Transcriptome analysis of ovary culture-induced embryogenesis in cucumber (*Cucumis sativus* L.)

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Background. Ovary culture is a useful technique used to generate double haploid (DH) cucumber (*Cucumis sativus* L.) plants. However, cucumber ovary culture have a low rate of embryo induction and plant regeneration. Moreover, the cucumber embryogenesis mechanism remains unclear. In this study, we explored the molecular basis of cucumber embryogenesis in order to establish a foundation for a more efficient ovary culture method. Using transcriptome sequencing, we also investigated the differentially expression of genes during the embryogenesis process.**Methods.** Cytological and morphological observations have divided cucumber ovary culture into three stages: early embryo development (T0), embryo morphogenesis (T1, T2, T3, and T4), and shoot formation (T5). We selected six key time points for transcriptome sequencing and analysis: T0 (the ovules were cultured for 0 d), T1 (the ovules were cultured for 2 d), T2 (the embryos were cultured for 10 d), T3 (the embryos were cultured for 20 d), T4 (the embryos were cultured for 30 d), and T5 (the shoots after 60 d culture).**Results.** We used cytology and morphology to observe the characteristics of the cucumber's developmental transformation during embryogenesis and plant regeneration. The differentially expressed genes(DEGs) at developmental transition points were analyzed using transcriptome sequencing. In the early embryogenesis stage, the cells expanded, which was the signal for gametophytes to switch to the sporophyte development pathway. RNA-seq revealed that when compared to the fresh unpollinated ovaries, there were 3,468 upregulated genes in the embryos, including hormone signal transduction genes, hormone response genes, and stress-induced genes. The reported embryogenesis-related genes *BBM, HSP90,* and *AGL* were also actively expressed during this stage. In the embryo morphogenesis stage (from cell division to cotyledon-embryo formation), 480 genes that functioned in protein complex binding, microtubule binding, tetrapyrrole binding, tubulin binding and other microtubule activities were continuously upregulated during the T1, T2, T3, and T4

time points. This indicated that the cytoskeleton structure was continuously being built and maintained by the action of microtubule-binding proteins and enzyme modification. In the shoot formation stage, 1,383 genes were upregulated that were mainly enriched in phenylpropanoid biosynthesis, plant hormone signal transduction, phenylalanine metabolism, and starch and sucrose metabolism. These upregulated genes included six transcription factors that contained a B3 domain, nine genes in the AP2/ERF family, and two genes encoding *WUS* homologous domain proteins. **Conclusions.** Evaluating of molecular gynogenesis events may contribute to a better understanding of the molecular mechanism of cucumber ovarian culture.

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2   (*Cucumis sativus* L.)

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Abstract

21   **Background.** Ovary culture is a useful technique used to generate double haploid (DH)

22   cucumber (*Cucumis sativus* L.) plants. However, cucumber ovary culture have a low rate of

23 embryo induction and plant regeneration. Moreover, the cucumber embryogenesis mechanism

24 remains unclear. In this study, we explored the molecular basis of cucumber embryogenesis in

25 order to establish a foundation for a more efficient ovary culture method. Using transcriptome

26 sequencing, we also investigated the differentially expression of genes during the embryogenesis

27 process.

28 **Methods.** Cytological and morphological observations have divided cucumber ovary culture into

29 three stages: early embryo development (T0), embryo morphogenesis (T1, T2, T3, and T4), and

30 shoot formation (T5). We selected six key time points for transcriptome sequencing and analysis:

31 T0 (the ovules were cultured for 0 d), T1 (the ovules were cultured for 2 d), T2 (the embryos

32 were cultured for 10 d), T3 (the embryos were cultured for 20 d), T4 (the embryos were cultured

33 for 30 d), and T5 (the shoots after 60 d culture).

34 **Results.** We used cytology and morphology to observe the characteristics of the cucumber's

35 developmental transformation during embryogenesis and plant regeneration. The differentially

36 expressed genes(DEGs) at developmental transition

37 points were analyzed using transcriptome sequencing. In the early embryogenesis stage, the cells

38 expanded, which was the signal for gametophytes to switch to the sporophyte development

39 pathway. RNA-seq revealed that when compared to the fresh unpollinated ovaries, there were

40 3,468 upregulated genes in the embryos, including hormone signal transduction genes, hormone

41 response genes, and stress-induced genes. The reported embryogenesis-related genes *BBM,*

42 *HSP90,* and *AGL* were also actively expressed during this stage. In the embryo morphogenesis

43 stage (from cell division to cotyledon-embryo formation), 480 genes that functioned in protein

44 complex binding, microtubule binding, tetrapyrrole binding, tubulin binding and other

45 microtubule activities were continuously upregulated during the T1, T2, T3, and T4 time points.

46 This indicated that the cytoskeleton structure was continuously being built and maintained by the

47 action of microtubule-binding proteins and enzyme modification. In the shoot formation stage,

48 1,383 genes were upregulated that were mainly enriched in phenylpropanoid biosynthesis, plant

49 hormone signal transduction, phenylalanine metabolism, and starch and sucrose metabolism.

50 These upregulated genes included six transcription factors that contained a B3 domain, nine

51 genes in the AP2/ERF family, and two genes encoding *WUS* homologous domain proteins.

52 **Conclusions.** *Evaluation* Eevaluating of molecular gynogenesis events may contribute to a better

53 understanding of the molecular mechanism of cucumber ovarian culture.

54 **Key words:** Cucumber; Ovary culture; Embryogenesis; Transcriptome; Differentially expressed

55 genes

Introduction

Gametophyte cultures involve the stress-induced reprogramming of male or female gametophytes that develop into embryo-like structures and can be directly regenerated into completely homozygous, doubled haploid (DH) plants. The study of male gametophyte (microspore) embryogenesis began in the 1960s (Guha et al., 1964). In vitro microspore embryogenesis can be easily monitored and provide a convenient experimental platform for large-scale physiological and biochemical analyses (Malik et al., 2007). However, the study of gynogenesis is more complicated. The embryo sac is small and embedded in the surrounding tissues, making early embryos difficult to observe and isolate. For plants that are recalcitrant to androgenesis (e.g., male-sterile and dioecious plants), gynogenesis is worthwhile (Pazuki et al. 2018). In vitro gynogenesis has mainly been used on the *Cucurbitaceae*, and previous studies have revealed that this technique is still imperfect with a low embryogenesis rate (Metwally et al., 1998; Gémes et al., 2002; Shalaby, 2007; Diao et al., 2009; Li et al., 2013; Plapung et al., 2014; Tantasawat, 2015). The study of the embryogenesis mechanism has therefore remained unexplored, and the understanding of the mechanisms underlying gynogenesis is extremely limited.

In general, embryogenesis induction using in vitro culture is a stress-induced phenomenon. Microspores are cultured and develop into embryos in vitro, and stress treatments involving cold shock, heat shock or hormones are used as trigger factors to induce gametocytes to follow the sporophyte development pathway (Touraev et al., 1997). The genes associated with the reprogramming phase and the early stage of embryogenesis were successfully characterized and include *AGAMOUS-LIKE15-related* (*AGL15-related*) genes and the *AGAMOUS-LIKE15* (*AGL15*) gene, the *BABY BOOM* (*BBM*) gene, and the *HEAT SHOCK PROTEIN* (*HSP*) genes (Perry et al.,1999; Boutilier et al., 2002; Maraschin, 2005). Recently, functional genomics has made it possible to identify more genes associated with different microspore embryogenesis stages. When analyzing in vitro microspore cultures of *Brassica napus*, it was found that genes related to embryogenesis, such as *BBM1*, *LEC1,* and *LEC2*, were expressed as early as 2-3 days after microspore culture. While embryogenesis was clearly established by 7 d of culture, the genes that were specifically expressed included *ABSCISIC ACID INSENSITIVE3, ATS1, LEC1, LEC2*, and *FUSCA3* (Malik et al., 2007). Genes expression associated with metabolism,

86 chromosome remodeling, transcription, and translation signaling was upregulated during the
87 stress treatment stage in tobacco (Hosp et al., 2007). Similarly, genes related to metabolism, cell
88 wall, cell membrane, cell tissue control, cell communication, and signal transduction were
89 detected in the early stage of rape embryogenesis (Rosa Angélica et al., 2013). High levels of
90 *BBM* and *LEC* gene expression were confirmed in the early embryonic development of sweet
91 pepper anther cultures (Irikova et al., 2012). The transition from microspores to developing
92 embryos is mainly manifested in the induction of transcription factor genes that play an
93 important role in early embryogenesis. Many genes involved in hormone biosynthesis and plant
94 hormone signal transduction are additionally involved in secondary metabolism (Bélanger et al.,
95 2018). Studies have shown that differentially expressed genes (DEGs) can be detected by gene
96 chips in an early stage of the cucumber's in vitro gynogenesis, suggesting that phenylalanine
97 metabolism and synthesis may play an important role in the early in vitro development of
98 cucumber in vitro (Zhang et al., 2013).

99 Cucumber (*Cucumis sativus* L.) is the fourth most important vegetable worldwide (Lv et al.,
100 2012), and accounted for more than 2.2 million hectares and a total production of approximately
101 83 million tons in 2017 (http://www.fao.org/faostat). Cucumber is one of the oldest vegetable
102 crops and was domesticated in China approximately 2,000 years ago (Golabadi et al., 2012). To
103 meet the needs of production, breeders are constantly looking for valuable germplasm
104 characteristics, particularly resistance to disease and environmental stresses such as cold, drought,
105 and salt (Wang et al., 2018). Although collecting of extensive germplasm resources for variety
106 improvements can greatly facilitate these breeding efforts, cucumber is a cross-pollinated plant
107 with clear heterosis. Therefore, almost all of the cucumber varieties currently in production are
108 hybrids with slow breeding processes. Breeding parents takes 6-8 years of artificial self-breeding
109 in order to develop a stable inbred line. To accelerate the purification of cucumber parents and
110 improve breeding efficiency, haploid gametophyte cultures can be used to induce embryoids,
111 since homozygous DH plants can be obtained in 1-2 years. DH technology is a powerful tool
112 used to speed up plant breeding. However, additional research is needed to improve our
113 understanding of genes and their roles in embryogenesis and haploid induction mechanism in
114 embryo sacs (Chen et al., 2011).

115 This study focused on the cucumber's highly efficient in vitro ovary culture technology
116 system, We divided the process from acquiring embryogenic potential to plant regeneration was

117 ~~divided~~ into three stages: early embryo development, embryo morphogenesis (from pro-embryos

118 to cotyledon embryos), and shoot formation. *Via* ovary culture, we evaluated the embryo

119 morphology and transcriptomes across   different developmental stages during cucumber

120 embryogenesis. The metabolic and biological process of embryogenesis in the cucumber

121 gynogenesis are discussed, and several key genes that regulated embryogenesis were identified.

Materials and methods

122

Plant materials

123

124 We used the F_1 cultivar 'SG033' (http://kunming056278.11467.com/) for ovary culture because

125 this material has a high embryo generation rate even after many screenings. Cucumber plants

126 from southern China were cultivated and stored in our lab. The plant growth protocol was

127 slightly adapted from Li et al. (2014). Specifically, the plants were grown in 12-hour photoperiod

128 greenhouse at the Horticulture Institute of Guizhou Academy of Agricultural Sciences, Guizhou,

129 China, with an average daily air temperature of 25/15 °C (day/night), a relative humidity of 85%,

130 and a photosynthetic luminous flux density of 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. We collected ovaries from

131 'SG033' after the first anthesis flower appeared.

Culture medium composition

132

133 For MS medium, we used a basal medium that was supplemented with 0.06 $mg \cdot L^{-1}$ thidiazuron

134 (TDZ, Solarbio, Beijing, China) and 3% (w/v) sucrose, and was solidified with 7% (w/v) agar.

135 The medium pH was adjusted to 5.9 before autoclaving at 116 °C and 1.1 kg/cm^2 for 30 min.

Ovary culture

136

137 Ovaries were harvested 6 h before anthesis, and were treated for 24 h in a refrigerator at 4 °C.

138 We removed their fruit tumor, sterilized ~~the~~ *with* 75% ethanol for 30-60 s, and them rinsed in sterile

139 distilled water three times, soaked in a 0.5% sodium hypochlorite for 20 min, and then rinsed

140 them in sterile distilled water three times. The ovaries were cut into small round 2-mm slices

141 under sterile conditions and placed on 30 mL of solid media in cylindrical flasks. All materials

142 were treated with a high temperature of 33 °C for 2 d, after which recovery growth was allowed

143 until shoot formation occurred at 25 °C under a 16/8 h (light/dark) photoperiod with a 4,000-lux

144 light intensity.

145

146

Observation

148 We observed embryo development according to the methods previously published by our team

149 (Deng et al., 2020). Specifically, SG033 explants (varian fragments) were collected before and

150 after culture, and resin sections were obtained to observe early, embryo development. Fifty

151 explants were collected separately at 0 d and 2 d fixed in formalin-acetic acid-alcohol (5 ml of

152 formalin with 38% formaldehyde, 5 ml of glacial acetic acid, and   90ml of 70% ethanol) (FAA)

153 at room temperature for 48 h, dehydrated with a graded ethanol series, and then embedded in the

154 spur resin. Nine-micrometer-thick sections were stained with safranine and fast green, and were

155 observed using a biological microscope (Leica DM2500, Wetzlar, Germany). After 10 d of

156 culture, we evaluated the embryonic morphology using a stereo microscope (Leica M165 C,

157 Wetzlar, Germany).

RNA isolation

159 The materials were harvested separately at the following time points: T0 (the fresh unpollinated

160 ovaries were cultured for 0 d), T1 (the ovules were cultured for 2 d), T2 (the embryos were

161 cultured for 10 d), T3 (the embryos were cultured for 20 d), T4 (the embryos were cultured for

162 30 d), and T5 (the shoots after 60 d of culture). We extracted ovules at T0 and T1 time points by

163 hand under a stereo microscope,   directly selected embryos at the time points from T2 to T4,

164 and selected the shoots from T5. We used 200 mg for each sample as the starting material for the

165 RNAseq and qPCR experiments.

166 These materials were stored at -80 °C for subsequent RNA extraction, and the remaining

167 materials were maintained in culture in order to observe plantlet regeneration. All further

168 experimental procedures, such as culture medium replacement and sample collection, were

169 performed under similar conditions to minimize possible circadian effects.

170 As described above, we collected samples from six ovary culture time points for RNA-seq

171 analysis. For each sample, the ovules or embryo-like structures were immersed in liquid nitrogen

172 in a mortar and were ground into powder (three biological replicates per sample). Total RNA

173 was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and DNase I (Fermentas, Waltham,

174 MA, USA) digestion was then performed for 30 min at 25 °C to remove DNA, according to the

175   manufacturer's instructions. We determined the integrity and quality of the total RNA using a

176   NanoDrop 1000 spectrophotometer and formaldehyde-agarose gel electrophoresis. RNA was

177   used only when the OD_{260}:OD_{280} ratio was above 1.8.

178   The total RNA concentration for all of the samples was ≥80 ng/μl, the OD：260/280>2,

179   260/230>2, and the rin value was close to 8.0 (see Fig. 1 for a agarose gel electrophoresis),

180   which is 28 s/18s greater than 1.0. This indicated that the total concentration and quality of the

181   prepared RNA was suitable.

182   **Library construction and sequencing**

183   We performed library construction and sequencing as previously described by Liu et al. (2014).

184   Specifically, the library was constructed with 3 micrograms of total RNA and enriched with

185   magnetic beads coated with oligo (dT). After adding fragment buffer, the mRNA was converted

186   to short fragments (200-300 bp). Next, we used mRNA fragments as templates to synthesize

187   cDNA. The double-stranded cDNA was purified with a QiaQuick PCR kit, washed with EB

188   buffer solution, and repaired at the end, and a single adenine (A) nucleotide was added. Finally,

189   the sequencing adapters were connected to the fragments. The fragments were purified using by

190   agarose gel electrophoresis and amplified by PCR. We then transferred the library products to an

191   Illumina HiSeq™ 2000 for sequencing analysis. It was important to use that to base calling to

192   convert the original image data into sequences in order to obtain clean reads before further

193   analysis.

194   **Transcriptome analysis**

195   We analyzed of the transcriptomes using the Illumina Hiseq™ 2000 platform. The raw ~~were~~ data *was*

196   uploaded to NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147282). Using *the*

197   Illumina GA Pipeline (v1.6) software, we removed more than 10% of unknown base and low-

198   quality base readings from the raw sequencing data. Using TopHat2 (http://tophat.cbcb.umd.edu/)

199   software, we aligned the filtered reads with the Chinese long v2 cucumber genome. Reference

200   genome and gene database information are publicly available at the website

201   http://cmb.bnu.edu.cn/Cucumis_ sativus_ V20 / (Huang et al., 2009). We performed differential

202   expression analysis using DESseq (version 1.18.0). Benjamini and Hochberg's methods were

203   used to control the false discovery rate and adjust the obtained P values. Genes with an adjusted

204   P value of <0.05 and an absolute value of $|\log_2 \text{fold change} \geq 1|$ according to DEseq were

205   considered differentially expressed. Gene ontology (GO) annotation was implemented by

206   Blast2GO software, and GO association was performed via a BLASTX search of the NCBI NR

207   database. We conducted the GO enrichment analysis of DEGs using the BiNGO plugin.

208   Over-represented GO terms were recognized using a hypergeometric test following

209   Benjamini-Hochberg false discovery rate (FDR) correction with a significance threshold of 0.05.

210   We used the KOBAS (2.0) software to carry out the KEGG enrichment analysis of theDEGs

211   (Mao et al., 2005). A p value $\leqslant 0.05$ was used as the threshold to judge significantly enriched

212   DEG pathways.

213   Verifying RNA-seq data using quantitative real-time PCR (qRT-PCR)

214   To test the reliability of RNA-seq, we selected nine up-regulated genes for qRT-PCR. The total

215   RNA of the RNA-seq samples were treated with DNase I enzyme, and were then transformed

216   into single-strand cDNA using the GoScriptTM Reverse

217   Transcription System (Promega, Madison, WI, USA). according to the manufacturer's protocol.

218   According to its cDNA sequence , primer 5.0 was used to design specific gene primers (Table

219   S1). Actin was used as an internal control to normalize minor differences in the number of

220   templates. qRT-PCR was performed with GoTaq qPCR Master Mix (Promega, Madison, WI,

221   USA) in a Bio-Rad iQ1 real-time PCR system (Bio-Rad, Hercules, CA, USA), The reaction

222   conditions were as follows: pre-denaturation at 95 ℃ for 10 min, followed by denaturation at 95

223   ℃ for 15 s, and renaturation at 55 ℃ for 1 min for a total of 40 cycles; and at the end of the

224   reaction, the system was kept at 95 ℃ for 15 s, followed by 60 ℃ for 1 min, and finally at last at

225   95 ℃ for 15 s. There were three biological replicates of each gene at each time point, qPCR

226   experimental samples were the same as the RNA sequencing samples. The expression ratio was

227   calculated using the $2^{-\Delta\Delta CT}$ method.

228   Results

229   Morphological and cytological characterization of in vitro-induced

230   embryogenesis in cucumber

231   We conducted a negative and positive contrast of the cucumber ovary culture. After 2 days of

232   culture, some ovules were clearly enlarged, the embryo sac was also significantly enlarged (Fig.

233   2a and c), but some ovules did not respond (Fig. 2a and b). After 10 days of culture, the non-

? presumed / detected as / found to be ?

234 embryogenic ovaries were ~~direct~~ dead. In our study, we used the cells that underwent

235 embryogenesis for sequencing analysis.(Fig. 2d).

236   We observed the early embryogenesis stage, embryo morphogenesis stage, and the shoot

237 formation stage in order to determine the cytological changes that occur   during embryogenesis

238 in cucumber ovary culture. Ovaries were collected 6 h before anthesis and were pretreated at 4

239 °C. The ovaries were then sterilized, sliced, inoculated into the medium, incubated for 2 d at 33

240 °C in the dark, and then transferred to 25 °C conditions until plants formed. Notably, our

241 morphological and cytological observations showed that critical developmental transitions in the

242 embryos occurred at 2 d (T1), 10 d (T2), 20 d (T3), 30 d (T4) and 60 d (T5) of culture. Fresh

243 unpollinated ovaries before culture were referred to as T0 (Fig. 3). At T0, we selected the fresh

244 unpollinated ovaries with clear ovules (Fig. 3a) and mature embryo sacs (Fig. 3g). At T1, heat

245 shock stress induced the embryogenesis of cucumber. The most obvious indicators of embryo

246 development were enlarged cells in the embryo sac (Fig. 3b and h). Enlarged of microspores

247 have shown a correlation with embryogenic potential acquisition during androgenesis induction

248 in many crop species (Hoekstra et al., 1996; Maraschin, 2005). After culturing for 2 d, 4 d, and 6

249 d, the expanded cells formed two (Fig. 3i), four (Fig. 3j), and multiple cell structures (Fig. 3k),

250 respectively. At T2 (10 d), the ovules had successfully protruded from the surrounding tissues

251 and a pro-embryo had formed (Fig. 3c). At T3 (20 d), the total number of embryos had increased

252 in one ovary slice (Fig. 3d). From T3 to T4 (30 d culture), the whole embryo had formed with

253 globular, heart-shaped, torpedo-shaped, and cotyledon-shaped (Fig. 3l). At the same time, the

254 color of the embryonic cells appeared green, indicating the beginning of plant development from

255 the embryo (Fig. 1e). At T5 (60 d after culture), shoots had formed (Fig. 3f).

256   At T1, ovule enlargement and cell enlargement were clearly observed, suggesting that the

257 transformation of the developmental pathway from the gametophyte to the sporophyte was

258 induced by stress, and that the stage between T0 and T1 was the key to inducing embryogenesis.

259 After the switch in cell development, embryogenic potential was acquired. Mitosis then occurred

260 to continuously form pro-embryos, globular embryos, heart-shaped embryos, torpedo-shaped

261 embryos, and cotyledon-shaped embryos, similar to those observed during zygote development,

262 and this process continued until T4. After 60 d of culture, the cotyledon-shaped embryos

263 developed into shoots. We divided the six culture time points into three stages: T0 to T1 (early

264 embryogenesis stage), T1 to T4 (embryo morphogenesis stage), and T4 to T5 (shoot formation

265   stage).

266   **Sequencing and aligning to the reference genome**

267   Based on our observation of the morphological and cytological characteristics, we selected

268   six time points *and* used three biological replicates for RNAseq, and   ultimately represented a total

269   of 18 libraries. The transcriptome data were generated using Illumina II HiSeq™ 2000

270   sequencing. Overall, more than 101.3 billion paired-end reads passing filter were generated, and

271   we used these to align to the reference genes, which provided the general information for the

272   project (Table S2). Reads counts were generated from Bam *BAM* alignment files using HTseq 0.6.1p2

273   software (http://www-huber.embl.de/users/anders/HTseq). Data normalization was implemented

274   with Reads Per Kilobase of transcript, pre *per* Million mapped reads (RPKM).

275   To analyze the ovary culture biochemical process on the cucumber transcriptome, we

276   conducted transcriptomic analysis of T0 vs. T1(the control group), and the experimental groups

277   T1 vs. T2, T2 vs. T3, T3 vs. T4, and T4 vs. T5 to reveal significantly genes. We determined

278   which genes were differentially expressed using   DESeq (version 1.18.0), and used P-

279   adjust≤0.05 and the absolute value of $|\log_2 \text{foldchange}| \geq 1$ as the threshold to judge the

280   significance of the differential gene expression. Between T0 vs. T1, T1 vs. T2, T2 vs. T3, T3 vs.

281   T4, and T4 vs. T5, there were 3,468, 2,341, 1,117, 1,478 and 1,383 up-regulated genes, and 3,065,

282   1,906, 574, 2,223 and 1,837 down-regulated genes, respectively (Fig. 4).

       use ";" to de-lineate values with "," in them; otherwise difficult to determine.

283   **Validation of differentially expressed genes**

284   Validation of the Illumina sequencing data and the DEG expression patterns   revealed by RNA-

285   seq was performed in order to examine the expression patterns of nine DEGs: including seven

286   genes involved in plant hormone signal transduction and two plant-pathogen interaction genes.

287   qRT-PCR results showed that seven genes were involved in plant hormone signal transduction,

288   *Csa7M452370.1, Csa1M006300.1, Csa5M223020.1, Csa5M434550.1, Csa5M623800.1,*

289   *Csa6M383530.1,* and *Csa3M389850.1,* with Pearson's r = 0.77, 0.57, 0.74, 0.79, 0.69, 0.81, and

290   0.86, respectively. The two genes involved in plant-pathogen interaction, *Csa7M420160.1* and

291   *Csa1M006320.1,* had Pearson's r values of 0.62 and 0.81, respectively. The results showed a

292   correlation between the relative expression levels revealed by RNA-seq and qRT-PCR. The fold

293   changes in the qRT-PCR analysis were different from those in the RNA-seq analysis, which may

294   have been due to the different   sensitivities between the qRT-PCR analysis and the RNA-seq

295   technique. However, the qRT-PCR analysis showed that the upregulation and downregulation

296   trends of the differential gene expression were consistent with those of the RNA-seq analysis

297   (Fig. 5).

Embryogenesis-related genes expression in cucumber ovary culture

299   Ovule enlargement was noticeable at the early stage of embryogenesis, following heat shock

300   stress (Fig. 3b). Cytological observations also showed that one of the cells in the embryo sac

301   expanded (Fig. 3h), suggesting acquisition of embryogenic potential.

302   We determined that the differentially expressed genes at this stage (T0 vs. T1) were   comprised

303   of 3,468 upregulated genes and 3,065 downregulated genes (Fig. 4). We performed a GO

304   enrichment analysis of these genes, and the results showed that the proteins encoded by these

305   genes were assigned to four biological processes, three molecular functions, and nine cellular

306   components (Table S3). The majority of DEGs were involved in the 'single-organism process'

307   (GO:0044699) and the 'oxidation-reduction process' (GO:0055114). They were mainly

308   distributed across the terms 'membrane' (GO:0016020), 'membrane part' (GO:0044425),

309   'intrinsic component of membrane' (GO:0031224), and 'integral component of membrane'

310   (GO:0016021), with good molecular oxidoreductase activity (GO:0016491). Among the DEGs,

311   the expression of related genes involved in functional classification might play an important role

312   in early embryogenesis.

313   To compare and summarize the results of this stage, we performed a pathway analysis to

314   identify potential target genes. Based on the KEGG database, the pathway enrichment analysis of

315   these genes revealed significant involvement in eight distinct pathways (Fig. 6a): pathways

316   involving plant hormone signal transduction, phenylpropanoid biosynthesis, plant-pathogen

317   interaction, glutathione metabolism, cysteine and methionine metabolism, drug metabolism-

318   cytochrome P450, phenylalanine metabolism, and metabolism of xenobiotics by cytochrome

319   P450.

Plant hormone signal transduction genes

321   The DEGs were significantly enriched in the plant hormone signal transduction pathway

322   (Ko04075), We detected a large number of genes related to cytokinin and ethylene signalling

323   pathways, histidine phosphotransferase protein encoders (*Csa2M373410.1*, *Csa6M067360.1*, and

324   *Csa7M452370.1*), response regulators (RR) (*Csa1M006300.1*, *Csa3M822100.1*, *Csa4M436980.1*,

325   *Csa5M223020.1*, *Csa5M434550.1*, *Csa5M603910.1*, *Csa5M623800.1*, and *Csa6M383530.1*),

326   EIN3 binding (F-box) proteins (EBFs) (*CsaUNG009930*), and ethylene-responsive protein

327   transcription factors (ERFs) (*Csa3M389850.1*) that were significantly upregulated after heat

328   treatment (Table S4). Moreover, the partially upregulated DEGs encoding auxin influx carriers,

329   auxin-responsive proteins, SAUR family proteins, abscisic acid receptor proteins,

330   serine/threonine-protein kinases, ABA-responsive element binding factors, and brassinosteroid

331   signal-responsive protein kinases were significantly enriched in eight pathways, some of which

332   involved tryptophan metabolism as well as zeatin, carotene, and brassinosteroid biosynthesis,

333   suggesting that hormone production and signal transduction were closely related to stress-

334   induced embryogenesis. The upregulated genes encoding major enzymes and the receptor

335   proteins involved in the plant hormone signal transduction pathway were expressed during the

336   whole ovary culture stage . Most of the genes were dynamically expressed after their upregulated

337   expression during embryogenesis. Six genes (*Csa6M125240.1*, *Csa6M147590.1*,

338   *Csa6M007440.1*, *Csa4M454150.1*, *CsaUNG009930, and Csa3M389850.1*) were upregulated

339   only at the embryo initiation stage, and then appeared downregulated in the later stages (Fig. 7).

340   These genes mainly encoded auxin synthetase and induced proteins, ABA protein kinase, and

341   ethylene-responsive transcription factors, indicating that they might be closely related to

342   embryogenesis.

343   **Stress response proteins related to plant defense mechanisms**

344   DEGs were significantly enriched in the plant-pathogen interaction pathway, which is related to

345   plant defense mechanisms and complex physiological responses to heat shock-induced

346   embryogenesis. In this regulatory pathway, the expression of *Pto-interacting protein 1* (*Pti1,*

347   *Csa7M420160.1*), *Heat shock protein 90* (*HSP90, Csa3M183950.1*), *Enhanced disease*

348   *susceptibility 1* (*EDS1* , *Csa1M006320.1*), and other related genes were upregulated (Table S4).

349   *HSP90* was upregulated under heat stress, had been highly conserved during evolution and has

350   plant defense functions.

351   **Embryogenesis-related transcription factors**

352   Transcription regulation through transcription factors is a key step in a plant's stress response. In

353   the embryogenesis stage, a total of 32 transcription factor families were upregulated after heat

354   shock stress (Table S4). Among these, the *AP2/ERF, WRKY, basic helix loop helix* (*bHLH*),

355   *MYB*, *GATA*, *heat stress factor* (*HSF*) and *NAM/ATAF/CUC* (*NAC*) families had the most

356   transcription factors. There were 26 transcription factors upregulated in the AP2/ERF family,

357   which plays an important role in ethylene response, brassinolide response, and biological and

358   abiotic stress responses. Two *BBM* genes (*Csa3M827310.1* and *Csa3M827320.1*) were

359   upregulated. Nineteen transcription factors were upregulated in the *WRKY* family, which are

360   involved in physiological embryo development, metabolism, environmental stress, and defense

361   response to pathogens. The *bHLH* family plays an active role in regulating environmental stress.

362   The *MYB* family controls different stages of cell division and promoting embryogenesis. After

363   heat shock stress, four *HSF* genes (*Csa2M356690.1*, *Csa6M517310.1*, *Csa1M629180.1,* and

364   *Csa3M822450.1*) were specifically expressed that could activate the *HSP90* gene in response to

365   heat stress. The upregulated expression of these transcription factors might be involved in

366   embryogenesis and the active regulation of biological and abiotic stress processes.

367   **Response of genes involved in microtubule organization in cucumber ovary culture**

368   In the embryo morphogenesis stage, the gametophyte development pathway switched to the

369   sporophyte development pathway, and the cells enlarged initiated mitosis, and formed two-cell,

370   four-cell, and multicellular pro-embryos after heat stress (Fig. 3h-k). Afterward, globular

371   embryos, heart-shaped embryos, torpedo-shaped embryos, and cotyledon embryos were formed

372   (Fig. 3l). The DEG analyses of T1 vs. T2. T2 vs. T3, and T3 vs. T4 revealed 480 continuously

373   expressed genes. Additionally, the numbers of uniquely expressed genes between T1 and T2, T2

374   and T3 and T3 and T4 were 2,236, 371 and 1,558, respectively (Fig. 8).

numerical commas and delineating commas confusing

375   The GO categories of the 480 continuously expressed genes were significantly assigned to

376   two biological processes, cell movement or subcellular component (GO:0006928) and

377   microtubule-based movement (GO:0007018), which are mainly associated with microtubules

378   (GO:0005874), supramolecular fibers (GO:0099512), and polymeric cytoskeletal fibers

379   (GO:0099513). Subsequently, the proteins encoded by these genes were classified into 11

380   functional categories: protein complex binding (GO:0032403), microtubule binding

381   (GO:0008017), tetrapyrrole binding (GO:0046906), tubulin binding (GO:0015631), microtubule

382   motor activity (GO:0003777), motor activity (GO:0003774), macromolecular complex binding

383   (GO:0044877), heme binding (GO:0020037), oxidoreductase activity (GO:0016491), iron ion

384   binding (GO:0005506), and oxidoreductase activity (GO:0016491), acting on paired donors or

385   the molecular incorporation of oxygen (GO:0016705) (Table S5). These findings indicated that

386   during certain stages, the cytoskeleton structure was continuously built and maintained by the

387 action of microtubule binding proteins and enzyme modifications. This provided the basis for
388 positioning the various organelles and implementation functions, in order to ensure the orderly
389 activities of various cells in time and space.

390   To determine the involvement of these DEGs in embryo development, we performed a
391 pathway analysis to identify the potential target genes. Seventeen significant pathways were
392 obtained by mapping to the KEGG database (Fig. 6b): protein processing in the endoplasmic
393 reticulum, phenylpropanoid biosynthesis, photosynthesis-antenna proteins, limonene and pinene
394 degradation, meiosis-yeast, the estrogen signaling pathway, cell cycle, cell cycle-yeast,
395 diterpenoid biosynthesis, chloroalkane and chloroalkene degradation, carotenoid biosynthesis,
396 progesterone-mediated oocyte maturation, glycerolipid metabolism, histidine metabolism, fatty
397 acid degradation, antigen processing and presentation, and ascorbate and aldarate metabolism.
398 Among these, the protein processing pathways in the endoplasmic reticulum and
399 phenylpropanoid biosynthesis were the most significant.

400 **Main oxidation-reduction and metabolic process-related genes expression in cucumber**
401 **ovary culture**
402 In the shoot formation stage, the embryos further differentiated into shoots (Fig. 3f). A   total of
403 3,320 genes were differentially expressed between T4, and T5, 1,383 of which genes were
404 upregulated and 1,837 were downregulated in T5 (Fig. 4). Next, we performed an enrichment
405 analysis of the genes using GO, which revealed eighteen biological processes, fifteen molecular
406 functions, and five cellular components (Table S6). The terms annotated under the biological
407 process category mainly included oxidation-reduction processes (GO: 0055114), the regulation
408 of primary metabolic processes (GO: 0080090), the regulation of cellular metabolic processes
409 (GO: 0031323), and the regulation of macromolecule metabolic processes (GO: 0060255). The
410 molecular function category mainly included the terms oxidoreductase activity (GO: 0016491)
411 and DNA binding (GO: 0003677). The cellular component category   included mainly
412 extracellular region (GO: 0005576). Ovary culture development was mainly focused on the
413 oxidation-reduction and metabolism processes.

414   The DEGs were subsequently annotated using the KEGG database in order to identify
415 pathway enrichments. A variety of pathways were found to be significantly enriched (Fig. 6c),
416 including those involved in phenylpropanoid biosynthesis; plant hormone signal transduction;

417   stilbenoid, diarylheptanoid, and gingerol biosynthesis; metabolism of xenobiotics by cytochrome

418   P450; drug metabolism-cytochrome P450; flavonoid biosynthesis; phenylalanine metabolism;

419   starch and sucrose metabolism; and zeatin biosynthesis. Most of the DEGs were enriched in

420   phenylpropanoid biosynthesis, plant hormone signal transduction, phenylalanine metabolism,

421   and starch and sucrose metabolism. Pathways involving phenylpropanoid biosynthesis and

422   phenylalanine are important for metabolizing secondary metabolites in plants and are closely

423   related to cell differentiation and pigmentation in plant development. In the plant hormone signal

424   transduction pathway, the expression of an auxin response factor, the gibberellin receptor *GID1*,

425   ethylene-responsive transcription factor 1 (*ERF1*) and cyclin D3 were upregulated, showing that

426   they promoted plant development, morphogenesis, cell enlargement, division and stem

427   developmen. Additionally, the DEGs involved in starch and sugar metabolism were also

428   significantly enriched and mainly encoded 3-βD glucosidase, T6Ps, GlgB, endoglucanase, and

429   alpha-trehalase, which satisfied the requirements of embryo growth and development.

430   **Shoot formation-related transcription factors**

431   In the shoot formation stage, the 62 transcription factors that were upregulated belonged to 23

432   transcription factor families, with an upregulation ratio between 2.03 and 214.13. The most

433   upregulated gene was the *TCACG motif-binding factor* (*TGA*) transcription factor

434   (*CSA4M625020.1*) in the *basic leucine zipper* (*bZIP*) family. It which can specifically bind to

435   the activation sequence with *TGACG* as the core in order to regulate the transcription level of

436   target genes, and it plays an important role in organ development and the defense response

437   against biological and abiotic stress. *AP2/EFR, bHLH, MYB, WRKY, ZIP* and *WUSCHEL-related*

438   *homeobox* (*WOX*) are the families with a large number of transcription factors, which may be an

439   important molecular indicator of shoot formation.

440   # Discussion

441   In this present study, the whole process from acquisition of embryogenic potential to plant

442   regeneration was investigated. We divided the ovary culture into three stages. In the early stage

443   of embryogenesis, the acquisition of embryogenic potential by stress (e.g., low or high

444   temperature or hormone induction) was observed together with the repression of gametophytic

445   development, leading to cell dedifferentiation. In the embryo morphogenesis stage, cell divisions

446   gave rise to the formation of pro-embryos (cell clumps), globular embryos, heart-shaped

447   embryos, torpedo-shaped embryos, and cotyledon embryos. In the shoot formation stage,

448   cotyledon embryos developed into shoots. There were molecular events that regulated embryo

449   development at different stages of development.

Embryogenesis-related genes are expressed in cucumber ovary culture

451   In the early stage of embryogenesis, the female gametophyte was regulated by a variety of

452   hormones, including auxin, abscisic acid, cytokinin and ethylene, similar to somatic cell

453   embryogenesis and transformation (Ikeda-Iwai et al., 2003). A large number of DEGs were

454   enriched in the plant hormone signal transduction pathway. Phytohormones such as cytokinin

455   and ethylene were significantly upregulated in multiple biosynthesis and metabolic pathways,

456   and upregulated and downregulated genes related to auxin and abscisic acid were both observed.

457   Generally, high-level endogenous cytokinin and low-level endogenous ethylene are beneficial

458   for the acquisition of embryogenic ability. Embryogenesis is the comprehensive performance of

459   interactions across different hormones, and all kinds of endogenous hormones show dynamic

460   changes. The upregulated expression of the main enzymes and receptor proteins in the plant

461   hormone signal transduction pathway could promote embryogenesis, indicating that the high

462   expression levels of related genes may play important roles in the process of switching from the

463   gametophyte to the sporophyte development pathway. In this pathway, we found six genes

464   related to hormone regulation (*Csa6M125240.1*, *Csa6M147590.1*, *Csa6M007440.1*,

465   *Csa4M454150.1*, *CsaUNG009930*, and *Csa3M389850.1*) that were highly expressed only at the

466   embryo initiation stage, then were downregulated in the later stages. Additionally, we

467   determined the functional categories and regulatory pathways of the specific functions that lay an

468   important theoretical foundation for understanding this mechanism.

469   In in vitro culture, a certain stress condition is needed to block the development direction of

470   the original gametophyte, which needs to be turned towards the direction of the sporophyte, and

471   continue symmetry splitting in order to eventually lead to embryogenesis (Fan et al.,1988). In

472   cucumber ovary culture , heat shock pretreatment, silver nitrate, genotype and hormone

473   combination factors can independently and simultaneously play key roles in embryo and callus

474   production (Golabadi et al., 2017). In this study, heat shock stress was an important factor in the

475   transformation from gametophyte to sporophyte. Previous studies have shown that *HSP* genes

476   are specifically expressed in the spore stress-induction process of many crop species (Zarsky et

477   al., 2010; Binarova et al., 1997; Sabehat et al., 1998). *HSP* genes play important roles in cell

478   cycle control, genomic silencing, protein transduction and signal transduction (Zhang et al, 2013).

479   We found that 26 upregulated genes encoded proteins and transcription factors related to heat

480   shock, and that *HSP90* (*Csa3M183950.1*) was involved in the regulation of the hypersensitive

481   response in the plant and pathogen interaction pathway. The expression of these genes might

482   play a regulatory role in the embryogenesis process. The *BBM* gene is preferentially expressed in

483   developing embryos and seeds (Boutilier et al., 2002), and is a key regulator of plant cell

484   totipotency because *BBM* ectopic overexpression induces the formation of somatic embryos in

485   *Arabidopsis* seedlings without exogenous growth regulators or stress treatments (Boutilier et

486   al.,2017). We found that two *BBM* genes (*Csa3M827310.1* and *Csa3M827320.1*) were

487   upregulated at the beginning of embryogenesis and were subsequently downregulated.

488   Additionally, *AGL15,* a member of the MADS family, is also considered a regulatory protein that

489   acts at the start of cell division (Perry et al., 1999). We found that the *AGL* gene

490   (*Csa3M258140.1*) was upregulated in our study, indicating that all three of these genes (*BBM*,

491   *HSP90 and AGL*) play important roles in the induction of embryogenesis by cucumber ovary

492   culture.

493   Gametophyte embryogenesis and zygotic embryogenesis are similar processes, and the only

494   difference is that the former occurs in sex cells while the latter occurs in fertilized egg cells

495   during the development and differentiation of zygotic embryos. Microtubules play an important

496   role in cells between the polarized diffuse growth and the acquisition stages (Kimata, et al.,

497   2016). Electron microscope-based observations of carrot culture showed that the appearance of

498   microtubules was accompanied by the formation of somatic embryos (Halperin et al., 1967). The

499   different patterns of microtubule organization in the cells of the mature embryo sac cells reflect

500   their structural adaptations for their future function (Huang et al., 1994). Similar to somatic

501   embryos, a large number of genes participated in microtubule movement and bind to tubulin

502   after embryonic potential is acquired, and the different embryo shapes form through active cell

503   metabolism and rapid cell division. We concluded that the cellular reprogramming and

504   morphological changes in embryos were controlled by microtubule organization genes.

505   Cotyledon embryos are further cultured to form shoots, and at this stage, a large number of

506   transcription factors were expressed. The first constitutes transcription factors that contain a B3

507   domain (Stone et al., 2001). These transcription factors encode regulatory proteins involved in

508   the embryonic development process, and maintaining embryo development during late

509 embryonic development (Boutilier et al., 2002; Riechmann & Meyerowitz, 1998). In our study, a

510 total of six transcription factors (*Csa2M359980.1*, *Csa2M292240.1*, *Csa5M608380.1*,

511 *Csa6M489980.1*, *Csa6M489940.1,* and   *CsaUNG031640*) containing a B3 domain were

512 upregulated. The second was the *AIL* gene from the AP2/ERF family, which is involved in key

513 developmental processes throughout the whole plant life cycle. Some genes in the AP2/ERF

514 gene family are expressed in ~~other many~~ *many other* tissues and participate in many plant developmental

515 processes, such as embryogenesis and shoot development (Boutilier et al., 2002; Riechmann &

516 Meyerowitz, 1998). Additionally, we found that the expression levels of nine genes

517 (*Csa1M423190.1*, *Csa3M114480.1*, *Csa3M652380.1*, *Csa3M114470.1*, *Csa4M644740.1*,

518 *Csa5M175970.1*, *Csa5M608380.1*, *Csa6M496390.1*, and *CsaUNG031640*) from the AP2/ERF

519 family were upregulated. Furthermore, *WUS* homologous domain proteins not only alter the cell

520 fate of the shoot and flower meristem, but they also promote the development of somatic

521 embryos into seedlings. *WUS* proteins have no direct connection to the embryo characteristics,

522 but they will alter the development state of the tissue by maintaining cells in an undifferentiated

523 state in response to different stimuli (Mayer et al., 1998; Gallois et al., 2004). *WUS's* ability to

524 transform the vegetative growth phase to the embryonic stage, as well as to eventually form

525 somatic cells, indicates that this homologous domain protein also plays an important role in

526 embryo morphogenesis and meristem (Palovaara & Hakman, 2008). In the shoot apical meristem,

527 *WUS* defines the organizing center and is critical for the induction of these cells (Dai et al., 2017;

528 Meng et al., 2017). During this stage, two genes (*Csa6M301060.1* and *Csa6M505860.1*)

529 encoding *WUS* homologous domain proteins were upregulated. We concluded that those

530 homologous domain proteins might directly or indirectly regulate shoot formation.

531 Because the ovary culture embryogenesis takes place in the surrounding tissue, it was

532 difficult to observe the embryo development process and the transcriptome analysis sample was

533 difficult to select. Additionally, this study sample contained other tissues, especially at the T0/T1

534 time points, and isolated ovules ~~were~~ *yielded* sequencing materials that may have impacted the

535 transcriptome data.

536

Conclusion

538 Our study revealed the molecular events of embryogenesis in cucumber ovary culture. The

539   early stage of embryogenesis was the turning point for embryos formation, and a large number of

540   hormone-related genes (*Csa6M125240.1*, *Csa6M147590.1*, *Csa6M007440.1*, *Csa4M454150.1*,

541   *CsaUNG009930*, *Csa3M389850.1.*, and *BBM*, *HSP90* and *AGL*) were expressed at the stage of

542   early embryo development. In the stage of embryo morphogenesis stage, GO analysis showed

543   that continuously expressed genes participated in microtubule-based movement, movement of

544   the cell, or subcellular component processes, indicating functional protein complex binding,

545   microtubule binding, tetrapyrrole binding, tubulin binding and other microtubule activity, which

546   are involved in protein processing in the endoplasmic reticulum and phenylpropanoid

547   biosynthesis. Shoot formation was regulated by six transcription factors that contained a B3

548   domain, a total of nine genes in the *AP2/ERF* family, and two genes that encoded *WUS*

549   homologous domain proteins. This evaluation of molecular events in gynogenesis may be useful

550   for understanding the molecular mechanism of cucumber ovarian culture.

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699

Figures

701 **Fig. 1. Total RNA agarose gel electrophoresis of cucumber ovary embryos**

702 1, 2, 3, 4, 5, 6......18 = T0-1, T0-2, T0-3, T1-1, T1-2, T1-3......T5-3.

703

704 **Fig. 2. Negative and positive contrast of cucumber ovary culture**

705 Ovule of culture 2 d, **a**- L and **b** non-responsive ovule, **a**- R and **c** Enlarged ovule; **d** Ovaries of culture 10 days.

706 **Ov** Ovule, **Vb** Vascular bundle, **Es** Embryo sac, **Pl** Placenta, **In** Intergument, **Ie** Inner epidermis. Enlarged

707 ovule was marked with a white arrow.

708

709 **Fig. 3. Embryogenic process of cucumber ovary culture**

710 **a**-**f** and **l** Observation under stereoscopic microscope. **a** Selected fresh unpollinated ovaries (0 d,

711 T0). **b** Ovules were treated with a high temperature of 33 °C for 2 d; **c** Embryos cultured for 10 d,

712 note the ovule enlargement and embryo initiation. **d** Embryos cultured for 10 d, with an average

713 of six embryoids in each slice of ovary; **e** Embryos cultured for 30 d, embryo morphogenesis and

714 cotyledon-embryo formation. **f** Cultured for 60 d, embryo differentiation into shoot. Histological

715 observations: the histological sections were stained with Delafield's hematoxylin. **g** Multicellular

716 embryo sac before culturing, **h** One of the cells expands in the embryo sac after 2 d of culture, **i**

717 Cell mitosis occurred at 4 d of culture. **j** Cells continued to be divided into four cells at 6 d of

718 culture. **k** A cell clumps structure was visible at 8 d of culture. **l** A globular-embryo switched

719 into a cotyledon shape-embryo. **Ov** ovule, **Ow** ovary wall,   **Es** Embryo sac, **Ec** Egg cell, **Sy**

720 synergid, **PN** Polar nucleus, **Ig** Integument, **Eb** Embryo, **Dc** Developing cells, **G-e** Globular

721 embryo, **H-se** Heart-shaped embryo, **T-se** Torpedo-shaped embryo, **C-se** Cotyledon-shaped

722 embryo, S Shoot. The solid white lines represent 2mm, and the black lines represent 200μm.

723

Fig. 4. The total number of upregulated and downregulated genes

Black-colored bars represent upregulated genes, and gray bars represent downregulated genes.

726

Fig. 5. Validation of differentially expressed genes and the correlation between RNA-seq and qRT-PCR

qRT-PCR analysis of nine upregulated genes involved in plant hormone signal transduction and plant-pathogen interaction genes in the whole ovary culture, **a** Csa7M452370.1, **b** Csa1M006300.1, **c** Csa5M223020.1 **d** Csa5M434550.1, **e** Csa5M623800.1, **f** Csa6M383530.1, **g** Csa3M389850.1, **h** Csa7M420160.1, **i** Csa1M006320.1. Close correlations (Person's r= 0.77, 0.57, 0.74, 0.79, 0.69, 0.81, 0.86, 0.62, and 0.81) were observed between relative expression levels in RNA-seq and qRT-PCR, validating the RNA-Seq methodology described here for quantitative analysis of the cucumber transcriptome. Three biological replicates of each gene at each time point,, and data were shown as mean ± SE (n = 3).

737

Fig. 6. KEGG pathway enrichment analysis based on the differentially expressed genes

a Early embryo development, **b** In the stage of embryo morphogenesis, **c** In shoot stage. The longitudinal coordinates are the enriched KEGG pathways, and the x-axis monitored the rich factor; The size of the dots in the graph represents the number of differentially expressed genes annotated to the mentioned pathway, and the color represents the significant P value of the pathway. The most significant pathways are shown in the picture.

744

Fig. 7. The matrix graph of upregulated expression genes in the plant hormone signal transduction pathway across six time points

The absolute values of the relative expression of upregulated gene expression are indicated by the depth of color. The x-axis of the matrix graph shows the six time points. The y-axis of the matrix graph shows the name of the upregulated genes. The upregulated genes were arrayed according to their hormonal signal transduction flows, which were classified into six groups: auxin, cytokinin, abscisic acid, ethylene, brassinosteroid, and salicylic acid.

752

753 **Fig. 8. Venn diagram of showed differentially expressed genes in embryo morphogenesis**
754 **stage**

755 The DEG sets (T1 vs. T2, T2 vs. T3, and T3 vs. T4) described in Fig. 4 were analyzed using the
756 Venn method. The numbers marked in the diagram indicated the number of genes significantly
757 expressed across the three DEGs sets.

758

Additional files

759

760 **Table S1.** Primers for real-time quantitative PCR

761 **Table S2.** Statistics of RNA-Seq alignment

762 **Table S3.** GO classification of common expressed genes in the early stage of embryogenesis

763 **Table S4.** Main differentially expressed genes in T0 vs T1

764 **Table S5.** GO classification of common expressed genes in the morphogenesis stage

765 **Table S6.** GO classification of common expressed genes in the shoot formed stage

766

Figure 1

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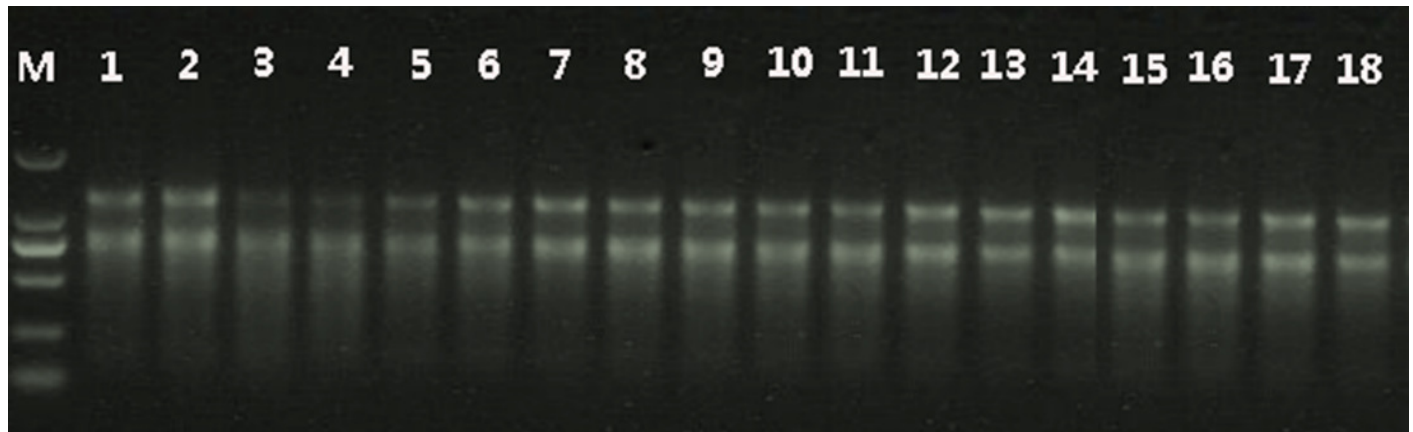
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Negative and positive contrast figure of cucumber ovary culture

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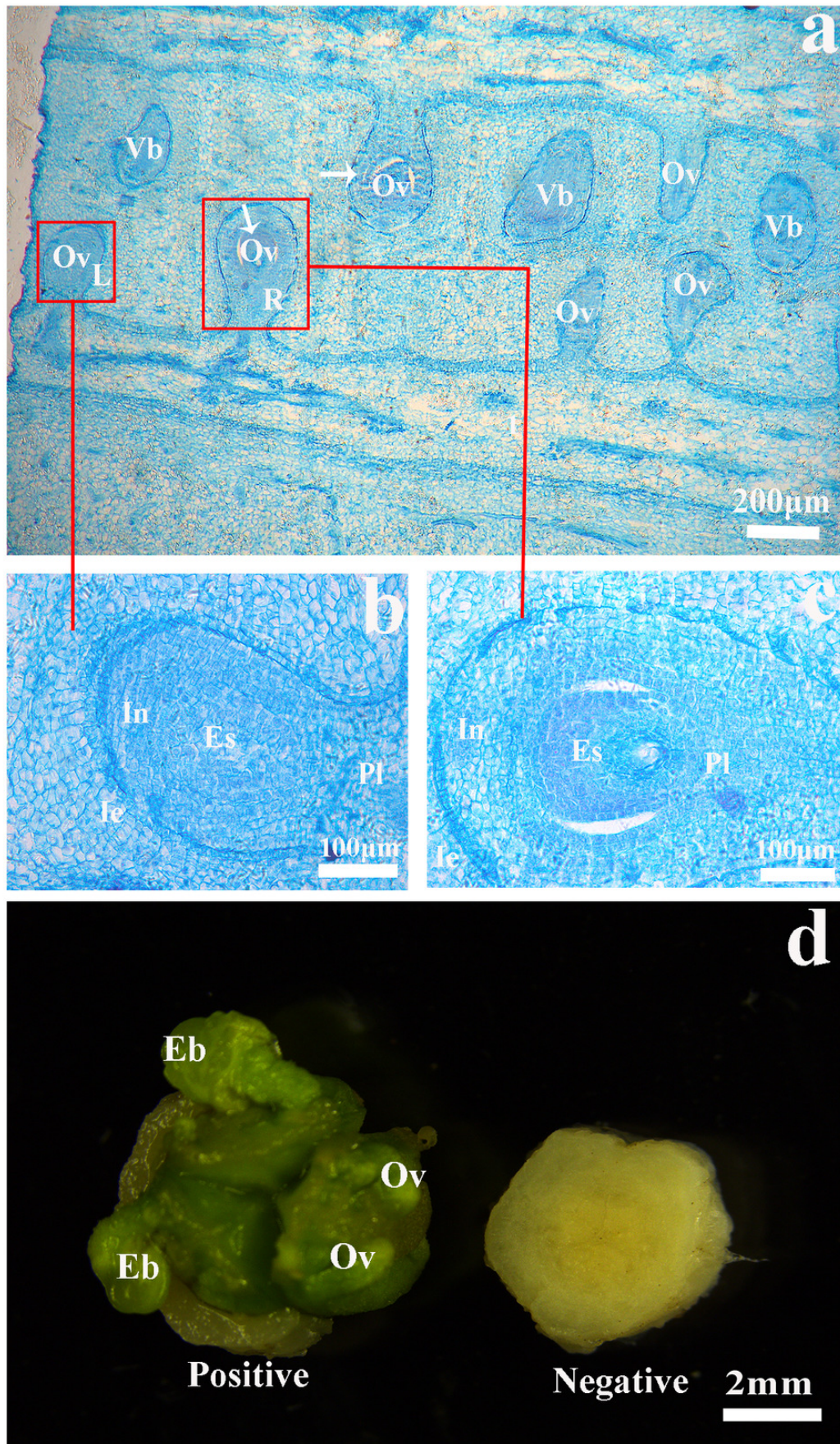
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Embryogenic process of ovary culture in cucumber

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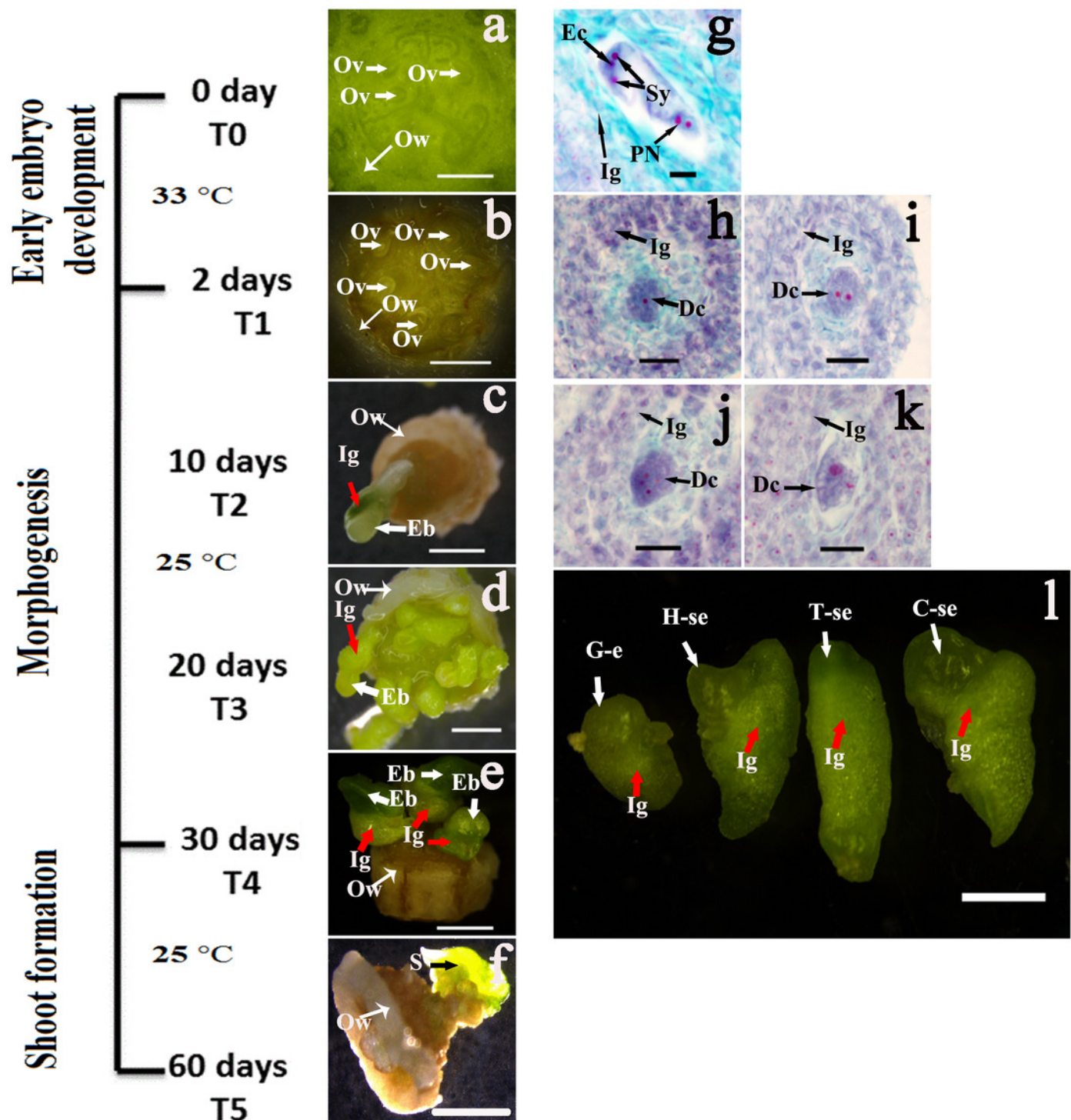
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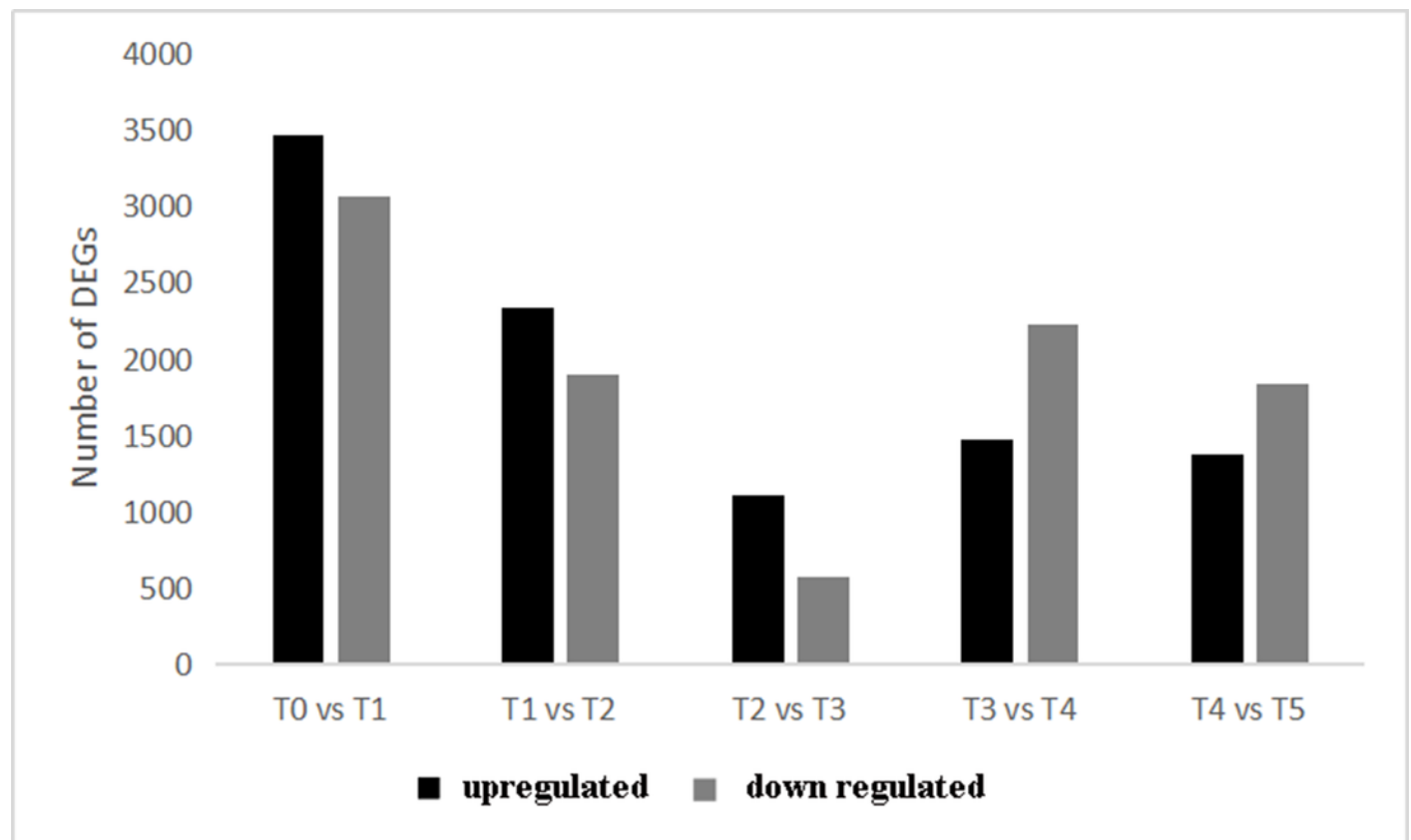
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Validation of differentially expressed genes and correlation between RNA-seq and qRT-PCR

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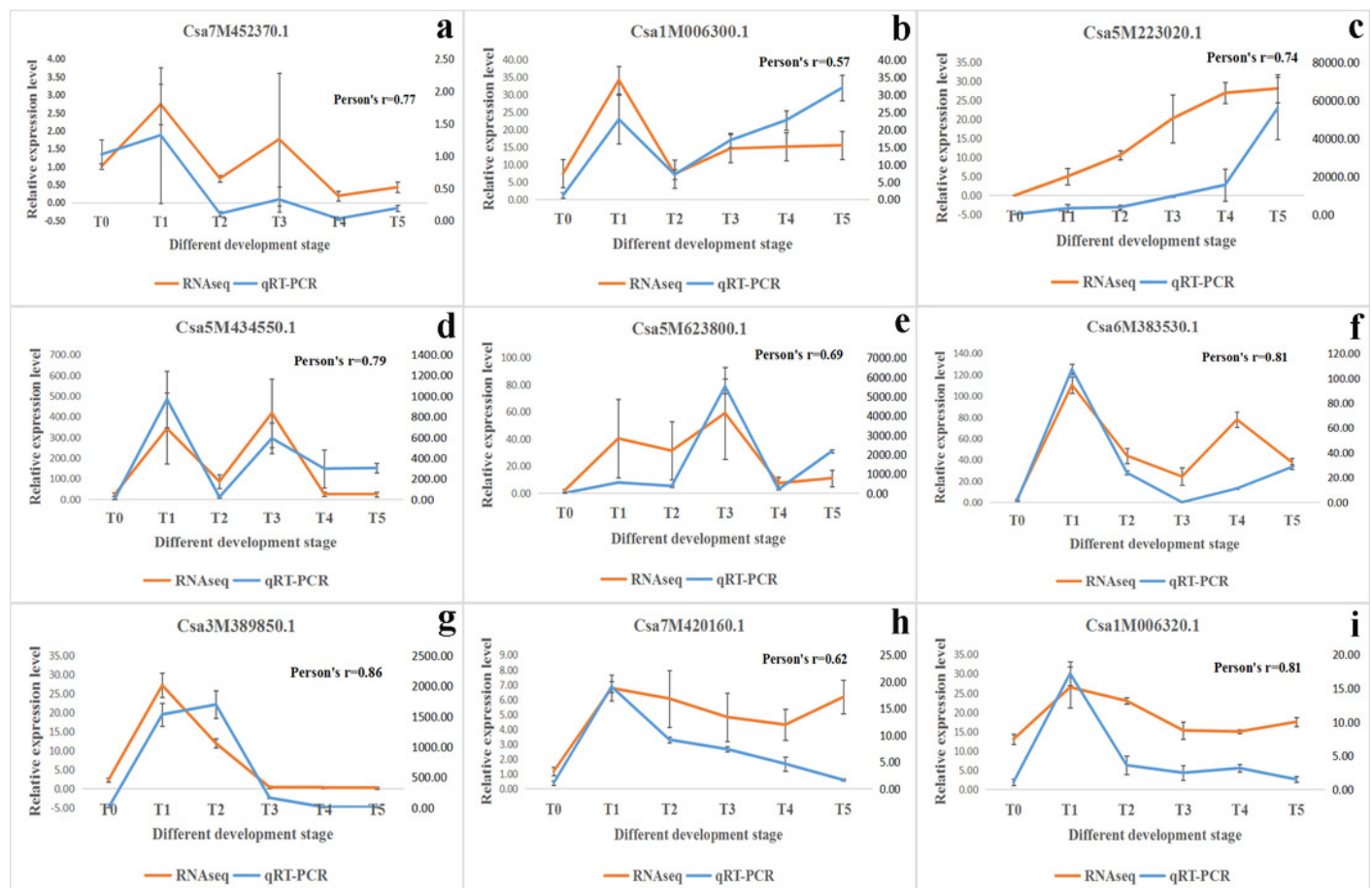
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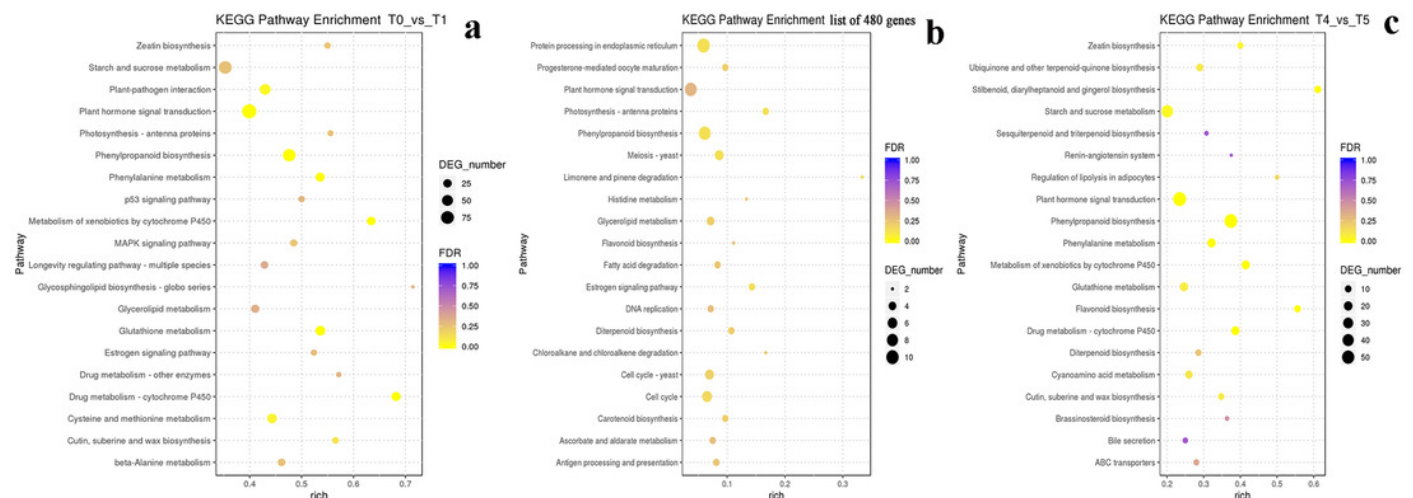
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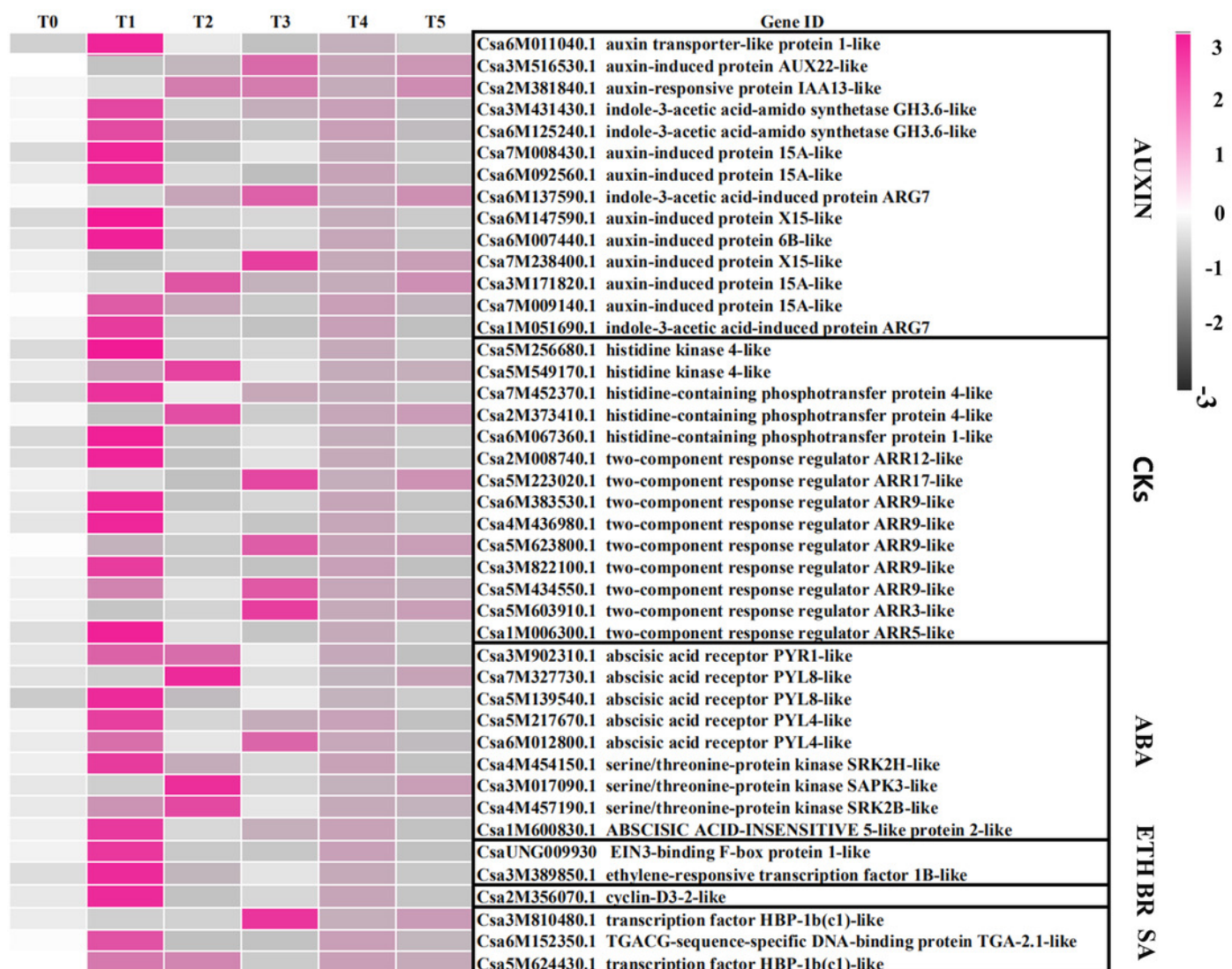
Figure 8

Venn diagram showed differentially expressed genes in the stage of embryo morphogenesis

The DEG sets (T1 vs T2, T2 vs T3 and T3 vs T4) described in Fig. 4 were analyzed using the Venn method. The numbers marked in the diagram indicated the number of genes significantly expressed among the three DEGs sets.