Introduction

Small non-coding RNAs play key roles in the regulation of gene expression and control of transposon activity in eukaryotes. These RNAs include microRNAs (miRNAs) that regulate gene expression, endogenous small interfering RNAs (endo siRNAs) that regulate gene expression and transposition in somatic cells, and Piwi-interacting RNAs (piRNAs) that silence transposons in germ cells. They are key components of the various small RNA silencing pathways and act within protein complexes including the Argonaute proteins, which represent a large protein family with distinct functions and small RNA specificities. The Argonaute protein family is subdivided into two subgroups, the Ago subfamily and the Piwi subfamily, according to their role in siRNA and miRNA silencing pathway; the Ago subfamily is composed of the miRNA-related Argonaute-1 (Ago1) and the siRNA-related Argonaute-2 (Ago2) subfamilies, respectively. These specific expression patterns suggest that expanded aphid piwi and ago3 genes have distinct roles in asexual and sexual reproduction.

Expansion of Genes Encoding piRNA-Associated Argonaute Proteins in the Pea Aphid: Diversification of Expression Profiles in Different Plastic Morphs

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Abstract

Piwi-interacting RNAs (piRNAs) are known to regulate transposon activity in germ cells of several animal models that propagate sexually. However, the role of piRNAs during asexual reproduction remains almost unknown. Aphi ds that can alternate sexual and asexual reproduction cycles in response to seasonal changes of photoperiod provide a unique opportunity to study piRNAs and the piRNA pathway in both reproductive modes. Taking advantage of the recently sequenced genome of the pea aphid Acyrthosiphon pisum, we found an unusually large lineage-specific expansion of genes encoding the Piwi sub-clade of Argonaute proteins. In situ hybridisation showed differential expressions between the duplicated piwi copies: while Api-piwi2 and Api-piwi6 are ‘specialised’ in germ cells their most closely related copy, respectively Api-piwi5 and Api-piwi3, are expressed in the somatic cells. The differential expression was also identified in duplicated ago3: Api-ago3a in germ cells and Api-ago3b in somatic cells. Moreover, analyses of expression profiles of the expanded piwi and ago3 genes by semi-quantitative RT-PCR showed that expressions varied according to the reproductive types. These specific expression patterns suggest that expanded aphid piwi and ago3 genes have distinct roles in asexual and sexual reproduction.

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questioned since Drosophila Piwi has been recently identified in both the germline and the ovarian somatic support cells [10–12], in contrast to Ago3 and Aub that are restricted to the germline [13,14].

To date, piRNAs in Drosophila can be categorized as two distinct types: the germline piRNAs and the somatic piRNAs. The germline piRNAs, which are associated with Aub, Piwi and Ago3, are processed by the "ping-pong mechanism". The somatic piRNAs, which are usually associated only with Piwi, are generated via the "primary piRNA model" [7,15]. It has been demonstrated that the germline piRNAs are involved in germline specification, maintenance of germ cells, meiosis and transposon movement [7]. In addition, roles for piRNAs in early embryo-genesis have been described for the Piwi subfamily and piRNAs in Drosophila [16,17] and zebrafish [18–21]. In Drosophila, maternally inherited piRNAs are present in early embryos where they provide resistance to transposition [16]. Moreover, Aub and Ago3, both of which are components of the piRNA pathway, are found to associate with Smaug and the CCR4 deadenylase complex to regulate the decay of the maternal nanos mRNA, a posterior determinant in Drosophila [17]. In zebrafish, both zivi and zili encode Piwi proteins that can bind piRNAs of opposite polarity and suppress the transposon activity in germ cells [18,19]. In addition to these conserved roles in transposon defense and germline development, recent reports show that zili is involved in axis patterning via transforming growth factor (TGF)-β and fibroblast growth factor (FGF) signaling pathways [20,21].

Here, we provide information on the piRNA pathway for the pea aphid Acyrthosiphon pisum, an emerging genomic model organism with a recently sequenced and annotated genome [22]. This phloem-sucking insect has an unusual capacity for phenotypic plasticity, displaying an ability to adapt its phenotype according to environmental conditions [23]. Aphids switch their reproductive mode in response to seasonal changes. During spring and summer, aphids reproduce by clonal viviparous parthenogenesis: a single female can give birth to approximately 80 larvae in 10 days, all of which are genetically identical to each other and to their mother. This reproductive mode allows a rapid and effective colonisation of host plants during the growing seasons. In autumn, the decrease of day length induces parthenogenetic viviparous females (named sexuparce) that produce sexual males and sexual oviparous females (named oviparce). Embryos in both virginoparce and sexuparce viviparous females complete development within the mother, and prior to larviposition germ cells within the embryos are specified and migrate to coalesce with the somatic gonads [24] – resulting in a phenomenon known as "telescoping of generations". The sexual males and oviparous sexual females then mate and produce overwintering eggs [25]. The pea aphid thus offers a great opportunity to analyse the molecular mechanisms of sexual and asexual reproduction, including the roles of small non-coding RNAs in the alternation of reproductive modes and in the development of the different sexual and asexual morphs. We describe here an expansion of the piRNA associated Argonaute family in the pea aphid. This expansion correlates to a diversification of developmental and spatial expression profiles in different reproductive morphs. Diversified expressions of these expanded copies suggest an adaptation of the piRNA pathways to contribute to the regulation of reproductive plasticity in aphids.

Results

Piwi-type Argonautes in the pea aphid genome

Aphid homologues of genes from the Piwi subgroup of the Argonaute family (Piwi and Ago3) were identified in the A. pisum genome. All of the eight Api-Piwi and the two Api-Ago3 deduced proteins possess the functional PAZ, MID and PIW domain (Data S1) characteristic of the Argonaute family. The main residues involved in the binding of the 5' end of small RNAs and the DDH catalytic triad are conserved in most of the A. pisum proteins, suggesting that these proteins are functional Argonautes. However, the mutation of the DDH triad into DDR in Api-Ago3 indicates that this protein may have lost slicer activity (Data S1).

In addition, we identified piwi homologues in the genome of another hemipteran, the blood-sucking bug Rhodinus prolixus. The genome of R. prolixus has been recently sequenced [26] and fibroblast development, recent reports show that zili is involved in axis patterning via transforming growth factor (TGF)-β and fibroblast growth factor (FGF) signaling pathways [20,21].

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PCR in the different reproductive morphs of the pea aphid (Figure S1): adult parthenogenetic virgoparae females (that produce parthenogenetic embryos), adult parthenogenetic sexuparae females (that produce sexual embryos), sexual oviparae females, and males. For each morph, RT-PCR was performed on RNA extracted from three independent batches of 10 adult whole bodies collected 48 h after adult moult. The ratio between the expression of the amplified gene and that of the \textit{Api-rpl7} reference gene was calculated to normalise the variation in experimental conditions. An average expression level was calculated for each reproductive morph based on the expression level obtained from the three independent batches (Figure 2). ANOVA tests were performed on the normalised values for each reproductive morph.

All the eight \textit{Api-piwi} and the two \textit{Api-ago3} genes were expressed in females but showed distinct expression profiles that varied between the morphs (Figure 2). Most \textit{Api-piwi} genes and \textit{Api-ago3a} genes were expressed at a lower level in males than in the three female morphs. For example, \textit{Api-piwi2}, 3, 4 and 6 were almost undetectable in males. However, \textit{Api-piwi5} was strongly expressed in males, and \textit{Api-piwi7} expression level is stronger in males than in virginoparae and oviparae females. As for genes that were preferentially expressed in some morphs, the following specific expression patterns were noteworthy: (1) The expression profiles of \textit{Api-piwi2} and \textit{Api-piwi8} discriminated between parthenogenetic females (virginoparae and sexuparae) and sexual females (ovi-

Figure 1. ML phylogenetic tree including protein Piwi-like and Ago3-like sequences from insects. Piwi-like and Ago3-like sequences were retrieved from insect species with a complete genome sequence. Prefixes correspond to abbreviated species names: Aae, \textit{Aedes aegypti}; Ada, \textit{Anopheles darlingi}; Aga, \textit{Anopheles gambiae}; Ame, \textit{Apis mellifera}; Api, \textit{Acrystosiphon pism}; Bmo, \textit{Bombyx mori}; Cfi, \textit{Camponotus floridanus}; Cqu, \textit{Culex quinquefasciatus}; Dme, \textit{Drosophila melanogaster}; Hsa, \textit{Harpegnathos saltator}; Nvi, \textit{Nasonia vitripennis}; Phu, \textit{Pediculus humanus}; Rpr, \textit{Rhodnius prolixus}; Tca, \textit{Tribolium castaneum}. Gene accession numbers are provided in Table S2. Bootstrap values are shown only for the most relevant groups discussed in the Materials and Methods section.

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Apipiwi8 in asexuals; (2) Apipiwi5 was preferentially expressed in males while it was only weakly expressed in the female morphs; (3) Apipiwi6 and Apiago38 were preferentially overexpressed in sexuparae females. Taken together, these results indicate that the expansion of the piRNA-related piwi and ago3 genes in the pea aphid appears to be linked to its reproductive plasticity.

Developmental expression of Api-piwi and Api-ago3 genes

The temporal and spatial distribution of the eight Api-piwi and two Api-ago3 transcripts were analysed by in situ hybridisation on dissected ovarioles containing adult germaria, oocytes, and developing embryos. For each gene, specific antisense riboprobe was designed to detect the distribution of the corresponding transcripts; sense riboprobes were applied in negative controls. Because of the high sequence identity between Api-piwi1, 4 and 7 (>92%), we were not able to synthesise specific probes that could discriminate individual expressions of these three genes. Nevertheless, in situ signals detected by antisense riboprobes synthesised from different template regions of Api-piwi1, 4, and 7 reflected consensus expression patterns.

In virgoparae, combinational expression of Api-piwi1, 4 and 7 was detected in germaria, oocytes, and early embryos before gastrulation (Figure 3 A–C). Preferential expression was identified in the follicle cells located between germaria and oocytes (Figure 3A, B) but newly-segregated germ cells were almost devoid of staining (Figure 3C). During mid-embryogenesis, expression patterns appeared dynamic (Figure 3D, E), but in situ signals were preferentially detected in germ cells residing in the gonads of late embryos (Figure 3F). Expression of Api-piwi8, a gene within the same phylogenetic subgroup of Api-piwi1, 4, and 7, was almost undetected during oogenesis and embryogenesis (Figure 3 G–I) except in early embryos before gastrulation (Figure 3F). During mid and late embryogenesis, Api-piwi8 was weakly expressed (Figure 3 J–L). Gene expression was not detected in ovarioles hybridised with sense riboprobes (Figure 3M, N), indicating that the in situ signals identified with antisense probes accurately represented the distributions of Api-piwi1, 4 and/or 7 and Api-piwi8. The developmental characteristics of embryos hybridised with probes of Api-piwi1, 4, 7, 8 and those stained with other gene probes are highlighted in the cartoon illustration shown in Figure 3O.

Coding regions with more than 25% sequence difference were used as templates to synthesise probes to distinguish expression of the closely related Api-piwi2/Api-piwi5 and Api-piwi3/Api-piwi6 genes in virgoparae embryos. During early development, transcripts of Api-piwi2 were restricted to the germlarial region and the anterior region of the oocytes (Figure 4A). Weak expression of Api-piwi2 was visible in the newly-segregated germ cells (Figure 4B), but strong intensity of the germline-specific signals of Api-piwi2 were identified from gastrulation onward (Figure 4 C–E). In comparison with Api-piwi2, a lower level of Api-piwi3 transcript abundance was identified in germaria and oocytes (Figure 4F). Expression of Api-piwi5 was evenly distributed in embryos throughout development and no preferential expression was identified in germ cells (Figure 4 G–J). Expression of Api-piwi3 was undetected in germania and gastrulating embryos (Figure 4A, C), and transcripts of Api-piwi3 appeared not specifically restricted to germ cells (Figure 5D, E). In contrast, the transcripts of its closely related copy Api-piwi6 were identified specifically in germ cells from mid embryogenesis onward (Figure 5 H–J) as was also observed for Api-piwi2 (Figure 4 C–E), another germline-specific piwi gene in the pea aphid. However, preferential expression of Api-piwi6 was detected neither in the oocyte anterior (Figure 5F) nor in the newly-segregated germ cells (Figure 5G), which distinguished its expression pattern from that of Api-piwi2 (Figure 4A, B) during early embryogenesis.

Localisation of these transcripts was also investigated in ovarioles containing the embryonic stages of parthenogenetic sexuparae and sexual oviparae. The expression patterns of Api-piwi genes in sexuparae and oviparae were similar to those obtained from virgoparae: Api-piwi2 and Api-piwi6 were germline-specific while their closely related copies Api-piwi3 and Api-piwi7 respectively were not (Figure 6 and Figure S2). However, Api-piwi6 showed a distinct localisation of expression between asexual and sexual embryos: it was preferentially restricted to germ cells in the virgoparae (Figure 5 H–J) and sexuparae (Figure S2K, L), but it was evenly expressed in the oviparae (Figure S2O, P).

In addition to the piwi genes, we also analysed the expression of ago3 genes (Api-ago3a and Api-ago3b) in the pea aphid. In virgoparae, Api-ago3a was expressed in the germlarial lumen as well as in the cytoplasm of oocytes (Figure 7A). During embryogenesis, the transcripts of Api-ago3a were preferentially restricted to the primordial germ cells (Figure 7B), migrating germ cells (Figure 7C, D), and germ cells in the gonads (Figure 7E). Expression of Api-ago3b was not detected in the germ cells of developing virgoparae, nor was it detected in any other cells throughout development (Figure 7 F–J). In strong contrast to the virgoparae, expression of Api-ago3a in sexuparae and oviparae was not restricted to the germ cells (Figure S3 A–H); but expression of Api-ago3b remained almost undetected (Figure S3 I–P).

In order to verify the expression of Api-piwi2, Api-piwi6 and Apiago3a are located in the embryonic germ cells, we performed double in situ hybridisation of these three genes with the germline marker gene vasa [24]. Experimental results show that transcripts of Api-piwi2, Api-piwi6 and Api-ago3a are colocalised with vasa to the germ cells (Figures S4, S5, S6), confirming that their preferential expressions are restricted to the germline.

Discussion

Evolutionary diversification of Api-piwi genes

We describe here an expansion of the genes encoding Piwi sub-clade of Argonaute proteins in the pea aphid A. pisum. Annotation of the A. pisum genome identified eight copies of the piwi genes and two copies of the Api-ago3. Conservation of the key residues of PAZ, MID and PIWI domains suggest that Api-Piw1-7 proteins...
are functional Argonautes while Api-piwi8 presented mutation within the DDH catalytic triad and may have lost slicer activity. Since Api-piwi8 results from an ancient duplication, this mutation appears to be evolutionary conserved. From the comparison of complete genomes in different insect orders, the duplication of ago3 in aphids appears to be unique (Figure 1). By contrast, duplications of piwi genes have been identified in the mosquitoes Aedes aegypti [26]; six piwi genes have been annotated in the plant Arabidopsis thaliana [27]; 12 and 15 piwi genes have been characterized in the ciliate protozoa Tetrahymena thermophila and Paramecium tetraurelia respectively [28–30]. The biological significance of the expansions in mosquitoes and Daphnia has not yet been examined in detail. However, in the protozoan T. thermophila and P. tetraurelia, the expansion of piwi genes has been associated with neo- or sub-functionalisation of these proteins [28–30], as has been observed previously for Argonaute proteins in plants [31] and in the nematode C. elegans [6]. In most cases, including the pea aphid, piwi expansions appear to be independent events and lineage-specific, as shown by the monophyletic grouping of copies from each species. Indeed, the pea aphid expansion is not only independent of the expansion in mosquitoes, but also of the expansion we identified in its closer relative R. prolixus. Thus the expansions of the Piwi family in different animal groups appear to represent the repeated unfolding of a similar scenario.

Diversification of developmental expression profiles in germline and in soma

In situ hybridisation on whole mounted ovarioles showed differential expression among some pea aphid Api-piwi and Api-ago3 genes (piwi expression profiles are summarised in Figure 8). In animals, expression of Piwi and Ago3 is mainly restricted to the germline [19,32,33] and to the gonadal somatic cells in Drosophila [13]. In pea aphid virginoparae, we observed differential expression of the closely-related piwi genes Api-piwi2/Api-piwi5 (Figure 4) and Api-piwi3/Api-piwi6 (Figure 5), suggesting that duplication of piwi genes in this instance has led to the

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**Figure 3. Comparison of developmental expressions of Api-piwi1,4,7 and Api-piwi8 in virginoparous embryos.** Samples were hybridised with Api-piwi1,4,7 (A–F) and Api-piwi8 (G–L) antisense riboprobes. Anterior is to the left; dorsal is upper. All views are lateral. Unless addressed specifically, locations of germ cells are labelled with double arrowheads. (A, G) Germaria and segregated oocytes (stage 1). Preferential expression was identified in the follicle cells between the germarium and the oocyte (arrowheads). Api-piwi8 transcripts were not detected. (B, H) Embryos undergoing nuclear divisions (stage 2, 3). Api-piwi1,4,7 expression remained restricted to the follicle cells (arrowheads). Api-piwi8 stayed undetected. (C, I) Embryos with newly-segregated germ cells (stage 6). Most Api-piwi1,4,7 transcripts were restricted to the central syncytium (arrow), but Api-piwi1,4,7 expression was not detected in germ cells. Api-piwi8 was weakly expressed in anterior two thirds of the egg. (F, J) Embryos undergoing gastrulation (stage 8). Api-piwi1,4,7 expression was preferentially identified in the invaginating germband (arrow). Api-piwi8 expression was almost undetected. (E, K) Extension of the germband (stage 12). Strong expression of Api-piwi1,4,7 was identified in the abdomen (arrow). Faint expression of Api-piwi8 was visible. (F, L) Completion of germband retraction (stage 18). Api-piwi1,4,7 transcripts were restricted to the gonadal germ cells (arrow); Api-piwi8 expression was almost undetected. (M, N) Negative control, ovarioles hybridised with sense riboprobes. No signals were detected. **(O)** Illustration displaying presented developmental stages. Abbreviations: Ab, abdomen; Ant, antennae; Bc, endosymbiotic bacteria (invading embryos during stage 7); Blc, blastodermal cells; CNS, central nerve system; Cs, central syncytium (central blastoderm containing multiplying nuclei); Dev, development; Dn, dividing nuclei; Fc, follicle cells; Gc, germ cells; Gl, germlar lumen (central cavity of the gerarium, where germline stem cells are derived); Gm, germarium; Hd, head; Ig, invaginating germband; L1-3, limbs 1–3; Nc, nurse cells; Nu, nuclei; Oc, oocyte; Ps, posterior syncytium; Sn, syncytial nuclei. St, stage; Th, thorax; T1-3, thoracic segments 1–3. Scale bar, 20 μm. doi:10.1371/journal.pone.0028051.g003
expression patterns of piwi genes in aphids, and also resulting from a duplication event. Like expanded piwi genes in aphids, piwi and aub show somatic/germline diversification of expression profiles in Drosophila. Piwi is expressed in somatic follicle cells as well as in germline while Aub and Ago3 are restricted to the germline. These two spatial expression patterns are associated with two distinct functions. In ovarian somatic cells Piwi alone is involved in primary linear piRNA biogenesis, but in germline Piwi, Aub and Ago3 are involved in a more complex piRNA biogenesis pathway - the “ping-pong mechanism”.}

Figure 4. Comparison of expressions of Api-piwi2 and Api-piwi5 in the virginoparous embryos. Dissected ovarioles were hybridised with DIG-labelled antisense riboprobes of Api-piwi2 (A–E) and Api-piwi5 (F–J), respectively. Orientations and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, G; Mid development: C, D, H, I; Late development: E, J. Germ cells stained with antisense Api-piwi2 and Api-piwi5 riboprobes are highlighted with arrows (with preferential expression) and double arrows (without preferential expression). (A, F) Germaria and segregated oocytes. (B, C, G, H) Extension of the germ band (stage 12). Transcripts of Api-piwi2 were specifically identified in the germ cells whilst transcripts of Api-piwi5 were evenly distributed except in bacteria. (E, J) Completion of germband retraction (stage 18). Specific expression of Api-piwi2 was identified in germ cells located in the dorsal region of the embryo, but universal expression of Api-piwi5 remained as it was detected in the stage-12 embryos. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm.

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Figure 5. Comparison of expressions of Api-piwi3 and Api-piwi6 in virginoparous embryos. Dissected ovarioles were hybridised with DIG-labelled antisense riboprobes of Api-piwi3 (A–E) and Api-piwi6 (F–J). For orientations and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, G; Mid development: C, D, H, I; Late development: E, J. Germ cells stained with antisense Api-piwi3 and Api-piwi6 riboprobes are indicated by arrows (with preferential expression) and double arrows (without preferential expression). (A) Germaria and segregated oocytes. (B, C, G, H) Extension of the germ band (stage 12). Weak expression of Api-piwi3 was not detected but preferential expression of Api-piwi6 could be identified in the germlinal lumen. (D, I) Limb bud formation (stage 13). Expression of Api-piwi3 was evenly distributed in the embryo. Preferential expression of Api-piwi6 was restricted to germ cells in the dorsal region and weak expression was identified in other regions of the embryo. (E, J) Completion of germband retraction (stage 18). Expression of Api-piwi3 was evenly distributed in the embryo but preferential expression was identified in the region of the head (arrow). Expression of Api-piwi6 remained detected specifically in germ cells. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm.

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Figure 6. Comparison of expressions of Api-piwi2 and Api-piwi5 in sexuparous and oviparous embryos. Ovarioles were dissected from adult virgoparous raised under short day conditions that induce the production of sexuparae, and from adult sexuparae that produce sexual oviparae females and males. Ovarioles were hybridised with DIG-labelled antisense riboprobes of Api-piwi2 (A–H) and Api-piwi5 (I–P), respectively. For orientations and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, E, F, I, J, M, N; Mid development: C, G, K, O; Late development: D, H, L, P. Germ cells stained with antisense Api-piwi2 and Api-piwi5 riboprobes are highlighted with arrows (with preferential expression) and double arrows (without preferential expression). (A, E, I, M) Germaria and segregated oocytes (stage 1). Transcripts of Api-piwi2 were detected in the germlia and the oocyte (arrowheads). Expression of Api-piwi5 remained the same pattern as that of Api-piwi2, but signal intensity in germlia was reduced. (B, F, J, N) Embryos with newly-segregated germ cells (stage 6). Weak expression of Api-piwi2 was specifically detected in the germ cells; expression of Api-piwi5 was evenly distributed in the embryo including the germ cells. (C, G, K, O) Extension of the germband and limb bud formation (stage 12-14). Transcripts of Api-piwi2 were preferentially identified in the germ cells of sexuparae and oviparae embryos but expression of Api-piwi5 was evenly distributed. In panel (O), germ cells are not presented in the shown focal plane. (D, H, L, P) Germband retraction (stage 17) and completion of germband retraction (stage 18). Specific expression of Api-piwi2 was identified in germ cells located in the dorsal region of the embryo, but germline-specific expression of Api-piwi5 was not detected. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm.

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Figure 7. Comparison of expressions of Api-ago3a and Api-ago3b in virgoparous embryos. Dissected ovarioles were hybridised with DIG-labelled antisense riboprobes of Api-ago3a (A–E) and Api-ago3b (F–J). For orientation and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, F, G; Mid development: C, D, H, I; Late development: E, J. Germ cells stained with antisense Api-ago3a and Api-ago3b riboprobes are highlighted with arrows (with preferential expression) and double arrows (without preferential expression). (A) Expression of Api-ago3a was identified in the germlia lumen and the cytoplasm of the segregated oocytes (arrowheads). (B–E) From stage 6 onward, specific expression of Api-ago3a was detected in germ cells. (F–J) Expression of Api-ago3b was not detected in germlia (F), segregated oocytes (F) and embryos (G–J). Background staining in late embryos (J) was also seen in embryos hybridised with sense riboprobe of Api-ago3b (data not shown). Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm.

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piwi genes in distantly related organisms including the aphids: some copies are “specialised” in germ cells whereas others are expressed in the somatic cells.

Somatic expression was reported for Api-piwi3 and Api-piwi5 in the embryonic stages of the three female morphs, Api-piwi6 in embryonic oviparae, and Api-ago3a in embryonic oviparae and sexuparae. Ubiquitous expression of piwi, aub and ago3 has also been observed during Drosophila embryogenesis [5]. However this somatic expression in Drosophila was restricted to early embryos, in which a significant proportion of mRNA is maternally inherited [10]. In aphid embryos, somatic signals were observed for Api-piwi5, Api-ago3a and Api-piwi6 in early and late embryo stages and specifically in late embryo stages for Api-piwi6. While signal observed until embryo blastula stage 6 can be imputed to maternal mRNAs [34], clear signal observed for these genes in late somatic stage (stage 12–18) reflects de novo somatic transcription (Table S1).

In adult Drosophila, until recently, somatic expression of Piwi and piRNAs has only been reported in a specific type of somatic tissues: ovarian follicle cells, where they are responsible for the maintenance of germline stem cells (GSCs) in ovaries [15,35]. Restriction of the Api-piwi1,4,7 transcripts to the follicle cells in the posterior margin of the germarium (Figure 3A, B), from which the GSCs are derived [36], hence appears to suggest a potential role in the development of GSCs, like that in Drosophila. Apart from the ovarian follicle cells, recent findings show that piRNA-like molecules have now been identified in head and imaginal discs of Drosophila [8], but the biogenesis of the “non-ovarian follicle” somatic piRNAs still needs to be understood. Similarly, it remains to be solved whether the somatic ubiquitous zygotic expression of Api-piwi3, Api-piwi5, Api-piwi6 in oviparae and Api-ago3a in sexuparae and oviparae reflects somatic expression in adults, as has been reported for several piwi genes in D. pulex [27]. Semi-quantitative RT-PCR realised on adult virginoparae heads did not evidence clear somatic expression of Api-piwi3 and Api-piwi5 (S. Jaubert-Possamai unpublished results). The role of such somatic piwi genes therefore requires further investigation.

Diversification of expression profiles is correlated with the reproductive mode

In response to seasonal modifications of photoperiod, aphids can switch from asexual parthenogenesis to sexual reproduction. Analyses of expression profiles of the expanded Api-piwi and Api-ago3 genes by semi-quantitative RT-PCR showed that these genes are differentially expressed in the various adult reproductive morphs of the pea aphid (piwi expression profiles are summarised in Figure 8). Every Api-piwi and Api-ago3 gene exhibits a distinct expression profile, including the closely-related genes Api-piwi2/Api-piwi5, Api-piwi3/Api-piwi6, and Api-ago3a/Api-ago3b. These specific expression patterns of mRNAs suggest that expanded aphid Piwi proteins may have distinct roles in asexual or sexual reproductive phases.

In situ hybridisation also provided evidence for differentiation among reproductive morphs in the spatial expression of duplicated Api-piwi and Api-ago3 genes. Interestingly, among all studied...
argonaute genes in the pea aphid, Api-piwi2 is the only one that remains germline-specific in three different reproduction morphs (Figures 4 and 6). The germline-specific expression of other argonaute genes, however, varies between morphs. For example, the association of two genes, Api-piwi6 and Api-ago3a, with germline tissue varied substantially among female morphs. Api-piwi6 expression was germline specific in virginoparous and sexuparous (Figure 5 H–J and Figure 52 K, L) but was not specific to germ cells in oviparous (Figure S2O, P). Similarly, Api-ago3a was specifically expressed in germ cells in virginoparous (Figure 7 B–E), but showed no germline-specific localisation in either sexuparous or oviparous (Figure S3 A–H). This difference in expression location between the morphs strongly suggests that expressions of Api-piwi6 and Api-ago3a are regulated by the change of photoperiods.

We did observe some inconsistencies between the results of our RT-PCR and in situ hybridisation analyses for Api-ago3b and Api-piwi6. These differences in the detection of expression between the two methods could have been caused by the increased sensitivity of the RT-PCR approach, or because the target tissue for RT-PCR was the whole adult body whilst the in situ hybridisation targeted only the dissected ovarioles. This latter explanation would require that these genes be expressed only in non-ovarian tissues.

To our knowledge, this is the first report implicating Piwi proteins in asexual reproduction in a metazoan. However, our results do parallel previous findings obtained for the protozoans T. thermophila and P. tetraurelia, which resemble aphids in that they alternate between sexual and asexual phases during their life cycle. Like in aphids, protozoan piwi genes showed a diversification of expression pattern during sexual and asexual phases of their life cycle. Despite having highly similar structures, these protozoan Piwi proteins show different specificity to distinct classes of small non-coding RNAs [28–30]. Functional studies of piwi genes must now be conducted in the pea aphid in order to understand the piRNA binding specificities.

Our results indicate that the expansion of genes encoding the Piwi sub-clade of Argonaute proteins in the pea aphid is associated with a diversification of expression profiles that correlates with the different reproductive modes displayed by this insect. Expanded Piwi and Ago3 of the pea aphid belong to the large Argonaute protein family, which also includes the microRNA specific Ago1 and the siRNA specific Ago2 in insects. We previously described a duplication of ago1 but no duplication of ago2 in the pea aphid genome [37]. Altogether our results show a global expansion of the Argonaute protein family with the exception of Ago2 in the pea aphid. Beside Argonaute proteins, other components of the microRNA and piRNA pathways are expanded in the pea aphid, such as Dicer-1 (2 copies) and Pasha (4 copies) for the microRNA pathway [37] and Pimet/Hen1 (2 copies) for the piRNA pathway (S. Jaubert-Possamai, personal communication). This duplication appears to be specific to the microRNA and piRNA pathways since no duplication of the genes of the siRNA pathway has been identified, suggesting an expansion of a part of several small RNA pathways in the pea aphid genome. The significance of this expansion remains to be understood. A crucial role for Piwi together with Dicer-1 and a protein named Fragile X Mental Retardation Protein (FMRP) has been proposed in germline fate determination during Drosophila embryogenesis [38]. In the pea aphid we have identified five copies of fmr, suggesting that duplication of Api-fmr, together with the duplicated Api-piwi and Api-dicer-1, may be critical to the specification of germ cells (S. Jaubert-Possamai unpublished data). Further functional analyses will be necessary to clarify the role of these expanded small RNAs machineries in the pea aphid A. pisum.

**Materials and Methods**

**Pea aphid clones and induction of sexual reproduction**

The LSR1-A1-G1 clone of the pea aphid A. pisum [22] was reared on broad bean (Vicia faba) at 18°C. Parthenogenetic reproduction was maintained at 16 hours (h) of light and aphids were reared at low density (1 to 5 individuals per plant). Production of sexual morphs was obtained by rearing aphids at 12 h light and 18°C for two generations [39]. Total RNA was extracted by using the RNeasy kit (QIAGEN) from each of the A. pisum reproductive morphs: adult parthenogenetic females reared under long day photoperiod (called virginoparous) and producing parthenogenetic clones, adult parthenogenetic females reared under short day photoperiod (called sexuparous) and producing males and sexual oviparous female clones, adult sexual female oviparous, and adult sexual males. Parthenogenetic and vicioparous pea aphid morphs used for in situ hybridisation were from an obligate parthenogenetic clone reared in the laboratory at the National Taiwan University [40].

**Annotation of genes encoding Piwi-type Argonaute Proteins**

Homologues of Piwi/Aub and Ago3 were identified by mining the genomic data in the A. pisum LSR1 genome (Acyr 1.2 version of the assembly) at AphidBac (www.aphidbase.com). This was performed using the corresponding D. melanogaster sequences as bait and A. pisum predicted proteins (program BlastP) and genomic scaffolds (program TBLASTN) as targets. The hits were included in a preliminary phylogenetic analysis by using Neighbour-Joining (NJ), which allowed an unambiguous identification of homologues. Gene models from prediction programs were checked, resulting in only a few manual curations. All annotated genes are listed in Table S3. Amino acid sequences were then deduced from the curated pea aphid gene models for all the candidate genes. The domain distribution of the deduced A. pisum proteins was predicted by using Pfam [41] and Interproscan [42] software. Because R. prolixus (another hemipteran) is to date the closest evolutionary parent with a complete genome sequence (which is not yet fully annotated), we performed a similar research of piwi-like and ago3-like genes in the scaffolds of that species (Blast at NCBI).

**Phylogenetic analysis**

Homologues of Piwi/Aub and Ago3 were also identified in other insects using Blast against GenBank; hit sequences were collected to form a file in which we included all similar A. pisum genes. Given the relatively large evolutionary distance between the different insect species represented in the data set (and even among copies of A. pisum), analysing protein rather than DNA sequences seemed to be most appropriate. The sequences were aligned using T-Coffee [43]. A few of the sequences (from some Diptera species), being incomplete, were discarded. The N-terminal part of the protein sequence, which is poorly conserved among the different piwi-like genes and ago3-like genes, was removed. An NJ analysis was performed using MEGA, with the pairwise deletion option and Poisson correction [44]. A Maximum Likelihood (ML) phylogeny analysis was also performed with PHYML on the same alignment, testing different models of substitution. Bootstrap support for the nodes was calculated using 100 replicates.

**Gene expression profiling in different reproductive morphs**

The expression level of the eight Api-piwi and the two Api-ago3 genes in the various reproductive morphs was compared by semi-
quantitative reverse transcription-polymerase chain reaction (RT-PCR). Expression of *Api-piwi* and *Api-ago3* genes in the different morphs was normalised against the gene encoding ribosomal protein T (*Api-rpl7*) as a reference gene [45]. For each morph, three batches of 10 individuals from three independent biological experiments were frozen in liquid nitrogen 48 h after adult moult and used for RNA extraction. The concentration and quality of the extracted RNA was estimated with a NanoDrop (Thermo Scientific). First strand cDNAs were produced from 500 ng total RNA using the SuperscriptIII reverse transcriptase (Invitrogen) and random nonamers (Promega) following the supplier’s instructions. DNA contamination was removed by treating RNA extraction products with RNase-free DNase (Promega). As a negative control, RT-PCR experiments were realised on total RNA without SuperscriptIII reverse transcriptase.

The standard PCR program comprised an initial step of 4 minutes (min) at 94°C, then multiple cycles composed of 2 min at 94°C, 30 seconds (sec) at the annealing temperature and 1 min 30 sec at 72°C, and a final elongation step of 5 min at 72°C. For quantification of the amplification products, the appropriate number of cycles corresponding to the exponential range was defined for every gene. The sequences of PCR primers, the corresponding annealing temperatures and the appropriate number of cycles used to measure expression level of the different *Api-piwi* and *Api-ago3* genes are listed in Table S3. Images of the RT-PCR Sybr-safe (Invitrogen) stained agarose gels were acquired with a G:BOX Imager (Syngene) and quantification of the bands was performed using Image-J (http://rsbweb.nih.gov/ij/). For each batch of each reproductive morph, the ratio between the band intensity of the amplified gene and *Api-rpl7* reference gene was calculated to normalise for initial variations in sample concentration. The data were subjected to an arc-sine transformation for analysis of variance (ANOVA) in order to test for the significance of expression level (p-value >0.05).

Cloning, amplification, and synthesising riboprobes of *Api-piwi* and *Api-ago3* genes

We cloned, then amplified, partial sequences of *Api-piwi* and *Api-ago3* genes through RT-PCR. PCR cloning of template sequences for synthesising riboprobes were performed for 40 thermal cycles. Complementary DNA (cDNA) templates were reversely transcribed using HiScript I reverse transcriptase (Bionovas) and poly dT18 primers from total RNA extracted from dissected ovaries of the adult virginoparous. Gene-specific primers were designed based upon sequences of putative homologues of *Api-piwi* and *Api-ago3* genes in AphidBase. PCR fragments containing verified sequences were then cloned into the pGEM-T Easy Vector (Promega) for subsequent in vitro transcription. Sense and antisense digoxigenin (DIG)-labelled riboprobes for *in situ* hybridisation were synthesised from linearised plasmids containing target sequences using the DIG RNA Labeling Kit (SP6/T7) (Roche). Probes of *vasa* subjected to double *in situ* hybridisation were labelled with fluorescein (FL) using the DIG/FL labelling mix (Roche). Primer sequences, lengths of PCR amplicorns, and annealing temperatures of PCR are summarized in Table S4.

Whole-mount *in situ* hybridisation and microscopy

Dissected ovarioles, which contain developing oocytes and embryos, were fixed in 3.8% formaldehyde in 1 x phosphate-buffered saline (PBS) at 4°C overnight. The steps for single and double *in situ* hybridisation followed the protocol described in [40]. The working concentration of each probe, including sense and antisense strands, ranged from 1.5 to 2.0 ng/μl. We optimised the hybridisation temperatures according to the best signal intensity that could be discriminated between experimental (antisense probes) and control (sense probes) groups. The hybridisation temperature of each probe varied (Table S3). In single *in situ* hybridisation, we applied Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche) as the substrates for developing *api-piwi* and *api-ago3* signals. For double *in situ* hybridisation, we used 4-benzoylalamine-2,5 diethoxybenzenediazonium chloride hemizinc chloride] (Fast Blue BB) salt/naphthol-AS-MXphosphate (NAMP) (Sigma) to develop signals of *api-piwi*2 but remained NBT/BCIP as the substrates for *api-piwi*6 and *api-ago3a*. After signals of *piwi* and *ago3a* were developed, *vasa* signals were then generated using the Fast Red substrate (Roche). In order to increase the signal specificity of *api-piwi*6 and *api-ago3a*, we briefly rinsed the embryos in methanol after signal development with NBT/BCIP. After *in situ* hybridisation, samples were mounted in 70% of glycerol/1 x PBS and photographed with a Leica DMR connected to Canon EOS 5D MarkII digital camera (Canon). Morphological characteristics of developmental stages were described according to Miura et al. [46] and locations of germ cells were described according to Chang et al. [24].

**Supporting Information**

**Figure S1** Expression levels of the *Api-piwi* and *Api-ago3* genes in the four reproductive morphs. Expressions of the eight *api-piwi* and the two *api-ago3* genes were quantified in the four reproductive morphs of the pea aphid by semi-quantitative RT-PCR. The figure shows agarose gels after electrophoresis of RT-PCR products. Quantification of gene expression was analysed by semi-quantitative PCR with copy specific primers in the four reproductive morphs of A. pisum parthenogenetic virginoapar, parthenogenetic sexuparapar, ovi- parapar sexual females and sexual males. The expression of the ribosomal *api-rpl7* gene was analysed as a reference gene [45]. For each morph, RT-PCR were realised on total RNA extracted from batches (R1, R2, R3) of 10 pooled adults resulting from three independent biological replicates. As a negative control, RT-PCR experiments were realised on each RNA sample without SuperscriptIII reverse transcriptase (–). Primers used to investigate the expression of *api-piwi* and co-amplified *api-piwi1*, so only the amplification product corresponding to *api-piwi4* (*) was considered for gene expression analysis. Abbreviations: R: replicates. (TIF)

**Figure S2** Comparison of expressions of *Api-piwi3* and *Api-piwi6* in sexuparous and oviparous embryos. Dissected ovarioles were hybridised with DIG-labelled antisense riboprobes of *Api-piwi3* (A–H) and *Api-piwi6* (I–P), respectively. For orientations and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, E, F, I, J, M, N; Mid development: C, G, K, O; Late development: D, H, L, P. Germ cells stained with antisense *api-piwi3* and *api-piwi6* riboprobes are highlighted with arrows (with preferential expression) and double arrows (without preferential expression). (*A, E, I, M*) Germaria and segregated oocytes (stage 1). Transcripts of *api-piwi3* were weakly expressed in the germaria and the oocyte of sexuparapar (arrowheads) whilst transcripts of *api-piwi3* were not detected in oviparapar. *Api-piwi6* expression was distributed in germaria and the oocytes of sexuparapar and oviparapar (arrowheads). (*B, F, J, N*) Embryos with newly-segregated germ cells (stage 6). Transcripts of *api-piwi3* were almost not identified in the embryos of sexuparapar and oviparapar. *Api-piwi6* expression was not detected in the embryos of sexuparapar but was evenly distributed in those of the oviparapar. (*C, G, K, O*) Extension of the germband and limb bud formation (stage 12–13). Transcripts of *api-piwi3* were not
identified in the embryos of sexuparous and oviparous but expression of Api-ago3b in sexuparous embryos was preferentially identified in germ cells. Api-ago3b transcripts were evenly distributed in the embryos of oviparae. In panel (G), germ cells are not presented in the shown focal plane. D, H, L, P: Germ band retraction (stage 17) and completion of germ band retraction (stage 18). Transcripts of Api-ago3a were almost not identified in the embryos of sexuparous and oviparous. In sexuparous specific expression of Api-ago3a was identified in germ cells located in the dorsal region of the embryo. Universal expression of Api-ago3a was detected in the embryos of oviparae. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm. (TIF)

Figure S3 Comparison of expressions of Api-ago3a and Api-ago3b in sexuparous and oviparous embryos. Dissected ovarioles were hybridised with DIG-labelled antisense riboprobes of Api-ago3a (A–H) and Api-ago3b (I–P), respectively. For orientation and morphological characteristics of embryos refer to Figure 3O. Early development: A, B, E, F, I, J, M, N; Mid development: C, G, K, O; Late development: D, H, L, P. Germ cells stained with antisense Api-ago3a and Api-ago3b riboprobes are highlighted with arrows (with preferential expression) and double arrows (without preferential expression). A, E, I, M: Germinaria and segregated oocytes (stage 1). Transcripts of Api-ago3a were identified in the germinaria and the oocytes of sexuparous and oviparous embryos (arrowheads) but transcripts of Api-ago3b were not detected. B, F, J, N: Embryos with newly-segregated germ cells (stage 6). Expression of Api-ago3a was evenly distributed in the embryos of sexuparous and oviparous. Transcripts of Api-ago3b were not identified in the embryos of both sexuparous and oviparous. C, G, K, O: Extension of the germand and limb bud formation (stage 12–14). Transcripts of Api-ago3a were evenly distributed in the embryos of sexuparous and oviparous. By contrast, transcripts of Api-ago3b were not almost identified in both morphs. D, H, L, P: Germ band retraction (stage 17) and completion of germ band retraction (stage 18). Expression of Api-ago3a was evenly distributed in embryos of sexuparous and oviparous. Transcripts of Api-ago3b were almost undetected in the embryos of both morphs. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm. (TIF)

Figure S4 Double in situ hybridisation of Api-piwi2 and vasa in virgino-parous embryos. A–D: Ovarioles hybridised with DIG-labelled antisense riboprobe of Api-piwi2; E–H: Ovarioles hybridised with FL-labelled antisense riboprobes of vasa; I–L: Ovarioles hybridised with both DIG-labelled Api-piwi2 and FL-labelled vasa riboprobes. Color keys indicating single and double in situ signals are highlighted below the figures. Orientation and developmental stages of embryos refer to Figure 3O. Single in situ hybridisations show that both Api-piwi2 and vasa were expressed in germinaria and oocytes during early development (panels A and E). From mid development onward, transcripts of Api-piwi2 were preferentially expressed in germ cells but universal expression of Api-piwi2 could be identified in somatic cells (B–D). Expression of vasa remained specific in germ cells (F–H). Panels (I) to (L) show co-localised signals of Api-piwi2 and vasa in germinaria, oocytes and embryonic germ cells. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm. (TIF)

Figure S5 Double in situ hybridisation of Api-piwi6 and vasa in virgino-parous embryos. A–D: Ovarioles hybridised with DIG-labelled antisense riboprobe of Api-piwi6; E–H: Ovarioles hybridised with FL-labelled antisense riboprobes of vasa; I–L: Ovarioles hybridised with both DIG-labelled Api-piwi6 and FL-labelled vasa riboprobes. Color keys indicating single and double in situ signals are highlighted below the figures. Orientation and developmental stages of embryos refer to Figure 3O. Locations of germ cells are indicated with arrows. Single in situ hybridisations show that both Api-piwi6 and vasa were expressed in germinaria and oocytes during early development (panels A and E). From mid development onward, transcripts of Api-piwi6 were preferentially expressed in germ cells but universal expression of Api-piwi6 could be identified in somatic cells (B–D). Expression of vasa remained specific in germ cells (F–H). Panels (I) to (L) show co-localised signals of Api-piwi6 and vasa in germinaria, oocytes and embryonic germ cells. Abbreviations: Bc, endosymbiotic bacteria; Dev, development; Hd, head; St, stage. Scale bar, 20 μm. (TIF)

Table S1 Summary of in situ hybridisation of Api-piwi and Api-ago3 genes during embryogenesis. (DOC)

Table S2 (DOC)

Table S3 Primers used for semi quantitative RT-PCR. (DOC)

Table S4 Primer pairs used for the synthesis of sense and antisense riboprobes. (DOC)

Data S1 Alignment of deduced protein sequences of the eight Api-piwi genes and the two Api-ago3 genes. Diced protein sequence of the eight Api-Piwi and the two Api-Ago3 proteins were aligned with their D. melanogaster orthologues Dm-Piwi, Dmc-Aub and Dmc-Ago3 by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The PAZ and PIWI domains [47] of each protein were deduced from protein sequence by Interproscan [42]. Amino acids (AA) that belong to the PAZ domain are underlined in yellow, AA that belong to the MID domain are underlined in gray. Key residues predicted in the PAZ domain to be involved in the binding of sRNA are indicated in pink. The DDH triad of the PIWI domain involved in slicer activity is indicated in red. (DOC)
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Author Contributions

Conceived and designed the experiments: SJ-P H-LL C-CC. Performed the experiments: SJ-P H-LL C-CC ST. Analyzed the data: SJ-P H-LL C-CC KG CR JPG. Contributed reagents/materials/analysis tools: TW. Wrote the paper: SJ-P C-CC DT CR OE.

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