serial homology. Most researches on appendage development have focused on cephalic (antennae and mouth parts) and thoracic (legs and wings) appendages. Less attention has been paid on abdominal appendages since the absence of abdominal appendages is one of the derived characteristics in insects. Nevertheless, primitive groups of species, such as the

springtails (Collembora), bristletails (Archaeognatha) and silverfish (Thysanura), retain abdominal appendages in adults (Uemiya and Ando, 1987; Palopoli and Patel, 1998; Machida, 1981; Grimaldi and Engel, 2005). Many holometabolous species indeed have abdominal appendages during embryogenesis, and in most cases they disappear before larval hatching. The abdominal appendages in embryos are therefore useful traits to understand morphological evolution (Angelini and Kaufman, 2005). The larvae of *A. rosae ruficornis* have remarkable abdominal appendages, known as prolegs, as well as other members of the symphytan Hymenoptera. Some lepidopteran larvae also have prolegs, whereas they appear in different arrangements in terms of number and distribution. We focus on the development of abdominal appendages during embryogenesis of *A. rosae ruficornis*. Embryos with synchronized developmental stages are obtained in *A. rosae ruficornis* because a number of eggs can be artificially activated at once. This is an advantage to investigate morphogenesis and underlying temporal molecular regulation.

The abdomen of the embryo of *A. rosae ruficornis* consists of eleven segments, A1 to A11 (Yamamoto *et al.*, 2004). A pair of appendage primordia first appears in all abdominal segments, except segment A10. The primordia of segments A2 to A8 elongate without further segmentation and develop to larval prolegs. The primordia of segments A1 and A9 are prominent initially, but diminish as embryonic development proceeds and finally disappear. Morphological observation reveals that the prolegs have serial homology with cephalic and thoracic appendages (Oka *et al.*, 2007). The gene expression pattern of *decapentaplegic (dpp)*, one of the essential upstream regulators of appendage development, supports this idea. *dpp* is expressed in segments where appendage primordia appear, corresponding to the primary determination of embryonic appendages (Yamamoto *et al.*, 2004). It has been demonstrated in two sawfly species that the *Distal-less (Dll)* gene product is absent from the prolegs, and the *Hox* genes, *Ultrabithorax (Ubx)* and *abdominal-A (abd-A)*, are expressed in all prolegs to repress *Dll* expression (Suzuki and Palopoli, 2001). The *Dll* gene orthologue is not expressed in the prolegs of *A. rosae ruficornis* (Oka *et al.*, unpublished results). These findings suggest that the prolegs of hymenopteran species correspond to the proximal parts (coxopodites) of the thoracic legs lacking distal (telopodite) regions. Our observation of embryonic appendage development reveals that the appearance of proleg primordia is delayed by about 15 h compared to those in cephalic and thoracic segments (Oka *et al.*, 2007). The appearance of proleg primordia corresponds to the time when the endites of the mandible (incisor and molar), maxilla (galea and lacinia) and labium (glossa-paraglossa composite) are formed from coxopodite

swellings in each cephalic appendage. In addition, the arrangement of the prolegs seems not to align with those of the cephalic and thoracic appendages, implying that the prolegs are equivalent to the endites of the coxopodites of appendages. Further investigations to figure out the identity of the prolegs of *A. rosae ruficornis* are under way.

The proleg of Hymenoptera corresponds to the coxopodite, whereas that of Lepidoptera corresponds to a whole limb (coxopodite and the distal telopodite) (Suzuki and Palopoli, 2001). In lepidopteran species, *Dll* is expressed in all prolegs to form distal structures. *Ubx* and *abd-A* repress *Dll* expression in the abdominal segments where prolegs are not formed (Panganiban *et al.*, 1994; Warren *et al.*, 1994; Ueno *et al.*, 1995; Suzuki and Palopoli, 2001). It is interesting that the *Hox* genes suppress only telopodites of prolegs in Hymenoptera, whereas they suppress whole prolegs (both coxopodites and telopodites) in Lepidoptera. Moreover, the *Hox* genes do not repress *Dll* expression in the abdominal appendages of the collembolans (Palopoli and Patel, 1998). Although abdominal appendages seem to share direct homology, the underlying molecular regulatory mechanisms for their development are different among different groups of insects. It is hypothesized that derepression of abdominal appendage development has occurred independently in holometabolous insects (Nagy and Grbic, 1999; Suzuki and Palopoli, 2001). It is not yet clear what factors are responsible for the derepression of proleg development, and how they interact with the essential genes involved in appendage development. Unraveling the molecular mechanisms of proleg development in Hymenoptera in which prolegs remain derepressed irrespective of *Hox* gene expression, will offer clues to understand the morphological evolution of the larval form.

A question raised considering morphological evolution in holometabolous insects is whether the molecular mechanisms to form larval and adult appendages are governed by a similar pathway with conserved genes (Tanaka and Truman, 2005; Niimi *et al.*, 2005). Comparative analysis of appendage development in embryos (larval appendage) and pupae (adult appendage) of one species is required. *D. melanogaster* seems to be an inappropriate model from this aspect. The molecular mechanisms of adult appendage development have been extensively studied and well understood in *D. melanogaster*, in which most adult structures are formed from the imaginal discs (Cohen, 1993; Rauskolb, 2001; Kojima, 2004). In contrast, information about appendage development in embryos is limited, since larvae of *D. melanogaster* lack appendages other than the sense organs, such as Keilin's organs (Panganiban, 2000). On the other hand, expression patterns of the genes involved in embryonic appendage development have

been studied in a variety of insects, whereas little is known about the formation of adult appendages during metamorphosis at the molecular level, other than *D. melanogaster* (Angelini and Kaufman, 2005). Recently, molecular patterning mechanisms of adult legs were examined in *Manduca sexta* (Tanaka and Truman, 2007). In Lepidoptera, adult legs are produced within the larval legs from non-imaginal disc-like primordia occupying a small region of larval legs (Svacha, 1992; Tanaka and Truman, 2005; Singh *et al.*, 2007). Tanaka and Truman (2007) demonstrated that some genes involved in patterning of the proximodistal axis in adult legs were not expressed in larval legs during embryogenesis. They concluded that larval legs are formed by transient arrest in the conserved adult leg patterning process. It is considered that adult leg development from imaginal discs observed in Diptera (Cohen, 1993) and the apocritan Hymenoptera (Wheeler and Nijhout, 1981) is the derived mode. In coleopteran species, adult leg primordia are not observed and the entire larval legs contribute to the adult legs (Truman and Riddiford, 2002). The adult legs of *A. rosae ruficornis* are formed from non-imaginal disc-like primordia under the larval leg cuticles (Sawa, personal communication), while the mode of development has not been ascertained in either the Lepidoptera-type or Coleoptera-type. It remains to be elucidated how adult leg development is regulated in *A. rosae ruficornis*, and comparison of the outcome with other holometabolous insects will provide information to understand the evolutionary aspect of larval-adult transition.

5. Development of tools for gene functional analysis

Several genes with critical roles in development and reproduction have been cloned and partially analyzed in *A. rosae ruficornis*, as mentioned above. In addition, the accumulation of genomic information by whole genome sequencing and expressed sequence tag (EST) analyses in insects enables the isolation of orthologous genes of interest relatively easily from *A. rosae ruficornis*. An effective approach for functional studies of these genes is to induce misexpression of the genes, namely loss-of-function and gain-of-function analyses. We have developed molecular tools for interfering with gene function (gene knockdown), introducing exogenous genes to the genome (transgenesis), and regulating expression of integrated genes (binary expression system) in *A. rosae ruficornis*.

RNAi is one of the widely used gene knockdown methods, introducing short double-stranded RNA (dsRNA) of the targeting gene into cells to degrade its transcripts (Fire *et al.*, 1998; Montgomery, 2004). RNAi has been employed in a variety of insects (Tomoyasu *et al.*, 2008). The effectiveness of RNAi in insects was first demonstrated in *D. melanogaster* by the injection

of dsRNA into blastoderms before cellularization (Kennerdell and Carthew, 1998). This method is termed embryonic RNAi since dsRNA is injected into eggs or early embryos. Embryonic RNAi works in *A. rosae ruficornis* (Sumitani *et al.*, 2005). The *white* gene orthologue of *A. rosae ruficornis*, that encodes an ATP-binding cassette membrane transporter responsible for the importation of pigment precursors to eye pigment cells (Ewart *et al.*, 1994), has been isolated and examined. The eye pigmentation of *A. rosae ruficornis* is detectable from the mid-embryonic stage (Lee *et al.*, 1998). Embryos that have been microinjected with dsRNA targeting *white* transcript show the phenocopy of *white* mutation in their eye pigmentation. Interfering effects are caused by the degradation of endogenous *white* mRNA in a dose-dependent manner.

Embryonic RNAi is applied for many insect species, though the interfering effects are not durable to allow analysis in post-embryonic stages. Alternative methods to embryonic RNAi have been developed in the red flour beetle, *Tribolium castaneum* and the ladybird beetle, *Harmonia axyridis*. In these coleopteran species, injection of dsRNA into larval hemocoel effectively interferes with the targeted gene functions in the entire body of pupae and adults (larval RNAi) (Tomoyasu and Denell, 2004; Niimi *et al.*, 2005). The method termed parental RNAi, in which dsRNA is injected into hemocoel of female pupae, resulting in gene silencing in their embryos, has been developed in *T. castaneum* (Bucher *et al.*, 2002). In each case of RNAi, dsRNA injected into extracellular regions is taken up into cells. Systemic uptake of dsRNA occurs in virtually all tissues in *T. castaneum* (Miller *et al.*, 2008). Larval RNAi is one of the desirable methods for *A. rosae ruficornis* to knock down the *mos* gene and the genes involved in the formation of adult appendages, such as *dpp* and *Dll*. *mos* is expressed during oogenesis in nurse cells of pupal ovaries (Yamamoto *et al.*, 2008). *dpp* and *Dll* are expressed during not only embryogenesis, but also metamorphosis (Yamamoto *et al.*, 2004; Oka *et al.*, 2007). We are now working on establishing larval RNAi using a transgenic strain in which the *green fluorescent protein (GFP)* gene is constitutively expressed (Sumitani *et al.*, 2003).

An important prerequisite for gain-of-function analyses is a method to introduce exogenous genes into individuals. Transgenesis that integrates the gene of interest into the host genome is the most favorable technique. The initial success of insect transgenesis has been demonstrated in *D. melanogaster* using a transposon, the *P* element (Rubin and Spradling, 1982), while *P* element-derived vectors do not work outside of drosophilid species. Broadly applicable systems for insect transgenesis have been established using vectors derived from transposons other than *P* element and marker fluorescent protein genes driven by an artificial

promoter, 3xP3 (three tandem repeat of the Pax6 binding site: P3) (Horn and Wimmer, 2000; Horn *et al.*, 2002). Among the transposon-based vectors, *piggyBac*-derived vectors are the most promising, and transgenesis has been achieved in more than 20 insect species (Fraser, 2000; Handler, 2002; Condon *et al.*, 2007). *A. rosae ruficornis* is one of these, and the only species in Hymenoptera (Sumitani *et al.*, 2003). As shown in Figure 6, a *piggyBac*-derived transformation vector carrying a gene of interest and a 3xP3-driven fluorescent protein gene, and a helper plasmid producing *piggyBac* transposase are injected together into mature eggs. These eggs are allowed parthenogenetic development to male adults, and crossed with females. About 5% of these males produce transgenic offspring as revealed by marker fluorescent exhibition. The 3xP3-driven fluorescent protein genes are detectable in mid-embryos, pupae and adults. Each fluorescent protein gene marker could be used to distinguish the respective transgenic strains. Once transgenesis becomes feasible, another issue would arise: how to maintain a large number of transgenic strains to be established with a minimal effort. We have an answer in *A. rosae ruficornis*. Artificial fertilization by ICSI employing cryopreserved sperm, instead of intact sperm, is practicable (Hatakeyama *et al.*, 1994b). It has been demonstrated that the transgene is stably inherited when sperm from transgenic males are frozen, thawed and used for ICSI. The transgene remains where it has been originally inserted (Hatakeyama and Sumitani, 2005).

Transgenesis is indeed the powerful tool for gain-of-function analysis, while it is not sufficient by itself for gene functional studies. Systems to regulate the expression of transgenes are required; however, there is the issue of the scarcity of endogenous promoters to drive spatial and temporal gene expression in *A. rosae ruficornis* as well as other non-model insects. One solution is to use well-characterized heterologous promoters, such as the *heat-shock protein 70 (hsp70)* gene promoter of *D. melanogaster*. The *hsp70* gene promoter works in *A. rosae ruficornis*, while it is not able to regulate gene expression strictly, but rather induces ubiquitous expression without heat-shock (Sumitani *et al.*, 2003). Then, binary expression systems in which gene expression is induced by crossing two separate transgenic strains (transactivator and responder) will overcome the issue of directed gene expression (McGuire *et al.*, 2004; Viktorinova and Wimmer, 2007). Binary expression systems also enable functional analysis of the genes that affect viability and fertility. A system based on the yeast transactivator Gal4 and its upstream activating sequence (UAS) is the well-known Gal4/UAS system commonly used in *D. melanogaster* (Brand and Perrimon, 1993). This popular system still requires specific promoters to direct the expression of

the transactivator. In contrast, specific promoters are not always necessary in the system based on a bacterial tetracycline-resistance operon, known as the Tet-Off system. The tetracycline-controlled transactivator (tTA) promotes gene expression by binding to the tTA-response element (TRE) placed upstream of a targeted gene (Gossen and Bujard, 1992). The advantage of this system is that the expression of the targeted gene can be negatively regulated by the application of tetracycline. Tetracycline forms a complex with tTA and the complex prevents tTA from binding to TRE, keeping the targeted gene unexpressed (Fig. 7).

Our preliminary results suggest that the Tet-Off system works in *A. rosae ruficornis*, although it seems to be limited to gene functional analysis during embryonic stages (Hatakeyama, *et al.*, 2007). The transactivator strain bearing the *hsp70* gene promoter-driven tTA and the responder strain bearing the reporter *enhanced GFP* (*EGFP*) gene placed downstream of TRE are produced. The respective strains are marked with different fluorescent protein genes driven by 3xP3 for differentiation. The tTA is expressed constitutively regardless of

heat-shock, as mentioned above. The reporter *EGFP* gene is expressed only in individuals carrying both transgenes (tTA and TRE-*EGFP*) produced by crossing the two strains. *EGFP* fluorescence is detectable from the mid-embryonic stage and thereafter. The system induces gene expression in the absence of tetracycline. Expression of the reporter *EGFP* gene can be suppressed when tetracycline is orally supplemented to the parental mothers of either strain; unfortunately however, this suppression does not continue to post-embryonic stages.

Although the Tet-Off system is very useful, promoters and enhancers of known expression patterns are indispensable to control the spatiotemporal expression of the targeted genes. A transgenesis-based enhancer trap has been developed in *D. melanogaster* (Bellen *et al.*, 1989) and improved for application in non-drosophilid species with a combination of *piggyBac*-mediated method and binary expression system (Horn *et al.*, 2003). The improved enhancer trap could immediately be applicable to *A. rosae ruficornis*. When promoters and enhancers available for targeted gene

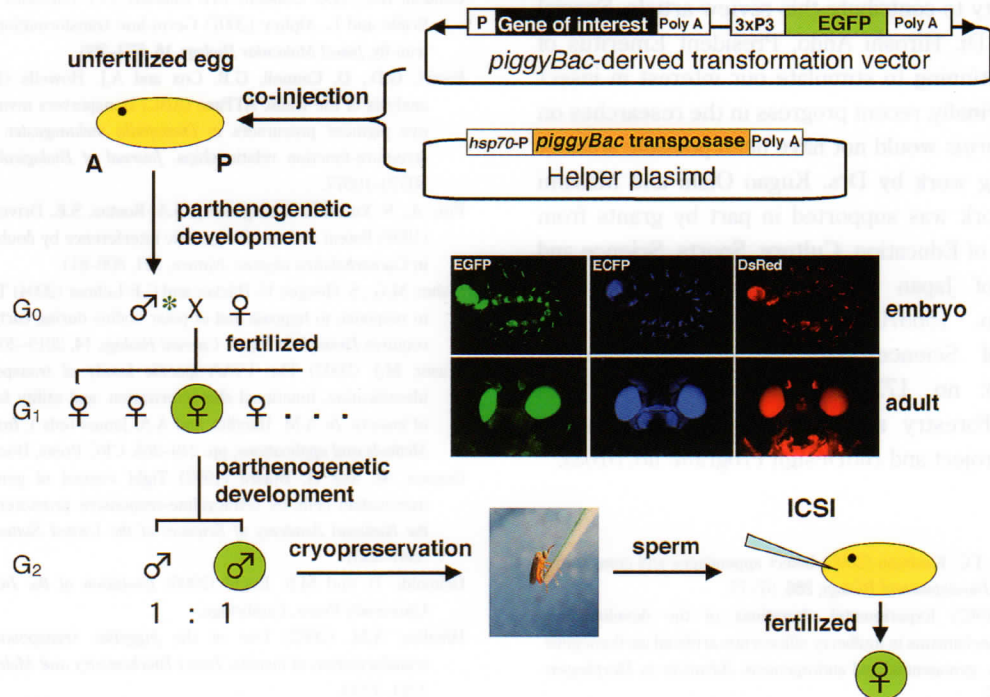


Fig. 6 Schematic presentation of the methods of germline transformation and preservation of transgenic strains. Two kinds of plasmids are prepared. One is a *piggyBac* transposon-based vector plasmid carrying a gene of interest and a 3xP3-driven marker fluorescent protein gene inserted between inverted terminal repeat (ITR) sequences. The other is a helper plasmid producing the *piggyBac* transposase. They are injected together into the posterior end of mature unfertilized eggs dissected from female adults. Injected eggs are allowed parthenogenetic development to become haploid males (G₀). Some G₀ males produce a fraction of sperm of which genome a transgene is integrated (germline chimeras). When the G₀ male is a germline chimera (asterisk in green) bearing a transgene, some of his daughters (G₁) show the expression of marker fluorescent protein gene (green circle). Middle panels show examples of transgenic sawflies expressing various marker fluorescent protein genes in embryo and adults. Transgenic males of the G₂ generation and thereafter can be cryopreserved by freezing directly in liquid nitrogen and stored at -80°C . Upon microinjection of sperm taken from thawed transgenic males into eggs, fertilized diploid females bearing the transgene are recovered.

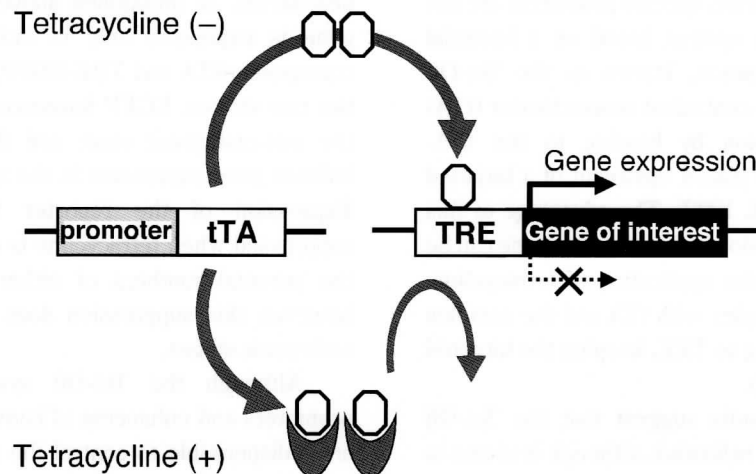


Fig. 7 Tet-Off binary expression system. In the absence of tetracycline, the targeting gene ("Gene of interest") is expressed by binding of the transactivator (tTA: open hexagon) to its response element (TRE) placed upstream of the targeting gene. In the presence of tetracycline, a complex of tetracycline (solid cup) and tTA is formed. The tetracycline/tTA complex is unable to bind to TRE, so that the targeting gene is not expressed.

expression are obtained, gene functional analysis in *A. rosae ruficornis* will advance significantly.

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