Gene expression profiles in rice gametes and zygotes: identification of gamete-enriched genes and up- or down-regulated genes in zygotes after fertilization

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Abstract

In angiosperms, fertilization and subsequent zygotic development occur in embryo sacs deeply embedded in the ovaries; therefore, these processes are poorly elucidated. In this study, microarray-based transcriptome analyses were conducted on rice sperm cells, egg cells, and zygotes isolated from flowers to identify candidate genes involved in gametic and/or early zygotic development. Cell type-specific transcriptomes were obtained, and up- or down-regulated genes in zygotes after fertilization were identified, in addition to genes enriched in male and female gametes. A total of 325 putatively up-regulated and 94 putatively down-regulated genes in zygotes were obtained. Interestingly, several genes encoding homeobox proteins or transcription factors were identified as highly up-regulated genes after fertilization, and the gene ontology for up-regulated genes was highly enriched in functions related to chromatin/DNA organization and assembly. Because a gene encoding methyltransferase 1 was identified as a highly up-regulated gene in zygotes after fertilization, the effect of an inhibitor of this enzyme on zygote development was monitored. The inhibitor appeared partially to affect polarity or division asymmetry in rice zygotes, but it did not block normal embryo generation.

Key words: Egg cell, fertilization, microarray, Oryza sativa, sperm cell, zygote.

Introduction

In angiosperms, the sporophytic generation is initiated by double fertilization, resulting in the formation of seeds (reviewed in Raghavan, 2003). In double fertilization, one sperm cell from the pollen grain fuses with the egg cell, and the resultant zygote develops into an embryo that transmits genetic material from the parents to the next generation. The central cell fuses with the second sperm cell to form a triploid primary endosperm cell, which develops into the endosperm that nourishes the developing embryo/seedling (Nawaschin, 1898; Guignard, 1899; Russell, 1992).

To date, three molecular factors, GENERATIVE CELL SPECIFIC 1/HAPLESS 2 (GCS1/HAP2), EGG CELL 1 (EC1), and ANKYRIN REPEAT PROTEIN 6 (ANK6), are known to play critical roles in male–female gamete recognition and/or fusion in angiosperms (Mori et al., 2006; von Besser et al., 2006; Yu et al., 2010; Sprunck et al., 2012). GCS1/HAP2 was identified as a key male membrane protein with a single transmembrane domain and a histidine-rich domain in the extracellular region. Sperm cells with a gcs mutation remained attached to the egg cell without cell fusion (Mori et al., 2006). Although the mechanism by which GCS1/HAP2 acts is not known, both the N- and C-terminal domains appear to be essential for its function (Mori et al., 2010; Wong et al., 2010). Recently, Sprunck et al. (2012) indicated that small cysteine-rich EC1 proteins accumulated in storage vesicles in Arabidopsis egg cells are secreted via...
exocytosis upon sperm cell attachment to the egg cell, and that the secreted EC1 proteins function in redistribution of GCS1/HAP2 proteins to the sperm cell surface, resulting in successful gamete fusion. ANK6 is a mitochondrial ankyrin-repeat protein with high expression in both male and female gametophytes and appears to play a role in male–female recognition by regulating mitochondrial gene expression (Yu et al., 2010). In addition to these three proteins, other players need to be identified in order to understand the mechanisms of gamete recognition and fusion.

When a sperm and egg cell fuse, plasmogamy is followed by the sperm nucleus moving toward the egg nucleus. Subsequently, karyogamy, the fusion of nuclei, occurs to form a zygotic nucleus. Ultrastructural and cytological observations of the zygote produced by in vitro fertilization (IVF) using maize gametes revealed that karyogamy in maize occurs within 1 h of gametic fusion (Faure et al., 1993). In angiosperms, it has been supposed that the zygotic genome appears to be activated during embryogenesis through ‘maternal to zygotic transition’, as in animals (Schier, 2007; Tadros and Lipshitz, 2009; Autran et al., 2011; Pilot et al., 2010). However, recent studies on early development in maize, tobacco, and Arabidopsis have indicated that the zygotic genome switches on within hours of fertilization (Meyer and Scholten, 2007; Zhao et al., 2011; Nodine and Bartel, 2012), and the zygotic genome is generally considered to be activated almost immediately after fertilization in angiosperms (Hale and Jacobsen, 2012; Nodine and Bartel, 2012).

In addition to de novo transcription/translation, a notable feature of zygote development is remodelling of cell polarity. For example, the vacuole in Arabidopsis zygotes becomes fragmented after fertilization, and the zygote elongates 2- to 3-fold before a large vacuole is re-assembled (Faure et al., 2002). In rice zygotes, the vacuoles and nucleus, which are localized at the apical pore (chalazal side) and basal region (micropyle side) in egg cells, respectively, are re-positioned to opposite poles in the cell (Sato et al., 2010). Although various polarity remodelling processes occur in zygotes of different species, each creates a cytologically polarized cell, with its nucleus positioned at the apical pore and its vacuoles at the basal region (reviewed in Jeong et al., 2011). The polarized zygote divides asymmetrically into a two-celled proembryo consisting of an apical and a basal cell, which develop into the embryo proper and the suspensor/hypophysis, respectively (Pritchard, 1964; Schulz and Jensen, 1968; Tykarska, 1976; Schel et al., 1984; reviewed in Lindsey and Topping, 1993). These facts strongly suggest that cellular polarity in the zygote is tightly linked to the establishment of the initial apical–basal axis in plants. However, the molecular machinery generating and maintaining cellular polarity in zygotes remains poorly understood (Abrash and Bergmann, 2010; Jeong et al., 2011).

In contrast to animals and lower plants, which have free-living gametes, angiosperm fertilization and subsequent events, such as embryogenesis and endosperm development, occur in the embryo sac, which is deeply embedded in ovular tissue. Difficulties in directly researching the biology of the embedded female gametophyte, zygote, and early embryo have impeded investigations into the molecular mechanisms of fertilization and embryogenesis. Therefore, such studies have been conducted predominantly through analyses of Arabidopsis mutants. Alternatively, direct analyses using isolated gametes or zygotes are possible, because procedures for isolating viable gametes have been reported for a wide range of plant species, including maize, wheat, tobacco, rape, rice, barley, Plumbago zeylanica, and Alstroemeria (Dupuis et al., 1987; Kranz et al., 1991; Holm et al., 1994; Kovács et al., 1994; Cao and Russell, 1997; Katoh et al., 1997; Tian and Russell, 1997; Hoshino et al., 2006; Uchiumi et al., 2006). Moreover, IVF using isolated gametes can be used to observe and analyse fertilization and post-fertilization processes directly (reviewed in Wang et al., 2006; Okamoto, 2011).

Using isolated gametes and/or embryos, previous studies have successfully identified genes specifically expressed in male gametes, female gametes, or early embryos (Kasahara et al., 2005; Márton et al., 2005; Sprunk et al., 2005; Ning et al., 2006; Yang et al., 2006; Steffen et al., 2007; Borges et al., 2008; Amien et al., 2010; Ohnishi et al., 2011; Wang et al., 2010; Wuest et al., 2010). However, as far as is known, microarray-based transcriptome analyses of sperm cells, egg cells, and zygotes using the same experimental platform have not been conducted, probably because of difficulties in preparing zygote samples. A procedure to isolate rice gametes and an IVF system to produce zygotes that could develop into fertile plants were established previously (Uchiumi et al., 2006, 2007b). In this study, microarrays analyses of rice gametes and zygotes were performed to examine their gene expression profiles and to monitor changes in gene expression from pre-fertilization to post-fertilization phases.

Materials and methods

Plant materials, isolation of gametes and zygotes, and electrofusion of gametes

Oryza sativa L. cv. Nipponbare was grown in environmental chambers (K30-7248, Koito Industries, Yokohama, Japan) at 26 °C in a 13/11 h light/dark cycle. Rice egg and sperm cells were isolated according to Uchiumi et al. (2006), except for using mannitol solution adjusted to 370 mOsmol/kg H₂O instead of 0.3 M mannitol. Electrofusion of egg and sperm cells and culture of the subsequent zygote were conducted as described by Uchiumi et al. (2007b).

To isolate zygotes from pollinated rice ovaries, ovaries were harvested from flowers 2–3 h after flowering, and zygotes were obtained by the above-mentioned procedure for egg cell isolation, with some modifications. Briefly, the pollinated ovaries were transferred into plastic dishes (9.5 cm) containing 3 ml of mannitol adjusted to 450 mOsmol/kg H₂O and cut transversely at their midpoints with razor blades (Supplementary Fig. S1 available at JXB online). The zygote was obtained from a dissected ovary by gently pushing the basal portion of the cut ovary with a glass needle under an inverted microscope. The isolated zygotes were either cultured or immediately used for RNA extraction.

RNA isolation from egg cells, zygotes, sperm cells, and pollen grains

Isolated egg cells, sperm cells, and zygotes were washed three times by transferring the cells into fresh droplets of mannitol solution on...
coverslips. The washed cells were submerged in 5 μl of the extraction buffer supplied in a PicoPure RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) and stored at −80 °C until use. Pollen grains were released from mature anthers, transferred into 5 μl of extraction buffer, and stored at −80 °C until use. For RNA extraction, cells stored in the extraction buffer were pooled, and total RNAs were isolated using the PicoPure RNA Isolation Kit according to the manufacturer’s instructions. The quality of the total RNAs used for microarray analyses was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Microarray and data analysis
Total RNAs prepared from 31–111 egg cells (31, 34, and 111 egg cells; three biological replicates), 30 and 33 zygotes (two biological replicates), ~3000 sperm cells (two biological replicates), and ~100 pollen grains (three biological replicates) were labelled using a Quick Amp Labeling Kit One-Color (Agilent) in the presence of cyanine-3 (Cy3)-CTP for 6 h according to the manufacturer’s protocol. The Cy3-labelled cRNA was purified with an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), and the quantity was examined with a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA). A total of 1000–1200 ng of Cy3-labelled cRNA was fragmented, and hybridized on a rice 4 × 44K oligoDNA microarray slide (G2519F#15241; Agilent) at 65 °C for 17 h. After washing according to the manufacturer’s protocol, the hybridized slides were scanned on a DNA microarray scanner (G2505BA; Agilent). Background correction of the Cy3 raw signals was performed using Feature Extraction software 10.5 (Agilent). Subsequent data processing was performed using GeneSpring GX 11.0 software (Agilent). Raw signal intensities of all probes were subjected to 75th percentile normalization and were log transformed. Next, probes were filtered by signal intensity values from the 20th to the 100th percentile and further filtered by Present/Absent flags and by standard deviation (SD <0.5). Two different cell types were compared with an unpaired Student’s t-test. To correct the false discovery rate (FDR), the F-value was adjusted for multiple testing by the Benjamini–Hochberg procedure. The cut-off-corrected F-value was 0.05.

A hierarchical clustering tree was constructed based on genes expressed in egg cells, zygotes, sperm cells, and pollen grains by the centred Pearson correlation algorithm with complete linkage. Gene ontology terms enriched in the 286 genes with 2-fold higher expression levels in egg cells than in zygotes and in the 325 genes whose expression levels in zygotes were 3-fold higher than in egg cells were obtained from the AgriGO database (http://bioinfo.cau.edu.cn/agriGO/index.php). A user-driven tool that displays large genomics data sets, MapMan (Thimm et al., 2004; Usadel et al., 2005), was used to overview the metabolism and regulation in zygotes, compared with egg cells.

RT–PCR and quantitative PCR
cDNAs were synthesized from total RNAs of 10 egg cells, 10 zygotes, and 100 sperm cells using the High Capacity RNA-to-cDNA™ Kit (Life Technologies) according to the manufacturer’s instructions. For reverse transcription–PCR (RT–PCR), 0.2 μl of first-strand cDNA was used as template in a 20 μl PCR with 0.3 μM primers using KOD-FX DNA polymerase (Toyobo, Osaka, Japan) as follows: 35 or 40 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. Expression of the ubiquitin gene (Os02g0161900) was monitored as an internal control. For quantitative PCR analysis, 0.5 μl of first-strand cDNA was used with LightCycler 480 SYBR Green I Master (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s protocol. Each PCR cycle was conducted as follows: 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s, and relative quantification was calculated with ubiquitin as a reference by the ΔΔCt method. Primer sequences used for PCR analyses are listed in Supplementary Table S1 at JXB online.

Vector construction and preparation of transformants
The genomic sequence of the promoter region positioned 2558 bp upstream of the translation start site of a ubiquitin gene (Os02g0161900) was PCR amplified using the primers 5’-AAGCTTGTATCGATAGCTGCCG-3' and 5’-TCTAGAC TGGCAAGAATAATCACCACCAACAG-3’. The amplified PCR product was subcloned into pGEM-T Easy Vector (Promega, Fitchburg, WI, USA) and its sequence verified. The plasmid harbouring the ubiquitin promoter was cut with HindIII and XhoI, and the excised DNA fragment was subcloned into the HindIII–XhoI site of pG121-Hm, a binary plasmid vector harbouring a β-glucuronidase (GUS) coding sequence. The resulting vector was termed the Ubi promoter::GUS vector.

cDNA for Arabidopsis histone H2B protein (AT1g22880.1) was amplified by PCR using gene-specific primers (5’-CACCAT GGCGAAGGCAGATAAAGAACAG-3' and 5’-AGAACCTCG TAAACTCTGAATACCG-3') and cloned into the entry vector pENTR/D-TOPO (Life Technologies). To generate the DNA construct for H2B-green fluorescent protein (GFP) fusion protein, the cloned cDNA was transferred from the entry vector to the destination vector pGW4B405 (Nakagawa et al., 2007) by an LR reaction using Gateway LR Clonase enzyme mix (Invitrogen) according to the manufacturer’s instructions. This constructed vector was used as template for PCR amplification of the DNA region encoding H2B-GFP fusion protein using the specific primers 5’-GCTAGCATGGCGAAGGCAGATAAAGAACAG-3' and 5’-GAGCTCTTACCTGTGACTACGTCGTCGA-3'. The amplified PCR product was subcloned into pGEM-T Easy Vector, and the plasmid harbouring the H2B-GFP sequence was cut with XhoI and SacI. Then, the excised DNA fragment was subcloned into the XbaI–SacI site of the Ubi promoter::GUS vector, replacing the GUS sequence with that of H2B-GFP. The vector was used as the Ubi promoter::H2B-GFP vector to transform rice plants.

Agrobacterium tumefaciens LBA4404 was transformed with the Ubi promoter::H2B-GFP vector, and transformed rice plants were prepared by co-cultivation of scutellum tissue with A. tumefaciens according to Toki et al. (2006).

Microscopic observation
Cells and embryos were observed using a BX-71 inverted microscope (Olympus, Tokyo, Japan). Fluorescent cells/embryos expressing H2B-GFP were visualized with a BX-71 inverted fluorescence microscope with 460–490 nm excitation and 510–550 nm emission wavelengths (U-MWIBA2 mirror unit; Olympus). Digital images of egg cells, zygotes, and their resulting embryos were obtained through a cooled charge-coupled device camera (Penguin 600CL; Pixcera, Los Gatos, CA, USA) and InStudio software (Pixcera).

Effects of RG108 on DNA methylation status of some transposon-related elements in cultured rice cells and zygotic development
Based on the methods of Yin et al. (2008), DNA methylation of transposon-related elements on chromosome 4 was assayed by MRCB digestion of genomic DNA and subsequent PCR amplification. A rice suspension cell culture was initiated from scutellum-derived callus according to Kyoizuka et al. (1987), and the suspension cells were cultured with or without 100 μM RG108 for 4 d. Genomic DNA (250ng) isolated from cultured cells was digested with 20U of MrcB for 16h at 37 °C. Water was substituted for enzyme as a negative control. PCR was performed with gene-specific primers (Supplementary Table S1 at JXB online) and the products were visualized by gel electrophoresis. Zygotes were produced by electrofusion of sperm cells with egg cells expressing H2B-GFP and cultured with or without 100 μM RG108.

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Results and Discussion

Isolation of gametes and zygotes

Rice egg cells and zygotes were isolated from transversely cut unpollinated and pollinated ovaries, respectively (Supplementary Fig. S1 at JXB online). The isolation efficiency of zygotes was much lower than that of egg cells; on average, 8–15 egg cells and 1–3 zygotes were obtained from 30 unpollinated and pollinated flowers, respectively. This difference can be explained thus: because egg cells have an incomplete cell wall (Russell, 1992), they were easily released from cut ovaries. However, upon fertilization, zygotes immediately begin forming a complete cell wall (Kranz et al., 1995), which tightly attaches the zygote to other cells in the embryo sac and/or to the embryo sac membrane. Such attachment could explain why zygotes were so much more difficult to isolate.

The isolated egg cells showed typical cytological characters, with the cytoplasm, mitochondria, and possible starch granules densely distributed around the nucleus, and with vacuoles at the peripheries of the cells (Fig. 1A; Uchiumi et al., 2006). In the zygotes isolated from pollinated ovaries, cytoplasm and granule structures were observed throughout the cells and vacuoles were undeveloped (Fig. 1B, C). When isolated zygotes were further cultured, they divided into globular-like embryos via asymmetric two-celled proembryos (Fig. 1C–E), as is known to occur in IVF-produced and in planta zygotes (Uchiumi et al., 2007b; Sato et al., 2010). This finding suggests that the gene expression profiles in the isolated zygotes probably reflect those of zygotes embedded in embryo sacs.

Rice pollen grains are tricellular, with one vegetative cell and two sperm cells (Raghavan, 1988). Sperm cells released by bursting pollen grains in mannitol solution were collected with almost homogeneity (Fig. 1F).

Microarrays using RNA from isolated gametes and zygotes

In total RNA fractions prepared from egg cells, zygotes, or sperm cells, 28S and 18S rRNAs were clearly visible (Supplementary Fig. S2 at JXB online). The RNAs were labelled and hybridized to a rice 44K oligo microarray. Correlation plots for the microarray data sets supported the correlation between samples of the same cell type (Supplementary Fig. S3). The correlation coefficient between two sperm cell sets (0.68) was lower than that between other data sets. It may be a reason that sperm cells degenerate after isolation more easily than egg cells and zygotes.

Fig. 1. Isolated rice cells, cultured zygotes, and hierarchical clustering of expressed genes in the cell types. (A) Egg cells. (B) Zygotes. Zygotes were further cultured for 0 (C), 14 (D), and 37 h (E). Black and white arrowheads indicate nucleoli and nuclei, respectively. (F) Sperm cells. (G) Hierarchical clustering tree of egg cells, zygotes, sperm cells, and pollen grains. Bars=50 μm in A–E; 10 μm in F.
Hierarchical sample clustering also grouped the biological replicates together (Fig. 1G), indicating that cell type-specific transcriptomes were obtained. Interestingly, the egg cells and zygotes formed a tighter cluster and showed higher correlation than did the sperm cells and pollen grains, consistent with previous reports that male gametes and gametophytes show very different gene expression profiles from somatic tissue (Borges et al., 2008) and female gametic cells, including eggs, synergids, and central cells (Wuest et al., 2010).

After processing, signals from 3998, 5153, and 8613 probes were judged to be positive in sperm cells, egg cells, and zygotes, respectively (Supplementary Tables S2–S4 at JXB online). Notably, 81% of positive probes in egg cells were also positive in zygotes (Fig. 2A). In contrast, only 26.5% of positive probes in egg cells were positive in sperm cells. These results were consistent with those of the clustering analysis (Fig. 1G) and correlation plots (Supplementary Fig. S3).

More than 5000 genes have been reported to be genes expressed in Arabidopsis sperm cells (Borges et al., 2008). Deveshwar et al. (2011) identified 4152 rice genes orthologous to those in Arabidopsis. Oligonucleotide probes for 3965 of these orthologous genes are printed on the Agilent rice 44K oligo microarray chip. In rice sperm cells, 807 of these 3965 genes were expressed (Supplementary Table S2 at JXB online).

To find genes enriched in sperm or egg cells, gene expression profiles were first compared between male and female gametes. A total of 573 genes had expression levels in sperm cells that were 10-fold higher than in egg cells (Supplementary Table S5 at JXB online), and 1067 genes had 10-fold higher expression levels in egg cells than in sperm cells (Supplementary Table S6). Next, to identify gamete-specific genes, these differentially expressed genes were compared with 4119 genes whose expression was clearly suppressed in somatic tissues in the whole-plantlet rice transcriptome of Ohnishi et al. (2011). This analysis found that 49 and 14 genes were enriched in sperm and egg cells, respectively (Fig. 2B). Thirty of the 49 sperm-specific genes had relatively high expression levels in pollen grains (>5000 raw signal value) and were omitted; the remaining 19 genes are listed in Table 1. Table 2 includes the 14 potentially egg-specific genes. Using quantitative PCR, expression levels of seven genes enriched in gametes and a control gene encoding glyceraldehyde 3-phosphate dehydrogenase in somatic tissues and gametes were monitored. All seven genes showed gamete-specific expression (Fig. 2C), supporting the possibility that the genes listed in Tables 1 and 2 have gamete-specific expression profiles.

Five of the 19 genes enriched in sperm cells (Os01g0180900, Os10g0550400, Os03g0661900, Os09g0483200, and Os11g0620800) were reported to be sperm cell specific by Russell et al. (2012). Nine of the 19 enriched genes were annotated as hypothetical proteins or genes, consistent with a previous report indicating enrichment of genes encoding protein with unknown function in sperm-specific genes (Russell et al., 2012). In animals, serine proteases in the trypsin family can be expressed in sperm and involved in fertilization, although
their molecular mechanisms during the fertilization process remain unknown (Sawada et al., 1984, 1996; Baba et al., 1994; Adham et al., 1997). Interestingly, Os03g0661900 encodes a trypsin-like serine protease (Table 1). Trypsin-like protease may be expressed in male gametes of both plants and animals and perhaps have similar roles in gamete attachment, recognition, or fusion, although the fertilization systems are largely divergent in the kingdoms.

Three genes in Table 2 (Os03g0296600, Os01g0299700, and Os11g0187600) were reported as enriched in egg cells by Ohnishi et al. (2011). Os03g0296600 encodes an EARLY CULTURE ABUNDANCE (ECA) family protein; ECA1 expression in egg cells was first revealed by their molecular mechanisms during the fertilization process.
expressed sequence tag analysis of wheat egg cells (Sprunck et al., 2005). Os11g0187600 encodes heat shock protein 70 (HSP70). In addition to HSP70, HSP90 was identified as a major protein component of rice egg cells by proteomic analysis (Uchiumi et al., 2007a). HSP90 proteins are evolutionarily conserved molecular chaperones that promote the folding of client proteins with various co-chaperones (reviewed in Sangster and Queitsch, 2005). Notably, among its pleiotropic functions, HSP90 plays a role in buffering the expression of genetic variation when divergent ecotypes are crossed, and profoundly affects developmental plasticity in response to environmental cues (Queitsch et al., 2002). Furthermore, Calvert et al. (2003) revealed that mouse eggs contain molecular chaperones, including HSP90, HSP70, and protein disulphide isomerase (PDI), as major protein components. Because HSP70 and HSP90 both appear to be major protein components of rice egg cells as well, an abundance of these proteins may be a common characteristic of mammalian and plant eggs. These HSPs in egg cells may function following fertilization by a sperm cell, because conversion of an egg cell into a zygote represents major genetic and environmental changes.

MADS-box proteins bind to specific DNA sites as homo- or heterodimers to regulate their own transcription and that of target genes (West et al., 1998), and act early in organ development (Riechmann and Meyerowitz, 1997; Theißen et al., 2000). Os07g0108900, encoding MADS-box transcription factor 15 (OsMADS15), was enriched in eggs in this study. In maize, ZmMADS3, which is orthologous to OsMADS15, is strongly expressed in maize egg cells, although its function in egg cells is still unclear (Heuer et al., 2001). MADS-box proteins that accumulate in female gametes may have roles in egg cell differentiation during gametophyte genesis and/or zygotic development after fertilization. A gene enriched in eggs, Os04g0289600, encodes allergen V5/Tpx-1-related protein, which belongs to the CAP superfamily, a family of cysteine-rich secretory proteins (Gibbs et al., 2008). The gene is known to be expressed in synergids as well as egg cells at a high level (Ohnishi et al., 2011), suggesting that the protein functions in the egg apparatus and may be related to the reproductive process.

**Down-regulated genes in zygotes**

Egg cells are developmentally quiescent, a state that is broken after fertilization and subsequent egg activation. Because the expression of genes involved in maintaining egg cell quiescence should be suppressed in zygotes, the gene expression profiles of egg cells and zygotes were compared to detect genes that were down-regulated after fertilization. A total of 94 genes that had 3-fold lower expression levels in zygotes than in egg cells were identified (Supplementary Table S7 at JXB online). No gene ontologies could be found for these genes, so 286 genes with 2-fold higher expression levels were examined. Most ontologies were related to metabolic or biosynthetic processes (Supplementary Table S8). A comprehensive overview of metabolism in zygotes, compared with egg cells, indicated that several metabolic pathways, including terpene, flavonoid, and amino acid synthetic pathways, appear to be down-regulated after fertilization (Supplementary Fig. S4A). Expression profiles of the 10 most strongly suppressed genes in zygotes (Table 3) were confirmed using semi-quantitative PCR (Fig. 3). Seven genes showed clear down-regulation in zygotes; however, the expression of three genes was not detected in egg cells, suggesting that they may have low expression levels in egg cells. Alternatively, this may be due to using a small amount of RNAs from gametes or zygotes for microarray and RT–PCR analyses.

**Up-regulated genes in zygotes**

Upon fertilization, the developmentally quiescent egg cell converts to an active zygote, and expression of genes involved in zygotic development should be induced. Comprehensive overviews of metabolism and regulation in zygotes, compared with egg cells, suggested that synthetic pathways for the cell wall, auxin, and ethylene appeared to be activated in zygotes after fertilization (Supplementary Fig. S4A). In addition, several receptor kinase-related genes appeared to be up-regulated in zygotes, suggesting that signal transduction pathways are also activated via fertilization. A search for genes that were up-regulated in zygotes identified 325 genes whose expression levels in zygotes were 3-fold higher than in egg cells (Supplementary Table S9). Enriched ontologies

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**Table 3.** Ten genes whose expression levels were most putatively down-regulated in zygotes after fertilization.

<table>
<thead>
<tr>
<th>Locus</th>
<th>RAPDB description</th>
<th>Fold change (egg/zygote)</th>
</tr>
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<tbody>
<tr>
<td>Os01g0360200</td>
<td>Similar to Respiratory burst oxidase homologue</td>
<td>15.44</td>
</tr>
<tr>
<td>Os01g0396200</td>
<td>Lipase, class 3 family protein</td>
<td>11.75</td>
</tr>
<tr>
<td>Os03g0760200</td>
<td>Cytochrome P450 family protein</td>
<td>11.32</td>
</tr>
<tr>
<td>Os03g0154000</td>
<td>Aromatic-ring hydroxylase family protein</td>
<td>10.08</td>
</tr>
<tr>
<td>Os08g0452000</td>
<td>Dormancyauxin associated family protein</td>
<td>8.30</td>
</tr>
<tr>
<td>Os02g0530100</td>
<td>Heavy metal transport/detoxification protein domain containing</td>
<td>8.82</td>
</tr>
<tr>
<td>Os06g0231700</td>
<td>Similar to Tonoplast membrane integral protein ZmTIP4-2</td>
<td>6.67</td>
</tr>
<tr>
<td>Os04g0505050</td>
<td>Leucine-rich repeat, cysteine-containing subtype-containing protein</td>
<td>6.52</td>
</tr>
<tr>
<td>Os03g0100900</td>
<td>Plasma membrane H⁺-ATPase</td>
<td>6.50</td>
</tr>
<tr>
<td>Os08g0299400</td>
<td>Similar to MGDG synthase type A</td>
<td>6.40</td>
</tr>
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</table>

Mean t-test P-values < 0.05.
were obtained for these genes (Supplementary Table S10). Interestingly, genes related to chromatin/DNA organization and assembly were well represented among the up-regulated genes. In addition, several genes encoding homeobox protein or transcription factors were strongly induced in zygotes (Table 4). Rice plants grown under the conditions used in this study undergo the first zygotic cleavage 14–19 h after pollination (Sato et al., 2010). In this study, zygotes were isolated 2–3 h post-pollination, implying that zygotes were in the early developmental stage. The developmental stage, the enriched gene ontologies, and the putative functions of up-regulated genes in zygotes together indicate that the zygotic genome is activated early in rice development, as reported for other plant zygotes (Meyer and Scholten, 2007; Zhao et al., 2011; Nodine and Bartel, 2012).

Semi-quantitative PCR was conducted for the up-regulated genes listed in Table 4. Although 16 were clearly induced in zygotes (Fig. 4), Os01g0840300 expression was detected at equal levels in egg cells and zygotes, and no expression was detected for three genes. Although why such a difference occurs between semi-quantitative PCR and microarray analyses cannot be explained, it may due to using a small amount of gamete/zygote RNA for these analyses.

Fertilization-induced expression of Os01g0840300, encoding a Wuschel-related homeobox (WOX) protein, was confirmed by PCR (Fig. 4). WOX proteins are key regulators in determining cell fate in plants (Haecker et al., 2004; Park et al., 2005; Mayer et al., 1998; Zhao et al., 2009), and 15 WOX genes, including WUSCHEL, have been identified in Arabidopsis. Interestingly, Os01g0840300 has been reported to be a rice orthologue of Arabidopsis WOX2 (Deveaux et al., 2008). WOX2 transcripts accumulate in Arabidopsis zygotes and are restricted to the apical cell of two-celled proembryos (Haecker et al., 2004). In addition, WOX2 has been proposed to be the predominant regulator of apical patterning (Jeong et al., 2011). In this study, Os01g0840300, a putative rice orthologue of AtWOX2, was identified as a fertilization-induced gene, which may have a role in determining cell fate during early embryogenesis in rice.

Effects of inhibitors of DNA methyltransferase on zygotic development and early embryogenesis

The gene Os07g0182900, putatively encoding DNA methyltransferase 1 (MET1), which functions in maintaining CG DNA methylation (Kankel et al., 2003), was identified among the highly up-regulated genes in early zygotes (Table 4, Fig. 4). In Arabidopsis, expression of MET1 is suppressed in egg and central cells at the end of female gametophyte development via the RETINOBLASTOMA-RELATED 1 (RBR1) pathway (Jullien et al., 2008, 2012). After fertilization, MET1 expression resumes in young embryos to maintain DNA methylation (Jullien and Berger, 2010; Jullien et al., 2012). In addition, Xiao et al. (2006) showed that Arabidopsis embryos with loss-of-function mutants in both MET1 and CHROMOMETHYLASE 3 (CMT3) develop incorrectly and that genes specifying cell identity are misexpressed in abnormal met1 embryos. These reports suggest that induction of MET1 expression in zygotes would be important for zygotic development and early embryogenesis in rice as well. Therefore, to observe the function of MET1 during zygotic development immediately after gamete fusion, zygotes produced by electrofusion of a rice sperm cell with an egg cell...
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were cultured with or without RG108, a specific inhibitor of MET1 (Brueckner et al., 2005; Stresemann et al., 2006).

Whether RG108 affected GC-methylation status was first checked using cultured rice cells. Two of seven tested loci appeared to show decreased methylation levels when RG108 was applied to the culture medium, although differences in methylation status with and without RG108 could not be detected for the remaining five loci (Supplementary Fig. S5 at JXB online). These data suggest that RG108 partially or weakly affected the CG-methylation status of some loci in cultured rice cells.

Next, rice zygotes were produced by IVF in which H2B–GFP fusion protein was heterologously expressed, and the nuclei were visualized (Fig. 5A). Expression of the putative MET1 gene (Os07g0182900) in IVF-produced zygotes was confirmed by PCR (Supplementary Fig. S6 at JXB online). The zygotes expressing H2B–GFP were cultured with or without 100 μM RG108, and zygotic development was compared. Two nucleoli were observed in zygotes cultured with and without inhibitor at 5 h after fusion (Fig. 5B). Approximately 1 d after fusion, the zygotes cultured with the inhibitor divided unequally into two-celled proembryos with small apical cells and large basal cells, similar to zygotes cultured without inhibitor. Notably, among 14 two-celled proembryos cultured with inhibitor, several proembryos clearly showed extreme asymmetry (Fig. 5B), and the apical and basal cells were of similar sizes.

**Table 4.** Twenty genes whose expression levels were most putatively up-regulated in zygotes after fertilization.

<table>
<thead>
<tr>
<th>Locus</th>
<th>RAPDB description</th>
<th>Fold change (zygote/egg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os01g0840300</td>
<td>Similar to WUSCHEL-related homeobox 5</td>
<td>44.9</td>
</tr>
<tr>
<td>Os05g0571200</td>
<td>Similar to WRKY transcription factor 19</td>
<td>41.1</td>
</tr>
<tr>
<td>Os01g0841700</td>
<td>Similar to Isoform ERG1b of Elicitor-responsive protein 1</td>
<td>39.6</td>
</tr>
<tr>
<td>Os07g0182900</td>
<td>Similar to Cytosine-5 DNA methyltransferase MET1</td>
<td>35.7</td>
</tr>
<tr>
<td>Os02g0258200</td>
<td>High mobility group, HMG1/HMG2 domain-containing protein</td>
<td>35.2</td>
</tr>
<tr>
<td>Os02g0462800</td>
<td>WRKY transcription factor 42</td>
<td>29.9</td>
</tr>
<tr>
<td>Os01g0895600</td>
<td>Similar to Calvinicul 3</td>
<td>29.1</td>
</tr>
<tr>
<td>Os03g0279200</td>
<td>Similar to Histone H2A</td>
<td>27.5</td>
</tr>
<tr>
<td>Os10g0580900</td>
<td>Conserved hypothetical protein</td>
<td>24.8</td>
</tr>
<tr>
<td>Os05g0127300</td>
<td>Serine/threonine protein kinase domain-containing protein</td>
<td>23.0</td>
</tr>
<tr>
<td>Os08g0562800</td>
<td>Similar to Transparent testa 12 protein</td>
<td>19.4</td>
</tr>
<tr>
<td>Os03g0214100</td>
<td>Replication protein A1</td>
<td>18.7</td>
</tr>
<tr>
<td>Os03g0188500</td>
<td>Glutelin family protein</td>
<td>18.6</td>
</tr>
<tr>
<td>Os01g0551000</td>
<td>Conserved hypothetical protein</td>
<td>16.3</td>
</tr>
<tr>
<td>Os02g0572600</td>
<td>Protein kinase PKN/PRK1, effector domain-containing protein</td>
<td>16.0</td>
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<tr>
<td>Os02g0467500</td>
<td>Hypothetical conserved gene</td>
<td>15.6</td>
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<tr>
<td>Os03g0116400</td>
<td>Similar to Membrane protein</td>
<td>15.2</td>
</tr>
<tr>
<td>Os04g0177300</td>
<td>DEAD-like helicase, N-terminal domain-containing protein</td>
<td>14.6</td>
</tr>
<tr>
<td>Os03g0268800</td>
<td>MAP kinase</td>
<td>14.6</td>
</tr>
<tr>
<td>Os01g0000000</td>
<td>3’–5’ exonuclease domain-containing protein</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Mean t-test P-values < 0.05.

**Fig. 4.** Expression patterns of 20 genes whose expression levels were putatively induced after fertilization in rice zygotes. Semi-quantitative RT–PCR was performed on total RNAs isolated from egg cells and zygotes using primers specific for the putatively up-regulated genes listed in Table 4. Ubiquitin mRNA was used as an internal control. The numbers in parentheses indicate the number of PCR cycles. See Supplementary Table S1 at JXB online for primer sequences.
basal cells appeared to be more asymmetric in volume in inhibitor-treated two-celled proembryos than in untreated proembryos. Therefore, the axis lengths of the apical and basal cells in two-celled proembryos were next measured. As for two-celled proembryos cultured without inhibitor, the ratio of axis length between the apical and the basal cells was determined as 0.44:0.56 (n=6) when the combined axis length of both cells was considered as 1 (Fig. 5C). In the case of proembryos cultured with inhibitor, the ratio of axis length was calculated to be 0.37:0.63 (n=14), indicating the effect of the inhibitor on asymmetry of the two-celled proembryo. The two-celled proembryos cultured with inhibitor,
however, grew into normal globular embryos of 10–20 cells during subsequent culture for 1–2 d (Fig. 5B). These results suggested that establishment of polarity or asymmetric cell division was partly affected by the MET inhibitor, but cells can recover from the unusual asymmetry during early embryonic development. These findings may be consistent with the phenotype of the homologous met1 mutant in Arabidopsis. In the mutant, ~13% of two-celled embryos had abnormal division patterns but eventually grew into mature embryos (Xiao et al., 2006). This phenotypic recovery during Arabidopsis and rice embryogenesis can be explained by redundancy of DNA methylation, including CNG methylation by CHROMOMETHYLASE 3 (Xiao et al., 2006; Jullien et al., 2012), de novo CHH methylation by DOMAINS REARRANGED METHYLTRANSFERASES (Jullien et al., 2012), and the RNA-directed DNA methylation (RdDM) pathway (Mathieu et al., 2007). Alternatively, it is also suggested that MET1 is not a key regulator for zygote development, although it is highly up-regulated in zygotes after fertilization.

**Conclusion**

In this study, up- or down-regulated genes in rice zygotes after fertilization and genes enriched in gametes were identified. Addressing the functions of these genes during gametic and/or early zygotic developmental processes will improve our knowledge of these processes. Analyses using mutant plants for several of these genes are currently underway in the authors’ laboratories.

**Supplementary data**

Supplementary data are available at *JXB* online.

- **Figure S1.** Isolation of rice egg cells from ovaries.
- **Figure S2.** Assessment of RNA extracted from rice gametes and zygotes.
- **Figure S3.** Microarray correlation plot within and among rice cell types.
- **Figure S4.** Change of transcript levels in zygotes, compared with egg cells. Overview display of genes assigned to metabolism and regulation.
- **Figure S5.** Effects of RG108 on DNA methylation status on some transposon-related elements in cultured rice cells.
- **Figure S6.** Expression of a putative MET1 gene in a zygote produced by IVF.
- **Table S1.** DNA primers used for RT–PCR and quantitative PCR of rice reproductive cells or cultured cells.
- **Table S2.** Genes expressed in rice sperm cells.
- **Table S3.** Genes expressed in rice egg cells.
- **Table S4.** Genes expressed in rice zygotes.
- **Table S5.** Rice genes whose expression levels in sperm cells were at least 10-fold higher than in egg cells.
- **Table S6.** Rice genes whose expression levels in egg cells were at least 10-fold higher than in sperm cells.
- **Table S7.** Rice genes putatively down-regulated in zygotes after fertilization.
- **Table S8.** Enriched gene ontologies among rice genes that are putatively down-regulated in zygotes.
- **Table S9.** Rice genes putatively up-regulated in zygotes after fertilization.
- **Table S10.** Enriched gene ontologies among rice genes that are putatively up-regulated in zygotes.

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