DOI:10.6661/TESFE.2013017 台灣昆蟲Formosan Entomol. 33: 237-252 (2013) 研究報告 Research report

Formosan Entomologist
Journal Homepage: entsocjournal.yabee.com.tw

Appum, a Drosophila Homolog of pumilio in the Pea Aphid: Cloning, Developmental Expression, and Presumptive Roles in Posterior Patterning【Research report】

Appum：果蠅 pumilio 同源基因在豌豆蚜之選殖、發育表現、參與胚胎後端形塑之角色探討【研究報告】

Chen-yo Chung1,3, Jou-han Chen1, and Chun-che Chang1,2,3,4*
鍾成侑 1,3、陳柔含 1、張俊哲 1,2,3,4

*通訊作者E-mail : chunche@ntu.edu.tw

Received: 2014/01/04 Accepted: 2014/01/22 Available online: 2014/02/01

Abstract

Gradients of morphogens act as positional information to set the body plan during early development in many animals. Formation of a morphogen gradient usually relies on translational control of mRNA. During early embryogenesis in Drosophila, for example, translational repression of maternal hunchback (hb) is mediated by the 3′ untranslated region (UTR) bound by the complex of Nanos (Nos) and Pumilio (Pum). The anteroposterior gradient of Hb thus forms in the syncytium because a reverse gradient of Nos is established from posterior to anterior. Apart from Drosophila, the Nos/Pum-mediated translational repression has been identified in other animal models. We thus assume that this mechanism is also conserved in the asexual pea aphid Acyrthosiphon pisum. In this study we cloned Appum, a Drosophila homolog of pum in the pea aphid, and analyzed its developmental expression in asexual oocytes and embryos. Whole-mount in situ hybridization showed that three isoforms of Appum mRNA were evenly expressed throughout oogenesis and embryogenesis, suggesting that function of Appum mRNA does not dependent on asymmetric localization or local signal induction. Combining the known expression patterns of Aphb/ApHb and Apnos/ApNos, we proposed potential roles of ApPum in posterior patterning of the asexual pea aphid. Taken together, we expect that this study can pave a path for functional assay of ApPum in the future.

摘要

已知形態發生素 (morphogens) 之濃度梯度為許多動物早期胚胎發育所使用之位置訊息。此一梯度之建立，端賴聚集在胚胎當中的信使核糖核酸 (mRNA) 轉譯是否受到準確的抑制調控。以果蠅早期胚胎發育為例，抑制母系 hunchback (hb) mRNA之轉譯乃透過 Nanos (Nos) 及 Pumilio (Pum) 這兩個蛋白質所組成的複合體，結合hb mRNA 的3′ 非轉譯區 (3′ untranslated region, 3′ UTR) 所致。由於 Nos 在合胞囊胚 (syncytial blastoderm) 的後端濃度最高，因此 hb 轉譯抑制之強度也由後端至前端逐次遞減，與 Nos 之梯度反向。這樣一來，Hb 蛋白的濃度梯度遂由前到後被建立起來。除了果蠅，以 Nos/Pum 複合體來抑制基因轉譯之機制也被發現在其它的模式動物當中。因此，我們推測豌豆蚜亦保有此一機制，並於其中選殖到果蠅 pumilio的同源基因 Appum，以及運用全體原位雜合技術偵測 Appum mRNA 在無性世代卵、胚的發育表現。實驗結果顯示：在卵與胚胎發育的過程中 Appum 的三個 mRNA異構物皆呈現均勻分布之現象。由此推測，Appum 之功能並不倚賴 mRNA 的非對稱聚集，或藉由局部訊息誘導使mRNA 集中。結合先前所發現之Aphb/ApHb 與Apnos/ ApNos 發育表現結果，我們剖析了 ApPum 蛋白在胚胎後端形成過程所可能扮演的角色。希冀上述針對 Appum 所進行之實驗和討論，能為日後 ApPum 之功能解析奠定重要基礎。

Key words: embryogenesis, gene isoforms, in situ hybridization, parthenogenetic viviparity, translational repression

關鍵詞: 胚胎發育、基因異構物、原位雜合、孤雌胎生、轉譯抑制。

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Appum, a *Drosophila* Homolog of *pumilio* in the Pea Aphid: Cloning, Developmental Expression, and Presumptive Roles in Posterior Patterning

Chen-yo Chung[1,3], Jou-han Chen[1], and Chun-che Chang[1,2,3,4*]

[1] Laboratory for Genetics and Development, Department of Entomology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan
[2] Institute of Biotechnology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan
[3] Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan
[4] Genome and Systems Biology Degree Program, National Taiwan University, Taipei, Taiwan

ABSTRACT

Gradients of morphogens act as positional information to set the body plan during early development in many animals. Formation of a morphogen gradient usually relies on translational control of mRNA. During early embryogenesis in *Drosophila*, for example, translational repression of maternal *hunchback* (*hb*) is mediated by the 3' untranslated region (UTR) bound by the complex of Nanos (Nos) and Pumilio (Pum). The anteroposterior gradient of Hb thus forms in the syncytium because a reverse gradient of Nos is established from posterior to anterior. Apart from *Drosophila*, the Nos/Pum-mediated translational repression has been identified in other animal models. We thus assume that this mechanism is also conserved in the asexual pea aphid *Acyrthosiphon pisum*. In this study we cloned *Appum*, a *Drosophila* homolog of *pum* in the pea aphid, and analyzed its developmental expression in asexual oocytes and embryos. Whole-mount *in situ* hybridization showed that three isoforms of *Appum* mRNA were evenly expressed throughout oogenesis and embryogenesis, suggesting that function of *Appum* mRNA does not dependent on asymmetric localization or local signal induction. Combining the known expression patterns of *Aphb*/ApHb and *Apnos*/ApNos, we proposed potential roles of ApPum in posterior patterning of the asexual pea aphid. Taken together, we expect that this study can pave a path for functional assay of ApPum in the future.

Key words: embryogenesis, gene isoforms, *in situ* hybridization, parthenogenetic viviparity, translational repression

*Corresponding email: chunche@ntu.edu.tw

Introduction

Gene expression can be regulated via transcriptional and translational control during animal development (Sonenberg *et al.*, 2000; Lagha *et al.*, 2012). In the fly *Drosophila melanogaster* (Diptera: Drosophilidae), maternal mRNA is usually synthesized in the nurse cells and then transported to the developing oocytes during oogenesis (Cummings *et al.*, 1971). It has been clear that the asymmetric localization of *bicoid* (*bcd*) mRNA to the anterior and *oskar* (*osk*) mRNA to the posterior in the oocytes specifies the anterior-posterior (AP) polarity of the egg as well as the future AP axis of the embryo (Lehmann and Nüsslein-Volhard, 1986; Berleth *et al.*, 1988; Kim-Ha *et al.*, 1991). After fertilization, translation of *bcd* mRNA forms an AP gradient of the Bcd protein, which thereby can activate the transcription of *hunchback* (*hb*) and inhibit the translation of *caudal* (*cad*) (Driever and Nüsslein-Volhard, 1988; Driever and Nüsslein-Volhard, 1989; Struhl *et al.*, 1989). In the posterior pole, meanwhile, translation of *nanos* (*nos*) mRNA whose posterior anchorage relies on the maternal Osk forms an opposing, namely, the posterior-anterior (PA) gradient (Ephrussi *et al.*, 1991; Wang and Lehmann, 1991). Consequently, gradients of Bcd, Hb, Cad and Nos act as positional information to orchestrate proper patterning along the AP body axis.

In the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae), by contrast, homologous sequences of *Drosophila bcd* and *osk* cannot be identified and how the AP axis is established is still poorly understood (The International Aphid Genomics Consortium, 2010; Shigenobu *et al.*, 2010). Previously we found that *hb* mRNA (*Aphb*) was localized to the anterior pole of the developing oocytes and early embryos undergoing nuclear cleavage—the syncytial blastoderm (syncytium; syncytia for plural form)—in the parthenogenetic and viviparous (asexual) pea aphid (Huang *et al.*, 2010). This implicates that anterior localization of *Aphb*, a reminiscence of the anteriorly-localized *bcd* in *Drosophila*, may specify the anterior axis in the pea aphid. However, neither *nos* (*Apnos*) nor *cad* (*Apcad*) can be localized to the posterior region - the transcripts of *Apnos* are evenly distributed and those of *Apcad* cannot even be detected prior to blastulation (Chang *et al.*, 2009; Chang *et al.*, 2013). Hence, if the asexual pea aphid also employs asymmetric localization of mRNAs to establish the posterior axis, apparently it adopts distinct molecules that exclude transcripts of *Apnos* and *Apcad*. At the protein level, nonetheless, we have identified posterior localization of Nos and Vasa (Vas) in the egg posterior from embryogenesis onward. After cellularization of the blastoderm, Nos and Vas are incorporated within the primordial germ cells and then enter into the germline cell fate (Chang *et al.*, 2006). Accordingly, Nos and Vas are components of the preformed germ plasm assembled in the posterior syncytia (Saffman and Lasko, 1999; Extavour and Akam, 2003) but whether they are involved in the specification or maintenance of the posterior axis remains an open question.

If Nos plays a role in posterior development in the asexual pea aphid, we assume that it requires an interaction with Pumilio (Pum) to suppress the translation of anterior genes in the egg posterior. Our hypothesis is based on a fact that the direct interaction between Nos and Pum for translational repression is highly conserved in eukaryotic cells (Wickens *et al.*, 2002; Wharton and Aggarwal, 2006). The Pum proteins belong to the Pumilio/Fem3-binding protein (PUF) family, whose sequences comprise the Pumilio-homology domain (PUM-HD) for binding RNA targets. From yeast to human, more and more evidence indicates that PUF proteins repress the translation of target mRNAs via recruiting deadenylases

for shortening and removing the poly(A) tail of mRNA. Lack of the poly(A) tail will thus inhibit the initiation of translation and induce the degradation of mRNA (Macdonald, 1992; Wreden *et al.*, 1997; Zamore *et al.*, 1997; Zhang *et al.*, 1997; Subramaniam and Seydoux, 1999; Jaruzelska *et al.*, 2003). For example, in *Drosophila* the self-renewal of germline stem cells (GSCs) requires Nos/Pum-mediated translational repression of *mei-P26* mRNA by recruiting the CCR4 deadenylase. As a consequence, products of *mei-P26* fail to promote the differentiation of GSCs (Joly *et al.*, 2013).

In this study, we cloned and characterized the developmental expression of a *Drosophila* homolog of *pum* (*Appum*) in the pea aphid. In the asexual pea aphid, we detected the expression of *Appum* in oogenesis and embryogenesis, both of which take place consecutively within the ovarian tubule (ovariole). We also analyzed the expression of *Appum* in sexual ovarioles, comparing expression patterns of *Appum* in oogenesis of both asexual and sexual morphs. Besides proposing potential roles of *Appum* during development, we expect that all of our efforts presented in this study can serve as important foundations for making antibody against ApPum as well as functional assay of *Appum* in the future.

Materials and Methods

Pea aphid

The local strain of parthenogenetic and viviparous pea aphids feeding on pea plants (*Pisum sativum*) were reared at 20°C under a 16 h light/8 h dark photoperiod. The dissected ovaries of sexual pea aphids (ApL strain) were provided by the Core Research Facilities of the National Institute for Basic Biology in Japan. Induction and maintenance of the sexual aphids were described in Ishikawa *et al.* (2012). Staging of aphid embryogenesis followed Miura *et al*. (2003).

Cloning and sequence analysis of *Appum*

Total RNA of pea aphids was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out with poly dT_{18} primers and the HiScript I Reverse Transcriptase (Bionovas Biotechnology, Toronto, Canada). Reactions of RNA purification and reverse transcription followed manufacturers' protocols. Polymerase chain reaction (PCR) for amplifying DNA sequences of *Appum* upstream the 3' untranslated region (3' UTR) was performed using a pair of gene-specific primers (forward primer: 5'-CTACAGTGGTGCCG CAATACTATGG-3', encoding PTVVPQYYG; reverse primer: 5'-TTAAAGCACTCCGTTT GTAGGAGGTC-3', encoding GPPTNGVL) and the *Taq* polymerase (Bioman, Taipei, Taiwan). Conditions of PCR: in a total volume of 10 μL, reaction was conducted at 94°C for 5 min, followed by 40 cycles (94°C for 30 s, 59°C for 30 s, 72°C for 1 min), and 72°C for 10 min. Sequence of the PCR primers was deduced from that of the annotated *Appum* gene (GenBank accession number: XM_001950613.2). Primer design and sequence alignment relied on a calculation by the MacVector version 7.2.2 (Accelrys Inc., San Diego, CA, USA). PCR amplicons were then subcloned into the T&A cloning vector (RBC Bioscience, Taipei, Taiwan) for plasmid amplification and sequencing.

Synthesis of riboprobes

Three riboprobes against coding sequences of distinct numbers of exons were synthesized: (1) probe 1, E10-13 (forward primer: 5'-CTACAGTGGTGCCG CAATACTATGG-3'; reverse primer: 5'-AA CCACCCAACGATGAAGCAC-3'); (2) probe 2, E12 and 13 (forward primer: 5'-ATCTC TAACCCCTCCACCAG-3'; reverse primer: 5'-TCTCTGACACCAACTGTTTC-3'); and (3) probe 3, E11 (forward primer: 5'-ACC CAGTACCAGGTCATAC-3'; reverse primer: 5'-ATGGACGGTGAAAAAGCGG-3'). Sequences

of these three amplicons were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) for *in vitro* transcription. Linearized plasmids containing sequences of *Appum* probes 1-3 were used as templates for the synthesis of sense and antisense digoxigenin (DIG)-labeled riboprobes using the DIG RNA Labeling Kit (SP6/T7; Roche, Basel, Switzerland). Synthesis of the antisense riboprobe of *Apvasa* (*Apvas1*) followed Chang *et al.* (2007).

Whole-mount *in situ* hybridization (WISH) and microscopy

We fixed the dissected ovarioles and performed the WISH experiments according to the protocols described in Chang *et al.* (2008). For a probe hybridization, working concentration of probes—including sense and antisense probes—ranged from 1.5 to 2.0 ng/μL, and the hybridization temperature was 68°C. After staining the bound probes with the alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche), we developed *in situ* signals using the AP substrate Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche). Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI; 2 ng/μL) (Sigma, St. Louis, MO, USA) for 2 h at room temperature in dark. A Leica DMR (Leica, Wetzlar, Germany) connected to a Canon EOS 5D MarkII digital camera (Canon, Tokyo, Japan) was used to capture Nomarski images. Images of DAPI staining were taken using a LSM 510 META laser-scanning microscope (Carl Zeiss, Jena, Germany).

Results

Cloning and characterization of *Appum*

Based upon the annotated sequence of *pumilio* (*Appum*) in the AphidBase (http://www.aphidbase.com), we designed a pair of primers that could amplify the coding sequence upstream the 3' untranslated region (3' UTR) of the *Appum* mRNA (Fig. 1A). Three amplicons with different sizes of 1,804, 1,322, and 996 base pairs (bp), surprisingly, were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR). Predicted length of the open reading frame of *Appum* in the AphidBase is 3,456 bp; however, we have not been able to verify the sequence upstream the longest 1,804-bp amplicon. Sequence alignment using the Basic Local Alignment Search Tool (BLAST) shows that amino acids encoded by these three amplicons had best similarity with insect Pum proteins: most similar to that of *Tribolium castaneum* (red flour beetle; similarity: 78%) and *Pediculus humanus* (body louse; similarity: 75%). Given that there is only one *pum* gene (*Appum*) being identified in the pea aphid genome (The International Aphid Genomics Consortium, 2010; Shigenobu *et al*., 2010), these three amplicons are likely derived from alternatively spliced mRNAs, rather from 3 gene loci, of the *Appum* gene.

Alignment of the nucleotide sequences of *Appum* isoforms 1-3 (*Appum* iso1-3) shows that they have 6 exons in common. Alternative splicing was not identified in the longest isoform (*Appum* iso1, 1,804 nucleotides (nt)) as it contained sequences of all 9 exons within this region (exons 10-18 (E10-18) out of the total 18 exons containing 3,456 nt). However, there are 2 (E12 and E13) and 3 (E11-13) exons missing in *Appum* iso2 and *Appum* iso3, respectively. This suggests that alternative splicing occurs to these two *Appum* isoforms (Fig. 1A). Like other Pum proteins, amino acid sequences encoded by communal exons of *Appum* iso1-3 (E14-18) contain a conserved Pumilio-homology domain (PUM-HD) with 8 tandem repeats and 2 flanking regions. Every repeat domain is contiguous with each other except a spacer containing 7 amino acids that is located between repeats 7 and 8. There are 24 amino acids in the repeat 6 in the zebrafish Pum protein, but it is not seen in ApPum as well as other Pum proteins. Within each repeat domain of the pea

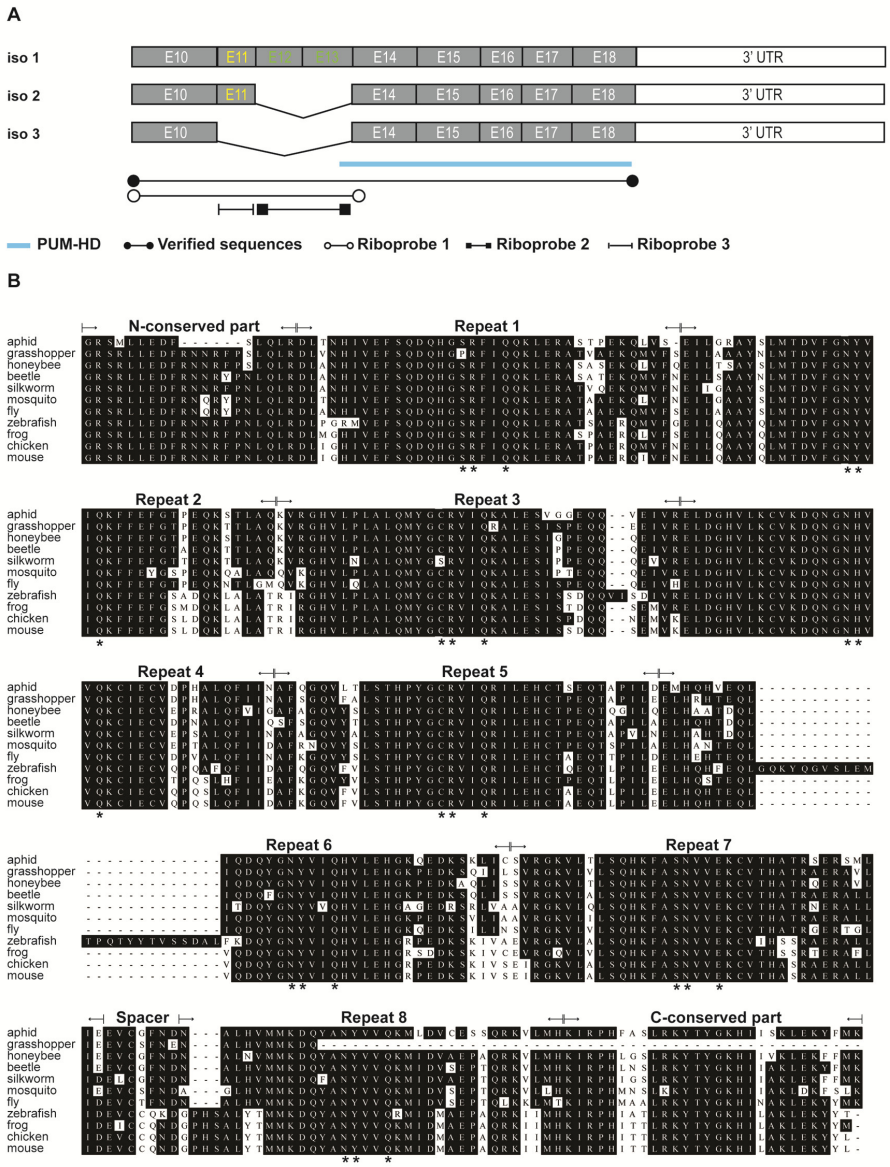

Fig. 1. Sequence characterization of the *Acyrthosiphon pisum pumilio* (*Appum*) gene. (A) Alignment of coding sequences of the *Appum* isoforms 1-3 (iso 1-3). Nine exons (E10-18) upstream the 3' UTR of *Appum* are aligned. Size of exons: E10, 273 nt; E11, 140 nt; E12, 168 nt; E13, 184 nt; E14, 229 nt; E15, 228 nt; E16, 163 nt; E17, 169 nt; E18, 250 nt. Lengths of exonal boxes presented in this figure are proportional to the exon sizes; box of the 3' UTR (2,802 nt) is not presented in proportion. Regions of verified sequence, probes 1-3, and sequence encoding PUM-HD are highlighted. (B) Alignment of the amino acid sequences of PUM-HD in ApPum as well as other 10 Pum proteins. All PUM-HDs contains 8 repeats and 2 conserved parts flanking the N and C termini of the PUM-HD. Sequences identical more than 6 amino acids are marked in black boxes. Asterisks indicate the conserved amino acid residues known to bind the target RNAs. Accession numbers of Pum proteins: beetle, EFA12956.1; chicken, NP_001012858.1; fly, NP_731314.1; frog, BAC57980.1; grasshopper, AAO38522.1; honeybee, XP_391849.4; mosquito, XP_001656036.1; mouse, NP_109647.2; silkworm, BAI77478.1; zebrafish, NP_001264048.1.

aphid PUM-HD, we could identify 3 conserved amino acids residues known to contact with target RNAs in *Drosophila* and human (Fig. 1B) (Zamore *et al.*, 1997; Wang *et al.*, 2002), implicating that ApPum may bind similar RNA sequences as those bound by the *Drosophila* Pum protein.

Expression of *Appum* mRNA during early embryogenesis

We synthesized 3 antisense riboprobes against *Appum* iso1-3: (1) probe 1: complementary sequence of E10-13; (2) probe 2: complementary sequence of E12 and 13; and (3) probe 3: complementary sequence of E11 (Fig. 1A). Consequently, we expected that: (1) probe 1 could detect expressions of *Appum* iso1-3 because its sequence comprised E10—a communal exon of these three isoforms; (2) probe 2 could only label the transcripts of *Appum* iso1 because E12 and 13 were *Appum* iso1-specific; and (3) probe 3 could monitor expressions of *Appum* iso1 and iso2 as E11 was shared by these two isoforms. Whole-mount *in situ* hybridization (WISH) showed that expression patterns generated by probes 1-3 were very similar to each other (compare Figs. 2, 3 and 4A-D with Fig. 4E). Because probe 1 provided the best intensity of *in situ* signals, we thus presented WISH results generated by probe 1 in this study.

For most insects that undergo sexual reproduction, eggs mature in the ovarioles and embryos develop in the laid eggs after fertilization. The parthenogenetic and viviparous (asexual) pea aphids, on the contrary, accommodate embryos in assembly line fashion in the ovarioles—an outcome of "embryogenesis within oogenesis" (Blackman, 1978; Miura *et al.*, 2003). It thus provides an excellent platform for consecutive observation of gene expression throughout embryogenesis in a single ovariole. In the germarium, a pear-shape structure located in the anteriormost region of the telotrophic ovariole, expression of *Appum* mRNA was identified in the nurse cells as wells as in the germarial lumen (Fig. 2A-C). Enrichment of transcripts of *Appum* was visualized in the germarial lumen as well as in the anterior area of the developing oocyte (Fig. 2A, C), suggesting that *Appum* mRNA was first aggregated to the germarial lumen and then transported to the oocyte anterior through the trophic cord (Fig. 2C). Nevertheless, unlike the anterior localization of *hunchback* mRNA (*Aphb*) in oocytes and early embryos (Huang *et al.*, 2010), preferential expression of *Appum* was not identified in the anterior part of egg chambers undergoing nuclear cleavage (Fig. 2D) and cellular proliferation (Fig. 2E, F). This indicates that transcripts of *Appum* may be diluted by the expanding volume of egg chambers.

In the asexual pea aphid, it has been clear that segregation of germ cells occurs soon after blastoderm formation (Fig. 2E, E') and later the endosymbionts invade into the egg chamber during blastoderm invagination (Chang *et al.*, 2006; Chang *et al.*, 2007) (Fig. 2F, F'). We did not identify preferential expression of *Appum* in the newly segregated germ cells (Fig. 2E) and embryonic germ cells in other developmental stages (Figs. 3 and 4). In the endosymbionts, *Appum* expression was devoid of staining throughout embryogenesis (Fig. 2F; Figs. 3 and 4). In ovarioles stained using the sense riboprobe of *Appum*, we did not identify the expression signals (Fig. 2H); in ovarioles probed with the antisense riboprobe of *Apvas1*, germ cells were specifically marked (Fig. 2G). Taken together, it shows that WISH experiments were carried out under optimal condition and that expression patterns are *Appum*-specific. Preferential expression of *Appum* in germarial lumina and anterior region of the oocytes is unlikely an artifact owing to the stacking of *Appum* iso1-3 transcripts, because the same expression pattern was also identified in the WISH results of probe 2,

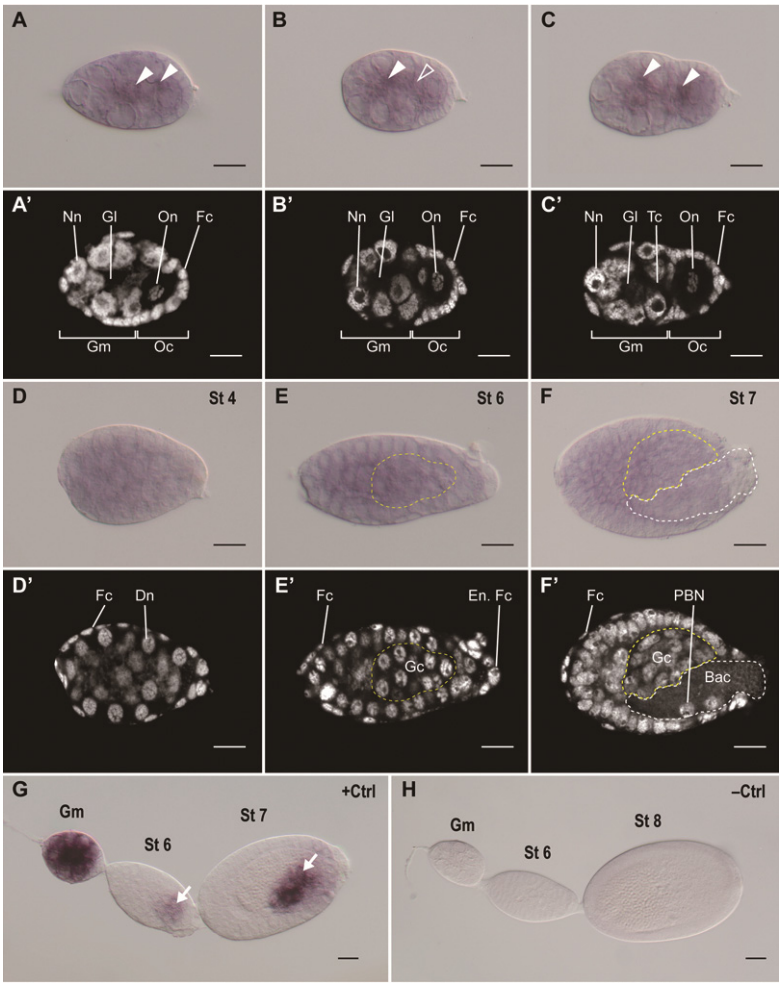

Fig. 2. Expression of *Appum* mRNA in germaria, oocytes and early embryos of the asexual pea aphid. Dissected ovarioles were stained with antisense riboprobes of *Appum* (A-F)/*Apvas1* (G), and sense riboprobe of *Appum* (H). Unless particularly addressed, WISH of *Appum* presented in this figure as well as other figures are performed using probe 1. Anterior of germaria and egg chambers is to the left. (A'-F') are nuclear staining of (A-F). (A-C) Expression of *Appum* during oogenesis. Degree of oocyte protrusion from the germarium increases from (A) to (C) - in (C), the oocyte is fully segregated. Arrowheads indicate preferential expression of *Appum* in germarial lumina and anterior region of oocytes. In (B), aggregation of *Appum* in the oocyte anterior is not obvious (hollow arrowhead). (D-F) Expression of *Appum* during early embryogenesis. (D) Stage 4 (syncytial blastoderm). Dividing nuclei are located in the inner periphery of the egg chamber. (E) Stage 6 (cellular blastoderm). (F) Stage 7 (invasion of the endosymbionts). Germ cells (yellow dashed line) containing germ granules and endosymbionts (white dashed line) are morphologically identifiable from stage 6 and 7, respectively. *Appum* was uniformly expressed in embryos, including somatic and germ cells. (G) Positive control. Germ cells were specifically labeled by the antisense riboprobe of *Apvas1* (arrows). From (A) to (F), follicle cells were devoid of staining. (H) Negative control. No signals were detected. Abbreviations: +Ctrl, positive control; -Ctrl, negative control; Bac, bacteria; Dn, dividing nuclei; En. Fc, enlarged follicle cells; Fc, follicle cells; Gc, germ cells; Gl, germarial lumen (plural form: germarial lumina); Gm, germarium (plural form: germaria); Nn, nuclei of the nurse cells; Oc, oocytes; On, oocyte nucleus; PBN, presumptive bacteriocyte nuclei; St, stage; Tc, trophic cord. Scale bars, 20 μm.

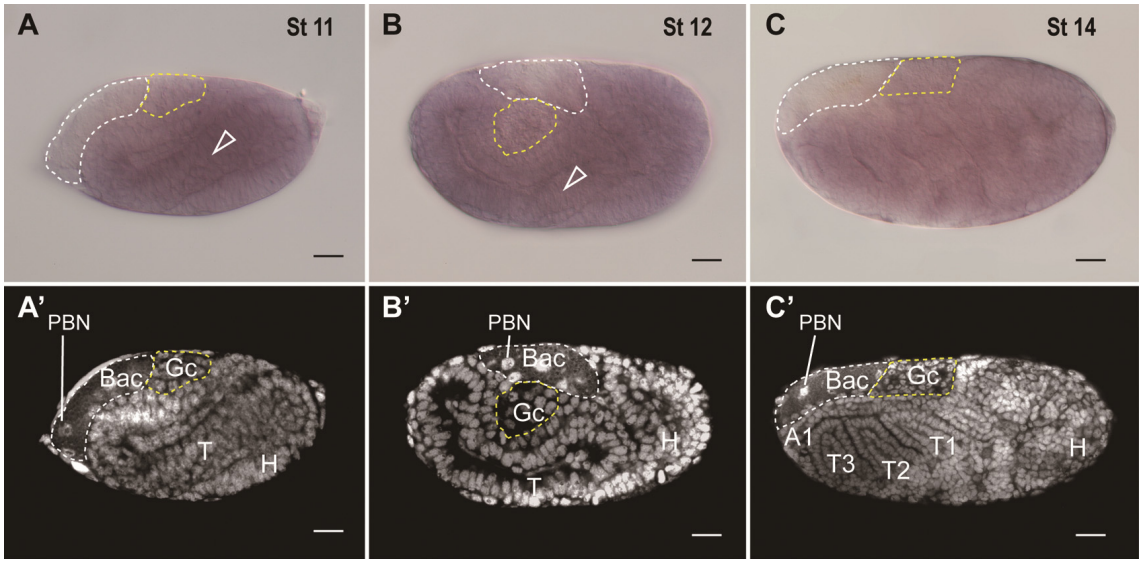

Fig. 3. Expression of *Appum* mRNA during mid embryogenesis of the asexual pea aphid. Elongation of the germ band from stages 11-14 of development prior to katatrepsis is presented. Anterior of the egg chamber is to the left whereas anterior of the embryos before katatrepsis is to the right. Dorsal is upper. Germ cells and endosymbionts are labeled with yellow and white dashed line, respectively. (A'-C') are nuclear staining of (A-C). (A) Stage 11 (S-shaped embryo). (B) Stage 12 (twisting embryo). (C) Stage 14 (extended germ band). Transcripts of *Appum* were evenly distributed in germ band and germ cells. Endosymbionts were devoid of staining. Owing to the thickening of tissues, signal intensity appeared higher in the thoracic region (hollow arrowheads). Abbreviations: A1, abdominal segment 1; Bac, Bacteria; Gc, germ cells; H, head; PBN, presumptive bacteriocyte nuclei; St, stage; T, thorax; T1-T3, thoracic segments 1-3. Scale bars, 20 μm.

which displays the sole expression of *Appum* iso1 (Fig. 4E).

Expression of *Appum* mRNA during mid and late embryogenesis

Transcripts of *Appum* were evenly distributed in embryos undergoing germband extension before katatrepsis (embryo flip) (Fig. 3). In the S-shape embryos (stage 11, Fig. 3A) and the twisting embryos (stage 12, Fig. 3B), preferential expression of *Appum* was visualized in the thoracic region. However, this was caused by the thickening of tissues. In late embryos undergoing or after katatrepsis, ubiquitous expression of *Appum* remained (Fig. 4A-D). Dark staining was detected in the tip of rostrum, but it had been proved to be non-specific (Chang *et al.*, 2009). In the primordial germaria located to the dorsal region of the embryo, aggregation of *Appum* in the germarial lumen, nevertheless, was not identified (Fig. 4C, D). Given that oocytes are not yet protruded from the primordial germaria in late embryos just after katatrepsis, accumulation of *Appum* transcripts to the germarial lumen ready for being transported by the trophic cord may not yet be required.

Expression of *Appum* during sexual oogenesis

In contrast to the asexual pea aphids, the sexual morphs display different morphological characteristics in oogenesis. Each adult sexual ovariole possesses only 1 to 3 egg chambers whilst a mature asexual ovariole can accommodate 7 to 9 egg chambers enclosing the embryos. However, size of the germaria and oocytes

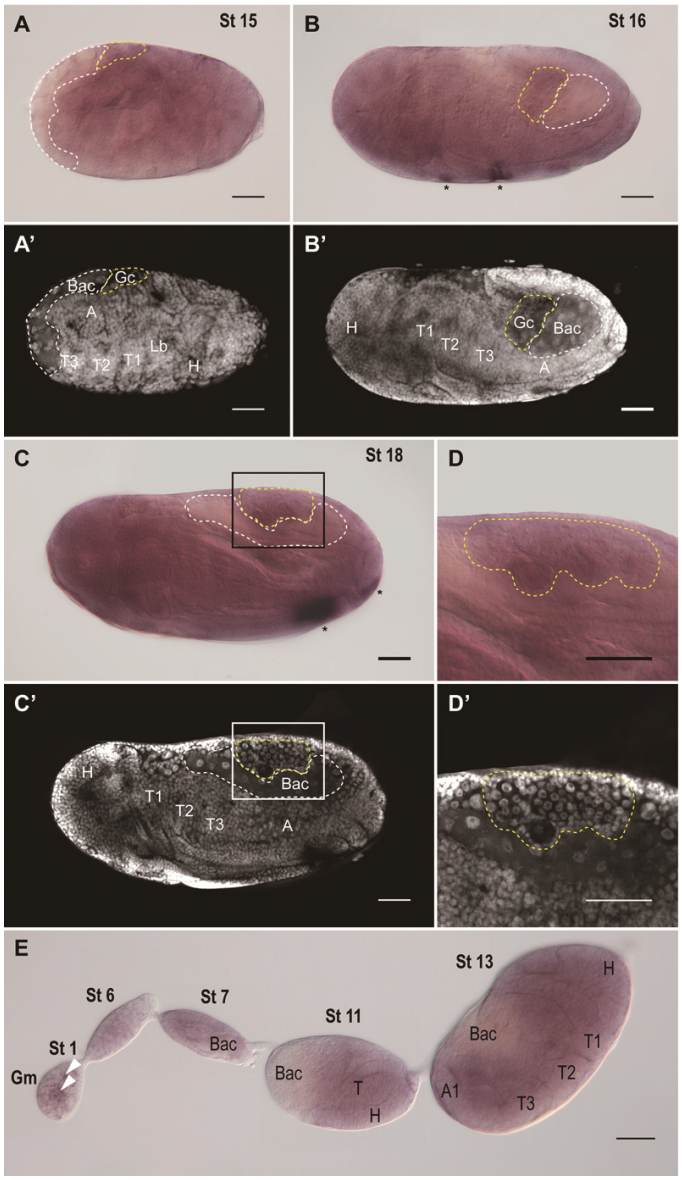

Fig. 4. Expression of *Appum* mRNA in embryos during and after katatrepsis of the asexual pea aphid. Embryos enter into katatrepsis from stage 15 of development. When embryos start flipping, they turn heads toward the egg anterior. Therefore, after katatrepsis the anterior region of the embryo is located to the egg anterior (the left side). Germ cells and endosymbionts are marked with yellow and white dashed line, respectively. (A'-D') are nuclear staining of (A-D). Embryos shown in (A) to (D) were labeled using the antisense probe 1 whereas those in (E) were stained with the antisense probe 2. Asterisks indicate the non-specific signals in the rostrum. (A) Stage 15 (embryo undergoing katatrepsis). (B) Stage 16 (embryo after katatrepsis). (C) Stage 18 (embryo after germ band retraction). (D) Magnification of inset shown in (C). (E) Dissected ovariole containing germarium, oocyte (stage 1), and embryos (stages 6, 7, 11, and 13). Expression patterns are similar to those stained by the probe 1. Arrowheads highlight the transient accumulation of *Appum* iso1 mRNA. Abbreviations: A, abdomen; A1, abdominal segment 1; Bac, Bacteria; Gc, germ cells; Gm, germaria; H, head; Lb, labrum; St. stage; T, thorax; T1-T3, thoracic segments 1-3. Scale bars, 50 μm.

can be up to fivefold larger in the sexual pea aphid (Davis, 2012). In the sexual ovarioles, we found that signal intensity of *Appum* mRNA in germaria was higher than that in oocytes. However, enrichment of *Appum* transcripts to the germarial lumen was not as significant as that seen in the asexual pea aphid and the accumulation of *Appum* to the anterior region of the oocyte was not detected (Fig. 5A-C). Perhaps rapid dilution of *Appum* mRNA occurs soon after it is transported to the oocyte anterior or, alternatively, the oocyte itself transcribes *Appum* mRNA so that the anterior-posterior gradient of *Appum* becomes unobvious.

Discussion

In this study, we cloned *Appum*—a *Drosophila* homolog of *pumilio* (*pum*) in the pea aphid—and analyzed its developmental expression in asexual viviparous oocytes and embryos. Based upon the expression patterns of *Appum* mRNA, we propose presumptive roles of *Appum* during development of the asexual the pea aphid, expecting to shed light on evolutionary and developmental aspects of *pum* genes in aphids.

Apart from the transient accumulation of *Appum* mRNA in germarial lumina and egg anterior during early embryogenesis, *Appum* was evenly distributed throughout developmental stages of the asexual pea aphid. Likewise, uniform expression of *pum* mRNA during embryogenesis also occurs in other insect models such as *Drosophila* and *Tribolium* (Macdonald, 1992; Schmitt-Engel *et al.*, 2012). Among insect species, however, immunostaining of Pum protein has been only reported in *Drosophila* and preferential expression of Pum protein, like that of *pum* mRNA, is not identified in this creature. Taken together, this suggests that functions of *pum*/Pum do not depend on asymmetric localization or local signal induction in *Drosophila* and perhaps in other insects including the pea aphid. In *Drosophila*, the Nanos/Pum-mediated translational repression mechanism plays an important role in the maintenance of germline stem cells during oogenesis and the development of germ cells during embryogenesis (Asaoka-Taguchi *et al.*, 1999; Parisi and Lin, 1999; Sonoda and Wharton, 1999). Similarly, in *Caenorhabditis elegans* (*C. elegans*) this mechanism is also critical to germline development (Kraemer *et al.*, 1999; Crittenden *et al.*, 2002). Given that *Drosophila* and *C. elegans* are evolutionarily distant species, we assume that the Nos/Pum-mediated translational repression is also conserved in the pea aphid.

That Nos interacts Pum to repress the translation of maternal *hunchback* (*hb*) mRNA is known to play a key role for posterior patterning in *Drosophila*. The posterior-anterior (PA) gradient of Nos protein in the syncytium thus forms a PA gradient of translational repression to the ubiquitously distributed maternal *hb* (Lehmann and Nüsslein-Volhard, 1991; Gavis and Lehmann, 1992; Sonoda and Wharton, 1999). Unlike *Drosophila*, *hb* mRNA (*Aphb*) is not uniformly patterned within the syncytia of the asexual pea aphid. *Aphb*, by contract, is anteriorly localized to the anterior pole of egg chambers (Huang *et al.*, 2010). Our recent staining shows that ApHb is evenly distributed within the central region of syncytia (Chang *et al.*, 2013). This implicates that *Aphb* is not a target of the Nos/Pum-mediated translational repression. Therefore, the asexual pea aphid may not rely on a PA gradient of ApHb to sustain posterior development as seen in *Drosophila*. Likewise, in *C. elegans hb* is neither a target of the Nos/Pum complex, and it is the *fem-3* mRNA whose translational repression is regulated by the Nos/*fem-3-binding* factor 1 (FBF-1, a Pum-like protein (Zhang *et al.*, 1997)). Moreover, instead of the posterior development, translational repression of *fem-3* is involved in the switch between spermatogenesis and

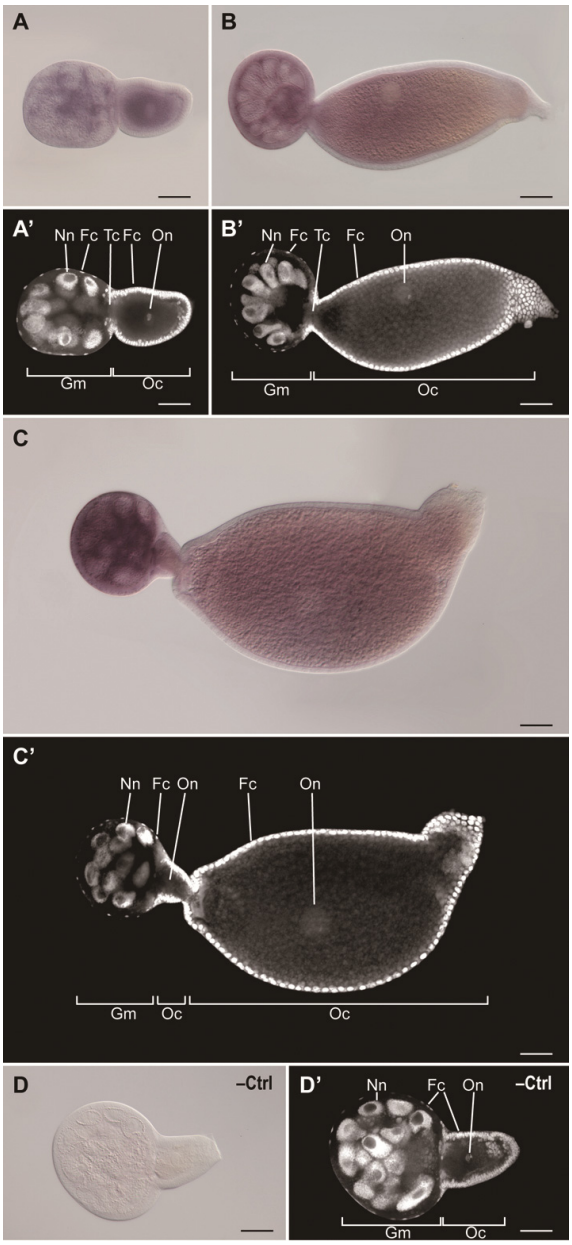

Fig. 5. Expression of *Appum* mRNA in ovarioles of the sexual pea aphid. Ovarioles were dissected from the sexual oviparous females. Like the asexual ovariole, the germarium is located to the anteriormost region of the sexual ovariole but the egg chambers only accommodate oocytes rather than embryos. (A'-D') are nuclear staining of (A-D). Anterior is to the left. (A) Previtellogenic oocytes. (B, C) Vitellogenic oocytes. There are two oocytes in the ovariole shown in (C): the big one is more mature than that in (B) whilst the small one—in between the germarium and the big oocyte—is still previtellogenic. Transcripts of *Appum* are evenly distributed in germaria and oocytes. Intensity of *Appum* signals in germaria appears higher than that in the oocytes. (D) Negative control. Ovarioles were stained with the sense probe 1. No signals were detected. Abbreviations: -Ctrl, negative control; Fc, follicle cells; Gm, germaria; Nn, nuclei of the nurse cells; Oc, oocytes; On, oocyte nuclei; Tc, trophic cord. Scale bars, 50 μm.

oogenesis in the *C. elegans* hermaphrodite (Zhang *et al.*, 1997). This suggests that the Nos/Pum-mediated translational repression is highly conserved but its targets may vary in distinct developmental events. At present, in the pea aphid we can identify *Drosophila* homologs of *epidermal growth factor receptor* (*EGFR*), *cyclin B*, *eukaryotic initiation factor 4E* (*eIF4E*) and *paralytic* (*para*), all of which are targets of Nos/Pum besides *hb* (Asaoka-Taguchi *et al.*, 1999; Mee *et al.*, 2004; Menon *et al.*, 2004; Kadyrova *et al.*, 2007; Muraro *et al.*, 2008; Kim *et al.*, 2012). Accordingly, we infer that mRNAs of these four homologous genes are potential targets of ApNos/ ApPum as well.

As a consequence, we suggest developmental roles of *Appum* as follows: (1) the *Appum* gene exerts its function at the protein level, and the alternative splicing of *Appum* is essential to providing sufficient amount of ApPum proteins via translation of the *Appum* isoforms; (2) like *Drosophila* and other animal models, the pea aphid can employ ApNos/ApPum-mediated translational repression for the development of germline stem cells in the germarium; (3) unlike *Drosophila*, the ApNos/ApPum complex may not suppress the translation of *Aphb* for posterior patterning because posterior expression of *Aphb* is not detectable with *in situ* hybridization; nevertheless, we cannot exclude the possibility that *in situ* hybridization is not sensitive enough to detect residual expression of *Aphb* in the egg posterior; and (4) whether ApPum can be involved in posterior patterning in the pea aphid requires assessment with functional tools such as RNA interference (RNAi) (Fire *et al.*, 1998), antisense morpholino (Summerton and Weller, 1997), and transcription activator-like effector nucleases (TALENs) (Christian *et al.*, 2010).

Acknowledgments

We thank Sue-Ping Lee for assistance with confocal microscopy, Shuji Shigenobu, Kota Ogawa and Miyuzu Suzuki for induction and dissection of ovaries from sexual pea aphids, and Gee-Way Lin, Hsiao-Ling Lu and Yi-min Hsiao for critical reading of the manuscript. This work was supported by National Science Council (97-2815-C-002-081-B for J-h. C.; 101-2313-B-002-059-MY3 and 101-2321-B-002-090-MY2 for C-y. C. and C-c. C.), BAPHIQ of the Agricultural Council (101.10.2.1-B3(4)), and National Taiwan University (NTU-CESRP 101R4602D3 and 102R7602D3).

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Received: January 4, 2014
Accepted: January 22, 2014

Appum：果蠅 *pumilio* 同源基因在豌豆蚜之選殖、發育表現、參與胚胎後端形塑之角色探討

鍾成侑 [1,3]、陳柔含 [1]、張俊哲 [1,2,3,4*]

[1] 國立臺灣大學生物資源暨農學院昆蟲學系遺傳暨發育生物學實驗室　10617 台北市大安區羅斯福路 4 段 113 巷 27 號
[2] 國立臺灣大學生物資源暨農學院生物科技研究所　10672 台北市大安區長興街 81 號 4 樓
[3] 國立臺灣大學發育生物學與再生醫學研究中心　10041 台北市中正區中山南路 8 號兒童醫療大樓 16 樓
[4] 國立臺灣大學基因體與系統生物學位學程　10617 台北市大安區羅斯福路 4 段 1 號

摘　　要

已知形態發生素（morphogens）之濃度梯度為許多動物早期胚胎發育所使用之位置訊息。此一梯度之建立，端賴聚集在胚胎當中的信使核糖核酸（mRNA）轉譯是否受到準確的抑制調控。以果蠅早期胚胎發育為例，抑制母系 *hunchback* (*hb*) mRNA 之轉譯乃透過 Nanos (Nos) 及 Pumilio (Pum) 這兩個蛋白質所組成的複合體，結合 *hb* mRNA 的 3' 非轉譯區（3' untranslated region, 3' UTR）所致。由於 Nos 在合胞囊胚（syncytial blastoderm）的後端濃度最高，因此 *hb* 轉譯抑制之強度也由後端至前端逐次遞減，與 Nos 之梯度反向。這樣一來，Hb 蛋白的濃度梯度遂由前到後被建立起來。除了果蠅，以 Nos/Pum 複合體來抑制基因轉譯之機制也被發現在其它的模式動物當中。因此，我們推測豌豆蚜亦保有此一機制，並於其中選殖到果蠅 *pumilio* 的同源基因 *Appum*，以及運用全體原位雜合技術偵測 *Appum* mRNA 在無性世代卵、胚的發育表現。實驗結果顯示：在卵與胚胎發育的過程中 *Appum* 的三個 mRNA 異構物皆呈現均勻分布之現象。由此推測，*Appum* 之功能並不倚賴 mRNA 的非對稱聚集，或藉由局部訊息誘導使 mRNA 集中。結合先前所發現之 *Aphb*/ApHb 與 *Apnos*/ApNos 發育表現結果，我們剖析了 ApPum 蛋白在胚胎後端形成過程所可能扮演的角色。希冀上述針對 *Appum* 所進行之實驗和討論，能為日後 ApPum 之功能解析奠定重要基礎。

關鍵詞：胚胎發育、基因異構物、原位雜合、孤雌胎生、轉譯抑制。

*論文聯繫人
Corresponding email: chunche@ntu.edu.tw