1 **LARGE-SCALE BIOLOGY ARTICLE**
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3 **Zygotic Genome Activation Occurs Shortly After Fertilization in**
4 **Maize**
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17 **Short title:** Zygotic gene activation in maize
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19 **One-sentence summary:** Transcription profiles generated from maize gametes and zygotes
20 at different stages reveal a highly dynamic zygotic genome activation pattern, providing
21 insights into early embryo development.
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28 **ABSTRACT**

29 The formation of a zygote via the fusion of an egg and sperm cell and its subsequent
30 asymmetric division (ACD) herald the start of the plant's life cycle. Zygotic genome activation
31 (ZGA) is thought to occur gradually, with the initial steps of zygote and embryo development
32 being primarily maternally controlled, and subsequent steps being governed by the zygotic
33 genome. Here, using maize (*Zea mays*) as a model plant system, we determined the timing
34 of zygote development and generated RNA-Seq transcriptome profiles of gametes, zygotes,
35 and apical and basal daughter cells. ZGA occurs shortly after fertilization and involves about
36 10% of the genome being activated in a highly dynamic pattern. In particular, genes
37 encoding transcriptional regulators of various families are activated shortly after fertilization.
38 Further analyses suggested that chromatin assembly is strongly modified after fertilization,
39 that the egg cell is primed to activate the translational machinery, and that hormones likely
40 play a minor role in the initial steps of early embryo development in maize. Our findings
41 provide important insights into gamete and zygote activity in plants, and our RNA-Seq
42 transcriptome profiles represent a comprehensive, unique RNA-Seq dataset that can be
43 used by the research community.
44

INTRODUCTION

46 The life cycles of animals and plants begin with the formation of a zygote, containing

47 the cytoplasm from two gametes, a large egg cell and a small sperm cell. This single

48 cell then develops via embryogenesis into an entire organism consisting of hundreds

49 of different cell types. In contrast to most animal systems, the flowering plant zygote

divides asymmetrically into daughter cells of completely different cell fates. While the small, cytoplasm-rich apical daughter cell further develops into the embryo proper, the highly vacuolated basal cell gives rise to the suspensor, which delivers nutrients to the embryo and positions the embryo proper in the surrounding endosperm tissue of the developing seed. Little is known about the establishment of zygote polarity and the gene regulatory network that leads to asymmetric cell division and cell fate determination in both daughter cells (Zhao et al., 2017). Due to the unequal distribution of their cytoplasm, it is generally thought that maternal factors contributed by the egg regulate zygote and early embryo development. The maternal-to-zygotic transition (MZT) depends on both zygotic genome activation (ZGA) and the degradation of maternal components. In animals, ZGA occurs after the first cell cycle in mammals and as late as the sixth to eighth round of the cell cycle in insects, fish, and amphibians (Schier, 2007; Lee et al., 2014). The time point at which ZGA occurs in plants has long been debated. Currently, the zygote is thought to be in a relatively quiescent transcriptional state, and ZGA is thought to occur gradually rather than as an all-or-none process initiated before the first division (Baroux and Grossniklaus, 2015; Zhao and Sun, 2015). However, analyses of a few candidate genes have indicated that ZGA in flowering plants occurs before zygotic division (Zhao et al., 2017).

To characterize the onset of ZGA at the whole-genome level, it is necessary to determine the transcriptome profiles of both gametes and to identify *de novo* generated transcripts from the zygotic genome. In the current study, we established methods to manually isolate living male and female gametes, zygotes at different stages, and their daughter cells using maize (*Zea mays*) as a model flowering plant. We then generated RNA-Seq data from these cells and investigated the transcriptome dynamics after fertilization. We compared the transcriptomes of maize and *Oryza sativa* (rice) gametes and explored how the cell cycle, chromatin, and auxin pathways are regulated after fertilization. Finally, we identified transcription factor (TF) and receptor-like kinase genes associated with the various cell types and zygotic stages and found that ZGA in maize occurs shortly after fertilization, displaying a highly dynamic pattern.

RESULTS AND DISCUSSION

Manual cell isolation, RNA-Seq, validation, and data quality

Maize plants were grown in indoor walk-in growth rooms to reduce seasonal variability in zygote development. We manually isolated cells of the inbred line B73 at 2-h intervals over a 2.5-day period as described in Methods. We used DAPI staining as a gross indicator to investigate cell cycle stages. As shown in **Figure 1A,** sperm cells consist mainly of the nucleus, containing highly condensed chromatin. Egg cell chromatin appeared less condensed than sperm cell chromatin and stained very weakly with DAPI. The strongest DAPI staining was detected in zygotes at 24 hours after pollination (HAP), indicating that S-phase was complete. Early anaphase occurred at ~30 HAP, and telophase began at 35 HAP. Cytokinesis was visible at 43 HAP, and asymmetric cell division (ACD) was completed by 48 HAP. The small cytoplasm-rich apical and vacuolated basal cells could be manually separated at ~52 HAP.

The protocol used to generate RNA-Seq data from only a few plant cells is described in Methods. Three biological replicates were prepared from approximately 1,000 sperm cells each and 13–20 cells each for the other stages (**Table 1**, see also **Supplemental Table 1** and **Supplemental Figures 1 and 2** for samples, sequencing details, and library quality). A gene expression master list, containing the median expression values of each cell type for the 39,469 annotated maize genes, protein annotations, and homologs from *Arabidopsis thaliana* and rice is provided in **Supplemental Data Set 1**.

For validation and initial analysis of the dynamics of gene expression patterns obtained from the RNA-Seq data, we focused on the transcript levels of 12 genes that were previously shown (by single-cell RT-PCR) to be highly or differentially expressed during fertilization and zygote development in maize (Engel et al., 2003). In agreement with previous results, the gamete-expressed membrane protein genes *ZmGEX1* (SP vs. all: $\log_2$FC >4.0*) and *ZmGEX2* (SP vs. all: $\log_2$FC >7.4*) were highly and specifically expressed in sperm cells, as were *Zmsp271* (SP vs. all: $\log_2$FC >3.8*) and *Zmsp041* (SP vs. all: $\log_2$FC >5.2*), which were identified in the same screen (**Figure 1I**, **Supplemental Data Sets 1–3**). *ZmEA1* (EC vs. SP: $\log_2$FC =8.7*) and *ZmES1-4* (EC vs. AC/BC: $\log_2$FC >2.9 to 9.7*, Zy24 vs. AC/BC: $\log_2$FC >2.4 to 8.7*), encoding secreted peptides required for micropylar pollen tube

guidance and pollen tube burst, respectively, were highly expressed in egg cells and synergids and were significantly downregulated after fertilization (Cordts et al., 2001; Marton et al., 2005; Amien et al., 2010) (**Supplemental Data Set 3**). The cell cycle genes *ZmMCM3, ZmMCM6, ZmCycB1;2*, and *ZmCycB2;1* were previously shown to be induced after fertilization (Sauter et al., 1998; Dresselhaus et al., 1999b; Dresselhaus et al., 2006). Expression of *ZmMCM3* (Zy12 vs. EC: $\log_2$FC =2.7*, AC vs. Zy24: $\log_2$FC =1.8*) and *ZmMCM6* (Zy12 vs. EC: $\log_2$FC =2.0*, AC vs. Zy24: $\log_2$FC =2.7*)*, marking the onset of DNA replication during S-phase (Maiorano et al., 2006), peaked in the zygote at 12 HAP, as well as after the first asymmetric zygote division in the apical cell, which divides more rapidly than the basal cell. The cell cycle regulatory genes *ZmCycB2;1* (Zy24 vs. Zy12 $\log_2$FC =3.6*) and *ZmCycB1;2* (Zy24 vs. Zy12 $\log_2$FC =5.0*), which mark the G2/M-transition (Maiorano et al., 2006), were strongly induced at 24 HAP. In contrast to *ZmCycB1;2* (AC/BC vs. Zy12 $\log_2$FC >1.9*), the expression levels of *ZmCycB2;1* (AC/BC vs. Zy12 $\log_2$FC >5.5*) were also high in apical and basal cells after zygote division (Sauter et al., 1998). In summary, these dynamic changes in gene expression (**Figure 1B**) are in perfect agreement with previous reports, which together with strong correlation between biological replicates (**Supplemental Figure 2**) assures the high quality and reliability of our data.

Contamination of transcriptomes by RNA from maternal tissues has recently been discussed as a serious issue that can result in poor reproducibility and misinterpretation of data sets (Schon and Nodine, 2017). We therefore investigated the presence of transcripts derived from genes expressed in maternal nucellus tissue surrounding embryo sacs (Chettoor et al., 2014) to evaluate the possibility of contamination. None of the nucellus-expressed genes, including GRMZM2G570791 (alpha subunit of DNA-directed RNA polymerase), GRMZM2G125823 (heparanase-like protein), GRMZM2G099420 (Cinnamoyl CoA reductase), and GRMZM5G803276 and GRMZM2G336859 (encoding unknown proteins), were detected in any of our data sets. These results indicate that our data sets are free of maternal RNA contamination and that the two washing steps were sufficient for removing maternal RNA from the burst maternal nucellus cells.

Comparison of transcriptomic data from maize and rice gametes

149   A comprehensive comparison of gene expression activity after fertilization has not
150   been reported yet for any plant species, and the present study thus represents the
151   first report of global gene expression patterns in gametes, zygotes, and daughter
152   cells. Therefore, we restricted our comparisons to the transcriptomes of maize and
153   rice gametes (egg and sperm cells). It was not possible to include the transcriptomes
154   of *Arabidopsis thaliana* gametes in the comparison, as RNA-Seq data were not
155   available, and the available microarray data (Borges et al., 2008; Wuest et al., 2010)
156   could not be accurately normalized to allow us to draw conclusions and lacked
157   information for thousands of genes. In addition, each gamete in the data set was
158   measured in a different experiment.

159   We used published RNA-Seq data from rice sperm and egg cells (Anderson et
160   al., 2013) and initially identified the rice homologs using public databases, i.e.,
161   EnsemblPlants and RiceAnnotationGenomeProject, which combine data from many
162   species to identify putative orthologs. If the identity of the homologs/orthologs was
163   unclear or unknown due to a lack of sequence information, we did not include them
164   in the comparison. To compare transcription patterns in rice versus maize gametes,
165   the gene expression values were binned into 200 expression level categories using
166   the 99th percentile per species as the highest category (see also **Supplemental Data**
167   **Set 4**). We selected the 80 most strongly expressed genes (TOP80 genes) in maize
168   sperm and egg cells and compared their expression levels with those of the
169   respective genes in rice (**Figure 2**). A summary of the TOP30 genes of all maize cell
170   types, including their annotation, is provided in **Supplemental Data Set 5**.

171   Many of the predicted rice homologs/orthologs displayed similar, strong
172   expression patterns. The proportion of genes with high expression levels was greater
173   in egg cells than in sperm cells (**Figure 2**). The observation that many predicted rice
174   homologs/orthologs displayed weaker expression patterns in rice than in maize
175   might be due to our methods (as we summarized rice ortholog data using the
176   median), the difficulty in predicting true orthologs within larger gene families, and/or
177   the lack of common controls to normalize these two studies. Tightly controlled
178   parallel RNA extraction from cells of different species and the identification of an
179   appropriate control cell type common to both plant species may improve interspecies
180   comparisons. However, our comparison pointed to some similarities and general
181   findings regarding genome activity. Among the TOP80 genes expressed in maize
182   sperm cells, 10% encode histones and high-mobility group (HMG) proteins (**Figure**

183   **2A**). This finding might explain the strongly condensed, compact sperm cell

184   chromatin (see also **Figure 1A**). Indeed, a similar observation was reported for rice

185   sperm cells (Russell et al., 2012b; Anderson et al., 2013). Notably, no chromatin

186   gene is among the TOP80 genes in maize egg cells. This finding correlates well with

187   the less condensed chromatin in these cells and the difficulty in staining egg cells

188   with DAPI (**Figure 1A**). However, in strong contrast to sperm cells, 20% of the

189   TOP80 genes in maize egg cells encode proteins of the translational machinery,

190   most of which also displayed similar expression patterns in rice egg cells. These

191   include two genes encoding the translation initiation factor IF5A (Dresselhaus et al.,

192   1999a), a gene encoding the translation initiation factor SUI1 (Cui et al., 1998), two

193   genes encoding the translation elongation factor EF1A (Budkevich et al., 2002), and

194   many ribosomal protein genes. These observations indicate that sperm cells are

195   translationally inactive, whereas egg cells are either highly active or well prepared to

196   strongly enhance translation after being activated during the fertilization process.

197   Another interesting observation relates to transcripts encoding polymorphic

198   EA1-box proteins and small secreted cysteine-rich proteins (CRPs), which we found

199   in maize egg cells, but not in sperm cells. The corresponding proteins play key roles

200   in female gamete cell identity, as well as pollen tube guidance and burst in maize (for

201   review, see (Dresselhaus et al., 2011; Dresselhaus et al., 2016). However, due to

202   their polymorphic nature, no unambiguous homologs of the individual members were

203   identified in the databases from Ensembl Compara or the Rice Genome Annotation

204   Project. Through manual searches, we identified similar genes in rice (Márton et al.,

205   2005; Uebler et al., 2015), but true orthologs could not be predicted based on protein

206   comparisons, and none of the candidate genes have been functionally tested in rice

207   (**Figure 2B**). However, this finding indicates that genes involved in processes directly

208   associated with fertilization appear to be polymorphic and specific-specific, thus

209   representing prime candidate genes involved in speciation mechanisms (Rieseberg

210   and Willis, 2007).

211

212   **Histone variants and chromatin-based gene regulation**

213   Histones are the major protein components of chromatin and, more importantly,

214   dynamic regulators of transcription. To begin to uncover the molecular basis of

215   chromatin remodeling and epigenetic reprogramming in plant gametes and the onset

216   of embryo development, we investigated the expression patterns of histone variants

and chromatin assembly factor genes in more detail (**Figure 3**, **Supplemental Data Set 3G**; important gene families).

As mentioned above, some canonical core histones, including two H3 (7 of 17 genes: SP vs. all: $\log_2$FC>1.5*) genes and at least three H4 (9 of 15 genes: SP vs. all: $\log_2$FC>1.4*) genes, were predominantly and highly expressed in sperm cells (**Figure 3B** and **C**), and may contribute to the compactness of the chromatin and suggesting that sperm cells are already prepared for S-phase. Moreover, the most highly expressed gene in sperm, *ZmHmgd1* (SP vs. all: $\log_2$FC>3.9*)*,* was expressed at much higher levels in sperm cells than in any other cell type examined (>~6-fold) (**Figure 3F**). *ZmHmgd1* encodes an HMG box protein that plays a role in chromosome condensation (Thomas and Travers, 2001) and is thought to possess a role similar to that of histone H1 in chromatin assembly, as both proteins bend linker DNA at the entry and exit points of the nucleosome. This hypothesis may explain why linker histone H1 genes are expressed at rather low levels in sperm cells, although their DNA is densely packed to ensure chromatin stability during sperm delivery. After fertilization, Hmgd1 appeared to be partially replaced by histone H1, as the expression of the H1 gene was strongly activated and peaked in 24 HAP zygotes (9 of 11 genes: Zy24 vs. EC: log2FC>1.8*) (**Figure 3A**), whereas *ZmHmgd1* (Zy24 vs. SP: $\log_2$FC= -5.0*) was strongly repressed.

The most abundant H3 gene in maize sperm (GRMZM2G145758, SP vs. all: $\log_2$FC>4.0*) encodes an unusual replication-independent (RI) H3.3-like variant that was also predominantly expressed in anthers and was previously designated as *ZmAPH3*. Phylogenetic analysis showed that *ZmAPH3* is most similar to the Arabidopsis male gamete-specific histone H3 variant gene *AtMGH3* (Okada et al., 2005) (**Supplemental Figure 3A**). However, the horizontal distance and the low number of histone variants and species included in the phylogenetic analysis suggest that they share a rather distant relationship. All other sperm cell-enriched H3 variants belong to the RI H3.3 group (**Supplemental Figure 3A**) (four orthologous genes, RI H3.3: SP vs. Zy24: log2FC>1.6*). In Arabidopsis, a limited subset of H3.3 variants referred to as the main HTR (HISTONE THREE-RELATED) protein genes are predominately expressed in the male gamete (Ingouff et al., 2010). Three *AtMGH3* homologs and two other RI H3.3 variants are also highly expressed in rice sperm cells, while other core H3 genes exhibit only limited expression in these cells

(Russell et al., 2012a). These findings suggest that RI H3.3 variants encoded by only a few genes act as major histone H3s in the sperm cells of flowering plants.

Two replication-coupled canonical H2As (GRMZM2G151826 and GRMZM2G041381) exhibited high transcript levels in maize sperm cells, but the most highly expressed H2A (GRMZM2G149775, SP vs. all, $log_2FC>2.7$) belongs to the RI H2A.Z class (**Supplemental Figure 3B**), which is associated with nucleosomes at transcription start sites, especially those also containing H3.3 (Deal and Henikoff, 2011). Therefore, together with histone H3.3 and H2A.Z, which mark active chromatin via RI nucleosome assembly (Ahmad and Henikoff, 2002; Deal and Henikoff, 2011), HMGD1 (encoded by *ZmHmgd1*), which likely represents the most abundant chromatin architectural protein in sperm that binds to highly accessible regulatory chromatin and active promoters, probably keeps the highly condensed sperm cell chromatin at least partially accessible. This hypothesis is supported by our RNA-Seq data set, which includes transcripts of approximately 11,000 differentially upregulated genes in sperm cells versus zygotes at 12 HAP (**Figure 4F**), including a subset of TF genes, despite the compact chromatin in sperm cells.

Upon fertilization, H3 variants from male and female gametes are actively removed in Arabidopsis (Ingouff et al., 2010), a scenario that likely also occurs in maize, as the expression of the canonical core histone repertoire was activated in conjunction with a dramatic reduction in *H2A.Z*, *H3.3*, and *Hmgd1* expression and a strong increase in *H1* expression (**Figure 3A**). Thus, it appears that the paternal chromatin was reprogrammed by newly synthesized and entirely different sets of histones in the zygote. This theme may even extend to apical and basal cells, since the list of expressed orthologs of H3 and H4 again shifted to genes different from those expressed in zygotes.

Finally, while the canonical core histones were mostly expressed at very low levels in egg cells (**Figure 3**), they were induced in zygotes at 12 HAP, indicating the G1 phase of the cell cycle. Expression peaked in zygotes at 24 HAP, suggesting that DNA replication was almost complete (**Figure 3**). These observations support the hypothesis that the egg cell is arrested and requires activation after gamete fusion. Thus, chromatin-based transcriptional reprogramming in the zygote may represent a key step in MZT and the initiation of the sporophytic generation.

ZGA occurs shortly after fertilization in maize

284   To identify the global onset of ZGA, it is important to examine the transcriptomes of

285   both gametes, i.e., egg and sperm cells, as well as zygotes at different stages. We

286   therefore investigated zygotes collected at 12 and 24 HAP. At 12 HAP, i.e., only ~4 h

287   after fertilization, all egg cells were fertilized, as indicated by the presence of

288   degenerated receptive synergid cells. These egg cells were considered to represent

289   early zygotes. We chose to investigate zygotes at 24 HAP, as they appeared to be

290   activated at this point, as indicated by the duplication of DNA content (**Figure 1A**). At

291   later time points after pollination, the zygotes underwent mitosis, but the stages

292   appeared less synchronous than those at earlier time points. We chose genes with

293   an abs(log$_2$FoldChange) >1 and adjusted p-value<0.05 in the respective comparison

294   as genes with a potential biological function and determined whether they were

295   induced or repressed. Based on these criteria, sperm cells formed the most distinct

296   group, expressing 4,090 differentially upregulated genes in all comparisons (**Figure**

297   **4F**). As all of the other cell types are similar, fewer genes were induced in these cells

298   compared to all other cell types; 482 differentially upregulated genes were detected

299   in egg cells in all comparisons, 109 were detected in zygotes at 12 HAP, 31 were

300   detected in zygotes at 24 HAP, and 6 and 8 were detected in apical and basal cells,

301   respectively (**Figure 4F**, **Supplemental Data Set 6)**.

302   We compared zygotes at 12 HAP to both sperm and egg cells and identified

303   3,605 induced genes (9.1% of the maize transcriptome of 39,469 annotated genes;

304   Schnable et al., 2009) over all chromosomes shortly after fertilization (**Figure 4B**).

305   This high number of activated genes indicates that global ZGA in maize already

306   occurs in the early zygote and not several days after fertilization, as previously

307   reported (Grimanelli et al., 2005). Although *de novo* transcription has also been

308   observed in Arabidopsis zygotes (e.g., Autran et al., 2011; Nodine and Bartel, 2012;

309   Del Toro-De León et al., 2014), the timing of global ZGA in this species is unclear,

310   and some reported results are currently under debate due to contamination from

311   maternal tissues (Schon and Nodine, 2017).

312   We compared zygotes at 12 HAP with sperm and egg cells and identified

313   7,356 and 1,933 induced genes, respectively (**Figure 4B**). Furthermore, we identified

314   1,730 differentially upregulated genes in sperm and egg cells versus zygotes at 12

315   HAP (**Figure 4A**), which can also be viewed as genes downregulated in zygotes at

316   12 HAP compared to gametes. These findings also suggest that major

317   rearrangements in the transcriptome occur following fertilization.

318   To obtain a global overview of transcriptome dynamics during zygote
319   development, we defined various gene expression profiles as capturing not only on
320   and off states, but also the induction or repression of genes during the transition from
321   gametes to zygotes and their descendant cells (**Figure 4E**, **Supplemental Data Set**
322   **6**). Very specific gene expression profiles were found for egg cells: 326 genes were
323   upregulated in this cell type, whereas 95 genes were downregulated. Shortly after
324   fertilization, 356 and 1,510 genes were transiently induced only in zygotes at 12 HAP
325   and in zygotes at both stages, respectively. Approximately 10% of the genes in each
326   group encode transcriptional regulators. Of the 3,808 genes induced after
327   fertilization, 223 were predominantly expressed in apical cells, whereas 182 were
328   predominantly expressed in basal cells. These results indicate that the expression
329   levels of many genes are higher in the zygote than in its progenitor cells, supporting
330   the notion of an early onset for ZGA. Few genes were transiently repressed after
331   fertilization (137 genes). Together, these findings reveal a highly dynamic
332   transcriptional landscape after fertilization in maize and demonstrate that ZGA
333   occurs shortly after fertilization in this plant. Studies involving pollination with other
334   inbred lines are now needed to distinguish between maternal and paternal
335   transcripts and thus to determine whether both genomes contribute equally to ZGA
336   in this species.
337

338   **Transcription factor activation schemes in gametes and zygotes**
339   As TFs are major regulators of gene expression, we next examined TF gene
340   expression levels in gametes and early zygotic embryos based on the maize TF
341   database Grassius (www.grassius.org). Across all cell types, 1,478 of 2,630 maize
342   TF genes were expressed in gametes and zygotes ($\log_2$FC>1 and padj<0.05 in at
343   least one comparison). Comparing their transcription levels during early development
344   (**Figure 5A**, **Supplemental Data Set 3**) showed that zygotes formed a group distinct
345   from apical and basal cells, and that both groups differed from gametes. We
346   identified 428 TF genes that were induced in zygotes (12 and 24 HAP), 189 of which
347   were strongly activated shortly after fertilization (Zy12 vs. SP and EC, $\log_2$FC>3). We
348   detected 25 TF genes specifically expressed in pro-embryonic cells. Only 23
349   paternal and 103 maternal TF genes were expressed at similar levels in gametes
350   and zygotes (**Supplemental Data Set 3**). While we could not distinguish between
351   paternal and maternal mRNAs in our assays and were thus unable to identify the

maternal-to-zygotic transition (MZT) (Baroux and Grossniklaus, 2015), the results suggest that approximately 8.6% (25+103/1,478) of expressed TF genes are parentally transmitted, while 29% (428/1,478) are newly or more intensely transcribed in maize zygotes. These data support the notion that TF genes are activated early in maize zygotes (at 12 HAP; 403 of 428 TF genes were induced in zygotes vs. 25 of 428 at 24 HAP) (**Figure 5A**).

We then identified important TF classes from the various expression profiles (**Supplemental Data Set 6**; profiles of TF classes are summarized in **Supplemental Data Set 7**). In sperm cells, many genes belong to the TF class AT-rich interactive domain (ARID 5/10) proteins. Members of this class have been implicated in sperm cell development in plants and mammals. In Arabidopsis, ARID1 is necessary for the appropriate expression of DUO1, a major TF required for sperm cell formation (Zheng et al., 2014). In mice, the loss of ARID4A combined with ARID4B haploinsufficiency leads to spermatogenic arrest (Wu et al., 2013). In egg cells, we detected high proportions of TFs from the classes FAR1-like (3/15), mTERF (8/30), Sigma70-like (2/9), S1Fa-like (2/2), and GeBP (5/21) compared to the other cell types analyzed. The first four classes are related to plastid development (Zhou et al., 1995; Ouyang et al., 2011; Kleine, 2012; Wei et al., 2012). This finding suggests that a tightly controlled regulatory network controls plastid development during the first steps in the plant life cycle. Members of the GL1 enhancer binding protein (GeBP) class of leucine-zipper TFs have been linked to the cytokinin response in Arabidopsis and are thought to be mainly expressed in vegetative meristems and in the primordia of young leaves (Chevalier et al., 2008). Our data suggest that this class of TFs also plays a role in the transition from egg cells to early zygotes.

As discussed above, at the first zygotic time point (12 HAP), genes for many TF classes were induced (**Supplemental Data Set 7**). The auxin-responsive ARF TFs (7/38) and the ethylene-responsive AP2-EREBP TFs (34/212) might be important at this stage, as crosstalk between these pathways is thought to be essential during zygote and pro-embryo development (see below). In addition, many genes from the TF classes C3H/CCCH (18/54), Trihelix (11/41), ZIM (10/36), MADS (15/77), NAC (26/134), bZIP (24/128), and Homeobox (26/133) were induced at this time point. MADS-box TFs are associated with reproductive organ development and play a role in gametes and in zygotic embryogenesis (Schreiber et al., 2004; Lehti-Shiu et al., 2005).

Activation of embryo patterning

After ACD, *WOX* genes encoding homeodomain TFs mark apical and basal cell fate upon zygote division in Arabidopsis (Breuninger et al., 2008). In maize, *ZmWOX9A* and *ZmWOX9B* likely represent the homologs of *AtWOX8* and *AtWOX9*, respectively (Salvo et al., 2014). Both *ZmWOX9A* and *ZmWOX9B* were induced shortly after fertilization (Zy12 vs. SP/EC:log2FC>5.9* and 7.0*, respectively) and were expressed at higher levels in basal cells than in apical cell, like their counterparts in Arabidopsis (**Figure 5B**, **Supplemental Data Set 3B**), indicating that they might play a similar role in early embryonic patterning. However, unlike *AtWOX2*, which marks apical descendants of the zygote, *ZmWOX2A* was expressed at very low levels in basal cells, with almost no expression in apical cells. Instead, this gene was expressed much later during seed development in the endosperm (Maize eFP Browser at bar.utoronto.ca). This finding indicates that pattern formation regulated by *ZmWOX2A* and other genes likely occurs later in maize embryogenesis than in Arabidopsis embryogenesis (Zhao et al., 2017). Notably, *ZmWOX13A* is the only *WOX* gene that was transcribed at high levels in sperm cells (SP vs. all: $\log_2$FC>4.3*); whether it marks cell identity of the male gamete or represents a zygote activator remains to be investigated. In Arabidopsis, *BBM* and *LEC* TF genes are key players in embryogenesis, and the presence of either gene is sufficient to induce competence for embryogenesis (Lotan et al., 1998; Boutilier et al., 2002). Overexpressing a combination of the maize homologs *WUS2* and *BBM* was recently shown to significantly increase embryogenic potential in tissue culture and thus represents a key mechanism to increase the transformation efficiency in maize (Lowe et al., 2016). Notably, the maize homologs *ZmBBML1* (Zy12 vs. SP/EC: $\log_2$FC>10.5*), *ZmBBML2* (Zy12 vs. SP/EC: $\log_2$FC>10.2*), and *ZmLEC1* (Zy12 vs. SP/EC: $\log_2$FC>7.9*) were already induced at 12 HAP (**Figure 5B**), suggesting that the egg cell quickly acquires embryogenic competence and that the characteristic embryogenic transcription program is activated shortly after fertilization. *ZmBBML3* was induced in zygotes at 24 HAP (Zy24 vs. Zy12: $\log_2$FC=6.5*) and after zygote division (AP/BC vs. Zy24: $\log_2$FC=3.5/3.9*), suggesting step-by-step activation of the embryonic program. A comparison of apical and basal cells with zygotes at 24 HAP revealed 2,228 genes induced in both cell types, 832 induced only in apical cells, and 485 induced only in basal cells (**Figure 4D**). This induction was accompanied by

420 the downregulation of 2,182 genes in apical and basal cells versus zygotes at 24

421 HAP (**Figure 4C**). These data suggest that global rearrangements also occur in the

422 transcriptomes of apical and basal cells compared to their predecessor.

423   The development of multicellular organisms often involves ACDs to generate

424 daughter cells with different cell fates. Spindle positioning is particularly associated

425 with the generation of symmetric or asymmetric cell fates (Siller and Doe, 2009). The

426 MATH-BTB domain protein ZmMAB1, a component of a CUL3-E3 ubiquitin ligase

427 complex, regulates spindle length during the development of the male and female

428 germline in maize (Juranic et al., 2012). ZmMAB1 may also function like its animal

429 homolog, the key ACD regulator MEL-26, a factor required for embryogenic

430 morphogenesis that regulates the formation of mitotic spindles in the early embryo

431 (Pintard et al., 2003). As shown in **Figure 5C,** MAB family genes were strongly

432 upregulated (*ZmMAB1-3*, *ZmMAB6* and *ZmMAB24,* Zy12 vs. SP: log$_2$FC >1.5 to

433 10.7*) after fertilization in maize. The highest expression levels of these genes were

434 detected in zygotes at 24 HAP (**Supplemental Data Set 3A**; important gene

435 families), suggesting that they play roles in processes such as spindle positioning

436 during the first asymmetric zygote division (ZmMAB2-3, Zy24 vs. Zy12 log$_2$FC=3.1*

437 and 1.8* respectively). Functional studies of *ZmMABs* are now needed to investigate

438 this hypothesis.

439

440 **Cell cycle regulation during zygote development**

441 Since previous reports provide only a glimpse of cell cycle regulation in plant

442 gametes and during zygote development, we investigated the expression patterns of

443 important cell cycle regulator genes. First, we searched for orthologs of Arabidopsis

444 cell cycle genes (Vandepoele et al., 2002) and then included cyclins and other cell

445 cycle-related factors described previously for maize (see Methods), resulting in a list

446 of 89 cell cycle genes (**Supplemental Data Set 3**). As shown in **Figure 6A***,

447 hierarchical clustering of these genes from different cell types clearly separated

448 sperm cell genes from a group of genes from egg cells and zygotes at 12 HAP. The

449 expression patterns of the genes from zygotes at 24 HAP were more similar to those

450 of apical and basal cells.

451   The G1-phase of sperm cells is characterized by high expression levels of

452 *Cdc27*-like genes (GRMZM2G148626, GRMZM2G005536, SP vs. all: log$_2$FC>5.7*)

453 encoding subunits of the APC complex, which controls CDK degradation in M- and

454 G1-phase, and relatively low levels of CDK gene expression. Furthermore,
455 retinoblastoma-related protein genes *RBR1* and *RBR2* were highly expressed in
456 sperm cells (**Figure 6A,** SP vs. all: $\log_2FC>2.9^*$). RBR1 and RBR2 mediate G1-
457 phase arrest by inhibiting E2F TFs, which in turn promote DNA replication (Sabelli et
458 al., 2013). Compared to the other cell types, egg cells showed by far the lowest
459 expression levels of cell cycle genes and lacked a typical cell cycle phase-specific
460 gene expression pattern. This finding, together with the results of cell cycle gene
461 expression analysis and the microscopy evidence reported above (**Figure 1**),
462 indicate that the egg cell is in a resting G0 stage rather than in G1 (**Figure 6B**).
463 Thus, the egg cell must be activated and its cell cycle synchronized with the sperm
464 cell cycle stage before karyogamy (fusion of both nuclei) is executed. A typical G1-
465 phase expression pattern was observed in zygotes at 12 HAP (**Figure 6A**), with
466 slightly upregulated expression of E2F TF genes (GRMZM2G060000,
467 GRMZM2G361659, GRMZM2G378665, Zy12 vs. EC, log2FC $>2.5^*$) and (especially)
468 their cell cycle target genes (**Supplemental Data Set 3**). The latter include mini-
469 chromosome maintenance genes (*ZmMCM3-6,* Zy12 vs. EC: log2FC $>2.0^*$),
470 encoding DNA-replication licensing factors required for replication initiation, and the
471 gene encoding proliferating cell nuclear antigen (PCNA), which acts as a scaffold to
472 recruit proteins involved in DNA replication and repair (Sabelli et al., 2009; Tuteja et
473 al., 2011). Notably, *RBR3*, encoding an activating factor of *MCM2-7* transcription,
474 was specifically induced in zygotes at 12 HAP (Zy12 vs. EC: $\log_2FC =2.2^*$), while
475 *RBR1* and *RBR2*, two genes encoding repressors of E2F TFs, were repressed in
476 these zygotes compared to sperm cells (Zy12 vs. SP: $\log_2FC< -3.8^*$). This
477 expression pattern coincided with the induction of CDK A genes (Zy12 vs. SP:
478 $\log_2FC> 1.1$ to 7.0^*) and low levels of expression of CDK B genes, indicating that
479 fertilized egg cells had been activated and zygotes at 12 HAP were in G1-phase.
480 *MCM2-7* expression levels were lower in zygotes at 24 HAP versus 12 HAP,
481 whereas CDK B2 genes (9 of 14 genes, Zy24 vs. Zy12: $\log_2FC >2.8^*$) and B-type
482 cyclin genes were induced (8 of 9 genes, Zy24 vs. Zy12: $\log_2FC >1.9^*$), especially
483 ZmCycB1;2, suggesting that S-phase was completed and the zygotes were prepared
484 for the G2/M-phase transition (Meijer and Murray, 2001). M-phase took place at 27–
485 35 HAP (**Figure 1A**). Notably, G1-phase markers such as *MCM2-7* were expressed
486 at slightly higher levels in apical versus basal cells, hinting at more rapid cell cycle
487 progression in the apical cell after ACD.

488   Taken together, our cell cycle analysis by microscopy of DNA staining

489   patterns, transcription data from selected gene sets, and global cell expression

490   analysis of cell cycle genes allowed us to determine the timing of zygote

491   development in maize (**Figure 6B**). On average, fertilization occurs at ~8 HAP.

492   Sperm cells appear in G1-phase, and egg cells are in a resting G0-phase of the cell

493   cycle. Activated zygotes are in G1 phase at 12 HAP (~4 h after fertilization) and at

494   G1/S-phase at 24 HAP. Mitosis typically occurs at 26–36 HAP, and cytokinesis lasts

495   until 44 HAP. ACD is completed between 44 and 50 HAP, generating both apical and

496   basal cells in G1-phase.

497

498   **The role of auxin in early embryogenesis in maize**

499   To demonstrate the utility of our data sets, we chose the auxin pathway, which plays

500   a key role in early embryo patterning in Arabidopsis, as an example for analysis.

501   Auxin gradients generated by PIN efflux carrier proteins establish the apical-basal

502   axis upon the first ACD in the Arabidopsis zygote (Friml et al., 2003). The molecular

503   mechanisms that determine axis formation during early embryogenesis in monocots

504   are largely unknown. We therefore analyzed the expression of auxin biosynthesis,

505   transport, and response genes, as shown in **Figure 7** (**Supplemental Data Set 3D**;

506   important gene families). In maize, the earliest ZmPIN1a localization was observed

507   at 6 DAP at the adaxial side of the embryo proper (Chen et al., 2014). To identify

508   potential PIN proteins that function at the earliest stage of embryo patterning, we

509   analyzed the expression of all *PIN* family genes. Among the 15 *PIN* genes in maize

510   (Yue et al., 2015), only *ZmPIN8* was weakly expressed in the zygote and

511   upregulated after ACD in both apical and basal cells (AC/BC vs. Zy12:

512    $\log_2FC=3.8/3.7*$). A number of *ZmABCBs*, representing potential auxin transporter

513   genes (Yue et al., 2015), were also active in maize gametes and/or zygotes. Auxin

514   biosynthesis genes *ZmTAR1* (Zy12 vs. SP/EC: $\log_2FC>1.2*$) and *ZmYUC3* (Zy12 vs.

515   EC: $\log_2FC=9.4*$) were significantly induced after fertilization, with higher expression

516   levels in basal cells versus apical cells (*ZmTAR1* BC vs. AC:$\log_2FC=1.4*$). Auxin

517   receptor genes *ZmABP1*, *ZmABP4*, and *ZmABPL* were also expressed in egg cells

518   (EC vs. SP: $\log_2FC>1.1*$) but not in sperm cells and became transiently activated

519   after fertilization (Zy12 vs. SP: $\log_2FC>1.9*$, AC/BC vs. Zy24: $\log_2FC< -1.4*$). Of the

520   auxin responsive factor (*ARF*) genes examined, *ZmARF7* had the highest

521   expression level in the egg cell and was almost completely switched off after

fertilization (EC vs. SP/Zy12/Zy24: $\log_2$FC>3.1*, AC/BC vs. EC: log2FC< -4.1*). Other *ARF* genes, such as *ZmARF8, 13, 17*, and *28*, were expressed at similar levels in all cells except sperm cells. In general, *ARF* transcript levels were higher in the apical daughter cell of the zygote than in the other daughter cell (**Figure 7B**: AC/BC vs. Zy24). A few AUX/IAA repressor genes encoding proteins that interact with ARF regulators were activated after fertilization (**Figure 7B**: Zy12 vs. SP/EC). In particular, *ZmIAA17* (Zy12 vs. SP/EC $\log_2$FC>4.3) was transiently expressed only shortly after fertilization; ZmIAA17 might be involved in the inactivation of ZmARF17-regulated gene expression patterns. Another highly upregulated gene, *ZmSAUR7* (Zy12 vs. SP/EC: $\log_2$FC> 5.2*, AC/BC vs. Zy12: $\log_2$FC< -2.6*), is one of 79 SAUR (*SMALL AUXIN UP RNAs*) genes in maize, representing the largest family of auxin response genes (Ren and Gray, 2015). Globally, we found that auxin pathway genes were highly induced in the early zygote at 12 HAP and expressed at decreasing levels from 24 HAP zygotes to apical and basal cells (**Figure 7B**). By contrast, in Arabidopsis, these genes continue to show a strong auxin response, especially in the apical cell after ACD (for review, see Petrášek and Friml, 2009). Moreover, transcripts encoding homologs of key players in auxin-regulated early embryo patterning in Arabidopsis, such as ARF5 (MP), IAA12 (BDL), PIN1, and PIN7, were absent in zygotes and their daughter cells in maize.

The observation that gametes and early zygotes are equipped with transcripts encoding proteins for auxin biosynthesis, transport, and perception, as well as the identification of strongly regulated auxin response genes (**Supplemental Data Set 8**), indicates that auxin-regulated early embryo patterning is likely different in maize and other grasses compared to Arabidopsis, providing an entry point for investigating the role of this important hormone during early embryogenesis in grasses. In addition to auxin signaling, we also obtained hints about brassinosteroid and ethylene signaling during early embryo development in maize, which will be investigated in the future.

Cell signaling during fertilization and early embryogenesis

Ca^{2+} signaling is thought to play a pivotal role in fertilization by regulating a plethora of cellular processes (Chen et al., 2015). Annexins are a class of Ca^{2+}-regulated proteins that link Ca^{2+} signaling to membrane and cytoskeleton dynamics (Gerke et al., 2005). Of the 12 genes encoding annexins in maize (Zhang et al., 2015),

ZmAnn6 and *ZmAnn7* exhibited very high, transient expression in egg cells (EC vs. SP: $\log_2 FC=8.5/2.1^*$, EC vs. Zy12: $\log 2FC=1.1/-^*$) and early zygotes (AC/BC vs. Zy12: $\log_2 FC< -4.1/-3.2^*$), respectively (**Figure 8A**, **Supplemental Data Set 3C;** important gene families). Thus, these proteins might function as key players in intra- and intercellular Ca^{2+} signaling during fertilization and early embryo development.

Signal perception and transduction through cell-surface receptor-like kinases (RLKs) likely play also roles in gamete interaction, fertilization, and early seed development in plants. We detected at least three RLK genes that were preferentially expressed in sperm cells (GRMZM2G011806, GRMZM2G016480, GRMZM2G428554, SP vs. EC/Zy12/Zy24: $\log_2 FC>2.3^*$) (**Figure 8B**, **Supplemental Data Set 3E;** important gene families), representing potential players in gamete recognition and/or sperm activation. We did not identify RLK genes that were preferentially expressed in egg cells. In Arabidopsis, *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*AtSERK1*) is expressed in developing ovules and early embryos, and enhances embryonic competence in cell culture (Hecht et al., 2001). While its maize ortholog *ZmSERK1* (Salvo et al., 2014) was expressed at very low levels in gametes and zygotes, *ZmSERK2* and *ZmSERK3* were expressed in zygotes (e.g., *ZmSERK3* Zy12 vs. EC: $\log 2FC=3.7^*$) and daughter cells (**Figure 8C**), indicating the involvement of similar signaling pathways in embryonic initiation. Moreover, several *RLK* genes (GRMZM2G038165, GRMZM2G428554, GRMZM2G089461) were upregulated and differentially expressed in apical and basal cells (AC/BC vs. Zy24: $\log 2FC>1.5^*$), thus representing exciting candidates for future functional studies investigating cellular communication during early embryo development in grasses.

Conclusions

Detailed analysis of global gene expression patterns in plant gametes, zygotes, and manually separated apical and basal cells has allowed the onset of global ZGA in maize to be determined for the first time. The observation that ZGA occurs soon after fertilization, displaying a highly dynamic and partially transient pattern, is surprising and contradicts previous studies using a limited number of genes. These studies indicated that the zygote is in a relatively quiescent transcriptional state, that only a few genes are *de novo* activated in the zygote, and that ZGA occurs gradually rather than all at once (Baroux and Grossniklaus, 2015; Zhao and Sun, 2015). The striking

590 differences in the expression patterns of cell cycle regulators between sperm and
591 egg cells coincide with a distinct chromatin state in sperm cells and define a
592 quiescent cell cycle state in egg cells, although egg cells appear to be translationally
593 highly active or well prepared to quickly activate the translational machinery after
594 fertilization. The chromatin state in sperm appears to depend on replication-
595 independent histone assembly and the HMG protein ZmHmgd1, which likely keeps
596 the highly condensed sperm cell chromatin at least partially accessible, as
597 demonstrated by the numerous transcribed genes. In addition, our data allowed us to
598 differentiate between the stages during G1-phase that occur in zygotes at 12 HAP,
599 apical cells, and basal cells, and they suggest a preference for certain CDKs and
600 cyclins during the first two cell cycles in plants. Analysis of the expression levels of
601 TFs, structural regulators, and signaling pathway genes allowed us to identify
602 relevant genes homologous to key, well-known Arabidopsis regulators as well as
603 novel candidate genes, which will serve as a starting point for many future studies.

604   In summary, our analyses of the genes described above represent only a few
605 examples of how our comprehensive dataset can be used. This gene expression
606 atlas should further accelerate the identification of key players involved in many
607 biological processes, including fertilization, early embryogenesis, and the cell cycle,
608 as well as the translational machinery. In addition, our data set could be used to
609 uncover genes (and their corresponding promoters) for use in future efforts aimed at
610 increasing seed yield and quality in maize and other crops.

611

612

METHODS
613

Plant materials and growth conditions
614

615 Maize (*Zea mays*) inbred line B73 was cultivated in a walk-in plant growth room at
616 26°C under illumination of 24,000 lux using alternating SON-T Agro and HPI-T Plus
617 bulbs with a 16 h light/8 h dark cycle and a relative humidity of 60%. Flowers at
618 anthesis and pollinated cobs were used to isolate cells for RNA-Seq.

619

Isolation of cells from male and female gametophytes
620

621 Hundreds of maize plants were grown to collect sufficient numbers of manually
622 isolated cells. Each biological replicate consisted of pooled cells from different

plants. Only the middle part of the cob was used for cell isolation from excised ovules. A whole cob was used to isolate cells at a defined developmental stage. Sperm cells were released from maize pollen grains (male gametophytes) by osmotic shock and separated using density gradient centrifugation on a discontinuous Percoll gradient. The detailed protocol is given below. Egg cells were isolated from embryo sacs (female gametophytes) of unpollinated ovules as described (Kranz et al., 1991). Early and late developmental stage zygotes were isolated from ovules at 12 hours after pollination (HAP) and 24 HAP, respectively, as previously described (Cordts et al., 2001). Apical and basal cells were dissected from two-celled proembryos isolated from ovules at between 48-52 HAP following the procedure used for zygotes with some modifications (described in detail below). All cells isolated from ovules were individually collected using a microcapillary and washed twice in mannitol solution (480 mOsmol·kg^{-1} H$_2$O). Cells showing cytoplasmic streaming were individually transferred to 0.5 ml Eppendorf RNA/DNA LoBind microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at −80°C for mRNA extraction. Three biological replicates (each representing an independent pool of cells) were carried out, each with ~1,000 sperm cells, 20 egg cells, 14 to 15 zygotes at 12 HAP, 16 to 17 zygotes at 24 HAP, 16 apical cells, and 13 to 14 basal cells (**Table 1**). All three biological replicates of each cell type were used for RNA-Seq and subsequent transcriptome analyses.

Sperm cell isolation

Sperm cells were isolated as described (Dupuis et al., 1987) with some modifications. Pollen grains were collected upon shedding, immersed in 550 mOsmol·kg^{-1} H$_2$O mannitol solution (100 mg pollen/ml solution), and incubated on a platform shaker with slow agitation (80 rpm) for 1 h. The resulting lysate was filtered through a 40 μm cell strainer to remove exines and unruptured pollen grains, resulting in a yellowish filtrate containing sperm cells and starch granules. A Percoll gradient was prepared in a 30 ml COREX tube, consisting of 5 ml 30% (v/v) Percoll in 550 mOsmol·kg^{-1} H$_2$O mannitol solution at the bottom, 6 ml 20% (v/v) Percoll in 550 mOsmol·kg^{-1} H$_2$O mannitol solution in the middle, and 6 ml 15% (v/v) Percoll in 550 mOsmol·kg^{-1} H$_2$O mannitol solution at the top. Sperm-containing filtrate (10 ml) was layered on top of the Percoll gradient and centrifuged in a swing-out rotor at

656 12,000xg for 1 h at 4°C. After centrifugation, distinct white layers were visible in the
657 15/20% Percoll interphase and the 20/30% Percoll interphase. The 20/30%
658 interphase, which was enriched in sperm cells, was carefully aspirated using a
659 Pasteur pipette and transferred to 15 ml or 50 ml Falcon tubes. At least 10 volumes
660 of fresh 550 mOsmol·kg^{-1} H_2O mannitol solution were added to the sperm cell-
661 enriched fraction, and the cells were washed by carefully inverting the tube several
662 times. The sperm cells were pelleted by centrifugation at 2,500xg for 15 minutes at
663 4°C and the supernatant was removed without disturbing the pellet, leaving a volume
664 of approximately 50–100 µl. The pellet was resuspended in the remaining
665 supernatant by careful pipetting, resulting in a solution highly enriched in sperm cells.
666 Cell counting was performed using a Neubauer counting chamber. Isolated sperm
667 cells were used immediately or shock-frozen in liquid nitrogen and stored at -80°C.
668

669 **Isolation of apical and basal cells**
670 To identify the time point of asymmetric cell division (ACD) of the zygote, several
671 cobs were pollinated and analyzed at different intervals after pollination. The first
672 zygotes were analyzed at 24 HAP. Subsequent examinations were performed at 1-h
673 intervals; on average, zygote ACD was observed at ~48 HAP. Apical and basal cells
674 were subsequently separated using cell wall degrading enzyme solution containing
675 1.5% Driselase (Sigma), 1.5% pectinase (Fluka), 0.5% pectolyase Y23 (Karlan),
676 1.0% hemicellulase (Sigma), 1.0% cellulase "Onozuka R10" (Serva), and 1.5%
677 maceroenzyme (Karlan) in mannitol solution (480 mOsmol·kg^{-1} H_2O). The enzyme
678 solution (100 µl) was combined with 1 ml mannitol solution (480 mOsmol·kg^{-1} H_2O),
679 and ovary sections containing embryo sacs at 48 HAP were incubated in the diluted
680 enzyme solution for 30 min at room temperature, followed by manual dissection of
681 two-celled proembryos. The attachment between the apical and basal cell
682 protoplasts was gently touched with a very thin glass needle to separate both cells.
683 The cells were washed twice in mannitol solution (480 mOsmol·kg^{-1} H_2O), collected
684 in 0.5 mL Eppendorf RNA/DNA LoBind microcentrifuge tubes, immediately frozen in
685 liquid nitrogen, and stored at -80°C for mRNA isolation.
686

687 **RNA extraction, cDNA preparation and purification**

688 The mRNA was extracted from cell samples using a Dynabeads mRNA DIRECT
689 Micro Kit (Life Technologies). A SMARTer Ultra Low RNA Kit for Illumina
690 Sequencing (Clontech Laboratories) was used to generate first-strand cDNA.
691 Double-stranded cDNA was amplified by LD PCR (15 cycles) and purified via
692 magnetic bead cleanup using an Agencourt AMPure PCR Purification Kit (Beckman
693 Coulter). The quality of the purified cDNA was measured using an Agilent 2100
694 Bioanalyzer with an Agilent High Sensitivity DNA Kit (Agilent Technologies), frozen in
695 liquid nitrogen, and stored at −80°C.
696

697 **Library construction and Illumina sequencing**
698 Library preparation was carried out as described in the Adapted Nextera Sample
699 Preparation protocol (Clontech Laboratories) for use with the SMARTer Ultra Low
700 RNA Kit for Illumina Sequencing. Input cDNA (5 ng) was tagmented (tagged and
701 fragmented) via the Nextera transposome. The products were purified and amplified
702 via a limited-cycle PCR program to generate multiplexed sequencing libraries. The
703 libraries were quantified using a KAPA SYBR FAST ABI Prism Library Quantification
704 Kit (Kapa Biosystems). Equimolar amounts of each library were pooled and used for
705 cluster generation on the cBot system with Illumina TruSeq PE Cluster v3 and
706 Illumina TruSeq Dual Index Sequencing Primer paired end kits. Sequencing runs
707 were performed on a HiSeq 1000 instrument using the dual indexed 2 x 100 cycles
708 paired end (PE) protocol and TruSeq SBS v3 reagents according to the Illumina
709 HiSeq 1000 System User Guide. Image analysis and base calling resulted in .bcl
710 files, which were converted into .fastq files with CASAVA1.8.2 software. Library
711 preparation and Illumina sequencing runs were performed at the Genomics Core
712 Facility "KFB - Center for Fluorescent Bioanalytics" (www.kfb-regensburg.de). The
713 raw data (.fastq) plus supplemental tables, including count and TPM data for all
714 replicates was uploaded to GEO and is available under accession number
715 GSE98379.
716

717 **Bioinformatic and statistical analyses**
718 *(I) Quality control and alignment:* The quality of sequencing data from the RNA-Seq
719 libraries   was   assessed   with   FASTQC
720 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Primer contamination
721 from the SMARTer Ultra Low RNA Kit was removed with cutadapt (ver.1.13; Martin,

2011). Size and quality trimming were then performed using Trimmomatic (ver. 0.32 (Am et al., 2014), ILLUMINACLIP: NexteraPE-PE_SMARTer.fa:2:30:10:2:true TRAILING:26 LEADING:26 MINLEN:25). The results from trimming are summarized in **Supplemental Table 1** (mapping information). Trimmed reads were aligned to the maize genome (AGPv3, INSEC Assembly GCA_000005005.5, release 23, ftp://ftp.ensemblgenomes.org/pub/plants/). STAR (Dobin et al., 2013) was used to align the mRNA reads. Alignment statistics are summarized in **Supplemental Table 1**. Duplicate reads were identified with picard MarkDuplicates (http://broadinstitute.github.io/picard). The remaining reads were then assessed on the gene level using featureCounts from the Rsubread R-library (Liao et al., 2014) using the annotation information supplied by Gramene, release 5b+, (http://ftp.gramene.org/maizesequence.org/release5b+/zea_mays.protein_coding.gff) . Gene annotation and ortholog information was retrieved from EnsemblPlants (www.plants.ensembl.org) via Biomart (BiomaRt[7], release 29, *Z. mays* genes (AGPv3 (5b)), *A. thaliana* genes (2010-09-TAIR10) and *O. sativa japonica* genes (IRGSP-1.0), restricting the gene model to AGPv3 (5b) from EnsemblPlants.

(II) Measurement of mRNA transcript abundance: Transcripts per million (TPM) was used as a measure of mRNA abundance, which takes into account the length of the RNA transcripts and the sequencing depth (Wagner et al., 2012). Gene length was approximated by determining the sum of the exon lengths of the gene model. The TPMs were calculated for each gene in each sample. The median TPM value was calculated from three biological replicates for each cell type. An annotated list of genes with their expression levels at each developmental stage (cell type) and orthologs (based on Ensembl Compara from *A. thaliana* and *O. sativa*) was assembled (**Supplemental Data Set 1**).

(III) *Differential expression analysis and Venn diagrams:* Count data at the gene level were analyzed with DESeq2 (Love et al., 2014). All 15 cell-type-to-cell-type comparisons were performed and corrected for multiple testing over all genes and cell type comparisons using FDR (Benjamini and Hochberg, 1995) (**Supplemental Data Set 2**). Genes with a log_2FoldChange>1 and FDR <0.05 ("padj" in supplemental tables) were considered significant differentially expressed, and Venn diagrams (R-library: venerable; https://r-forge.r-project.org/projects/venerable) were generated for certain comparisons (**Figure 4**).

Expression profiles and pathway analysis

Genes showing differential expression ($\log_2$FoldChange>1 and FDR <0.05) in at least one comparison were subjected to further analyses. To visualize gene expression values in heatmaps and to compare gene expression profiles during different stages of very early plant development, square root transformed TPM values were utilized. The median expression values were then transformed to standard units to follow the expression values of one gene across different developmental stages in the heatmaps. The dendrogram of the samples is based on Euclidean distances combined with hierarchical clustering with complete linkage. To classify genes into specific expression profiles, Pearson correlation analysis of the gene expression vector (square root transformed median TPM values as above) versus a binary vector encoding the different expression profiles, i.e., expressed only in egg cells and during fertilization (0,1,1,1,0,0)/(SP,EC,ZY12,ZY24,AC,BC) was performed (**Supplemental Data Set 1**). A stringent cutoff value of >0.9 (Pearson correlation coefficient) was used to define a positive correlation with the respective profile.

Comparison of gene expression levels in maize and rice gametes

To compare the expression of the 80 most highly expressed genes from maize sperm and egg cells with their orthologs in rice (*Oryza sativa* ssp. *japonica*), data from Ensembl Compara (http://plants.ensembl.org, based on protein information for 53 species) and the Rice Genome Annotation project (RGAP) (http://rapdb.dna.affrc.go.go.jp/download/archive/ RAP_MSU_2016-08-05.txt.gz) and orthologs from RGAP based on OrthoMCL were used (Li et al., 2003; Ouyang et al., 2007; Vilella et al., 2009). While OrthoMCL is based only on a comparison of protein sequences, Ensembl Compara also uses information from phylogenetic trees and therefore interprets sequence similarities based on the evolutionary development of a gene. Rice gene expression data (TPM) were obtained from Anderson et al. (2013). Maize and rice TPMs were square root transformed, and plastid transcripts were removed. Then, for each organism, the data were binned into 200 equally spaced expression categories, with the 99th percentile representing the maximum expression level. The color scale for the heatmaps shown in **Figure 2** represents the expression bin, indicating the relative expression level in the organism shown. For each maize gene, the respective rice orthologs in both resources were identified and

790 compared. If there were common orthologs predicted by both resources, these were
791 chosen, and if not, all predictions were used. From this selected set of orthologs, the
792 most highly expressed ortholog in rice was used for plotting. All orthologs are listed
793 in **Supplemental Data Set 4**. In the heatmaps (**Figure 2**), the maximum of the color
794 scale represents the 99th percentile, resulting in the same color for all maize genes
795 shown (because all of the top 80 genes are within the most highly expressed 1% of
796 the genes). Dark red bars in the rice column indicate that the gene was also in the
797 top 1% most highly expressed genes in the rice data, and the lighter color indicates
798 lower expression. Cyan lines represent expression levels as bar charts, with the
799 dotted line indicating the median value of the column.
800

801 **Transcription factor gene list**

802 A list of potential TFs in maize based on conserved domains at the protein level was
803 retrieved from Grassius (http://grassius.org/tfomecollection.html,
804 Maize_TFome_Bulk_data.txt). A list of median expression values (TPM>1) of all TF
805 genes, including their gene names and TF families, is shown in **Supplemental Data**
806 **Set 7**. Important TF classes were identified by analyzing the fraction of expressed
807 genes (TPM>1 in percent). Whenever the fraction of expressed genes of one cell
808 type in one TF class exceeded the fractions in the other cell types, this class was
809 discussed in the main text.
810

811 **Cell cycle gene list**
812 A list of cell cycle regulators identified in Arabidopsis (Vandepoele et al., 2002) was
813 used to retrieve maize orthologs from the Rice Genome Annotation Project Database
814 (University of Michigan), also comprising a full data set for maize (Kawahara et al.,
815 2013) (http://rice.plantbiology.msu.edu/annotation_pseudo_apk.shtml). Predicted
816 cyclins (Hu et al., 2010) were added to the list, as well as genes reported to be
817 involved in cell-cycle regulation in previous publications (Sauter et al., 1998;
818 Dresselhaus et al., 1999b; Dresselhaus et al., 2006; Buendía-Monreal et al., 2011;
819 Dante et al., 2014). Finally, the DNA replication-indicating genes encoding PCNA
820 and MCM homologous proteins were added to the list manually based on Ensemble
821 plants (www.plants.ensemble.org) (Sabelli et al., 2009). The genes were sorted into

822 different classes: CDKs and cyclins, followed by additional cell cycle-related factors
823 (**Supplemental Data Set 3**).

824

825 **Auxin pathway analysis**

826 Information about auxin pathways was downloaded from
827 (http://www.genome.jp/dbget-bin/www_bget?ath04075). Maize genes in the different
828 categories were obtained from the literature based on the gene list **Supplemental**
829 **Data Set 3D** (important gene families). For each category (i.e., SAURs), all
830 significantly (p-adjusted <0.05) differentially expressed genes from the respective
831 comparisons were selected. The median of the $\log_2$FoldChanges of these genes was
832 calculated and represented by a color scale ranging from green (-2) to red (2). All
833 log2FoldChanges above or below these values were set to 2 (-2).

834

835 **Accession Numbers:**

836 The raw data (.fastq) plus supplemental tables, including count and TPM data for all
837 replicates was uploaded to GEO and is available under accession number
838 GSE98379. Gene identifiers are listed in Supplemental Data Set 1.

839

840

841 **SUPPLEMENTAL DATA**

842 **Supplemental Figure 1.** cDNA prepared from maize gametes/zygotes and initial
843   validation based on the presence of selected transcripts.

844 **Supplemental Figure 2.** RNA-seq correlation plot and hierarchical clustering of
845   maize gamete and zygote samples.

846 **Supplemental Figure 3.** Phylogenetic analysis of protein sequences of histone H3
847   and H2A variants.

848 **Supplemental Table 1.** Summary of NGS runs, alignment process to Ensembl
849   genome (AGPv3, ver: 82.6) and annotation to Ensembl genebuild (AGPv3_5b,
850   ver: 82.6).

851 **Supplemental Data Set 1.** Master table: binary identifier for venn diagram category
852   membership. Median gene expression value (TPM, transcripts per million) of three
853   biological replicates, for the indicated cell types, followed by genome coordinates,
854   description, GO and InterPro annotation as well as information on homologous
855   genes from Arabidopsis and rice.

856 **Supplemental Data Set 2.** List of differentially expressed genes for all 15 cell type
857   comparisons, with abs($\log_2$FC)>1 and p-adjusted <0.05.
858 **Supplemental Data Set 3.** Lists of transcript levels of known genes required for
859   fertilization and zygote development (see Figure 1B), expression profiles of
860   transcription factors, cell cycle genes, and important gene families (see Figures 5,
861   6, 7, and 8).
862 **Supplemental Data Set 4.** Lists of TOP80 maize sperm and egg cell genes
863   compared to their predicted homologs/orthologs in rice gametes (see also Figure
864   2).
865 **Supplemental Data Set 5.** Lists of 30 most highly expressed genes in maize sperm
866   cell, egg cell, zygote 12HAP, zygote 24HAP, apical cell and basal cell,
867   respectively (for detailed annotation see Suppl. Data Set 1).
868 **Supplemental Data Set 6.** Lists of gene expression profiles selected for Figure 4E.
869 **Supplemental Data Set 7.** List of expressed transcription factor classes (see also
870 Figure 5A and B).
871 **Supplemental Data Set 8.** List of genes in the auxin pathway (see also Figure 7).
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884 **AUTHOR CONTRIBUTIONS**
885 TD initiated and designed the project. JC, NGK, and PC performed wet lab
886 experiments and interpreted results. JC, NS, SS, and JCE performed bioinformatics
887 analyses. JC, NS, and TD wrote the manuscript with input from all authors.
888
889

FIGURE LEGENDS

Figure 1. Time course of zygote development and validation of RNA-Seq data. (**A**) Sperm cells. (**B**) Egg cell. (**C**) Zygote at 24 hours after pollination (HAP). (**D**) Zygote at 30 HAP at anaphase. (**E**) Zygote at 35 HAP at telophase. Arrowheads indicate phragmoplast between daughter chromosome sets. (**F**) Zygote at 43 HAP during asymmetric cell division (ACD). (**G**) Zygote at 48 HAP. Cytokinesis is completed. Dotted line indicates the cell division plane. (**H**) Apical (AC) and basal cell (BC) have been separated at 54 HAP. Bright field microscopy images are shown on the left and UV images of DAPI stained cells on the right. Scale bars: 10 μm. (**I**) Validation of RNA-Seq data with known genes, as indicated. Top row: Genes preferentially expressed in sperm cells; middle row: Genes highly expressed in egg cells and downregulated after fertilization; bottom row: Expression of cell cycle regulators. Transcript levels are shown as TPM (transcripts per million) values (means ± SD) of three biological replicates.

Figure 2. Heatmap showing a comparison of the 80 most highly expressed genes in maize gametes and their predicted orthologs in the corresponding rice gametes. (**A**) TOP80 most highly expressed genes in maize sperm cells and (**B**) TOP80 genes in maize egg cells. Note that all maize genes display a TPM>200 and are thus indicated by red bars. For better visualization, rice and maize gene expression values (TPM) were square root transformed and classified into 80 expression categories using the 99th percentile of the data to summarize all higher expression values. Plastid genomes were excluded, as they showed overshooting of expression in the rice data. Non-expressed genes in the rice data and genes lacking a clear homolog are marked by black bars. Orthologous gene information is based on the EnsemblPlants Compara database, The Rice Genome Annotation Project (RGAP), and orthologs from RGAP based on OrthoMCL. Maize genes encoding histones and high-mobility group genes are shaded in purple, proteins involved in translation are in green, and EA1-box proteins and predicted secreted CRPs are in yellow. Proteins were classified according to InterPro.

Figure 3. Expression of major chromatin structure protein genes in maize gametes, zygotes, and daughter cells. (**A**) Histone H1, (**B**) histone H3, (**C**) histone

923 H4, (**D**) histone H2A, (**E**) histone H2B, and (**F**) high mobility group protein Hmgd1. All
924 significantly expressed genes of the various gene families are presented. See
925 Supplemental Figure 3 for phylogenetic analysis of male-specific and active
926 chromatin-specific variants of histones H3 and H2A. Gene identifiers are listed in
927 Supplemental Data Set 7 (important gene families). Transcript levels in the cells
928 indicated are given as TPM values (means ± SD).

929

930 **Figure 4. Gene expression dynamics in maize gametes, zygotes, and two-**
931 **celled pro-embryo cells.** Genes with a abs(log2FC)>1 and p-adjusted <0.05 were
932 considered to be biologically meaningful. (**A**) Comparison of the number of genes
933 induced in sperm cells (SP) and egg cells (EC) versus zygotes at 12 HAP (Zy12). (**B**)
934 Comparisons of genes upregulated in zygotes at 12 HAP versus sperm and egg
935 cells. (**C**) Number of genes induced in zygotes at 24 HAP compared to apical (AC)
936 and basal (BC) cells. (**D**) Comparison of genes upregulated in apical and basal cells
937 versus zygotes at 24 HAP (Zy24). (**E**) Selected gene expression profiles across the
938 specific cell types analyzed (see Supplemental Data Set 6). The Pearson correlation
939 (>0.9) of square root transformed TPM values per gene and binary profile vectors
940 were used to identify genes belonging to a specific profile. The total number of genes
941 including TFs contained in each profile is shown at the bottom. The mean expression
942 level of all genes per specific cell type is plotted in each profile (black line). (**F**) Table
943 of differentially upregulated genes (log$_2$FC>1, p-adjusted<0.05) of row cell type
944 versus column cell type. The last column show the number of differentially
945 upregulated genes in a row cell type versus all other cell types.

946

947 **Figure 5. Expression levels of transcription factor (TF) and MAB genes in**
948 **gametes, zygotes, and early two-celled pro-embryo cells in maize.** (**A**)
949 Expression levels of TF genes in gametes and at early developmental stages after
950 fertilization. Genes with abs(log$_2$FC)>1 and p-adjusted <0.05 in at least one cell type
951 comparison were considered. To make the expression levels comparable across
952 multiple cell types, z-scores were calculated from the square root transformed TPM
953 values corresponding to the number of standard deviations between the cell type-
954 specific expression value and the mean expression value of the respective gene.
955 Genes were ordered by TF classes (black/blue bars). Black and blue fonts were
956 used alternatively to distinguish the various classes. See Supplemental Data Sets 2

and 7 for details. (**B**) Expression analysis of selected maize genes encoding homologs of Arabidopsis early embryogenesis-related TFs as well as (**C**) MATH-BTB (MAB) family genes involved in ACD.

Figure 6. Gene expression analysis of cell cycle regulators. (**A**) Major regulators of the maize cell cycle (Supplemental Data Set 2). Transformation of gene expression values as described above. Genes were ordered into protein classes based on data from the literature (see Methods for details). (**B**) Summary of the time course of pollen tube (PT) growth, fertilization, and zygote development. Cell cycle stages of the selected cells are indicated based on the currently reported expression data.

Figure 7. **Analysis of the auxin pathway in gametes, zygotes, and during early embryogenesis.** (**A**) Expression analysis of the most highly expressed auxin biosynthesis, transport, and auxin response-related genes. (**B**) Auxin pathway analysis using three developmental transitions during zygote development. The median values over the $\log_2$FoldChanges of all differentially expressed genes (p-adjusted <0.05) from the respective comparison are color-coded in green (downregulated) and red (upregulated). All $\log_2$FoldChanges above 2 or below –2 were set to 2/–2 to improve visualization. *White boxes*: no significant $\log_2$FC found, *+u*: ubiquitination, *blue line*: inhibition, *dotted gray line*: dissociation, *red dashed line*: expression. Gene pathway based on KEGG analysis. See Supplemental Data Set 2 for genes.

Figure 8. Expression analysis of selected maize genes with putative roles in signaling during gamete interaction and early embryogenesis. (**A**) Ca^{2+}-dependent phospholipid-binding annexin family proteins, (**B**) sperm-specific receptor-like kinases, and (**C**) fertilization-regulated receptor-like kinases. Gene identifiers are listed in Supplemental Data Set 2 (important gene families). Transcript levels in the cells indicated are given as TPM values (means ± SD).

Table 1. Summary of samples, NGS runs, alignment to the Ensembl genome (AGPv3, ver. 82.6), and annotation to Ensembl genebuild (AGPv3_5b, ver. 82.6). The number of genes expressed in at least two of three replicates are given. See Supplemental Table 1 for details.

Sample	Cell number per replicate	All reads after trimming	Aligned pairs mapped unique	% Mapped reads per trimmed reads	Expressed genes (TPM>1)
Sperm Cell1	~1,000	68,988,450	26,514,492	77	
Sperm Cell2	~1,000	102,929,736	38,689,716	75	**11,819**
Sperm Cell3	~1,000	86,448,904	33,271,851	77	
Egg Cell1	20	57,338,078	13,901,359	48	
Egg Cell2	20	63,488,722	22,978,462	72	**16,026**
Egg Cell3	20	69,199,124	25,147,141	73	
Zygote_12HAP1	14	70,632,412	15,761,484	45	
Zygote_12HAP2	15	76,840,694	19,297,245	50	**19,865**
Zygote_12HAP3	15	55,130,854	11,323,005	41	
Zygote_24HAP1	16	82,825,752	20,424,191	49	
Zygote_24HAP2	16	80,911,418	20,173,448	50	**19,171**
Zygote_24HAP3	17	87,134,202	19,443,455	45	
Apical Cell1	16	94,779,064	29,713,124	63	
Apical Cell2	16	68,636,668	20,927,946	61	**17,747**
Apical Cell3	16	74,074,566	23,422,549	63	
Basal Cell1	13	71,456,410	22,440,462	63	
Basal Cell2	13	68,963,988	21,238,495	62	**18,069**
Basal Cell3	14	67,950,552	20,886,111	61	

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Figure 1. Time course of zygote development and validation of RNA-Seq data. (**A**) Sperm cells. (**B**) Egg cell. (**C**) Zygote at 24 hours after pollination (HAP). (**D**) Zygote at 30 HAP at anaphase. (**E**) Zygote at 35 HAP at telophase. Arrowheads indicate phragmoplast between daughter chromosome sets. (**F**) Zygote at 43 HAP during asymmetric cell division (ACD). (**G**) Zygote at 48 HAP. Cytokinesis is completed. Dotted line indicates the cell division plane. (**H**) Apical (AC) and basal cell (BC) have been separated at 54 HAP. Bright field microscopy images are shown on the left and UV images of DAPI stained cells on the right. Scale bars: 10 μm. (**I**) Validation of RNA-Seq data with known genes, as indicated. Top row: Genes preferentially expressed in sperm cells; middle row: Genes highly expressed in egg cells and downregulated after fertilization; bottom row: Expression of cell cycle regulators. Transcript levels are shown as TPM (transcripts per million) values (means ± SD) of three biological replicates.

Figure 2. Heatmap showing a comparison of the 80 most highly expressed genes in maize gametes and their predicted orthologs in the corresponding rice gametes. (**A**) TOP80 most highly expressed genes in maize sperm cells and (**B**) TOP80 genes in maize egg cells. Note that all maize genes display a TPM>200 and are thus indicated by red bars. For better visualization, rice and maize gene expression values (TPM) were square root transformed and classified into 80 expression categories using the 0.99th percentile of the data to summarize all higher expression values. Plastid genomes were excluded, as they showed overshooting of expression in the rice data. Non-expressed genes in the rice data and genes lacking a clear homolog are marked by black bars. Orthologous gene information is based on the EnsemblPlants Compara database, The Rice Genome Annotation Project (RGAP), and orthologs from RGAP based on OrthoMCL. Maize genes encoding histones and high-mobility group genes are shaded in purple, proteins involved in translation are in green, and EA1-box proteins and predicted secreted CRPs are in yellow. Proteins were classified according to InterPro.

Figure 3. Expression of major chromatin structure protein genes in maize gametes, zygotes, and daughter cells. (**A**) Histone H1, (**B**) histone H3, (**C**) histone H4, (**D**) histone H2A, (**E**) histone H2B, and (**F**) high mobility group protein Hmgd1. All significantly expressed genes of the various gene families are presented. See Supplemental Figure S3 for phylogenetic analysis of male-specific and active chromatin-specific variants of histones H3 and H2A. Gene identifiers are listed in Supplemental Table S8 (important gene families). Transcript levels in the cells indicated are given as TPM values (means ± SD).

A

SP vs Zy12

EC vs Zy12

4671 | 1730 | 1759

31309

B

Zy12 vs SP

Zy12 vs EC

7356 | 3605 | 1933

26575

C

Zy24 vs AC

Zy24 vs BC

1606 | 2182 | 791

34890

D

AC vs Zy24

BC vs Zy24

832 | 2228 | 485

35924

E

SP EC Zy12 Zy24 AC BC

induced shortly after fertilization
(1469/TF:129)

induced transiently after fertilization
(1510/TF:176)

induced shortly after fertilization then preferentially in apical cells
(223/TF:30)

induced later after fertilization
(293/TF:20)

preferentially expressed in egg cell
(326/TF:14)

SP EC Zy12 Zy24 AC BC

induced transiently shortly after fertilization
(356/TF:44)

down−regulated transiently after fertilization
(137/TF:8)

induced shortly after fertilization then preferentially in basal cells
(182/TF:18)

expressed transiently before 1st asymmetric zygote division (+SP)
(137/TF:4)

down−regulated in egg cell
(95/TF:5)

$\sqrt{\text{TPM}}$ gene expression levels

F

	SP	EC	Zy12	Zy24	AC	BC	overall
SP	0	6494	6401	6437	6485	6414	4090
EC	8608	0	3489	3247	3993	3682	482
Zy12	10961	5538	0	571	4939	4443	109
Zy24	10450	4931	813	0	3788	2973	31
AC	9638	4752	3655	3060	0	243	6
BC	9713	4574	3407	2713	364	0	8

Figure 4. Gene expression dynamics in maize gametes, zygotes, and two-celled pro-embryo cells. Genes with a abs(log2FC)>1 and p-adjusted <0.05 were considered to be biologically meaningful. (**A**) Comparison of the number of genes induced in sperm cells (SP) and egg cells (EC) versus zygotes at 12 HAP (Zy12). (**B**) Comparisons of genes upregulated in zygotes at 12 HAP versus sperm and egg cells. (**C**) Number of genes induced in zygotes at 24 HAP compared to apical (AC) and basal (BC) cells. (**D**) Comparison of genes upregulated in apical and basal cells versus zygotes at 24 HAP (Zy24). (**E**) Selected gene expression profiles across the specific cell types analyzed (see Supplemental Table S6). The Pearson correlation (>0.9) of square root transformed TPM values per gene and binary profile vectors were used to identify genes belonging to a specific profile. The total number of genes including TFs contained in each profile is shown at the bottom. The mean expression level of all genes per specific cell type is plotted in each profile (black line). (**F**) Table of differentially upregulated genes (log2FC>1, p-adjusted<0.05) of row cell type versus column cell type. The last column show the number of differentially upregulated genes in a row cell type versus all other cell types.
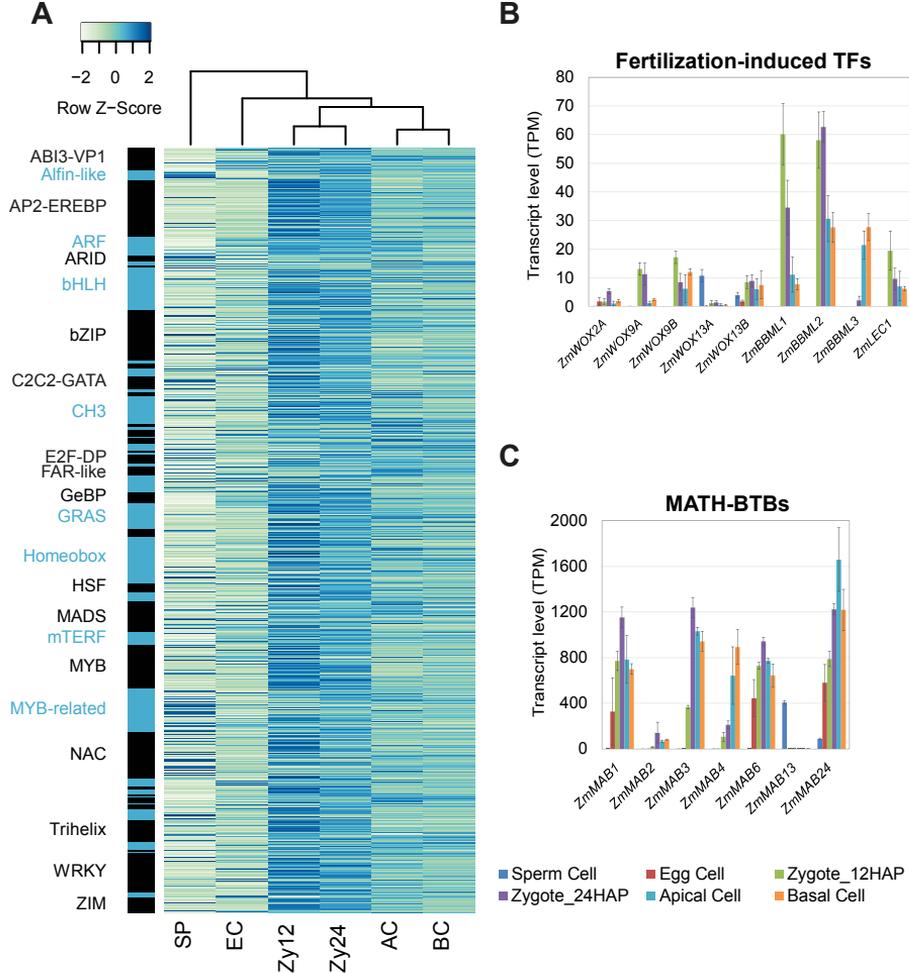
Figure 5. Expression levels of transcription factor (TF) and MAB genes in gametes, zygotes, and early two-celled pro-embryo cells in maize. (**A**) Expression levels of TF genes in gametes and at early developmental stages after fertilization. Genes with abs(log2FC)>1 and p-adjusted <0.05 in at least one cell type comparison were considered. To make the expression levels comparable across multiple cell types, z-scores were calculated from the square root transformed TPM values corresponding to the number of standard deviations between the cell type-specific expression value and the mean expression value of the respective gene. Genes were ordered by TF classes (black/blue bars). Black and blue fonts were used alternatively to distinguish the various classes. See Supplemental Tables S7 and S8 for details. (**B**) Expression analysis of selected maize genes encoding homologs of Arabidopsis early embryogenesis-related TFs as well as (**C**) MATH-BTB (MAB) family genes involved in ACD.
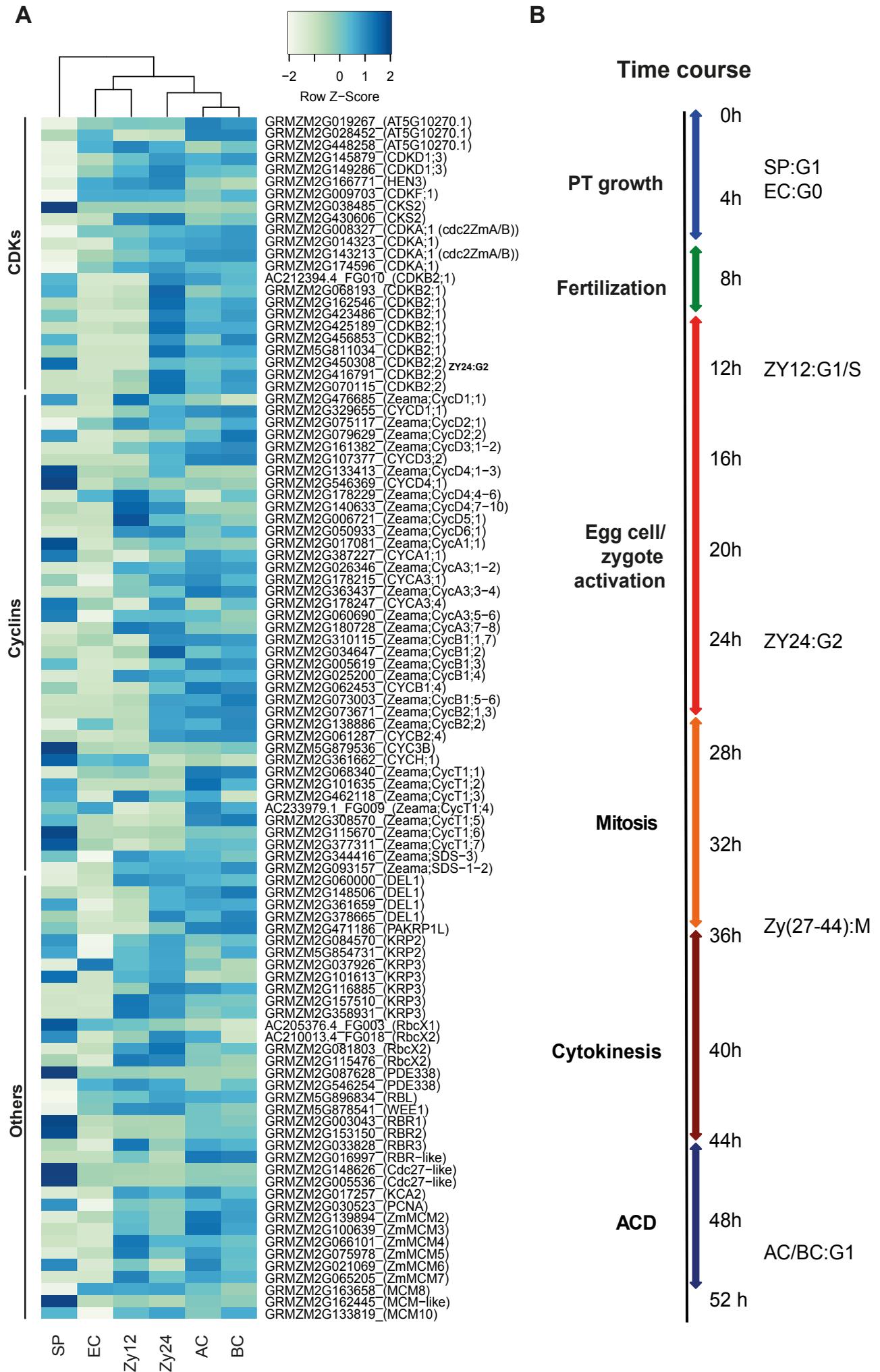
Figure 6. Gene expression analysis of cell cycle regulators. (**A**) Major regulators of the maize cell cycle (Supplemental Table S7). Transformation of gene expression values as described above. Genes were ordered into protein classes based on data from the literature (see Methods for details). (**B**) Summary of the time course of pollen tube (PT) growth, fertilization, and zygote development. Cell cycle stages of the selected cells are indicated based on the currently reported expression data.

Figure 7. Analysis of the auxin pathway in gametes, zygotes, and during early embryogenesis. (**A**) Expression analysis of the most highly expressed auxin biosynthesis, transport, and auxin response-related genes. (**B**) Auxin pathway analysis using three developmental transitions during zygote development. The median values over the log2FoldChanges of all differentially expressed genes (p-adjusted <0.05) from the respective comparison are color-coded in green (downregulated) and red (upregulated). All log2FoldChanges above 2 or below –2 were set to 2/–2 to improve visualization. White boxes: no significant log2FC found, +u: ubiquitination, blue line: inhibition, dotted gray line: dissociation, red dashed line: expression. Gene pathway based on KEGG analysis. See Supplementary Table 7 for genes.

Figure 8. Expression analysis of selected maize genes with putative roles in signaling during gamete interaction and early embryogenesis. (**A**) Ca^{2+}-dependent phospholipid-binding annexin family proteins, (**B**) sperm-specific receptor-like kinases, and (**C**) fertilization-regulated receptor-like kinases. Gene identifiers are listed in Supplemental Table S7 (important gene families). Transcript levels in the cells indicated are given as TPM values (means ± SD).

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