The transcriptomic signature of cyclical parthenogenesis

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Abstract

Cyclical parthenogenesis, where females can engage in sexual or asexual reproduction depending on environmental conditions, represents a novel reproductive phenotype that emerged during eukaryotic evolution. The fact that environmental conditions can trigger cyclical parthenogens to engage in distinct reproductive modes strongly suggests that gene expression plays a key role in the origin of cyclical parthenogenesis. However, the genetic basis underlying cyclical parthenogenesis remains understudied. In this study we characterize the female transcriptomic signature of sexual vs. asexual reproduction in the cyclically parthenogenetic microcrustacean *Daphnia pulex* and *D. pulicaria*. Our analyses of differentially expressed genes, pathway

enrichment, and GO term enrichment clearly show that compared to sexual reproduction the asexual reproductive stage is characterized by both the under-regulation of meiosis and cell-cycle genes and the up-regulation of metabolic genes. The consensus set of differentially expressed genes that this study identifies within the meiotic, cell-cycle, and metabolic pathways serve as candidate genes for future studies investigating how the two reproductive cycles in cyclical parthenogenesis are mediated at a molecular level. Furthermore, our analyses identify some cases of divergent expression among gene family members (e.g., doublesex, NOTCH2) associated with asexual or sexual reproductive stage, suggesting potential functional divergence among gene family members.

Significance statement

In some eukaryotic species, individuals can alternate sexual or asexual reproduction depending on the environmental conditions (i.e., engaging in cyclically parthenogenetic reproduction). However, the genetic mechanisms underlying cyclical parthenogenesis remains understudied. This study reveals the gene expression changes associated with sexual and asexual reproduction in a cyclical parthenogen, the microcrustacean *Daphnia pulex*.

Introduction

The establishment of sexual reproduction is a defining event of early eukaryotic evolution (Cavalier-Smith 2002). It is characterized by the (1) evolution of meiosis, (2) uniparental transmission of the organelle genome, (3) selective cell-cell fusion of gametes, and (4) the regulation of diploid resting spore formation by such coupling (reviewed in Goodenough and Heitman 2014). In the ~2.5-billion-year evolution of eukaryotes, most eukaryotic lineages remain sexual in spite of the evolutionary costs of sex (Otto 2009). However, the transition from

sexual, meiotic reproduction to parthenogenetic reproduction has occurred independently in many phylogenetic groups except birds and mammals (Bell 1982; Neiman *et al.* 2014; Simon *et al.* 2003). With ~1 in every 1,000 eukaryotic species reproducing parthenogenetically (Vrijenhoek 1998), chromosomally unreduced gametes can occur through various, distinct modified forms of meiosis (Maynard Smith 1978; Neiman *et al.* 2014; Suomalainen *et al.* 1987).

Interestingly, in contrast to obligately asexual reproduction where sex is completely abandoned, some eukaryotic lineages (e.g., rotifers, aphids, cladoceran crustaceans) have evolved cyclical parthenogenesis that can confer the evolutionary benefits of both sexual and asexual reproduction (Bell 1982). Cyclically parthenogenetic females can alternate between sexual and asexual reproduction depending on the environmental conditions. The fact that the same genome can engage in meiotic or parthenogenetic production of gametes under different environmental conditions strongly suggests that environment-mediated transcriptomic changes play a critical role in the origin of cyclical parthenogenesis. However, to date the transcriptomic signatures associated with the distinct phases of cyclical parthenogenesis remain unclear, resulting in a lack of understanding of the possible underlying genetic mechanisms.

In this study we experimentally investigate transcriptomics of cyclical parthenogenesis in Cladoceran microcrustaceans with *Daphnia* as a representative. There are about 600 species within the order Cladocera, occupying all kinds of aquatic habitats: freshwater ponds, lakes, and brackish and marine waters (Forro *et al.* 2008; Korovchinsky 1996). While in other major crustacean clades the emergence of asexual lineages does occur, e.g., asexual ostracods (Butlin *et al.* 1998), all cladoceran species reproduce through cyclical parthenogenesis with some lineages even having become obligately asexual. This suggests that cyclical parthenogenesis is a derived

trait in the Cladocera and the underlying genetic mechanism is most likely conserved in all cladoceran lineages, although lineage-specific modifications are possible.

Daphnia is one of the best known cladocerans because of its world-wide distribution in freshwater habitats. *Daphnia* females reproduce asexually under favorable environmental conditions (e.g., excellent food availability, low population density), producing numerous subitaneous eggs that directly develop into neonates in the females' brood chamber (**Fig. 1A**). The production of subitaneous eggs occurs through an ameiotic cell division in the germline. In this ameiotic division, the meiotic cellular machinery is modified so that recombination is suppressed, meiosis I is finished prematurely before the onset of anaphase I without the segregation of homologous chromosomes into daughter cells, and no cytokinesis occurs at the end of meiosis I (Hiruta *et al.* 2010; Ojima 1958; Zaffagnini & Sabelli 1972). Therefore, the ameiotic division is mitosis-like, leading to the production of genetically identical daughters (barring spontaneous mutations).

The sex determination of the asexually produced offspring is environmentally controlled. Stressful conditions (e.g., high population density) and associated release of juvenoid hormone methyl farnesoate can trigger the male developmental program in the asexual progeny (Olmstead & Leblanc 2002). Therefore, males only appear in the *Daphnia* population under environmental stress. Furthermore, environmental stress also stimulates female *Daphnia* to switch to sexual production, producing eggs through meiosis (**Figure 1A**). Upon fertilization by sperm, the meiotic eggs (usually two such eggs are produced) develop into dormant embryos that are encapsulated in a protective case (ephippium). These dormant embryos can sustain harsh environmental conditions (i.e., desiccation of habitats) and hatch under suitable conditions.

Environmental conditions are thus the primary driver of reproduction mode and sex determination (two related but distinct aspects of reproduction) in *Daphnia*. For environmental signals to direct the choice of reproductive mode, environment-mediated gene expression changes most likely play a pivotal role in initiating different reproductive modes. To identify the genetic mechanisms underlying cyclical parthenogenesis in *Daphnia*, we examine gene expression profiles in two species of the North American *Daphnia pulex* species complex, *D. pulex* and *D. pulicaria*. Although they share the same taxonomic name as the European *D. pulex* and *D. pulicaria*, they are distinct lineages (Cornetti *et al.* 2019), and we refer only to the North American *D. pulex* and *D. pulicaria* in this study. These two species are considered an ecological species pair which started their divergence about 0.8-1.2 million years ago (Omilian & Lynch 2009). Despite their extremely similar morphology and overlapping distribution range (Benzie 2005; Brandlova *et al.* 1972), they show habitat segregation and distinct ecological attributes (Dudycha & Tessier 1999), with *D. pulex* exclusively living in ephemeral ponds and *D. pulicaria* inhabiting stratified permanent lakes.

Because environment can trigger *Daphnia* females to alternate between meiotic and ameiotic production of eggs, we hypothesize that meiotic and ameiotic division are characterized by distinct gene expression profiles of meiosis and cell cycle genes. Furthermore, as *Daphnia* genomes contain many gene duplicates involved in meiosis and the cell cycle (Schurko *et al.* 2009), we ask whether these duplicates have divergent expression patterns in the meiotic and ameiotic reproduction phase, which would indicate functional divergence. To answer these questions, we assessed the transcriptomes of early and late stages of meiotic *vs.* ameiotic females in multiple natural isolates of *D. pulex* and *D. pulicaria*. Our transcriptome data across isolates

and species reveal conserved transcriptomic signatures that clearly distinguish meiosis and ameiosis in *Daphnia*.

Results

Overview of transcriptomic data

To discover the transcriptomic programs that associate with the different reproductive modes in *Daphnia*, we collected transcriptomic data from four distinct reproductive stages – early meiosis (EM), late meiosis (LM), early ameiosis (EA), and late ameiosis (LA) (**Figure 1B**). Furthermore, to identify the common genetic mechanisms underlying cyclical parthenogenesis in *Daphnia*, we sampled multiple isolates from two *Daphnia* species (3 isolates in *D. pulex* and 2 in *D. pulicaria*), yielding a total of 60 RNA-seq samples **(see Supplementary Table S2)**.

We visualized the transcriptomic variation in our dataset by conducting a principal component analysis (PCA) using normalized RNA-seq read counts from the top 500 most variable genes (**Figure 1C**). The first principal component, largely corresponding to inter-specific variation in our dataset, separates the two species and explains 27% of the total variance. The second principal component that explains 16% of the total variance. While a global separation of the reproductive modes and timepoints is not obvious at first sight, when concentrating on each genotype one by one, we can see a separation between the two reproductive modes (meiosis versus ameiosis). Within each genotype, we also see the presence of a clear clustering for each timepoints (EA versus LA; EM versus LM), showing the existence of transcriptomic differences among the different stages. Separation between reproductive modes is greater than the separation observed between different timepoints of the same reproductive mode. This is especially true of the samples at meiotic stages, in which EM and LM show strong

clustering. These observations validate that our data capture the inter-specific and between-isolate transcriptomic diversity as well as the transcriptomic signatures of distinct reproductive stages.

Transcriptomic signature of early meiosis vs. early ameiosis

Because there is no clear "control" or "treatment" group in the analysis below, we referred to DEGs as having higher expression in meiosis (i.e., upregulated during meiosis) or higher expression in ameiosis (i.e., downregulated during meiosis) . Differential expression analysis between the early stage of meiosis and ameiosis (EM vs EA) in each genotype resulted in a consensus set of 455 genes with higher EA expression and 319 with higher EM expression (**Figure 2A; see Supplementary Table S3 for gene list)**. After mapping these consensus differentially expressed genes (DEGs) to KEGG pathways (304 annotated genes out of 774 mapped to 326 pathways), we found a striking trend that nearly all DEGs in meiosis and cell-cycle related pathways have higher expression in EM when compared to EA (**Figure 2B; Figure 3**), clearly deviating from a random distribution of down- and up-regulated DEGs (one proportion t-test with Holm correction $p = 3.31 \times 10^{-6}$). For example, all 18 DEGs in the cell cycle pathway had higher expression during in EM (**Supplementary Figure 2**), and 12 genes in the progesterone-mediated oocyte maturation pathway were had higher expression during EM, whereas only 1 gene (protein kinase A) had lower expression in EM (**Supplementary Figure 3**). An equally noticeable pattern is that DEGs mapped to metabolic pathways had overwhelmingly lower expressions in EM compared to EA (**Figure 2B**; **Figure 3**), again deviating from the random distribution of up- and downregulated DEGs (one proportion t-test with Holm correction $p = 1.46 \times 10^{-3}$).

These observations from consensus set of DEGs are supported by KEGG pathway and GO term enrichment analyses using DEGs identified through our analyses with pooled samples. Pathways involved in meiosis and cell proliferation such as the oocyte meiosis pathway ($p = 3.85x10^{-5}$) and cell-cycle pathway ($p = 5.04x10^{-11}$) were significantly enriched with DEGs with higher expression during EM compared to EA (**Figure 4A**). The higher expression of meiosis and cell-cycle elements during EM was further supported by the enrichment of the DNA replication initiation ($p = 0.011$) and cell division ($p = 0.097$) GO terms during EM (**Figure 4B**). On the other hand, pathways and GO terms that were found to be enriched with DEGs with lower expression during EM were metabolism related. Among the metabolic pathways that were most enriched with DEGs with lower EM expression were pathways involved in sugar metabolism ($p = 0.0013$), especially amino sugars like UDP that are important in chitin metabolism (**Supplementary Figure 4)**. In addition to metabolic pathways, we also observe the hormone signaling renin-angiotensin pathway ($p = 0.071$) containing substantially lowered expression during EM compared to EA (**Supplementary Figure 5**).

Taken together, these results strongly suggest that onset of meiotic reproduction is associated with higher expression of meiosis and cell-cycle genes and with lower expression of metabolic genes, whereas the transcriptomic signature of early ameiotic reproduction is lower expression of meiosis and cell cycle genes and higher expression of metabolic genes (**Figure 3**).

Transcriptomic signature of late meiosis vs. ameiosis

The consensus differentially expressed genes (DEGs) for the comparison between LM (late meiosis) and LA (late ameiosis) stage consisted of 98 genes with higher LM expression and 71 genes with higher LA expression (**Figure 2A; see Supplementary Table 4**). Although this represents a 4- to 5-fold decrease in the number of consensus genes compared to the analysis of

early reproductive stages, we noted that a total of 51 and 20 genes maintained their differential expression pattern between early- and late- ameiosis and early- and late-meiosis stages (**Figure 2C**), respectively, including the persistent ameiotic-biased (higher expression found in ameiotic reproduction) expression of methyl farnesoate epoxidase and the persistent meiotic-biased (up-regulated) expression of NIMA (never in mitosis gene a) - related kinase 7. The former is an important enzyme in the methyl farnesoate signaling pathway, whereas the latter is an important regulator of mitosis (Fry *et al.* 2012).

Along with this decrease of consensus genes, KEGG enrichment analysis with pooled samples also showed fewer enriched pathways from 27 in the early-stage analysis to only 12 pathways at late reproductive stages. This marked decrease is largely due to the loss of enriched pathways (from 12 during early stages to 1 during late stages) that are enriched for higher expression during meiosis (**Figure 4A, C**). Our analyses reveal that metabolic pathways are still enriched with genes exhibiting high expression during late ameiosis when compared to late meiosis (**Figure 4C**).

The only pathway that was enriched of genes with higher expression during late meiosis is the ECM-receptor interaction pathway (**Figure 4C**). This is consistent with the loss of the cell division and DNA replication GO terms that were found during early meiosis using pooled sample analysis, although oogenesis remains in the top 10 enriched GO terms (not significant after correcting for multiple comparisons) (**Figure 4B, D**). Instead, we observed that many amino acid, lipid, and complex carbohydrate catabolism and biosynthesis related GO terms were enriched with DEGs possessing higher expression during late meiosis (**Figure 4D**).

Thus, while transcription is strikingly less conserved among genotypes during the late stages of the meiotic and ameiotic cycles (i.e., lower number of consensus genes), many of the

same metabolic and hormone (e.g., renin-angiotensin) pathways persistently had higher expression throughout the early and late ameiosis stages.

Differential expression within gene family

In total, 6443 genes were placed into 1691 gene families. While more than half of these families consist of only two genes (i.e., paralogs), around 13% of families are more expansive and contain over 5 genes (**Supplementary Figure 6**). These gene families containing over 5 genes represent broad biochemical functional groups rather than paralogs and were therefore excluded from downstream analysis. The results presented below are solely based on our analyses using pooled samples.

Although our analysis of the expression patterns of genes in each family revealed no differential expression in 656 (44.8%) and 879 (60%) gene families in early- and late-stage analyses, respectively (**Figure 5A, B**), two notable divergent expression patterns within gene family were identified. In the remaining gene families with at least one gene showing differential expression, most consisted of both DEGs and non-DEGs members (represented in regions marked with an arrow followed by a dash in **Figure 5A, B**). For example, 349 (19.9%) and 292 (23.8%) gene families contained member genes that were up-regulated and some non-differentially expressed member genes in early ameiosis and early meiosis, respectively; 330 (22.5%) and 164 (11.2%) gene families had paralogs that were up-regulated and non-differentially expressed in late ameiosis and late meiosis, respectively.

Furthermore, some of these gene families exhibiting divergent expression contained some genes with significantly increased expression during ameiosis and other genes with significant differential expression in a different direction (i.e., increased expression during meiosis) . We

will refer to this divergent expression pattern as bi-directional expression (represented in regions containing two arrows in opposing directions in **Figure 5A, B**). In total, 96 unique gene families showed this bi-directional expression pattern in at least one stage of reproduction (**Supplementary Table S5**). 50 families were found exhibiting bi-directional expression exclusively at early stages, 24 at lates stages, and 22 families maintained this pattern at both early and late stages. Especially interesting are the gene families involved in upstream transcriptional and signaling processes that have a paralog whose expression was specific to each reproductive cycle (**Figure 5C**).

Two such gene families represent transcription factors – *DMRT4_5* (doublesex gene) and *TFIIB* (transcription factor II B). *Daphnia* possesses two copies of the doublesex gene with one copy (gene15158) being upregulated during the sexual cycle and the other (gene4231) being upregulated during the asexual cycle. A previous study has identified one copy of the doublesex gene to be essential to sex determination in *Daphnia*, while the function of other copy remains unknown (Kato *et al.* 2011). Furthermore, another family with this bi-directional expression pattern is the NOTCH2 receptor in the NOTCH signaling pathway (**Figure 5C**), previously found to modulate reproduction in another arthropod system (Duncan et al. 2016). We also identified three gene families related to the glutamate and GABA signaling, including two neurotransmitter receptors (i.e., *GRIK1* and *GABRB*) and a modulator of neuroreceptors (i.e., *DBI*).

On the other hand, we found a small percentage of gene families whose member genes were all differentially expressed in the same direction between ameiosis and meiosis (represented by the region described as unidirectional and marked with a single arrow in **Figure 5A, B**). For gene families whose expression were higher during early- (60 gene families) and late-ameiosis

(37 families) stages, they contain 18 overlapping gene families. For gene families with higher expression in early- (36 families) and late-meiosis (9 families) stages, 6 gene families were in common. Consistent with the general trend identified for enriched pathways, most gene families with higher expression in ameiosis were associated with digestion and metabolism (with genes such as alpha-amylase, lactase-phlorizin hydrolase, and alpha-L-fucosidase), while gene families with higher expression in meiosis included several cyclins and other cell cycle regulators (**Supplementary Table S6 and Supplementary File 1**).

Discussion

Cyclical parthenogenesis represents a novel reproductive phenotype during the evolution of eukaryotes. Cladoceran crustaceans are the only clade in Crustacea that exclusively reproduce by cyclical parthenogenesis (McLaughlin 1980). To understand the genetic mechanisms underlying the evolution of cyclical parthenogenesis from sexual reproduction, we investigate the transcriptomic signature of sexual (meiotic) *vs*. asexual (ameiotic) reproduction stages in two *Daphnia* species, *D. pulex* and *D. pulicaria*.

A novelty of this study is that we distinguish and compare the transcriptomes of early and late stages of meiotic *vs.* ameiotic female *Daphnia pulex and D. pulicaria*, which has not been done in previous studies (e.g., Raborn *et al.* 2016; Zhang *et al.* 2016). This strategy allows us to investigate the transcriptomic signature close to the initiation of sexual/asexual reproduction and assess the persistence and divergence of transcriptomic signatures as *Daphnia* females advance in reproductive state. Furthermore, as transcriptomes are heavily affected by environment and genotype interaction, we use a consensus and pooled analytic approach across the five genotypes of *D. pulex* and *D. pulicaria* to remove transcriptomic noise and unveil the conserved transcriptomic program associated with the initiation of sexual/asexual reproduction. We focus

on genes annotated with a KO number or GO term to understand how transcriptional changes in these genes may be associated with reproductive mode switching.

Taking advantage of these experimental and analytical procedures, this study provides novel insights into the transcriptomic signature associated with sexual vs. asexual reproduction in *Daphnia*, which has not been observed in previous studies assessing transcriptomes of parthenogens (Hanson *et al.* 2013; Parker *et al.* 2019; Srinivasan *et al.* 2014; Warren *et al.* 2018). In comparison to ameiosis (i.e., asexual reproduction), the early stage of meiotic, sexual reproduction features genes with higher expression being enriched in meiosis and cell-cycle pathways and genes with lower expression were enriched in metabolism pathways. In contrast, early asexual reproduction (e.g., ameiosis) is characterized by the abundant genes in meiosis and cell-cycle pathways with lower expression and genes in metabolism pathways with higher expression.

Although our RNA-seq data are derived from female whole-body tissue, these patterns provide important clues as to genetic mechanisms underlying cyclical parthenogenesis in *Daphnia*. *Daphnia* females deposit all primary germ cells in the posterior end of ovary (Kato *et al.* 2012), and several primary germ cells can embark on either meiotic or ameiotic division as the females enter sexual or asexual reproduction. It is likely that the up- and down-regulation of meiosis and cell cycle genes are involved in determining the oogenesis pathway of primary germ cells. Among the under-expressed meiosis genes in asexual stage, we note an interesting candidate gene Cdc20, which is a member of the anaphase promoting complex and spindle checkpoint assembly. Cdc20 plays an important role in promoting homologous chromosome segregation, the onset of anaphase, and in maintaining ploidy during meiosis (Cooper & Strich 2011; Jin *et al.* 2010; Yin *et al.* 2007). The under-expression of Cdc20 in the ameiosis of

Daphnia could be primarily responsible for the observed cytological observations of no homologous chromosome segregation and no cytokinesis in ameiosis I.

Furthermore, the up- and down-regulation of transcription related to metabolism in early asexual and sexual reproduction could be due to the food quality and availability in favorable and adverse environmental conditions. Because it is well known that nutrients and amino acids play an important role in regulating gene expression (Haro *et al.* 2019; Moir & Willis 2013), we suggest that these metabolism- and biosynthesis-related differentially expressed genes may contain the master regulator controlling the reproductive mode of *Daphnia*. Pinpointing the master regulators will be highly interesting, but certainly remains a technically challenging task as it would involve intricate gene manipulation to modify the gene expression levels of genes of interests.

We note that the underexpression of meiosis genes and overexpression of metabolism genes in the early ameiosis for production of directly developing embryos in the cyclically parthenogenetic *Daphnia* is highly similar to the transcriptomic profile of early resting embryo production in obligately parthenogenetic *Daphnia* (Xu *et al.* 2021). Obligately parthenogenesis in *D. pulex* is distinct from cyclical parthenogenesis in that females parthenogenetically produce resting embryos, rather than through sexual reproduction. Notably these obligately parthenogenetic *D. pulex* originated through complex hybridization and backcrossing between cyclically parthenogenetic *D. pulex* and *D. pulicaria*. By comparing the transcriptomes of early parthenogenetic resting embryo production in obligate parthenogens with that of the cyclically parthenogenetic parental species *D. pulex* and *D. pulicaria* (i.e., meiosis), Xu et al. identified that the early embryo production in obligate parthenogens is characterized with underexpression of meiosis and cell-cycle genes and overexpression of metabolism genes compared to the parental

species. This observation coincides well with the cytological evidence that parthenogenetic production of resting embryo and parthenogenetic production of directly developing embryos in obligately parthenogenetic *Daphnia* share the same ameiotic cell division (Zaffagnini & Sabelli 1972).

Lastly, our analyses of the transcriptional differences between members of the same gene families provide initial evidence of possible functional divergence of gene duplicates. For example, we identified that paralogs of the doublesex, TFIIB, NOTCH2 receptor gene show meiosis- and ameiosis-specific up-regulation, indicating possible neofunctionalization of the paralogs. Detailed phylogenetic and functional analyses of these paralogs in *Daphnia*, cladocerans, and other crustacean outgroup lineages would be necessary to determine whether these paralogs experienced neofunctionalization.

Materials and Methods

Daphnia *culture and sampling*

Three isolates of cyclically parthenogenetic *D. pulex* and two of *D. pulicaria* were used in this study to identify the transcriptomic changes associated with ameiotic (asexual) vs. meiotic (sexual) reproduction in females. These isolates were originally collected from ephemeral ponds and lakes in North America (**Supplementary Table S1**). Each isolate was maintained as a clonal culture in COMBO artificial lake water (Kilham *et al.* 1998) at 18°C under a 12:12 hour light/dark photoperiod and was fed with the algae *Scenedesmus obliquus*.

Collection of females at different reproductive stages

Under a dissection microscope we collected females of each isolate at early ameiosis (EA), late ameiosis (LA), early meiosis (EM), and late meiosis (LM) stages (**Figure 1B**) that show distinct

size, color, and texture of the ovary (Rossi 1980). Females at EA stage possessed ovaries that are small, light, and clear. Ovaries at EM stage have a creamy color in contrast to the clear ovaries of early ameiosis. Ovaries of LA and LM stage are large and dark, but they can be distinguished from each other by their texture. Ovaries in LM are smooth because they typically only contain two embryos, while LA ovaries have a lumpy texture due to the presence of many embryos. Some *Daphnia* females were carrying offspring from a previous clutch in their brood pouch. In such cases, those offspring were removed from the brood pouch after the whole female was suspended in RNAlater® (ThermoFisher), and the offspring were excluded from the RNA extraction step. For each *Daphnia* isolate, three biological replicates of around 20 females each were collected for each reproductive stage, yielding a total of 60 whole-body samples for RNA-seq.

To eliminate the impact of environmental factors on gene expression, all the isolates were acclimatized to the same culture conditions for two generations before we started collecting females. All isolates were exposed to crowding at a concentration of around 30 animals per 25 mL to induce some of the females to enter the sexual cycle. Both sexual and asexual females were sampled from the same culture where they co-existed, minimizing the impact of environmental conditions on our gene expression data.

RNA isolation, library preparation, and sequencing

We extracted total RNA from each sample using the Quick-RNA tissue/Insect RNA extraction kit (Zymo Research). The RNA quality of each sample was assessed on an Agilent Bioanalyzer. We purified mRNA from each total RNA sample using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and prepared sequencing libraries using NEBNext® Ultra™ RNA Library Prep Kit following the manufacturer's instruction. Sequencing

of the libraries was performed on an Illumina HiSeq2500 platform with 150bp paired-end reads. The raw data are deposited at NCBI SRA PRJNA764929.

Transcriptomic analyses of ameiosis vs. meiosis

The raw transcript abundance of genes in each RNA-seq sample was quantified using the quasi-mapping approach in the software package Salmon (Patro *et al.* 2017) on its default parameters, with *D. pulex* PA42-3.0 transcriptome (Ye *et al.* 2017) as the reference. To validate whether our transcriptomic data correctly captured the transcriptomic diversity of different reproductive stages, isolates, and species, we performed principal component analysis using transcript count data normalized in the software DESeq2 (Love *et al.* 2014). The PCA analysis was performed with the plotPCA function of the DESeq2 package, using the top 500 genes with the most variance.

To characterize the transcriptomic profiles of ameiosis vs. meiosis, we examined the gene expression differences contrasting early/late stage of meiosis vs ameiosis (i.e., EM *vs.* EA, LM *vs.* LA) and contrasting early and late stage within meiosis and ameiosis by pooling all the *Daphnia* genotypes. Specifically, we identified the differentially expressed genes (DEGs) for each of the four reproductive stage comparisons, using the Wald negative binominal test with the design formulae ~clone + stage in DESeq2 (Love et al. 2014). A gene was considered differentially expressed if it had an adjusted p-value < 0.05 (Benjamini and Hochberg method) and a fold change > 1.5.

In addition to the analyses based on pooling all sampled genotypes, we also identified DEGs contrasting EM *vs.* EA and LM *vs.* LA in each individual genotype. We then generated a

consensus set of the genes that are differentially expressed in the same comparison across all the examined *Daphnia* isolates and species.

Enrichment analysis of KEGG pathways and GO terms

Mapping differentially expressed genes (DEGs) into functional pathways can provide stronger power for making biological discoveries compared to examining the functional significance of individual genes alone. We therefore produced KEGG pathway maps to visualize how differentially expressed genes may interact and to understand their collective functional significance.

First, we reconstructed the KEGG pathway maps for the *D. pulex* PA42-3.0 transcriptome (Ye *et al.* 2017). We queried all the protein sequences in the KEGG Automatic Annotation Server (KAAS) using the GHOSTX program (Moriya et al. 2007). 10,135 out of the 18,440 genes in the transcriptome were assigned a KO (KEGG ortholog) number, and 6,282 genes annotated with a KO number were placed to a KEGG pathway map.

In order to increase the power to detect biologically relevant pathways, up- and down-regulated DEGs were analyzed separately (Hong et al. 2014). We identified the KEGG pathways enriched for up- or down-regulated genes using hypergeometric tests with Holm–Bonferroni corrected p values. Pathways significantly enriched for up- or down-regulated genes ($p < 0.05$) were then visually inspected using the software Gene Annotation Easy Viewer (GAEV) (Huynh and Xu 2019), which displays DEGs on KEGG pathway maps with colors indicating the direction and magnitude of the differential expression.

Insight from KEGG pathways were supplemented with information from gene ontology (GO) enrichment analysis to find differentially activated biological processes between

reproductive stages. 11,447 genes out of the 18,440 total genes were annotated with at least one GO term. All GO enrichment tests were performed using the R package topGO (Alexa and Rahnenfuhrer 2020) with the weight01 method, its default combination of elim and weight algorithms. P values were adjusted with the Holm method with a cutoff of 0.05.

Divergence of expression within gene family

Divergence of expression within gene family associated with ameiosis and meiosis may indicate functional divergence between paralogous genes. We define a gene family as a group of at least two genes that have been annotated with the same KEGG ortholog number by the KEGG Automatic Annotation Server (KAAS). We investigated whether genes within the same gene family are differentially expressed in the same direction between meiosis and ameiosis using the differential expression pattern of the consensus gene set.

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Author contributions

SX and ASH designed this study. ASH performed the tissue collection and molecular experiments. TVH performed data analyses. TVH and SX wrote the manuscript with input from ASH.

Data Accessibility

The raw reads are deposited at NCBI SRA PRJNA764929. The scripts used in this study are available at https://github.com/UtaDaphniaLab/The-transcriptomic-signature-of-cyclical-parthenogenesis

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Figure 1. (A) Cyclically parthenogenetic life history of *Daphnia*. **(B)** Female *Daphnia* at the four reproductive stages where RNA was collected. Reproductive stages were identified based on the size, color, and texture of the ovaries (red circles). **(C)** PCA of the transcriptomic data.

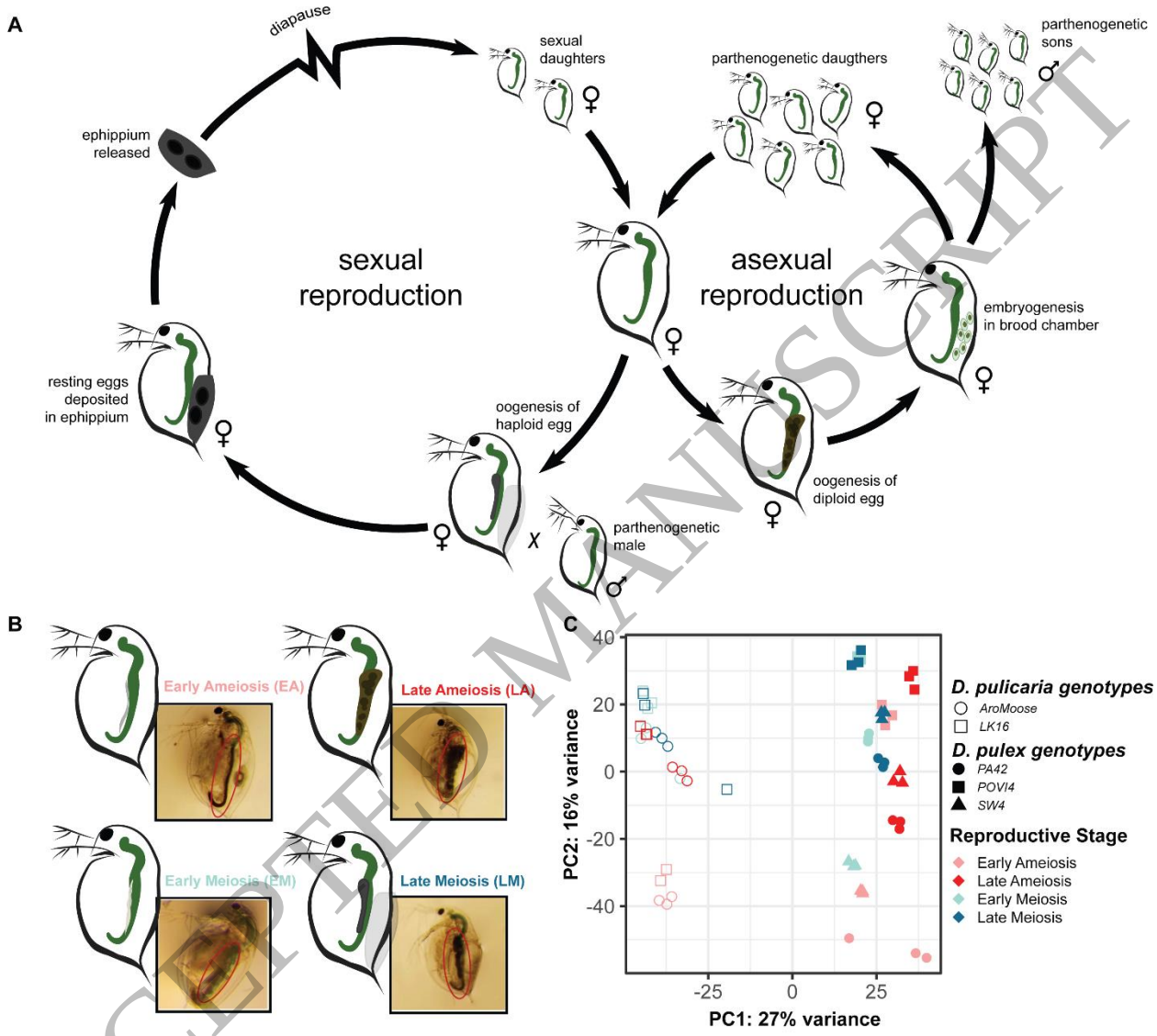

Figure 2. (A) Venn diagrams showing the number of differentially expressed genes (DEGs) in each genotype for the comparison between early meiosis vs. early ameiosis and late meiosis vs. late ameiosis. The top panels represents the number of DEGs with higher expression in ameiosis, whereas the bottom panels show DEGs with higher expression in meiosis (bottom) when the two reproductive modes were compared against each other at similar timepoints. **(B)** Distribution of consensus genes among the top enriched KEGG pathways. **(C)** Alluvium graph illustrating the direction of differential expression of consensus DEGs that are concordant among all genotypes between early and late stages.

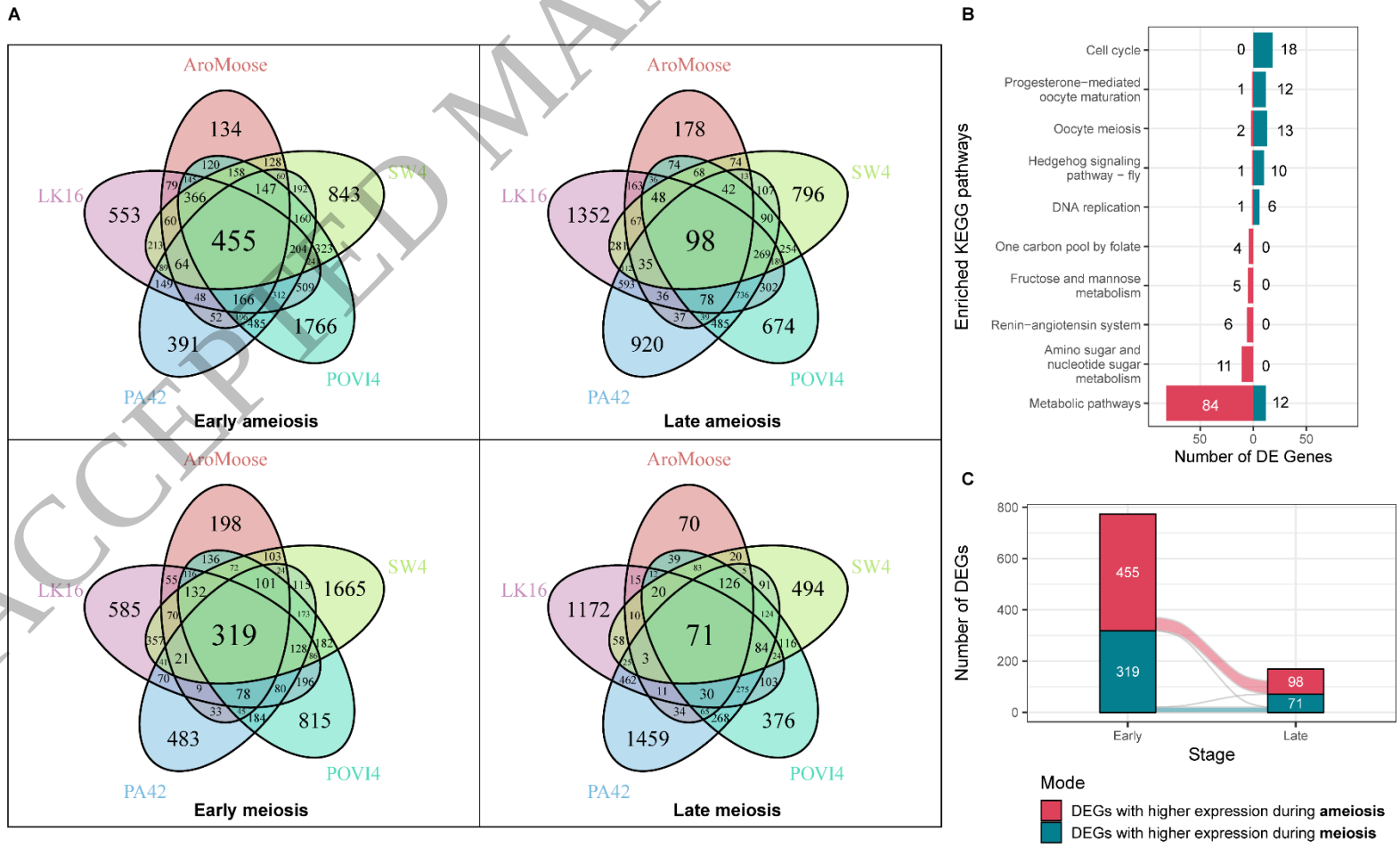

Figure 3. Expression heat map of KEGG pathways enriched with the consensus set of differentially expressed genes.

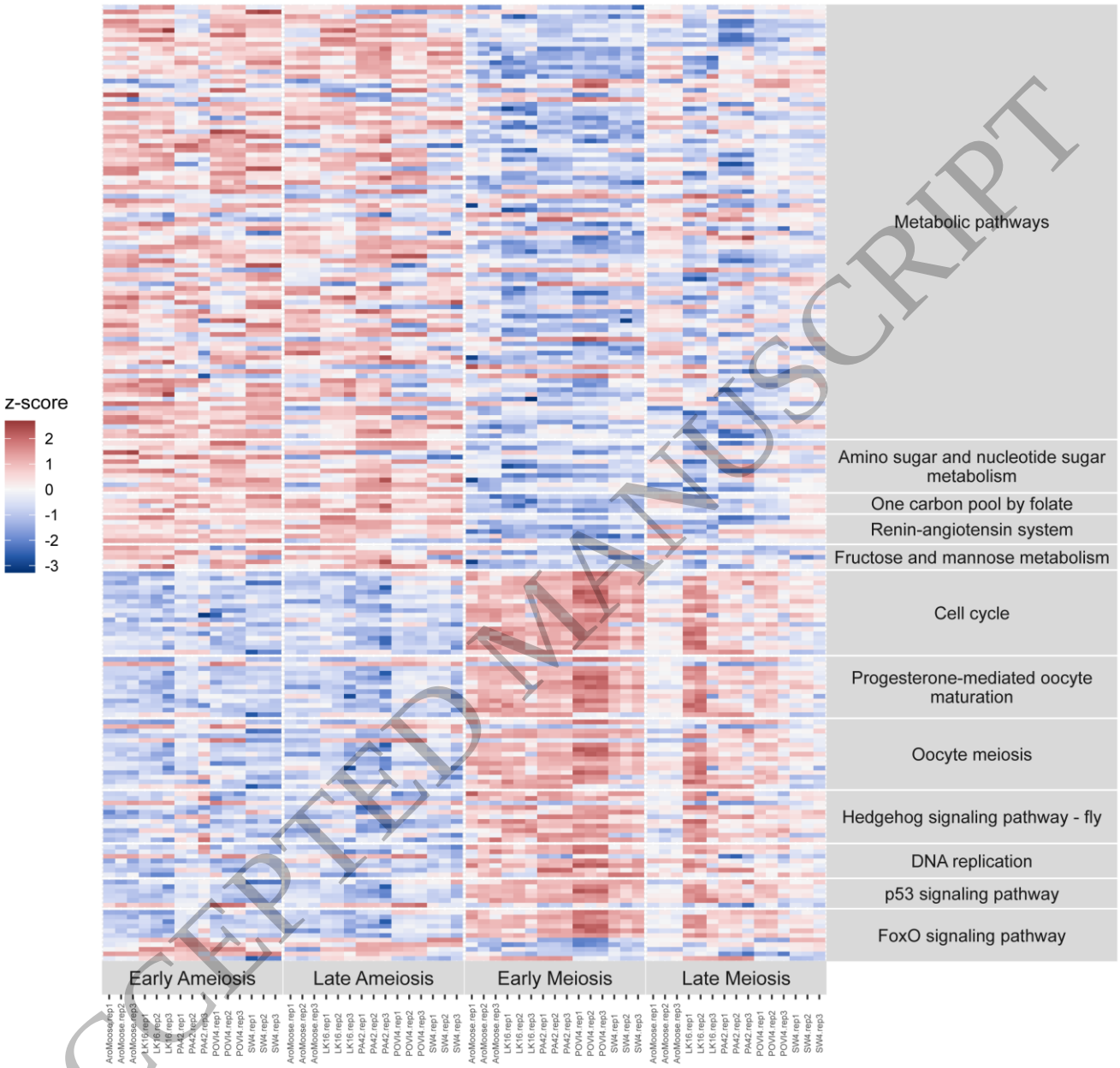

Figure 4. Functional analysis of differentially expressed genes (DEGs) from the pooled sample analysis contrasting meiosis against ameiosis, including significantly enriched KEGG pathways at (A) early and (C) late stages (vertical dash line represents no expression change) and top 10 enriched GO terms in (B) early-stage and (D) late-stage analysis.

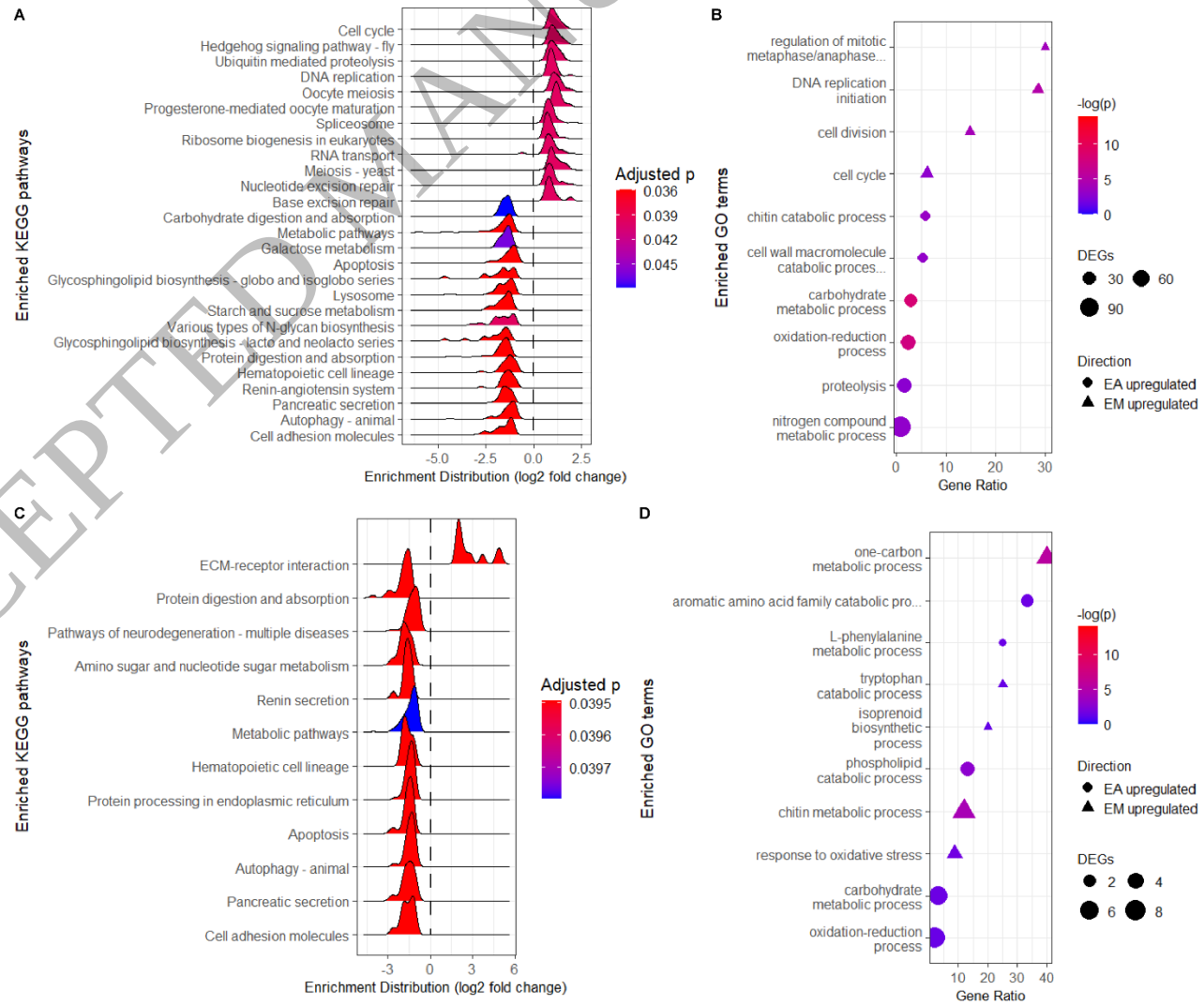

Figure 5. Venn diagrams showing gene families of different differential expression direction between member genes in early stage (A) and late stage (B) analysis. (C) Dot plots representing gene families with a bi-directional expression pattern in the early-stage comparison. Expression patterns at late stage is similar to the early stage shown here. Each gene is plotted by its log2 fold change, and significance is defined as adjusted p-value < 0.05 and fold change > 1.5. DEG: differentially expressed genes.