Comparative gene expression profile analysis of ovules provides insights into *Jatropha curcas* L. ovule development

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*Jatropha curcas*, an economically important biofuel feedstock with oil-rich seeds, has attracted considerable attention among researchers in recent years. Nevertheless, valuable information on the yield component of this plant, particularly regarding ovule development, remains scarce. In this study, transcriptome profiles of anther and ovule development were established to investigate the ovule development mechanism of *J. curcas*. In total, 64,325 unigenes with annotation were obtained, and 1723 differentially expressed genes (DEGs) were identified between different stages. The DEG analysis showed the participation of five transcription factor families (bHLH, WRKY, MYB, NAC and ERF), five hormone signaling pathways (auxin, gibberellic acid (GA), cytokinin, brassinosteroids (BR) and jasmonic acid (JA)), five MADS-box genes (*AGAMOUS*-2, *AGAMOUS*-1, *AGL1*, *AGL11*, and *AGL14*), SUP and SLK3 in ovule development. The role of GA and JA in ovule development was evident with increases in flower buds during ovule development: GA was increased approximately twofold, and JA was increased approximately sevenfold. In addition, the expression pattern analysis using qRT-PCR revealed that *CRABS CLAW* and *AGAMOUS*-2 were also involved in ovule development. The upregulation of BR signaling genes during ovule development might have been regulated by other phytohormone signaling pathways through crosstalk. This study provides a valuable framework for investigating the regulatory networks of ovule development in *J. curcas*.

*Jatropha curcas* L., a species native to tropical regions in the Western Hemisphere, is now found prevalently distributed in Africa and Asia. *J. curcas* is described as an ideal bioenergy crop for its oil-rich seeds, high unsaturated fatty acid content in seed oil (the oil with high unsaturated fatty acid content is suitable for producing biodiesel), low nutrient requirements and high drought tolerance. In addition, *J. curcas* also has potential in medical applications such as anti-tumor, anti-microbial and anti-parasitic1–3. However, due to low seed yield, this plant has limited economic benefit for exploitation and further expansion of the Jatropha-based biodiesel industry. The low ratio of female to male flowers (1/10—1/30) is one of the critical factors attributed to the low seed yield of *J. curcas*4; therefore, in *J. curcas*, gene expression profile analysis was employed to study the development of seeds5, the response of seedlings to drought and salt stress6,7, and lipid metabolism in seeds and other tissues8. For high-throughput discovery of novel Jatropha genes, *de novo* assembly and transcriptome analysis of various tissues of *J. curcas* were performed, obtaining 17,457 assembled transcripts (contigs) and 54,002 singletons9. However, genomics studies on flower development in *J. curcas* are relatively scarce. Pan *et al.* analyzed the transcriptome of the inflorescence meristems of *J curcas* treated with cytokinin, obtained 81,736 unigenes, and identified a series of cytokinin-responsive genes, such as *JcCycA3;2, JcCycD3;1, JcCycD3;2* and *JcTSO1*, which were thought to contribute to the increase in flower number10. Xu *et al.* analyzed the transcriptome of flower buds at different phases of sex differentiation in *J. curcas*, obtained 57962 unigenes, and found that the gibberellin-regulated protein 4-like gene and the AMP-activated protein kinase gene are associated with stamen differentiation11.

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J. curcas is a monoecious plant. Sex differentiation occurs after the initiation of five petal primordia. Unlike male flowers, the female flowers undergo a hermaphrodite period: stamens first develop carpels and then gradually degenerate as the three carpels to be formed are fused. Ovule development is an important process for the development of female flowers and is crucial for fruit yield. Any disturbances during macrosporogenesis or female gametogenesis may result in female flower abortion. Molecular studies on sporogenesis and gametogenesis of J. curcas are not available; therefore, transcriptome profiles spanning the developmental processes of pollen and ovule in J. curcas were constructed. In this study, we focused on macrosporogenesis and female gametogenesis during ovule development, and differentially expressed gene analysis was then performed on the expression profiles of flower buds at different developmental stages of the ovule in female flowers.

Results

Development of the ovule. After the fusion of the three carpels, the ovule primordium was formed. When the ovary was approximately 0.3 mm long, archesporial cells occurred. When the ovary was approximately 0.6 mm long, archesporial cells gave rise to primary sporogenous cells. When the ovary was approximately 0.9 mm long, the style began to develop, and the primary sporogenous cells developed into macrospore mother cells (MMC). When the ovary was approximately 1.2 mm long, the style was deep green, and the stigmas appeared; MMC underwent two consecutive meioses with a result of 4 cells, of which three were degenerated, and the remaining one was developed into a functional macrospore. When the ovary was approximately 3.2 mm long, functional macrospores experienced three consecutive mitoses, resulting in an embryo sac with 8 nuclei. When the whole ovary became green, the stigmas were mature; of these 8 nuclei, two moved to the center of the embryo sac and formed a polar, and the remaining 6 formed an egg, two synergids, and three antipodal cells, respectively (Figs 1 and 2).

Endogenous phytohormone level in flower buds at different development stages. The concentrations of endogenous gibberellic acid (GA) and jasmonic acid (JA) in flower buds began to significantly increase in JCFII, but JA further experienced a dramatic increase in JCFIV. Additionally, the concentration of endogenous...
SA in flower buds significantly declined only at the stage of JCFIV and only slightly changed in the stages of JCFl, JCFII and JCFIII (Fig. 3).

**Identification of differently expressed genes.** The sequencing results of the twelve sample DGE libraries are shown in Table 1. The depth, coverage and homogenization of sequencing were high, which suggested that these results could reflect the actual expression of genes, and the data were thus suitable for further analysis (Fig.
In total, 1723 genes that were differentially expressed during ovule development in female flower buds were screened out and are shown in Fig. 4. Of these differentially expressed genes (DEGs), 9 genes were downregulated and 12 genes were upregulated throughout the entire process of ovule development; 579 genes were downregulated and 1036 genes were upregulated in one stage; 42 genes were downregulated and 100 genes were upregulated at two stages (Fig. 5).

Gene expression patterns of selected genes. In total, 16 DEGs were selected for qRT-PCR analysis to validate the expression profiles obtained by digital gene expression analysis, and the results showed that the expression patterns of these genes were consistent with the results obtained by digital gene expression analysis, except AUX22 (c11413_g1) (Fig. 6), indicating that transcriptome data in this study were reliable.

KEGG pathway enrichment analysis of differentially expressed genes. After KEGG analysis, the significantly enriched pathways were selected for further analysis. The results indicated that sugar metabolism and protein biosynthesis/processing were respectively enhanced during the maturation of EC and the formation of MES, and the metabolisms of auxin and JA were also upregulated during the formation of MMC and the maturation of EC, but the metabolism of SA declined during ovule development. Among the significantly enriched pathways (Fig. 7), plant hormone signal transduction was downregulated in pairwise JCFI vs JCFII and JCFII vs JCFIII but showed a trend to upregulate in pairwise JCFIII vs JCFIV; starch and sucrose metabolism was upregulated in pairwise JCFIII vs JCFIV; protein processing in endoplasmic reticulum was upregulated in pairwise JCFIII vs JCFIV; tryptophan metabolism associated with auxin metabolism was upregulated in pairwise JCFI vs JCFIV; and protein processing in endoplasmic reticulum was upregulated in pairwise JCFIII vs JCFIV; plant hormone signaling-responsive DEGs were identified, 61 of which were screened out by transcriptome analysis, and the results showed that the expression of these genes was consistent with the results obtained by qRT-PCR analysis (Fig. 9). These results suggested that bHLHs, WRKYs, MYBs, NACs, and MYBs would extensively participate in ovule development.

Prediction of gene regulating ovule development. In total, 9 genes annotated as ovule development-related genes were upregulated during ovule formation as suggested by transcriptome analysis, including 5 MADS-box protein genes (AGAMOUS-2 (AG-2), AG-1, AGL11/SHP1, AGL11/STK and AGL14), YABBY 5, UPPerMAN (SUP), SLK3 and Argonaute 2 (Table 1). These five MADS-box proteins were then subjected to sequence alignment with their homologs in the protein database UniProt. The results showed that these five MADS-box proteins have a MADS-MEF2-LIKE and a K-box domain and belong to the MADS-MEF2-LIKE subfamily of MADS (Fig. 9). The MADS-MEF2-LIKE domain showed high homology between these five MADS-box proteins and their homologs (Fig. 9). Of these 9 genes, AG-2 was upregulated throughout ovule development, as suggested by both transcriptome analysis and qRT-PCR analysis (Fig. 10; Table 1); AGL1, AGL14, AG-1 and SUP were upregulated in pairwise JCFI vs JCFII; SLK3 was upregulated in pairwise JCFIV vs JCFIII; AGL11/STK was continuously upregulated from JCFI to JCFIV; YABBY 5 was continuously upregulated from JCFI to JCFIV; argonaute 2 was upregulated in pairwise JCFI vs JCFIV and JCFI vs JCFIV. On the other hand, CRABS CLAW (CRC), annotated as an ovule development-related gene, was upregulated from JCFI to JCFIV, as suggested by qRT-PCR analysis (Fig. 10).

Phytohormone signaling responsive genes associated with ovule development. In all, 71 phytohormone signaling-responsive DEGs were identified, 61 of which were screened out by transcriptome analysis, 9 by qRT-PCR analysis, and IAA1 by both qRT-PCR and transcriptome analysis (Figs 10, 11). The expression

| Gene_id   | Annotation description                  | Protein ID   | log2(JCFI/ JCFII) | log2(JCFII/ JCFIII) | log2(JCFIV/ JCFIII) | log2(JCFI/ JCFII) | log2(JCFI/ JCFII) | log2(JCFI/ JCFII) |
|-----------|-----------------------------------------|--------------|------------------|---------------------|---------------------|------------------|------------------|------------------|
| c26856_g5 | Argonaute 2 (Arabidopsis thaliana)      | Q9SHF3       | 0.092            | 1.407               | 1.078              | 0.692            | 0.814            |
| c27618_g1 | AGAMOUS (AGAMOUS-1) (Panax ginseng)    | Q40872       | 0.618            | 0.561               | 1.179              | 1.023            |
| c29108_g3 | AGAMOUS (Arabidopsis thaliana)          | Q38838       | 1.073            | 1.895               | 1.742              | 0.670            |
| c30128_g4 | AGL11 (Arabidopsis thaliana)            | Q38836       | 1.351            | 4.105               | 4.115              | 1.367            |
| c24381_g1 | YABBY 5 (Arabidopsis thaliana)          | P29381       | 1.126            | 1.470               | 1.344              |                  |
| c24288_g1 | AGAMOUS (AGAMOUS-2) (Nicotiana tabacum)| Q43585       | 1.329            | 1.210               | 0.730              | 2.541            | 3.271            | 1.946            |

Table 1. Expression level of ovule development related genes during ovule development.

S2). In these twelve libraries, approximately 83.78–87.00% of the clean reads could be mapped to the assembled transcriptome (Table S4). In total, 1723 genes that were differentially expressed during ovule development in female flower buds were screened out and are shown in Fig. 4. Of these differentially expressed genes (DEGs), 9 genes were downregulated and 12 genes were upregulated throughout the entire process of ovule development; 579 genes were downregulated and 1036 genes were upregulated in one stage; 42 genes were downregulated and 100 genes were upregulated at two stages (Fig. 5).
of *IAA1* was first downregulated at JCFII and then significantly upregulated during the following development stages (Fig. 10B,L).

Of these 61 DEGs screened out by transcriptome analysis, 24 were from auxin, 2 from BR, 10 from CK, 8 from ABA, 5 from ETH, 3 from GA, 6 from JA, and 3 from SA. In pairwise CFI vs JCFII, the expression of 4 *SAUR* genes (*22D* protein, *15A-1* and two *10A5* (*10A5-1* and *10A5-2*)) in auxin and histidine-containing phosphotransfer proteins 4 (*HP4-2*) in CK were upregulated, whereas *ARF 11*, *ARF 18* and *GH3.5* in auxin were downregulated. In pairwise JCFII vs JCFIII, the expression of *GH3.17* in auxin was upregulated, whereas *IAA30* and *X10A* in auxin and *GH3.5* in JA were downregulated. In pairwise JCFIII vs JCFIV, the expression of *GH3.1* in auxin, *ARR9-2* and *ARR5* in CK, gibberellin receptor *GID1B* in GA, xyloglucan endotransglucosylase/hydrolase protein

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**Figure 4.** Genes differently expressed during ovule development.
22 (XTH22) in BR, pathogenesis-related protein 1 in SA, and two TIFY10B and a MYC2 in JA were upregulated, whereas ARG7-3 in auxin was downregulated. In pairwise JCFI vs JCFIII, JCFI vs JCFIV or JCFII vs JCFIV, the expression of auxin transporter-like protein 2 (ATLP2), IAA4, ARF3 and ARG7-1 in auxin, HP5, HP1-2, ARR3 and ARR9-1 in CK, BES1/BRZ1 homolog protein 2 in BR, and TIFY 6B and hypothetical protein JCGZ_04744 in JA were upregulated, whereas ARG7-2, ARG7-4, 15A-2, GH3.6, ARF 5 and ARF 1 in auxin, GID1C and GID2 in GA, HP1 in CK, NPR4 and PERIANTHIA in SA were downregulated. On the other hand, HK 4 was continuously downregulated during ovule development, and HP4-1 was downregulated from JCFI to JCFIII. The genes in ETH signaling were all downregulated during the development of the ovule. Of the 8 genes involved in ABA signaling, only ABA receptor PYL9 was downregulated in pairwise JCFIII vs JCFIV, while the other genes gradually changed their expression during ovule development (Fig. 11).

Of these 9 DEGs screened by qRT-PCR analysis, 3 genes (GASA3, GAI and GASL7) were from the GA response signaling pathway, 2 (BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) and (BRASSINAZOLE-RESISTANT 1 protein (BRZ1)) from BR, 4 (ARF5, 5NG4, AUX22 and auxin-induced in root cultures protein 12 (AIT12)) from auxin. Of these genes, GASA3 was upregulated from JCFII to JCFIV, and GASL7 from JCFI to JCFII; BZR1, AIT12 and GAI were upregulated from JCFII to JCFIV; 5NG4, ARF5 and BAK1 were from JCFII to JCFIII; AUX22 was greatly downregulated at the stages after JCFI (Fig. 10).

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Differentially expressed genes related to phytohormone metabolism. The DEGs related to phytohormone metabolism were screened out as follows: auxin metabolism-related DEGs were screened out from the DEGs enriched in the tryptophan metabolism pathway, CK metabolism-related DEGs from the DEGs enriched in the enriched zeatin biosynthesis pathway, gibberellin metabolism-related DEGs from the DEGs enriched in the diterpenoid biosynthesis pathway, BR metabolism-related DEGs from the DEGs enriched in metabolism pathway, JA metabolism-related DEGs from the DEGs enriched in the alpha-linolenic acid metabolism pathway, and SA biosynthesis-related DEGs from the DEGs enriched in phenylalanine biosynthesis (Table 2).

In total, 26 differentially expressed genes were identified, of which 8 were from auxin, 2 from BR, 2 from CK, 3 from GA, 10 from JA, and 1 from SA. In pairwise JCFI vs JCFII, the expression levels of YUCCA8, aldehyde dehydrogenase family 3 member H1 (ALDH 3H1) and ALDH 2B4-1 in auxin were upregulated, whereas cyto-kinin dehydrogenase 3 in CK was downregulated. In pairwise JCFII vs JCFIII, the expression of Ent-kaurene oxidase (KO) in GA was upregulated, whereas ALDH 2 B4-2 in auxin and gibberellin 2-beta-dioxigenase 2 in GA were downregulated. In pairwise JCFIII vs JCFIV, the expression of linoleate 13 S-lipoxygenase 3-1, allene
oxide synthase, and 12-oxophytodienoate reductase 3 in JA was upregulated. In pairwise JCFI vs JCFII and JCFIII vs JCFIV, the expression of adenylate isopentenyltransferase 5 (IPT5) in CK was upregulated. In pairwise JCFII vs JCFIII and JCFIII vs JCFIV, the expression of 3-ketoacyl-CoA thiolase 2 in JA was upregulated. Additionally, the expression of several genes gradually changed during ovule development. In pairwise JCFI vs JCFIII, CFI vs JCFIV or JCFII vs JCFIV, the expression of ent-kaurenoic acid oxidase 1 (KAO1) in GA and fatty acid beta-oxidation multifunctional protein AIM1 gene, 4-coumarate-CoA ligase-like 5, allene oxide cyclase 4 gene, linoleate 13 S-lipoxygenase 2-1 gene, and 12-oxophytodienoate reductase 11 gene in JA were upregulated, whereas cytosolic sulfotransferase 16, ALDH3F1, YUCCA2 and YUCCA4 in auxin, acyl-coenzyme A oxidase 2 gene in JA, phenylalanine ammonia-lyase gene in SA, and CYP90D1 and CYP90B1 in BR were downregulated (Table 2).

Discussion

The embryo sac of J. curcas belongs to the polygonum type. A macrospore mother cell (MMC) only produces one functional macrospore that then develops into an embryo sac with 8 nuclei after three consecutive mitoses. Of these 8 nuclei, two move to the center of the embryo sac and form a polar, and the remaining six form an egg, two synergids, and three antipodal cells, respectively (Figs 1 and 2). A transcriptome covering the entire development process of sporogenesis and gametogenesis in both male and female flowers was constructed using Illumina sequencing.

Overall, plant hormone signal transduction declined during ovule development but showed a trend toward enhancement during the maturation of ES. Protein biosynthesis and processing were enhanced during the formation of MES. Sugar metabolism seemed to be enhanced during the development process of the ovule. A strong relationship exists between flower development and carbohydrates12–15. In particular, the concentration of starch in the ovule at specific steps of development is closely correlated with fertility16.

Transcription factors participating in ovule development.

Five TF families, such as bHLH, WRKY, MYB, NAC and ERF, would extensively contribute to ovule development, as suggested by the upregulation of 17 bHLH, 11 WRKY, 12 ERF, 10 NAC and 10 MYB during ovule development. The members of these five TF families are thought to be involved in ovule development. bHLHs play important roles in carpel, stigma and anther development and phytochrome signaling17–19. MYBs can regulate the development of anther, petal, and embryogenesis20–22. R2R3-MYBs are the largest group of plant MYB factors, and several R2R3-MYBs have been documented to function in ovule development. FOURLIPS and MYB88 are thought to regulate megasporogenesis23, and MYB98 is specifically expressed in synergid cells24. For WRKY, WRKY71 can accelerate flowering via the direct

Figure 7. KEGG classification analysis of the differently expressed genes.
activation of *FT* and *LEAFY*\textsuperscript{25}, and WRKY34 and WRKY2 are required for male gametogenesis in *Arabidopsis*\textsuperscript{26}. Several members of the AP2/ERF family are thought to be involved in microspore, somatic and zygotic embryogenesis\textsuperscript{27}. Members of the NAC family, such as CUC genes, contribute to ovule primordial development\textsuperscript{28,29}. MADS-BOX, *SUP* and *SLK3* genes participate in ovule development. In the present study, five MADS-BOX genes (*AG-1, AG-2, AGL1, AGL11* and *AGL14*), *SUP* and *SLK3* are expected to function in the development of the ovule in *J. curcas*. *AG* has been reported to determine stamen, carpel and ovule identity\textsuperscript{30–32}. In this study, *AG-1* functioned during the stage of occurrence of MMC and *AG-2* throughout the entire development.

Figure 8. Cluster analysis showing the differentially expressed transcriptor factor genes during ovule development.
of the ovule. SHP1 (AGL1) and STK (AGL11) are known as ovule identity genes that control ovule cell fate and regulate sporophyte and gametophyte development13–35. SHP1 (AGL1) is also reported to function in regulating cell divisions in the ovule and in promoting stigma, style and medial tissue development36,37. Our results show that AGL1 and AGL11 play roles during the stage of MMC, controlling ovule cell fate, and regulating sporophyte development and cell divisions in the ovule. However, AGL11 is also predicted to function during the formation of MES and regulate gametophyte development. AGL14 can control auxin transport via PIN transcriptional regulation38, and the PIN protein-dependent auxin gradient is responsible for pattern formation during early embryogenesis, organogenesis and ovule development39. Our results suggest that AGL14 functions during the
occurrence of MMC and regulates the early development of the ovule. SUP is known to function in the direct regulation of ovule development by dictating the growth of the adaxial outer-ovule integument by downregulating cell division\textsuperscript{40–42}. In \textit{J. curcas}, SUP may function during the occurrence of MMC when outer-ovule integument is

Figure 10. One-way ANOVA analysis of expression level of 15 selected genes in samples at different ovule development stages, as detected by real-time QPCR. Different letters in the same row indicate significant differences ($P \leq 0.005$). c28570\_g1: R2R3-type MYB; c24288\_g1: CRABS CLAW; c30007\_g3: CRAB S CLAW; c28499\_g1: Auxin-induced protein 5NG4; c21569\_g1: IAA1; c15640\_g1: GASA3; c8047\_g1: BZR1; C21626\_g1: Auxin-induced in root cultures protein 12; c29827\_g2: GAI; c26065\_g1: GASL7; c27617\_g8: ARF5; c25603\_g2: BAK1; c11413\_g1: AUX22.
not generated, and a new role of SUP in ovule development is predicted. Members of the YABBY family are active in determining abaxial-adaxial identity in Arabidopsis lateral organs\(^4^3\) and involved in amorphous or arrested growth of the integument\(^4^4\). However, YABBY 5, a member of the YABBY family, functions during the stages after the occurrence of MMC. The SLK gene is required for the proper development of vital female reproductive tissues derived from the carpel margin by maintaining meristematic potential in both the carpel margin meristem and the shoot apical meristem\(^4^5\). In the present study, SLK3, a member of the SLK gene, is predicted to function during the maturation of ES (a late stage of ovule development). Although these genes are predicted to function in certain developmental stages of the ovule, the details of their roles in ovule development are not clear and
Table 2. Phytohormone biosynthesis genes differently expressed during the ovule development.

| Genes       | Swissprot Description                          | Protein ID | log2(JCFII/JCFI) | log2(JCFIII/JCFII) | log2(JCFIV/JCFIII) | log2(JCFIV/JCFI) | log2(JCFIV/JCFI I) |
|-------------|------------------------------------------------|------------|------------------|--------------------|--------------------|-----------------|-------------------|
| Auxin       |                                                |            |                  |                    |                    |                 |                   |
| c24045_g2   | Probable YUCCA8 (Arabidopsis thaliana)         | Q9SVU0     | 1.504            |                    |                    |                 |                   |
| c25692_g3   | Alddehyde dehydrogenase family 3 member H1(ALDH3 H1) (Arabidopsis thaliana) | Q70DU8     | 1.096            |                    |                    |                 |                   |
| c14699_g1   | Alddehyde dehydrogenase family 2 member B4(ALDH2 B4-1), (Arabidopsis thaliana) | Q9SU63     | 0.737            |                    |                    |                 |                   |
| c20408_g1   | Alddehyde dehydrogenase family 2 member B4(ALDH2 B4-2), (Arabidopsis thaliana) | Q9SU63     | −0.688           |                    |                    |                 |                   |
| c29730_g3   | Cytosolic sulphatransferase 16 (Arabidopsis thaliana) | Q9C9D0     | −1.383           | −0.884             |                    |                 |                   |
| c24704_g1   | Alddehyde dehydrogenase family 3 member F1(ALDH3 F1) (Arabidopsis thaliana) | Q70E96     | −0.625           |                    |                    |                 |                   |
| c26913_g1   | YUCCA2 (Arabidopsis thaliana)                  | Q9SVQ1     | −2.025           |                    |                    |                 |                   |
| c28407_g1   | YUCCA4 (Arabidopsis thaliana)                  | Q9LFM5     | −0.937           |                    |                    |                 |                   |
| Gibberellins |                                                |            |                  |                    |                    |                 |                   |
| c15206_g1   | Gibberellin 2-beta-dioxygenase 2 (Pisum sativum) | Q9XHMS     | −2.105           |                    |                    | −2.100           |                   |
| c25340_g1   | Ent-kaurene oxidase (Arabidopsis thaliana)     | Q93ZB2     | 0.749            | 1.394              | 2.109              | 1.469            |                   |
| c28071_g1   | Ent-kaurenoic acid oxidase 1 (Arabidopsis thaliana) | O23051     | 0.558            | 0.585              |                    |                 |                   |
| Cytokinin   |                                                |            |                  |                    |                    |                 |                   |
| c39116_g1   | Cytokinin dehydrogenase 3 (Arabidopsis thaliana) | Q9LTS3     | −2.138           |                    |                    |                 |                   |
| c29016_g9   | Adenylate isopentenytransferase 5 (Arabidopsis thaliana) | Q941D2     | 2.246            | 1.099              | 2.785              | 3.884            | 1.642             |
| JA          |                                                |            |                  |                    |                    |                 |                   |
| c27373_g1   | 3-ketocarbonyl-CoA thiolase 2 (Arabidopsis thaliana) | Q56WD9     | 0.746            | 1.155              | 1.679              | 1.905            |                   |
| c27880_g2   | Linoleate 13S-lipoxigenase 3-1 (Solanum tuberosum) | O24371     | 1.015            | 1.275              | 1.182              |                 |                   |
| c17968_g1   | Allene oxide synthase (Linum usitatissimum)     | P48417     | 1.385            | 1.404              | 1.483              |                 |                   |
| c29952_g2   | 12-oxophytodienoate reductase 3 (Solanum lycopersicum) | Q9FEW9     | 1.040            | 0.957              |                    |                 |                   |
| c29272_g6   | Acyl-CoA oxidase A oxidase 2 (Arabidopsis thaliana) | O65201     | −0.733           |                    |                    |                 |                   |
| c29400_g1   | Fatty acid beta-oxidation multifunctional protein AIM1 (Arabidopsis thaliana) | Q9ZP16     | 0.522            | 0.942              | 0.723              |                 |                   |
| c24535_g1   | 4-carboxy-CoA ligase-like 5 (Arabidopsis thaliana) | Q84P21     | 0.520            | 0.476              |                    |                 |                   |
| c27608_g6   | Allene oxide cyclase 4 (Arabidopsis thaliana)   | Q93ZC5     | 1.095            | 1.203              |                    |                 |                   |
| c28745_g1   | Linoleate 13S-lipoxigenase 2-1 (Solanum tuberosum) | O24370     | 1.350            | 0.903              |                    |                 |                   |
| c29303_g1   | Putative 12-oxophytodienoate reductase 11 (Oryza sativa subsp. Japonica) | B9FSC8     |                 |                   |                    |                 |                   |
| SA          |                                                |            |                  |                    |                    |                 |                   |
| c29339_g2   | Phenylalanine ammonia-lyase (Populus trichocarpa) | P45730     | −1.040           | −0.617             |                    |                 |                   |
| BR          |                                                |            |                  |                    |                    |                 |                   |
| c28090_g2   | CYP90D1 (Arabidopsis thaliana)                  | Q9M066     | −0.672           |                    |                    |                 |                   |
| c24187_g1   | CYP90B1 (Arabidopsis thaliana)                  | O64989     | −0.990           |                    |                    |                 |                   |

warrant further exploration. CRC has been thought to function in carpel morphogenesis and in the development of ovules33,46. Similarly, CRC was upregulated with the development of ovules in J. curcas and may function in the entire process of ovule development47.
GA plays important roles at the stage from MMC to ovule development. GA is known to play various roles in plant reproductive development, and different species respond differently to GA. The GA signaling pathway is involved in the development of stamens and pollen in Arabidopsis and tomato. GA is a negative regulator in the development of stamens in maize but a positive regulator in Arabidopsis and rice. GA is thought to play essential and complicated roles in floral organ development in Arabidopsis. In Arabidopsis, GA treatment increased both female and male flowers in the studies of Pan et al. and Gayakvad et al. The development of stamens to produce bisexual flowers in research of Pi et al. and the number of female flowers in the research of Makwana et al. suggest that auxin contributes to ovule development. The auxin biosynthesis in flower buds is expected to be increased during the occurrence of MMC, as suggested by the upregulation of auxin biosynthesis genes during the stages from the formation of MMC to the maturation of SE. In cotton, GASL7 is predominantly expressed in cotyledons, and GASA3 is expressed in fiber. In Arabidopsis thaliana, GAS3 accumulates in root, meristems, and flower seeds. In Arabidopsis thaliana, 3 GA hormone receptors, such as GID1a, GID1b, and GID1c, have partially specialized functions in both proteolytic and nonproteolytic GA signaling. GID1b plays a stronger role in nonproteolytic GA signaling. As a member of DELLA, GAI is a negative regulator of GA signaling.

JA plays important roles in the stage of maturation of EC. JA is thought to play critical roles in regulating reproductive development in plants, such as sex determination and stamen development in maize, and the development of inflorescences and flowers in rice and Arabidopsis. In Arabidopsis, JAs are members of the JA family that are responsible for pattern formation during ovule development. JA biosynthesis, suggesting the upregulation of four JA biosynthesis genes, such as 3-ketoacyl-CoA thiolase 2, linoleate 13S-lipoxygenase 3-1, allene oxide synthase, and 12-oxophytodienoic reductase 3. On the other hand, several JA-responsive genes, such as MYC2, JAZ, TIFY 6B and TIFY 10B, were upregulated at the maturation of EC, supporting our results. TIFY 10B and TIFY 6B belong to the JAZ subfamilies of the TIFY family and could be induced by JA. In Arabidopsis, JA1/TIFY10b can work as a transcriptional repressor in the models of JA signaling. Activation of JA response genes is mediated in part by MYC2; in the absence of JA, MYC2's function as a transcriptional activator is repressed by members of the JAZ family proteins, and elevated levels of JA induce the release of MYC2 from this repression by increasing the rate of degradation of JAZs. Furthermore, MYC2 is a “master switch” in the cross talk between JA and the other hormone signaling pathways.

Auxin may regulate the occurrence of MMC in ovule development. Auxin is thought to regulate ovule development, possibly by determining the cell identity of the female gametophyte cell. The gradient of auxin is responsible for pattern formation during ovule development. Similarly, our results suggest that auxin contributes to ovule development. The auxin biosynthesis in flower buds is expected to be enhanced during the occurrence of MMC, as suggested by the upregulation of UYCCA, ALDH 3H1 and ALDH 2B4-1. In Arabidopsis thaliana, the UYCCA gene encodes a flavin monooxygenase-like enzyme that appears to oxidize tryptamine to N-hydroxytryptamine. The ALDH family is thought to function in IAA biosynthesis by catalyzing the NAD-dependent formation of IAA from indole-3-acetaldehyde. The expression levels of SAURs (22D, 10A5 and two 15A), two GH3s (GH3.17 and GH3.1), two AUX/IAA (IAA1, IAA4), AFR3, ARG7-1, AT1T2, ATLP2 and SNG4 were upregulated during the development of the ovule, which would be associated with the elevated level of GA. Also, 10A5 and 15A are members of SAURs and could be rapidly induced by auxin treatment within 2.5 min. The AUX/IAA family is short-lived transcriptional factors and repressors of early auxin response genes at low auxin concentrations, and increased auxin could reduce the level of AUX/IAA proteins by accelerating their degradation. Different GH3s respond differentially to auxin treatment. In Arabidopsis, under IAA treatment, GH3.1 showed strong transcriptional induction, while GH3.5 and GH3.6 showed weaker responses, and GH3.17 showed little or no induction. AFR3 is a member of group II AFRs and is thought to be involved in floral meristem, gynoecium, stamen and perianth patterning in Arabidopsis. Exogenous auxin treatment upregulates AFR3.

Cytokinin may regulate the occurrence of MMC and the maturation of ES in ovule development. Cytokinin is reported to regulate ovule formation and ovule number and to specify the functional megaspore in the female gametophyte. In Arabidopsis, the application of CK increased the number of female flowers. Similarly, our results suggest that CK contributes to female development. CK biosynthesis in female flower buds would be enhanced at stages of MMC occurrence and ES maturation, as suggested by the upregulation of IPT and the downregulation of cytokinin dehydrogenase at these two stages. IPT catalyzes the initial step in the de novo biosynthesis of cytokinin in higher plants, whereas cytokinin dehydrogenase catalyzes the degradation of cytokinin. In response to the increase in CK, several CK response genes, such as HPA4-2, HPS, HPI-2, and five A-type AARRs (ARR9-2, AARR5, AARR9-1 and two AARR3) were upregulated. The cytokine signaling cascade typically consists of three functional modules: IK, HP, and response regulator (ARR). HP proteins serve as phosphorelay carriers between cytokinin receptors and downstream nuclear responses, activating B-type AARR proteins, which in turn, activate A-type AARR. Additionally, A-type AARRs are reported to participate in ovule development. Megagametophyte defects could be observed in the Arabidopsis mutants arr7 and arr15. In ovules lacking a functional embryo sac, AARR7 and other A-type AARRs (ARR4, AARR5 and AARR6) are
Arabidopsis regulate the ovule number in development by regulating the transcription of genes, such as (ANT)

**Brassinosteroids may function in the stages from meiosis of MMC to ES maturation.**
Brassinosteroids (BRs) suppress the development of the female flower in cucumber, whereas they positively regulate the ovule number in *Arabidopsis* by regulating related gene expression by BRZ1. BRs influence ovule development by regulating the transcription of genes, such as *HUELLENLOS* (HLL) and *ANTIGEMENTA* (ANT), which are redundant in the control of the ovule primordial growth. ANT is a direct target of BRZ1, while HLL is regulated in an indirect way. However, in this research, BR biosynthesis in flower buds is expected to decline during ovule development, as suggested by the downregulation of *CYP90D1* and *CYP90B1*. In *Arabidopsis*, *CYP90D1* plays an important role in the early C-22 oxidation of BR biosynthesis, and *CYP90B1* catalyzes multiple 22 α-hydroxylation steps in BR biosynthesis. In contrast to the decline of BR biosynthesis, BR signaling-responsive DEGs were all upregulated during ovule development, such as *XTH22*, *BZR1*, *BAK1* and *BES1/BZR1*, indicating that cross-talk might exist between BRs and other phytohormone signaling pathways. Several reports proposed that BRs and GAs could interact at the signaling levele-108. The DELLA protein GAI is thought to inactivate BZR1 by inhibiting the ability of BZR1 to bind to target promoters and negatively regulate BR signaling. Additionally, auxin can also crosstalk with BRs through G-protein signaling. XTHs, which can be upregulated by BRs, are thought to modify the length of xyloglucans, enabling the cell wall to expand. *BAK1* is a coreceptor of *BR1*, and the binding of BRs to *BR1* relieves the repression of *BKI* and causes the association of *BR1* with *BAK1*, as well as a series of phosphorylation events. *BZR1* and *BES1* are two major transcription factors regulated by BIN2 and mediate BR-regulated gene expression.

**Conclusion**
To the best of our knowledge, this report is the first to perform a transcriptome analysis of ovule development in *J. curcas*. A set of ovule development-related genes has been identified in female flower buds through expression profiling analysis, which provided comprehensive information on the ovule development of *J. curcas*, revealing that five TF families (*bHLH*, *WRKY*, *MYB*, *NAC* and *ERF*), five hormones signaling (auxin, GA, CK, BR and JA), five MADS-box genes (*AG-1*, *AG-2*, *AGL1*, *AGL11* and *AGL14*), *SUP* and *SLK3* participate in ovule development. GA and JA are involved in ovule development, as confirmed by their accumulation in flower buds during ovule development. Additionally, *CRC* and *SUP* are demonstrated to be involved in ovule development by qRT-PCR analysis. Transcriptome analysis is only an initial step in the exploration of the molecular mechanism of ovule development in *J. curcas*, but the ovule development-related genes identified in this study will establish a foundation for investigations into the molecular mechanisms of ovule development in *J. curcas*, and further analyses of these genes are needed to elucidate their roles in ovule development in *J. curcas*.

**Materials and Methods**

**Flower bud samples collected.** The flower bud samples of different development stages were collected from thirty *J. curcas* trees in May 2015 in Zhenfeng, southwestern China (36°14′50.2″N, 87°51′47.8″E). Each flower bud sample was separated into two portions. The portion used for the anatomic structure analysis of the ovary was fixed immediately in formaldehyde-acetic acid-50% alcohol mixtures (4: 6: 90, v/v), and the other portion used for transcriptome analysis was dipped immediately in RNAlocker (Tianz, Inc., Beijing China) on ice. The flower bud samples used for the phytohormone quantification were stored in liquid nitrogen.

**Analysis of paraffin sections of ovaries.** The flower bud samples used for the anatomic structure analysis of ovaries were fixed in formaldehyde-acetic acid-50% alcohol mixtures (4: 6: 90, v/v) for 24–48 h and then stored in 70% ethanol. The ovaries cut from fixed flower samples were dehydrated by gradient alcohol and then embedded in paraffin. Serial sections (8–10 μm thick) were cut using a Leica CM1800 rotary microtome and stained in Safranin-Fast Green. The stained paraffin sections were analyzed under an Olympus – CX41 light microscope. The anatomic structure analysis of another development will be discussed in detail in further research.

**Grouping on the flower bud samples and total RNA extraction.** The flower bud samples stored in RNAlocker were then divided into eight development phases according to the morphology of flower buds at different development phases using an anatomical lens as follows: JCMI, the formation of ten stamen primordia; JCMII, from the occurrence of sporogenous cells to the formation of microspore mother cell; JCMIII, from the meiosis of microspore mother cell to the formation of meiotic tetrad; JCMIV, from free microspore stage to the maturation of pollen grain; JCFI, the growth of three carpels; JCFII, from the occurrence of sporogenous cells (SC) to the occurrence of macrospore mother cell (MMC); JCFIII, from the meiosis of MMC to the formation of mononuclear embryo sac (MES); JCFIV, from the formation of MES to the maturation of embryo sac (ES)(Figs 1 and 2).

For total RNA extraction, the flower buds from those eight development stages (each phase has three replicates) were ground in liquid nitrogen, and total RNA was isolated using the E.A.N. A Plant RNA Kit (Omega, USA) according to the manufacturer’s manual. The obtained RNA samples were then used for library construction.

**Library construction and Illumina sequencing.** The RNA of flower buds from those eight flower development stages was applied for library construction. A total amount of 3 μg RNA per sample was used for the RNA sample preparations. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries were generated using the
μm, 2.0

extracted with 0.1 mL methanol. Quantification of phytohormones was performed as described previously using
the resulting subnatant was collected for drying by liquid nitrogen. Phytohormones in the dried residue were
added. After incubation at 4 °C for 30 min, the sample was then centrifuged for 15 min at 13,000 g and 4 °C, and

Ortholog database) (E-value

using AMV RNA PCR Kit 3.0 (Takara). qPCR was performed on an ABI StepOnePlus Real-Time PCR System

pathways118.

analysis, and KOBAS software was used to test the statistical enrichment of differential expression genes in KEGG

P

Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted
was performed using the DESeq R package (1.10.1). The resulting P values were adjusted using Benjamini and

Expression profile of genes involved in ovule development. Total RNA samples from flower buds at
different development phases (JCFI, JCFII, JCFIII and JCFIV) (Fig. 2) were used for expression pattern analysis
of 16 selected DEGs (Table S5). The total RNA (1 μg) of each sample was used for first-strand cDNA synthesis
using AMV RNA PCR Kit 3.0 (Takara). qPCR was performed on an ABI StepOnePlus Real-Time PCR System
(Applied Biosystems, Inc. USA) using 2 × SYBR green PCR mix (QIAGEN, Shanghai, China). The β-tubulin
gene was chosen as the endogenous reference gene for the qPCR analysis. The primers used for qPCR analysis
are listed in Supplementary Table S1. Each sample has three biological replicates. The average threshold cycle (Ct)
was calculated, and the relative expression level of each gene was then calculated according to the 2−ΔΔCt method.
A one-way ANOVA was performed on the gene expression level of the samples at different development stages
using the software Statistical Package for the Social Science (SPSS) version 11.5 (SPSS Inc., Chicago, IL, USA).
The individual treatment means were compared using the LSD (least significance difference) test.

Quantification of phytohormone. The female flower bud samples at four development phases, JCFI, JCFII, JCFIII and JCFIV, were used for quantification of phytohormones. A 0.1-g sample frozen in liquid nitrogen
was ground in liquid nitrogen and then dipped in 1 mL mixture composed of cold acetone: deionized water:
hydrochloric acid (37%) = 2:1:0.002 (V:V:V). After incubation at 4 °C for 30 min, the sample was then centrifuged for 15 min at 13,000 g and 4 °C, and

hydrochloric acid (37%) = 2:1:0.002 (V:V:V). After incubation at 4 °C for 30 min, 1 mL dichloromethane was
added. After incubation at 4 °C for 30 min, the sample was then centrifuged for 15 min at 13,000 g and 4 °C, and

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Author contributions
Gang Xu designed and managed this study and wrote this manuscript. Shi-kang Lei performed the real-time q-PCR analysis. Jian Huang participated in the design and management of this study. Xue-guang Sun participated in the design and management of this study. Xue Li collected the flower bud samples and performed the transcriptome data analysis.

Competing interests
The authors declare no competing interests.

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