Reproductive pattern in the solanum mealybug, *Phenacoccus solani*: A new perspective

Jun Huang [Corresp., 1], **Fuying Zhi** [1, 2], **Juan Zhang** [3], **Muhammad Hafeez** [1], **Xiaowei Li** [1], **Jinming Zhang** [1], **Zhijun Zhang** [1], **Likun Wang** [1], **Yaobin lu** [Corresp. 1]

[1] Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

[2] College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, China

[3] Institute of Garden Plants and Flowers, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Corresponding Authors: Jun Huang, Yaobin lu
Email address: junhuang1981@aliyun.com, luybcn@163.com

Background. The reproductive pattern of most scale insects is ovoviviparity. The solanum mealybug, *Phenacoccus solani* (Hemiptera: Pseudococcidae), is known as a thelytokous parthenogenetic species, but there is still debate about the reproductive strategies of this species.

Methods. Here, we investigated the oviposition characteristics of *P. solani* and used scanning/transmission electron microscopy and RNA-seq to identify the differences between two types of eggs.

Results. We found that *P. solani* laid two types of eggs in one batch, with no significant difference in apparent size: one with eyespots that hatched and another without eyespots that failed to hatch. Furthermore, the physiological and molecular differences between the two types of eggs were highly significant. KEGG enrichment analysis revealed significant enrichment for the JAK-STAT, Notch, Hippo, and Wnt signaling pathways and dorsoventral axis formation, wax biosynthesis, cell cycle, insulin secretion, and nitrogen metabolism pathways. The results suggest that the embryo of the egg undergoes development inside the mother and only a short molting period outside the mother.

Discussion. Ovoviviparous species produce eggs and keep them inside the mother's body until they are ready to hatch, and the offspring exits the egg shell during or immediately following oviposition. Therefore, we suggest that the reproductive pattern of *P. solani* can be described as ovoviviparity.

Reproductive pattern in the solanum mealybug,

Phenacoccus solani: A new perspective

Jun Huang[1], Fuying Zhi[1,2], Juan Zhang[3], Muhammad Hafeez[1], Xiaowei Li[1], Jinming Zhang[1], Zhijun Zhang[1], Likun Wang[1], Yaobin LU[1]

[1] Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;

[2] College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua 321004, China;

[3] Institute of Garden Plants and Flowers, Zhejiang Academy of Agricultural Sciences, Hangzhou 311202, China;

Corresponding Author:

Jun Huang

NO. 298, Desheng Road, Hangzhou City, Zhejiang Province, 310021, China.

Junhuang1981@aliyun.com

Yaobin LU

NO. 298, Desheng Road, Hangzhou City, Zhejiang Province, 310021, China.

luybcn@163.com

Abstract

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Subjects Agricultural Science, Entomology, Insect Bioecology

Keywords mealybug, reproductive pattern, oviposition, egg, ovoviviparity, *Phenacoccus solani*

Introduction

The reproductive strategies of most insects have been described with three main patterns: oviparity, viviparity and ovoviviparity (*Meier et al., 1999*; *Wheeler, 2003*; *Gullan & Granston, 2014*). In oviparous species, egg development occurs in the external environment after oviposition, and hatching occurs outside the mother's body, whereas in viviparous species (which are relatively rare among insects), egg development occurs inside the mother's body, which provides gas exchange and, more importantly, nourishment for the embryos which are born alive (*Andrews & Rose, 1994*; *Tworzydlo et al., 2013*). Based on nutritional relationships between maternal and embryonic organisms, two modes of viviparity are recognized: matrotrophic and lecidotrophic (*Ostrovsky et al., 2016)*. Ovoviviparity is in fact a specific type of viviparity where developing eggs are retained within the body of the mother, and the offspring are nourished by the reserve materials accumulated in the eggs during oogenesis (*Blackburn, 1999*; *Gaino & Rebora, 2005*; *Lodé, 2012*). Therefore, the recent view is that there are truly only two main reproductive strategies: viviparity and oviparity (*Ostrovsky, 2013; Ostrovsky et al., 2016*). In previous studies, some insects, such as cockroaches (*Warnecke & Hintze-Podufal, 1996*), aphids (*Ortiz-Rivas et al., 2004*), tsetse flies (*Meier et al., 1999*), thrips (*Kranz et al., 2002*), and scale insects (*Gavrilov & Kuznetsova 2007*; *Ngernsiri et al., 2015*), were described as ovoviviparous species. Scale insects (Hemiptera: Sternorrhyncha: Coccoidea), like many Hemiptera, feed on sap drawn directly from the plant vascular system and secrete a waxy coating for defense; in addition, many scale insect species are major quarantine pests of agricultural or ornamental plants in tropical/subtropical climates as well as in greenhouses in temperate zones worldwide (*Gullan & Kosztarab 1997*; *Gavrilov-Zimin, 2018*). All previous relevant studies suggest that the phenomenon of ovoviviparity is widely distributed among scale insects (*Tremblay, 1997*; *Gavrilov & Kuznetsova, 2007*). Gavrilov-Zimin (*2018*) provided an overview of the distribution of different variants of ovoviviparity/viviparity among scale insect families and demonstrated that the evolution of scale insects shows multiple cyclic conversions of the oviparous reproduction pattern to ovoviviparity/viviparity, with the appearance of new and interesting adaptations for egg protection. In other words, the reproductive modes of scale insects may be rich and

95   variable; however, the understanding of the course of evolution of reproductive patterns

96   in these insects is not complete (*Gavrilov & Kuznetsova, 2007*). Therefore, the

97   identification of reproductive patterns has great heuristic value in terms of both

98   reproductive and evolutionary biology.

99   The solanum mealybug, *Phenacoccus solani* (Hemiptera: Pseudococcidae), is native to

100 North America (*Chatzidimitriou et al., 2016*) and is a newly recorded species in China.

101 Furthermore, this mealybug species is quite polyphagous and considered to be a major

102 threat to agricultural production and the ecological environment, causing significant

103 problems (*Ben-Dov, 2005a*; *Zhi et al., 2018*). *P. solani* is a thelytokous parthenogenetic

104 species, and no male individuals are found in its populations (*Lloyd, 1952*; *Ben-Dov,*

105 *2005b*; *Zhi et al., 2018*). Regarding the birth strategies of *P. solani*, McKenzie reported

106 that this species was viviparous (*McKenzie, 1967*); however, Kosztarab (*1996*) and

107 Chatzidimitriou *et al*. (*2016*) considered this species to be ovoviviparous. Vennila *et al*.

108 (*2010*) found that in another species of mealybug in the same genus, parthenogenesis

109 via ovoviviparity (96.5%) was dominant over oviparity (3.5%). Many scholars have found

110 that the hatching period of eggs laid by mealybugs is very short and that the hatching

111 process is relatively concealed (beneath the abdomen), as a result, short and concealed

112 hatching has been suggested as the main reason for the divergence of reproductive

113 modes in mealybugs (*Tremblay, 1997*; *Lagowska & Golan, 2009*; *Vennila et al., 2010*;

114 *Zhi et al., 2018*). According to our previous observations, we believe that the

115 reproductive mode in mealybugs, at least in *P. solani,* is complex and not simple to

116 define.

117 Here, we investigated the oviposition characteristics of *P. solani* and used scanning

118 electron microscopy (SEM) and transmission electron microscopy (TEM) to distinguish

119 between two types of eggs (i.e., reproductive products putatively considered to be eggs)

120 laid in one batch. Finally, we further investigated miRNA and mRNA expression in the

121 two types of eggs. We aimed to answer the following questions: 1) What life stage is *P.*

122 *solani* laying – an egg, a nymph, or something in between? 2) If different reproductive

123 "products" coexist, do they differ in appearance, physiology and molecular biology? 3)

124 According to the above findings, what is the reproductive mode of *P. solani*?

125

Materials & Methods

Oviposition characteristics

The oviposition process of female adults of *P. solani* was observed on two transparent, single-well, concave slides (Fig. 1) (length * width = 76.2 mm * 25.4 mm; the thickness of each concave slide was 1.3 mm; the diameter and depth of the circular hole were 15 mm and 0.5 mm, respectively). The steps of the procedure were as follows: 1) a concave slide was placed face up on a flat table and a female adult was gently placed into the middle of the hole of the concave slide with a brush; 2) quickly another concave slide was placed face down, on top of the bottom concave slide; and 3) finally, rubber bands were used to bind the ends of the two concave slides to secure them. Preliminary experiments showed that the thickness of female adults was 0.91 ± 0.03 mm, so the above observation method did not harm the bodies of female adults. The egg hatching process was further tracked and observed under a NikonSMZ1500 zoom stereomicroscope (Nikon, Japan), and photographs were taken every 5 minutes from the start of the egg laying process.

Egg morphology

The number, length, width and hatching rate of eggs with eyespots (hereafter eggs with-ES) or eggs without eyespots (hereafter eggs without-ES) were further observed in the same batch of eggs under a NikonSMZ1500 zoom stereomicroscope. There were two kinds of treatments: one employed the concave slide method (placing a female adult between two concave slides as mentioned above), and the other employed the blade method, i.e., placing a female adult on detached potato leaves, with the petiole wrapped with defatted cotton to maintain leaf freshness and the placing the whole treated leaves in Petri dishes (diameter = 9 cm, thickness = 1.4 cm). The oviposition of female adults was observed every 30 minutes from 9.00 a.m. to 4.00 p.m, and the numbers of the two kinds of eggs were counted. Each female adult was biometrically tested once, and each treatment was repeated 15 times. A total of 25 eggs were randomly selected from the two kinds of eggs, and their lengths and widths were measured. After 72 hours, the hatching of the two kinds of eggs was observed. Eggs with-ES hatched 114 individuals, while eggs without-ES hatched 45 individuals. The experiment was carried out in an

156   artificial climate chamber with a temperature of 27 ± 1°C, a humidity of 70% ± 5% and a

157   photoperiod of 16 L: 8D.

158   **Does the mother's body affect the hatching of eggs?**

159   Two treatments were established: 1) eggs with-ES were incubated under the mother's

160   body (Fig. 2A), and 2) eggs with-ES were artificially removed from the mother and kept

161   on the concave slide (Fig. 2C). Then, the female adults were continuously observed

162   through a NikonSMZ1500 zoom stereomicroscope every five minutes. Continuous

163   stretching of the abdomen by female adults indicated that they were about to lay eggs.

164   For treatment 2, the upper concave slide was removed immediately, and the mother

165   was carefully removed with an insect pin. Hatching time was recorded as the time from

166   when the female adult laid eggs to the time when the eggshell was completely

167   detached. Each egg was bioassayed once; experiment 1 included 30 replicates, and

168   experiment 2 included 47 replicates.

169   **Microscopic differences between the two kinds of eggs**

170   **1 Egg surface and internal structure**

171   The surface and internal structure of the two kinds of eggs were observed by SEM

172   (SU8010, Hitachi, Japan) and TEM (H7650, Hitachi, Japan). The collected eggs with

173   and without ES were pretreated with liquid nitrogen immediately. The follow-up

174   procedures followed the instrument operation methods for the scanning electron

175   microscope. TEM was performed as follows: 1) fixation, eggs were immersed in 2.5%

176   glutaraldehyde fixative solution and then rinsed with buffer solution; 2) gradient

177   dehydration, the fixed samples were dehydrated with an ethanol; 3) gradient osmosis,

178   the samples were permeated with a mixture of acetone and Spurr resin penetrant (1:1);

179   4) embedding and polymerization, 100% Spurr embedding agent was added, followed

180   by polymerization for 24 hours; 5) ultrathin sectioning, the samples were cut to

181   approximately 90 nm; and 6) observation and photography.

182   **2 RNA sequencing and data analysis**

183   **RNA extraction, library construction and RNA sequencing**

184   Samples of newly laid eggs (with-ES or without-ES) were collected and immediately

185   placed in a 0.5 MIEP tube and then frozen in liquid nitrogen for RNA extraction. Total

186   RNA was extracted from 6 samples, and a library was constructed as previously

187   described (*Yin et al., 2018*). The libraries were sequenced on the Illumina HiSeq X Ten

188   platform, and 150 bp paired-end reads were generated. Raw data (raw reads) in fastq

189   format were first processed using Trimmomatic (*Bolger et al., 2014*). Reads containing

190   poly-N stretches and reads oflow quality were removed to obtain clean reads. After

191   removing adaptor and low-quality sequences, the clean reads were assembled into

192   expressed sequence tag clusters (contigs) and de novo assembled into transcripts by

193   Trinity (*Grabherr et al., 2011*) (version: 2.4) with the paired-end method. The longest

194   transcript was chosen as a unigene based on similarity and length for subsequent

195   analyses. Raw data were deposited in the National Center for Biotechnology

196   Information (NCBI) Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra)

197   under accession number PRJNA554708.

198   **Unigene de novo assembly, functional annotation and data analysis**

199   Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd.

200   (Shanghai, China). The functions of the unigenes were annotated by alignment of the

201   unigenes with the NCBI nonredundant (NR), SwissProt, EuKaryotic Orthologous Groups

202   (KOG), Gene Ontology (GO) and Kyoto-Encyclopedia of Genes and Genomes (KEGG)

203   databases. Differentially expressed unigenes (DEGs) were identified using the DESeq

204   (*Anders & Huber, 2013*) functions "estimate size factors" and "nbinom test". A p value <

205   0.05 and fold change > 2 or < 0.5 were set as the thresholds for significant differential

206   expression. Hierarchical cluster analysis of DEGs was performed to explore transcript

207   expression patterns, and KEGG pathway enrichment analysis of DEGs was performed

208   using R based on a hypergeometric distribution.

209

210   # Results

211   **Oviposition characteristics**

212   Female adults of *P. solani* produced eggs in one generation through thelytokous

213   parthenogenesis, and the eggs were long and oval, similar to those described by Zhi *et*

214   *al*. (*2018*). Female adults secreted silken wax, but it never formed an ovisac. After egg

215   laying, the eggshell was detached from the nymph, followed by the appearance of 1st-

216   instar nymphs that quickly crawled away from the lower part of the mother. Moreover, in

217   a batch of eggs laid by female adults of *P. solani*, two kinds of eggs with distinct

218 morphological differences appeared (Fig. 3A/B): two reddish-brown eyespots were seen
219 on one type of egg (eggs with-ES; Fig. 3D), while the other type did not display these
220 eyespots (eggs without-ES; Fig. 3C). The hatching process of eggs with-ES was as
221 follows: 1) in the first 5 minutes, the eggs began to show considerable peristalsis
222 beneath the mother, and at 10-15 minutes, the eggshell was gradually detached, and
223 wax powder appeared on the surface of the body; and 2) at approximately 20 minutes,
224 antennae and feet began to appear, and the hatching process was generally completed
225 within 25 minutes, while the detached eggshell could be seen at the abdominal end of
226 the 1st instar nymph (Fig. 4).

227 **Egg morphology**
228 In the present study, we observed that female adults could lay two types of eggs, those
229 with-ES and those without-ES, in either treatment. The ratio of eggs with-ES was
230 significantly larger than that of eggs without-ES (on concave slides, 91.56 ± 2.14 vs.
231 8.44 ± 2.14, respectively, $\chi2$ = 86.01, n = 15, p< 0.001; on leaves, 87.31 ± 2.90 vs.
232 12.69 ± 2.90, respectively, $\chi2$ = 13.07, n = 15, p< 0.001) (Fig. 5A). The eggs were long
233 and elliptical, with lengths of 0.320 ± 0.006 mm (with-ES) and 0.305 ± 0.008 mm
234 (without-ES) (t = 1.42, n = 25; Fig. 5C) and widths of 0.146 ± 0.002 mm (with-ES) and
235 0.147 ± 0.003 mm (without-ES) (t = 0.24, n = 25; Fig. 5D). Furthermore, the hatching
236 rate of eggs with-ES was 100%, and no eggs without-ES hatched (Fig. 5B).

237 **Does the mother's body affect the hatching of eggs?**
238 To investigate the extent to which the mother's body affected the hatching of eggs, eggs
239 without-ES were removed from the area beneath the mother's body after they were laid.
240 Therefore, we further tested whether eggs with-ES hatched after they were removed
241 from the area beneath the mother's body. We found that eggs with-ES could hatch
242 normally under any treatment (Fig. 2 A/B/C), but the hatching times were different.
243 Inside the mother's body, the hatching time was 24.30 ± 0.60 minutes (Fig. 2D), but
244 when the mother's body was removed, the hatching time was reduced by nearly 5
245 minutes to 19.47 ± 0.45 minutes (Fig. 2E). Therefore, we suggest that the mother's
246 body has no effect on the success of egg hatching, or it could be inferred that the failure
247 of eggs without-ES to hatch was largely due to internal factors. Many species of scale
248 insects secrete abundant wax and form an ovisac that covers eggs and prevents their

249   adhesion, but in some species, ovisacs are not built, and the time of egg development

250   outside of the maternal body is decreased (*Gavrilov-Zimin, 2018*). *P. solani* belongs to

251   the latter group, secreting a small amount of wax and never forming an ovisac. Thus, it

252   is not surprising that the eggs of *P. solani* hatched so quickly, especially when the eggs

253   were isolated.

254   **Microscopic differences between the two types of eggs**

255   **1 Egg surface and internal structure**

256   The surface and internal structure of eggs with and without-ES were observed using

257   SEM and TEM. The contour of appendages could be clearly seen across the eggshell,

258   and the bristles, tubular glands and six conical receptors (which were symmetric, with

259   three on each side) could be seen on the surfaces of eggs with-ES (Fig. 6A); moreover,

260   complete blood cells, cytoplasm, mitochondria and myocutaneous filaments were

261   observed inside (Fig. 6B). Eggs without-ES had a smooth surface (Fig. 6C) and

262   contained only lipid droplets, endoplasmic reticulum and free ribosomes (Fig. 6D). **2**

263   **RNA sequencing and data analysis**

264   Illumina sequencing generated approximately 45 M reads per sample after the removal

265   of low-quality reads. These reads were assembled randomly and produced 55,558

266   unigenes with an N50 of 1,026 nt. After annotating unigenes with several databases and

267   calculating the expression of unigenes as fragments per kilobase of exon model per

268   million reads mapped (FPKM), correlation coefficients between samples were

269   calculated and used to estimate biological repeatability and differences between groups.

270   The correlation coefficient of 3 biological replicates in the group with-ES and the group

271   without-ES was > 0.8, and the sample correlation coefficient between these two groups

272   was only 0.4, showing an obvious difference between these two groups (Fig. 7A). DEGs

273   were identified and screened with the thresholds of *p* < 0.05 and fold change > 2 (or fold

274   change < 0.5) (Fig. 7B/C). There were 13,164 DEGs between the with-ES and without-

275   ES groups, including 9,243 up regulated DEGs and 3,921 down regulated DEGs. The

276   DEGs are shown with a volcano plot, and a heat map was generated from hierarchical

277   cluster analysis to show the expression patterns of the DEGs (Fig. 7B). KEGG

278   enrichment analysis of the DEGs was performed to determine the main pathways

279   associated with these DEGs. We found that the JAK-STAT, Notch, Hippo, and Wnt

280 signaling pathways and dorso-ventral axis formation, wax biosynthesis, progesterone-

281 mediated oocyte maturation, cell cycle, eukaryote ribosome biogenesis, insulin

282 secretion, and nitrogen metabolism pathways were significantly enriched (Fig. 8).

283

Discussion

284

Oviposition and hatching characteristics

285

286 In the present study, we found eggs laid by parthenogenetic *P. solani*, but soon

287 emerged 1st-instar nymphs following hatch. Because this hatching process is fast (< 30

288 minutes), researchers might think that 1st-instar nymphs are produced by *P. solani*

289 female adults. A similar phenomenon was reported in another invasive mealybug,

290 *Phenacoccus solenopsis*, and because both eggs and 1st-instar nymphs were found in

291 oocysts, Vennila *et al*. (*2010*) considered the scale insect to be able to reproduce in

292 both ways, i.e., via ovoviviparity and oviparity. In *Coccus hesperidum*, naked nymphs

293 appeared from the vulvar orifice, but thin eggshells were shown to remain in the female

294 reproductive tract (*Hagan, 1951*), and in *P. solani*, the eggshells were kept beneath the

295 mother.

296 In *Matsucoccus matsumurae*, there are two types of eggs, those with and without

297 eyespots, but the eggs with eyespots are similar to those without eyespots, and the egg

298 types show different developmental periods with normal hatching (*Xie et al., 2014*).

299 However, *P. solani* laid two types of eggs in one batch, with no significant difference in

300 apparent size: one with eyespots that hatched and another without eyespots that failed

301 to hatch. Generally, eggs are under stress from external factors, which may prevent

302 them from hatching properly. For example, some of the eggs laid by heat-treated

303 females of *Nilaparvata lugens*were unable to hatch due to failure during blastokinesis

304 (*Lee & Roger, 1987*). Further research revealed that yeast-like symbiotes in *N. lugens*

305 play an important role in the embryonic and postembryonic development of eggs,

306 especially the ventral differentiation of the embryo (*Wilkinson & Ishikawa, 2001*; *Nan et*

307 *al., 2016*). The symbiotic bacteria in the bodies of most mealybug subfamily insects are

308 *Tremblaya princeps* (*Gruwell et al., 2010*), play a substantive role in the host plant

309 specificity of their hosts (*Baumann, 2005; Hansen & Moran, 2014*), and are correlated

310 with host development (*Huang et al., 2015*); therefore, we suggest that the absence of

311   symbiotes might explain the presence of nondeveloping eggs. Another type of insect

312   egg that does not hatch is the nutritive egg common in social insects, such as ants. The

313   nutritive eggs of ants are unfertilized eggs that cannot hatch and are eaten in colonies

314   containing a queen (*Heinze et al., 1996, 1999*). However, for this parthenogenetic and

315   thelyotokous species of mealybug, the factors causing *P. solani* to lay eggs that cannot

316   hatch require further study.

317   **Physiological and molecular differences between the two types of eggs**

318   According to these observations of egg surface and internal structure, we suggest that

319   eggs with-ES are alive, with features such as conical receptors, blood cells and

320   myocutaneous filaments, and that they still have an eggshell and are close to hatching,

321   even though they no longer resemble an egg on a microscopic level. Although some

322   important organelles such as endoplasmic reticulum and free ribosomes were present in

323   the eggs without-ES, mitochondria had never been found. Mitochondria are a special

324   organelle that contain their own genomes and plays an important role in oocyte

325   maturation and embryo development (*Ferenz, 1993; Lieber et al., 2019*). Moreover,

326   mitochondria are inherited only in the maternal line, i.e., mitochondrial DNA is passed

327   only through the mother's egg cell (*Ma et al., 2014; Lieber et al., 2019*). Lack of

328   mitochondrial redistribution in cytoplasm was a sign of immature oocyte and was closely

329   related to low developmental of eggs (*Bavister & Squirrell, 2000*). If mitochondria were

330   missing, it could be fatal to the development of egg cell and later embryonic

331   development. Therefore, we hypothesized that the development of egg cell might be

332   arrested for the eggs without-ES in the absence of mitochondria.

333   We further determined the differences between eggs with and without ES at the

334   molecular level. The result revealed that the differences in terms of unigene expression

335   between two types of eggs were highly significant, and we also found that the JAK-

336   STAT, Notch, Hippo, and Wnt signaling pathways and some important pathways related

337   to metabolism and nutrition were significantly enriched. Recently, the JAK-STAT, Notch,

338   Hippo and Wnt signaling pathways were found to independently or interactively

339   participate in the regulation of egg production (*Hombria & Brown, 2002*; *McGregor et al.,*

340   *2002*). For example, mutual antagonism between the Notch and JAK/STAT signaling

341   pathways provides a crucial facet of follicle cell patterning and ultimately helps establish

342 the polarity of the egg chamber (*Assa-Kunik et al., 2007*), and the Hippo pathway

343 controls polar cell specification by repressing Notch activity (*Chen et al., 2011*).

344 Moreover, some of these important signaling pathways are involved in aspects of cell

345 development and metabolic function, such as dorsal-ventral axis formation, wax

346 biosynthesis, insulin secretion and nitrogen metabolism.

347

348 Conclusions

349 Although the reproductive mode of *P. solani* has been described previously, there is still

350 no clear agreement on its definition. We found no differences between the two types of

351 eggs by visual observation, but the physiological and molecular differences were highly

352 significant. The results suggest that the embryonic development of eggs with-ES is

353 complete when the eggs are laid beneath the abdomen, i.e., the embryo of the egg

354 develops inside the mother. However, the embryonic development of eggs without-ES

355 seems to be incomplete. Although there are no direct data on the entire process of

356 embryonic development, we can at least be sure that the cell development and

357 physiological metabolism of eggs without-ES are hindered or arrested.. Moreover, we

358 found that eggs with-ES begin to hatch and shed their eggshell (immediately) after

359 leaving the mother's body: i.e., this species lays eggs and does not experience live

360 birth. Ovoviviparous species oviposit eggs at an advanced stage of embryological

361 development, and the larva exits the eggshell during or immediately following

362 oviposition (*Meier et al., 1999*). Therefore, we suggest that the reproductive pattern of

363 *P. solani* can be described as ovoviviparity.

364

365

366 Acknowledgements

367 We would like to thank He Ziyi (Senior Experimentalist, Nanjing Agricultural University),

368 and Liao Zhenfeng (Zhejiang Academy of Agricultural Sciences) for their support of

369 electron microscope samples making and photos taking, and Dr. Wenya Lu and Dr. Fan

370 Zhang (OE Biotech, Shanghai, China) for assistance with the bioinformatics analysis of

371 the sequencing data. We are so grateful to Zhou Zhongshi (Chinese Academy of

372 Agricultural Sciences), Jiang Mingxing (Zhejiang University), Xu Yijuan (South China

373   Agricultural University), Ma Jun (Guangzhou Customs), and Fu Jianwei (Fujian

374   Academy of Agricultural Sciences) for their comments and suggestions on the

375   manuscript.

376

377

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Figure 1

The device used for the observation of egg-laying and egg hatching of adult females of *Phenacoccus solani* (Double-concavity slide method)

(A) Position and operation diagram. Two concave slides overlapped seamlessly, and adult female was placed into the middle of the hole of the concave slide; concave slides were fixed with rubber bands at both ends. (B) The final observation device contained two separate adult females. (C) Multiple devices together.
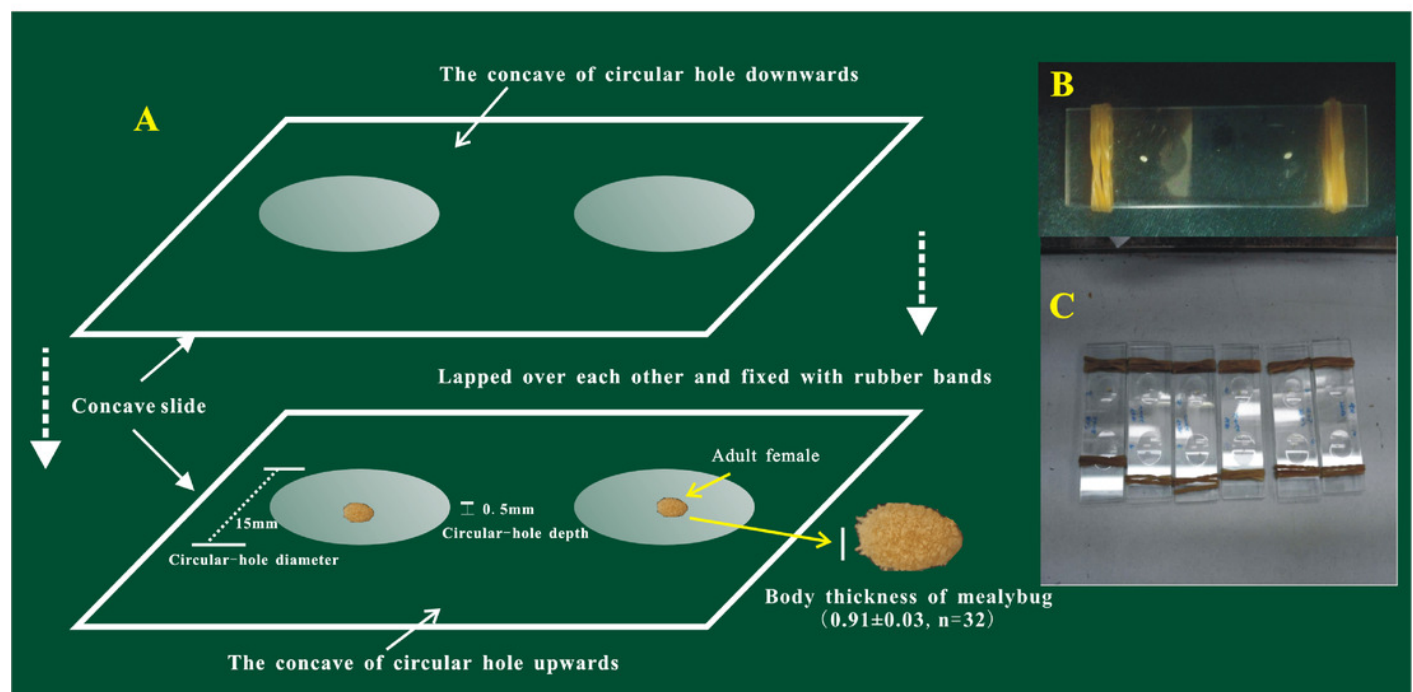
Figure 2

The hatching time of eggs beneath the abdomen of adult female or in isolation

(A) Egg hatching beneath the abdomen of adult female, at the point at which the eggshell detached; (B) Eggs with eyespots; (C) Egg hatching in isolation (without mother's body); (D) Hatching time of eggs beneath the abdomen of adult female; (E) Hatching time of eggs without the mother's body or in isolation.
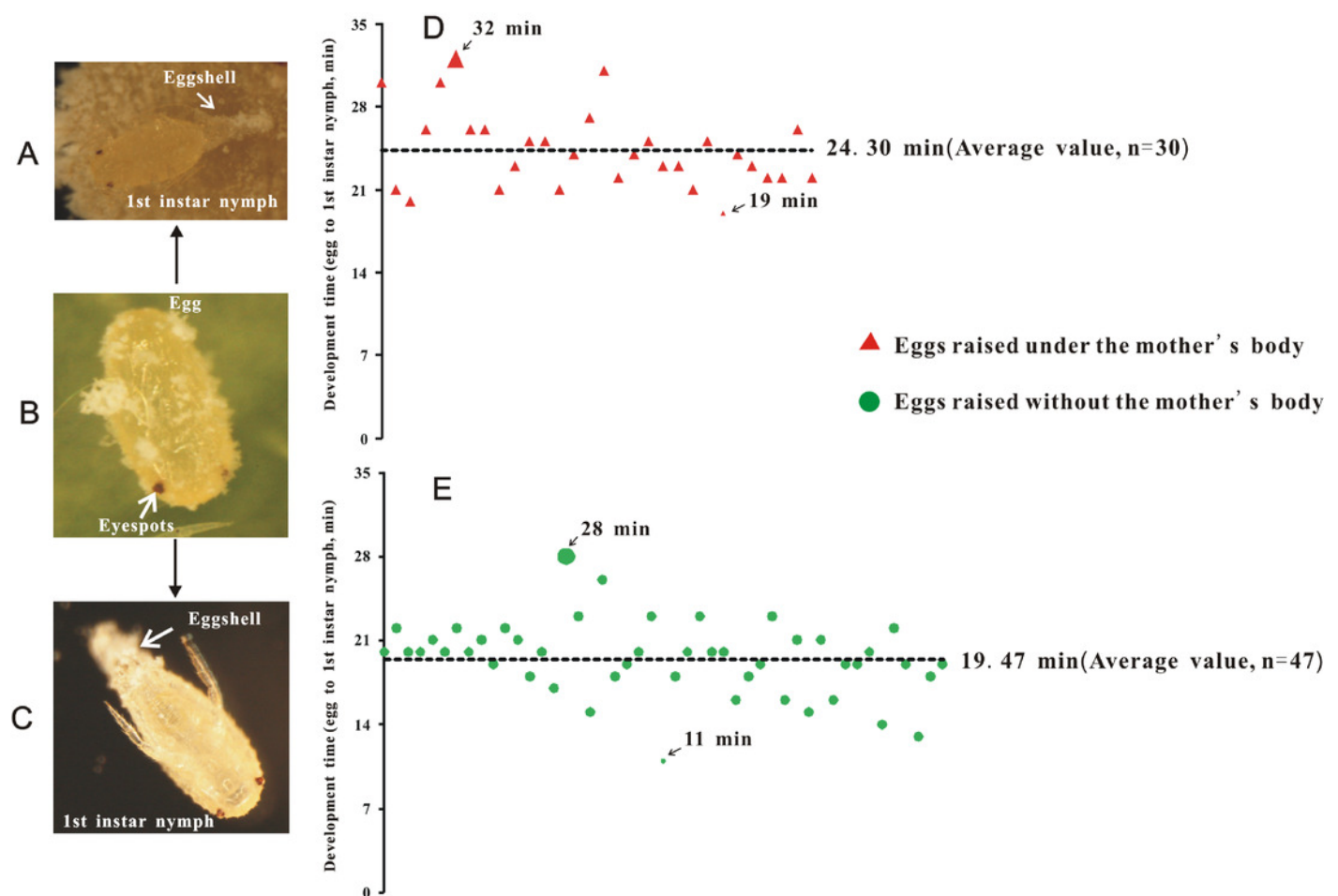
Figure 3

Adult female of *Phenacoccus solani* laid two types of eggs in one batch, one with eyespots (type 1), and another without eyespots (type 2)

(A) Adult female laying eggs, and some eggs have rapidly hatched into 1st instar nymphs; (B) Newly laid two types of eggs below the abdomen of adult female; (C) The eyespots of eggs (type 2) were not visible; (D) Eggs (type 1) before hatching, the eyespots were clearly visible.
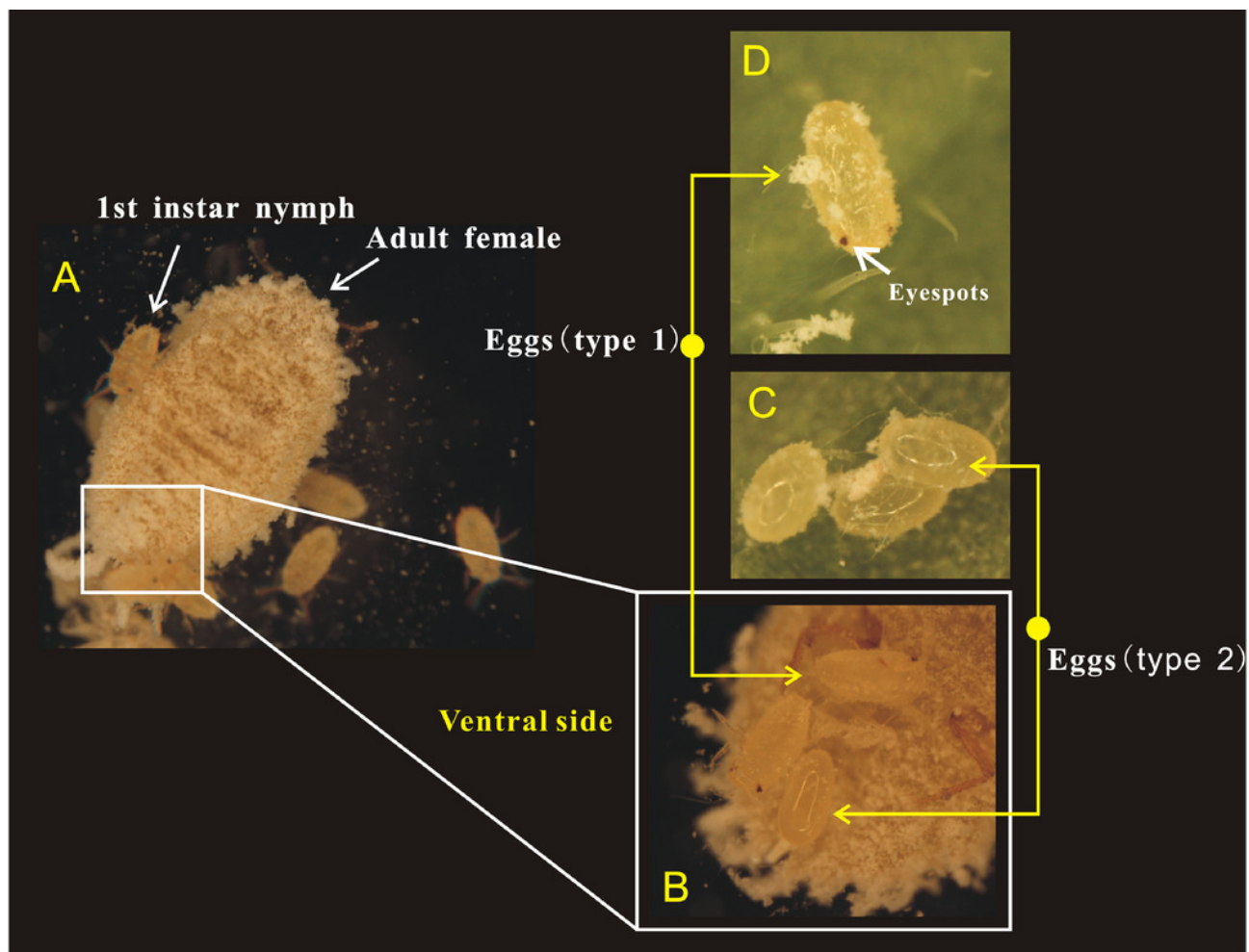
Figure 4

The hatching process of eggs

A) After the first 5 minutes, eggs began to show considerable peristalsis beneath the mother; B/C) At 10-15 minutes, the eggshell was gradually detached and wax powder appeared on the surface of body; D) At 20 minutes, antennae and feet were starting to become visible; E/F) The hatching was basically completed, while the detached eggshell could be seen at the abdominal end of the 1st instar nymph.
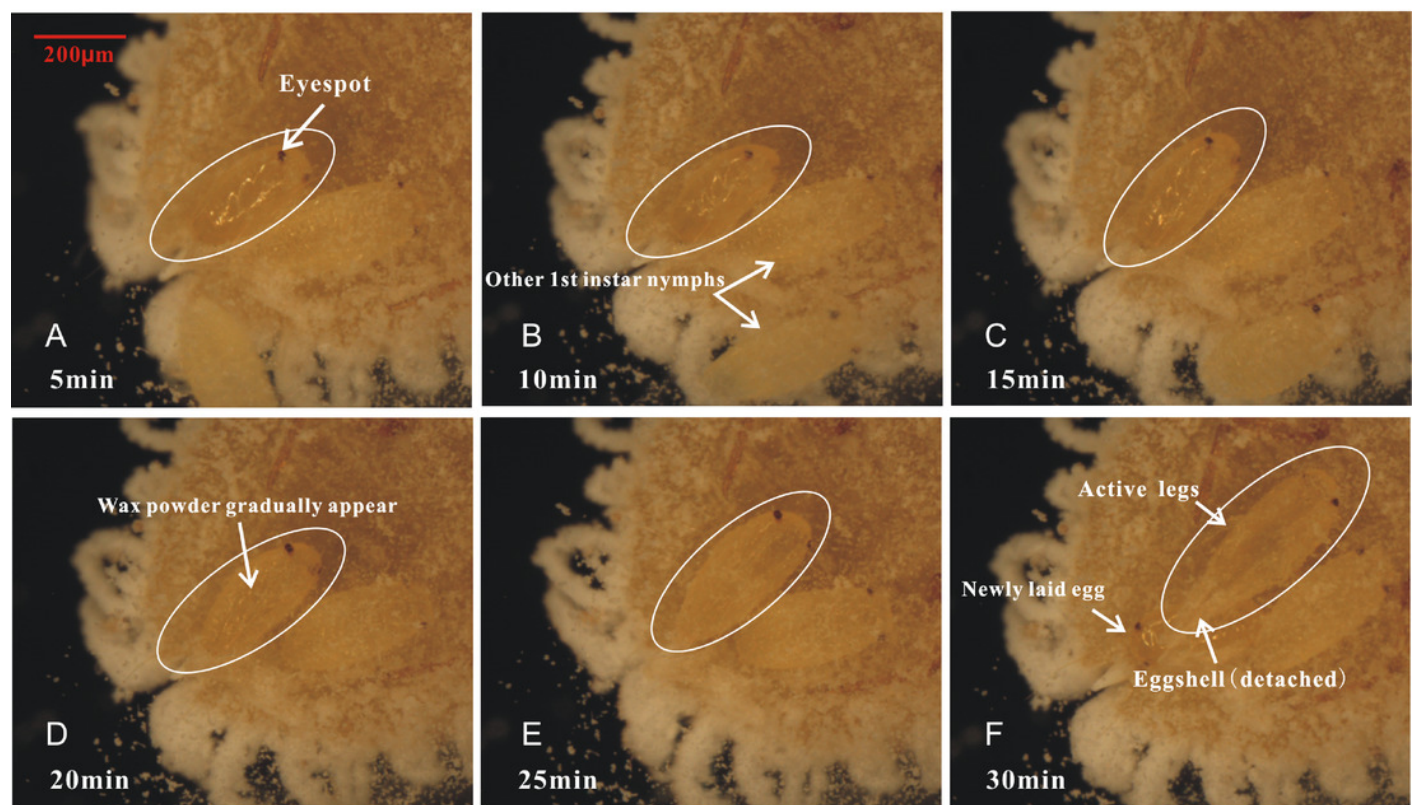
Figure 5

The percentage, morphology and hatching of the two types of eggs with different treatments

Percentage of the total for the two types of eggs with the treatment of placing female adults on leaves or concave slide (A), and hatching rate of eggs (B); the difference between the two types of eggs with same treatment was analyzed using Chi-SquareTest. The length (C) and width (D) of two types of eggs; the difference between the two types of eggs was analyzed using T-Test, and "*NS*" on the two bars indicate not significantly different from each other (*p* > 0.05).

$\chi^2 = 86.01,\ P < 0.001$

$\chi^2 = 13.07,\ P < 0.001$

A

100

80

60

Percentage of total (%)

40

0

Eggs
With eyespots
($n=15$)

Eggs
Without eyespots
($n=15$)

B

100

75

Hatching rate (%)

50

0

Eggs
With eyespots
($n=114$)

Eggs
Without eyespots
(n=45)

::::: On the leaf

On the concave slide

↓ Value=0

C

0.5

0.4

0.3

Egg length (mm)

0.2

0.1

0

NS

Eggs
With eyespots

Eggs
Without eyespots
($n=25$ each)

D

0.5

0.4

0.3

Egg width (mm)

0.2

0.1

0

NS

Eggs
With eyespots

Eggs
Without eyespots
($n=25$ each)

Figure 6

The surface and internal structure of eggs with eyespots and without eyespots

The surface of eggs with eyespots (A) and without eyespots (C) was observed by scanning electron microscope, and the internal structure of eggs with eyespots (B) and without eyespots (D) was observed by transmission electron microscope.
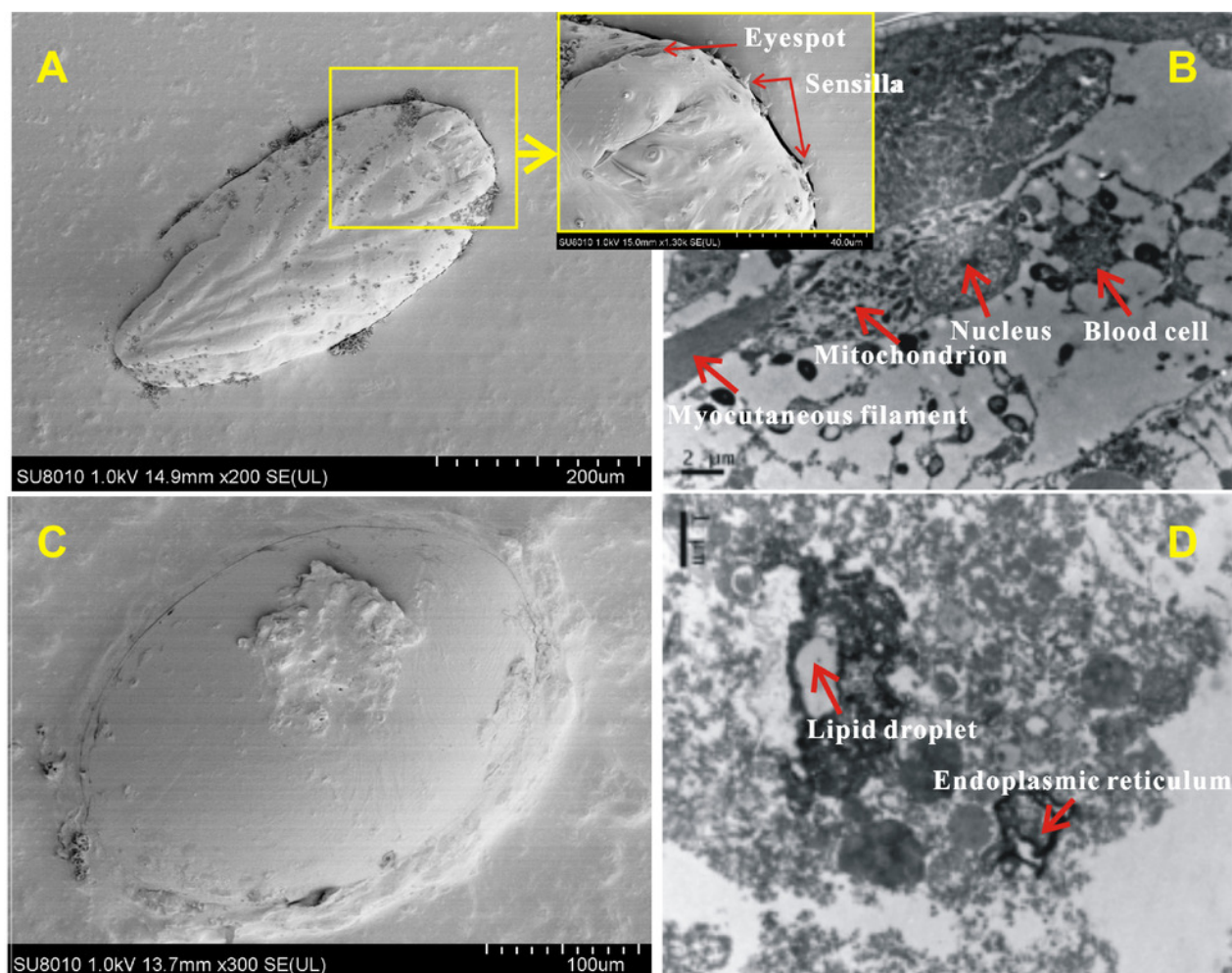
Figure 7

RNA-seq to distinguish the differences between two types of eggs.

Heat-map coefficient matrix (A); the abscissa indicates the sample name, the ordinate indicates the corresponding sample name, and the color indicates the correlation coefficient. The closer the correlation coefficient is to 1, the higher the similarity in expression patterns between samples. Volcano plots of different differentially expressed unigenes (DEGs) between two groups. Green dots indicate down-regulated unigenes, red dots indicate upregulated unigenes, and grey dots indicate no differential unigenes (B). Cluster analysis of DEG levels. Expression differences are shown in different colors. Red and blue indicate up-regulation and down-regulation, respectively (C).

A

B

Number of up genes is 9243
Number of down genes is 3921
p-value<0. 05, |log2FC|>1

C

p-value<0. 05, |log2FC|>1
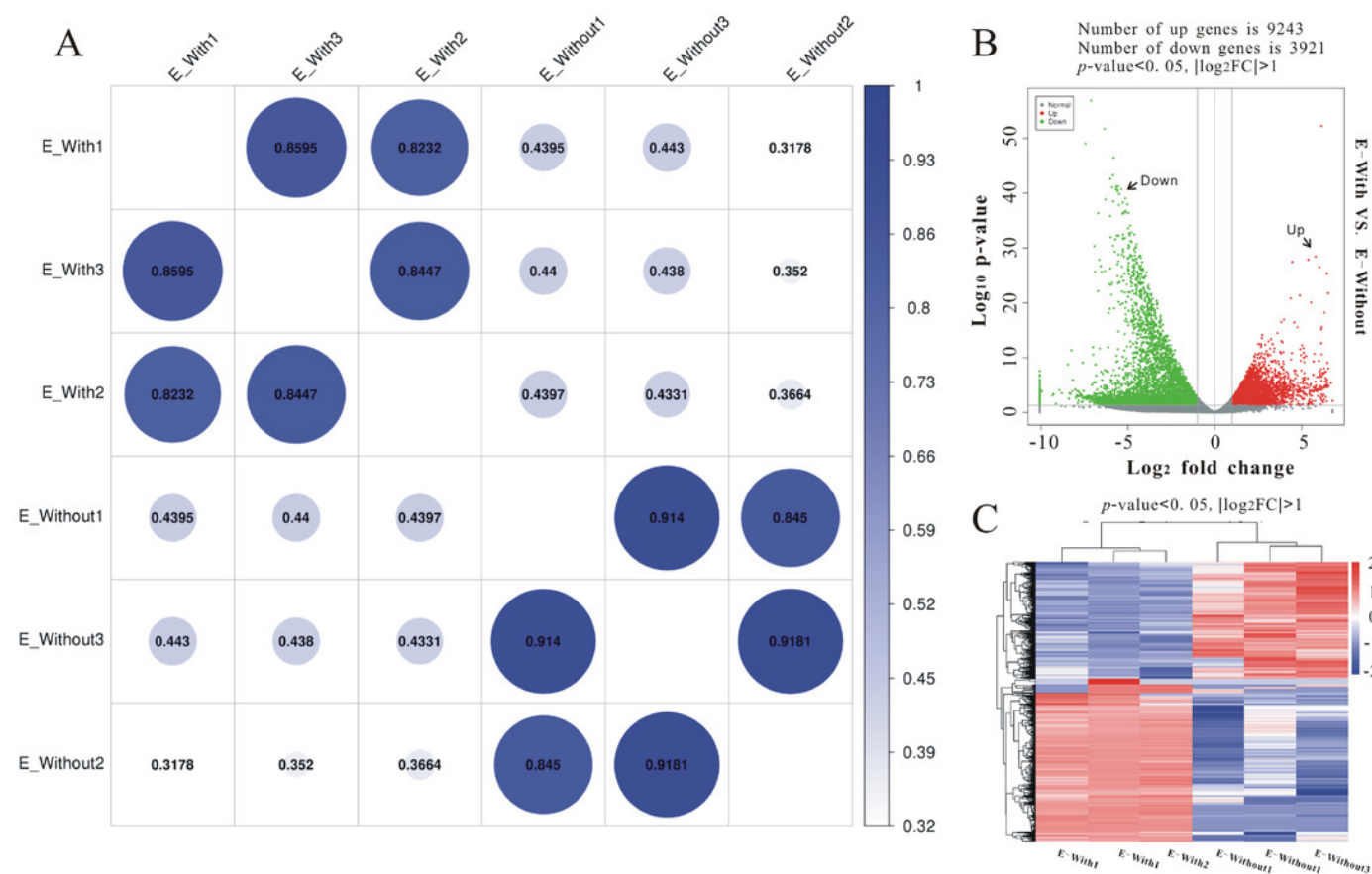
Figure 8

KEGG pathway enrichment analysis of differentially expressed genes (DEGs)

Only the top 20 pathways in KEGG enrichment function were listed in the figure. The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the Enrichment Score to the pathway. The larger the bubble is, the more different the number of Unigene is contained, and the bubble color changes from purple-blue-green-red, with smaller Enrichment *p* value and greater significance.