Chapter 30
Gene and Protein Expression Profiles in Rice Gametes and Zygotes: A Cue for Understanding the Mechanisms of Gametic and Early Zygotic Development in Angiosperms

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Abstract In angiosperms, female gamete differentiation, fertilization, and subsequent zygotic development occur in embryo sacs deeply embedded in the ovaries. Despite their importance in plant reproduction and development, how the egg cell is specialized, fuses with the sperm cell, and converts into an active zygote for early embryogenesis remains unclear. This lack of knowledge is partly attributable to the difficulty of direct analyses of gametes and zygotes in angiosperms. Cell type-specific transcriptomes were obtained by microarray analyses for egg cells, sperm cells and zygotes isolated from rice flowers, and up- or down-regulated genes in zygotes after fertilization were identified as well as genes enriched in male and female gametes. In addition to transcriptome, proteins expressing in egg and sperm cells were globally detected by highly sensitive liquid chromatography coupled with tandem mass spectroscopy technology, and proteins that are specifically or predominantly expressing in gametes were also identified by comparison of protein expression profiles between gametes and somatic cells/pollen grains. Several rice or Arabidopsis lines with mutations in genes identified by these proteome/transcriptome analyses showed clear phenotypic defects in seed set or seed development. These findings suggest that the cell type-specific proteome/transcriptome data for gametes/zygotes are foundational information toward understanding the mechanisms of gametic and early zygotic development in angiosperms.

Keywords Egg cell • Fertilization • Oryza sativa • Proteome • Sperm cell • Transcriptome • Zygote
30.1 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). The conversion of the egg cell into the zygote is completed by two serial gametic processes: plasmogamy, the fusion of the plasma membrane between male and female gametes, and karyogamy, fusion of the nuclei of the male and female gametes in the fused gamete. Thereafter, the zygotic genome switches on within hours of fertilization for subsequent development of zygotes (Meyer and Scholten 2007; Zhao et al. 2011; Nodine and Bartel 2012).

As for molecular players of plasmogamy, GENERATIVE CELL-SPECIFIC 1/HAPLESS 2 (GCS1/HAP2) and EGG CELL 1 (EC1) have been identified as putative fusigens for male and female gametes, respectively (Mori et al. 2006; von Besser et al. 2006; 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. Recently, Sprunck et al. (2012) indicated that small cysteine-rich EC1 proteins accumulated in storage vesicles in the Arabidopsis egg cell 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. In addition to these two possible fusigens, other players should be identified to understand the mechanisms in plasmogamy.

Karyogamy is accompanied by the congression of the male nucleus to the female nucleus and subsequent nuclear fusion. Yeast mating is the most intensively investigated karyogamy event, and cytoskeleton-dependent nuclear congression and chaperone/ER-protein-dependent nuclear fusion have been well studied (Kurihara et al. 1994; Melloy et al. 2009; Tartakoff and Jaiswal 2009). However, in angiosperms, the mechanism in karyogamy is poorly understood, except that Bip, a chaperone in the lumen of endoplasmic reticulum, and NFD1, a component of the mitochondrial ribosome, function in polar nuclei fusion in Arabidopsis (Portereiko et al. 2006; Maruyama et al. 2010).

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 or transformants coupled with live-imaging (Berger 2011; Hamamura et al. 2012). Alternatively, direct analyses using isolated gametes or zygotes are possible because procedures for isolating viable gametes have been established, and an in vitro fertilization (IVF)
system using the isolated gametes can be used to observe and analyze fertilization and postfertilization processes directly (Wang et al. 2006).

It has been supposed that genes specifically/predominantly expressing in gamete function in reproductive or developmental processes such as gamete differentiation, gamete fusion, and early zygotic development. Therefore, using isolated gametes or embryos, several studies have successfully identified genes specifically expressed in male gametes, female gametes, or early embryos (Kasahara et al. 2005; Márton et al. 2005; Sprunck et al. 2005; Ning et al. 2006; Yang et al. 2006; Steffen et al. 2007; Borges et al. 2008; Amien et al. 2010; Wang et al. 2010; Wuest et al. 2010; Ohnishi et al. 2011). Moreover, changes in gene expression from prefertilization to postfertilization phases were recently monitored using microarray-based transcriptome analyses of rice sperm cells, egg cells, and zygotes using the same experimental platform (Abiko et al. 2013b). In addition to gene expression profiles, single-cell-type proteomic approaches have been widely employed to dissect the functions of specific cells, because cellular-level information is diluted when organs or tissues, which comprise various differentiated cells, are used as starting materials (Dai and Chen 2012). However, such global proteomic analyses have not been conducted for plant gametes, possibly because of the difficulty in obtaining sufficient highly pure homogeneous cells, especially for egg cells. However, state-of-the-art proteomics technologies enable high-throughput and high-resolution analyses using such limited numbers of cells, and recently proteins expressing in the rice gamete were globally identified (Abiko et al. 2013a).

### 30.2 Isolation of Plant Gametes and IVF

Procedures for isolating viable gametes have been reported for a wide range of plant species, including maize, wheat, tobacco, rape, rice, barley, *Plumbago zeylanica*, *Alstroemeria*, and *Arabidopsis* (Kranz 1999; Okamoto 2011). IVF systems using isolated male and female gametes have been utilized to dissect fertilization-induced events. The IVF system used for angiosperms includes a combination of three basic microtechniques: (1) the isolation and selection of male and female gametes, (2) the fusion of pairs of gametes, and (3) single-cell culture (Kranz 1999). A complete IVF system was first developed by Kranz and Lörz (1993) using maize gametes and electrical fusion, and a rice IVF system was also established to take advantage of the abundant resources stemming from rice research, including the whole genome sequence and abundant mutant stocks (Fig. 30.1; Uchiumi et al. 2007b). These IVF systems have been successfully used to observe and analyze postfertilization events, such as karyogamy in zygotes (Faure et al. 1993), egg/zygote activation and development (Kranz et al. 1995), decondensation of paternal chromatin in zygotes (Scholten et al. 2002), changes in the microtubular architecture in zygotes (Hoshino et al. 2004), fertilization-induced/suppressed gene expression (Okamoto et al. 2005), epigenetic resetting in early embryos (Jahnke and Scholten 2009), positional relationship between gamete fusion point and zygotic development (Nakajima et al. 2010), and asymmetrical division of zygotes (Sato et al. 2010).
30.3 Gene Expression Profiles in Rice Gametes and Zygotes

30.3.1 Genes Enriched in Rice Gametes

Cell type-specific transcriptomes were obtained by microarray analyses using 33 to 111 egg cells (33, 34, and 111 egg cells; three biological replicates), 30 and 34 zygotes (two biological replicates), approximately 3,000 sperm cells (two biological replicates), and approximately 100 pollen grains (three biological replicates); subsequent
data processing resulted in identification of 14 and 19 genes with expression profiles specific to egg cells and sperm cells, respectively (Tables 30.1, 30.2).

A gene enriched in egg cells, Os11g0187600, encodes heat shock protein 70 (HSP70). In addition to HSP70, HSP90 was identified as a major protein component of rice egg cells by previous proteomic analysis (Uchiumi et al. 2007a). Interestingly, Calvert et al. (2003) revealed that mouse eggs contain molecular chaperones, including HSP90, HSP70, and protein disulfide isomerase (PDI), as major protein components. An abundance of HSP proteins may be a common characteristic of mammalian and plant eggs. Among pleiotropic functions of HSPs, it will be notable that they play a role in buffering the expression of genetic variation when divergent ecotypes are crossed and profoundly affect developmental plasticity in response to environmental cues (Queitsch et al. 2002; Sangster and Queitsch 2005). 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, the DNA-binding proteins that regulate their own transcription and that of target genes (West et al. 1998), act early in organ development (Riechmann and Meyerowitz 1997; Theissen et al. 2000). Os07g0108900, encoding MADS-box transcription factor 15 (OsMADS15), was egg enriched, and, ZmMADS3, which is orthologous gene to OsMADS15, was also strongly expressed in maize egg cells (Heuer et al. 2001). Although their function in egg cells is still unclear, MADS-box proteins that accumulate in female gametes may have roles in egg-cell differentiation during gametophytogenesis or zygotic development after fertilization.

Among 19 genes enriched in sperm cells, 9 were annotated as hypothetical proteins or genes (Table 30.2), being consistent with a previous report indicating

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**Table 30.1 Genes enriched in rice egg cells**

| Gene locus       | Annotation                                           | Expression in egg cell |
|------------------|------------------------------------------------------|------------------------|
| Os03g0296600     | Similar to ECA1 protein                             | 8.82                   |
| Os05g0491400     | Similar to LRR protein                              | 6.25                   |
| Os07g0574500     | Ubiquitin domain-containing protein                 | 5.58                   |
| Os04g0289600     | Allergen V5/Tpx-1-related family protein            | 4.65                   |
| Os01g0299700     | 3′-5′-Exonuclease domain-containing protein         | 4.45                   |
| Os06g0602400     | Similar to DEAD-box protein 3, X-chromosomal        | 4.26                   |
| Os11g0187600     | Similar to heat shock protein 70                    | 3.65                   |
| Os07g0108900     | Similar to MADS-box transcription factor 15         | 3.02                   |
| Os07g0136300     | Conserved hypothetical protein                       | 2.80                   |
| Os03g0679800     | Similar to TPR domain-containing protein            | 2.95                   |
| Os10g0560200     | Protein of unknown function Cys-rich family protein | 2.93                   |
| Os01g0350500     | Conserved hypothetical protein                       | 2.66                   |
| Os11g0579900     | Armadillo-like helical domain-containing protein     | 2.38                   |
| Os05g0153200     | Region of unknown function XH domain-containing     | 2.28                   |
|                  | protein                                              |                        |

Values are average of binary log values of two biological replicates
Annotations are referred from The Rice Annotation Project Database (RAPDB)
Mean t test P values < 0.05
enrichment of genes encoding proteins with unknown function in sperm-specific genes (Russell et al. 2012). Os03g0661900 encodes a trypsin-like serine protease, and, 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). 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.

### 30.3.2 Genes Down- or Up-Regulated in Rice Zygotes After Fertilization

Egg cells are developmentally quiescent, a state that is broken after fertilization and subsequent egg activation. Genes down-regulated after fertilization in zygotes were searched because the expression of genes involved in maintaining egg-cell quiescence should be suppressed in zygotes. Ninety-four genes that had threefold-lower

| Gene locus     | Annotation                                      | Expression in sperm cell |
|----------------|-------------------------------------------------|--------------------------|
| Os01g0605400   | Quinon protein alcohol dehydrogenase-like domain-containing protein | 6.21                     |
| Os06g0715300   | Similar to CEL5                                  | 5.72                     |
| Os02g0664400   | Hypothetical conserved gene                      | 5.04                     |
| Os01g0180900   | Similar to oxidoreductase                        | 5.08                     |
| Os07g0634100   | Hypothetical conserved gene                      | 4.60                     |
| Os10g0550400   | Hypothetical conserved gene                      | 4.13                     |
| Os03g0809200   | Similar to transcription factor EmBP-1           | 4.22                     |
| Os01g0876100   | Similar to chloride channel                      | 3.61                     |
| Os01g0855600   | Conserved hypothetical protein                   | 3.47                     |
| Os03g0661900   | Peptidase, trypsin-like serine, and cysteine domain-containing protein | 3.18                     |
| Os11g0601600   | Protein of unknown function DUF248              | 3.14                     |
| Os02g0177400   | Conserved hypothetical protein                   | 3.03                     |
| Os05g0393800   | Protein of unknown function DUF221 domain-containing protein | 2.45                     |
| Os08g0266700   | Rad21/Rec8-like protein, C-terminal domain-containing protein | 2.28                     |
| Os09g0483200   | Similar to UBQ13 (ubiquitin 13)                  | 2.12                     |
| Os09g0244200   | Conserved hypothetical protein                   | 1.96                     |
| Os11g0620800   | Hypothetical conserved gene                      | 1.46                     |
| Os02g0628100   | Hypothetical gene                                | 1.22                     |
| Os08g0474400   | Hypothetical conserved gene                      | 1.19                     |

Values are average of binary log values of two biological replicates
Annotations are referred from The Rice Annotation Project Database (RAPDB)
Mean t test P values < 0.05
expression levels in zygotes than in egg cells were obtained, and most ontologies for these genes were related to metabolic or biosynthetic processes, including terpene, flavonoid, and amino-acid synthetic pathways (Abiko et al. 2013b).

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 to egg cells, indicated that synthetic pathways for cell wall, auxin and ethylene and signal transduction pathways appeared to be activated via fertilization. A total of 325 genes whose expression levels in zygotes were threefold higher than those in egg cells were identified, and genes related to chromatin and DNA organization and assembly were well represented among these up-regulated genes (Table 30.3). The gene Os07g0182900, encoding DNA methyltransferase 1 (MET1), which functions in maintaining CG DNA methylation (Kankel et al. 2003), was identified among the highly up-regulated genes, and the specific inhibitor for the enzyme partly affected polarity or division asymmetry in rice zygotes (Abiko et al. 2013b). In addition, several genes encoding homeobox protein or transcription factors were strongly induced in zygotes. Os01g0840300 encodes a Wuschel-related homeobox (WOX) protein, the key regulator in determining cell fate in plants (Mayer et al. 1998; Haeker et al. 2004; Zhao et al. 2009), and 15 WOX genes, including *WUSCHEL*, have been identified in *Arabidopsis*. Interestingly, Os01g0840300 has been reported as the rice orthologue of *Arabidopsis* WOX2 (Deveaux et al. 2008), whose

| Gene locus    | Annotations                                                                 | Fold change (zygote/egg) |
|--------------|-----------------------------------------------------------------------------|--------------------------|
| Os01g0840300 | Similar to WUSCHEL-related homebox 5                                        | 44.9                     |
| Os05g0571200 | Similar to WRKY transcription factor 19                                     | 41.1                     |
| Os01g0841700 | Similar to isoform ERG1b of elicitor-responsive protein 1                    | 39.6                     |
| Os07g0182900 | Similar to cytosine-5-DNA methyltransferase MET1                            | 35.7                     |
| Os02g0258200 | High-mobility group, HMG1/HMG2 domain-containing protein                    | 35.2                     |
| Os02g0462800 | WRKY transcription factor 42                                                | 29.9                     |
| Os01g0895600 | Similar to calreticulin 3                                                   | 29.1                     |
| Os03g0279200 | Similar to histone H2A                                                      | 27.5                     |
| Os10g0580900 | Conserved hypothetical protein                                              | 24.8                     |
| Os05g0127300 | Serine/threonine protein kinase domain-containing protein                   | 23.0                     |
| Os08g0562800 | Similar to transparent testa 12 protein                                     | 19.4                     |
| Os03g0214100 | Replication protein A1                                                     | 18.7                     |
| Os03g0188500 | Glutelin family protein                                                     | 18.6                     |
| Os01g0551000 | Conserved hypothetical protein                                              | 16.3                     |
| Os02g0572600 | Protein kinase PKN/PRK1, effector domain-containing protein                 | 16.0                     |

Annotations are referred from The Rice Annotation Project Database (RAPDB)
Mean t test P values < 0.05
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), suggesting WOX proteins encoded by *Os01g0840300* may have a role in determining cell fate during early embryogenesis in rice.

### 30.4 Protein Expression Profiles in Rice Gametes

Lysates from 500 egg cells and $3 \times 10^4$ sperm cells were separated by one-dimensional polyacrylamide gel electrophoresis. Proteins in gel were digested with trypsin and identified by a direct nanoflow LC-MS system equipped with an Orbi Trap XL mass spectrometer. The proteins were judged as “identified” if at least two peptides were identified from the protein. Proteome analyses were also conducted for seedlings, callus, and pollen grains to compare their protein expression profiles to those of gametes. By analyzing proteins from egg and sperm cell lysates, 1,276 and 1,076 proteins were identified, respectively. In callus, seedlings, and pollen grains, 1,641, 1,329, and 1,274 proteins were detected, respectively. Putative proteins specifically or predominantly expressing in egg or sperm cells were chosen on a basis of comparison of the number of matched peptides in egg or sperm cells with those in other cell types. In total, 102 and 73 putative proteins were identified as egg- or sperm-specific or predominant proteins, respectively (Abiko et al. 2013a). Table 30.4 presents putative gamete-specific/predominant proteins with more than five matched peptides. Notably, except for HSP 70 (HSP70), none of these proteins has been reported to play a role in reproductive or developmental processes, suggesting that investigating these proteins further may uncover novel molecular mechanisms during gametic development and fusion and early embryogenesis.

### 30.5 Conclusion

Functional defects in proteins, whose expressions are specific/predominant in gametes or up- or down-regulated after fertilization, are supposed to affect reproductive or developmental processes. In fact, several rice or *Arabidopsis* lines with mutations in genes encoding the putative gamete-specific or -predominant proteins showed clear phenotypic defects in seed set or seed development (Fig. 30.2), suggesting that the cell type-specific proteome and transcriptome data for gametes and zygotes are foundational information toward understanding the mechanisms of gametic and zygotic development in angiosperms.
### Table 30.4  Proteins specifically or predominantly expressed in egg or sperm cells with more than five matched peptides

| cDNA accession | Gene locus     | Number of matched peptide | Annotations                                              |
|----------------|----------------|----------------------------|----------------------------------------------------------|
| AK106474       | Os06g0602400   | 15 0 0 0 1                | Similar to DEAD-box protein 3, X-chromosomal             |
| AK101183       | Os05g0168800   | 11 0 1 0 0                | KIP1-like domain-containing protein                      |
| AK065887       | Os03g0283100   | 11 0 1 0 0                | Similar to In2-1 protein                                 |
| AK063589       | Os05g0115600   | 9 0 0 0 0                 | Protein of unknown function DUF674 family protein        |
| AK106371       | Os03g0276800   | 9 0 0 0 0                 | Heat shock protein Hsp70 family protein                  |
| AK063560       | Os12g0600100   | 8 0 0 0 0                 | Tetra-tripeptide-like helical domain-containing protein  |
| AK067215       | Os01g0698000   | 8 0 0 0 0                 | Conserved hypothetical protein                           |
| AK121612       | Os02g0717400   | 8 0 0 0 1                 | Tetra-tripeptide-like helical domain-containing protein  |
| AK058611       | Os01g0895100   | 7 0 0 0 0                 | Similar to membrane-associated 30-kDa protein, chloroplast|
| Os06t0706700-01| Os06g0706700   | 7 0 0 0 0                 | Similar to PsAD1                                          |
| AK073477       | Os01g0369200   | 7 0 0 0 0                 | Similar to cullin-1                                     |
| AK106478       | Os01g0771100   | 7 0 1 0 0                 | Mitochondrial glycoprotein family protein                |
| AK107844       | Os05g0143600   | 6 0 0 0 0                 | Similar to jasmonate-induced protein                     |
| AK072587       | Os05g0164900   | 6 0 0 0 0                 | Galactose oxidase kelch, beta-propeller domain-containing protein|
| AK072334       | Os03g0583900   | 5 0 0 0 0                 | DEAD-like helicase, N-terminal domain-containing protein |
| AK119521       | Os06g0175800   | 5 0 0 0 0                 | Similar to cystathionine beta-lyase, chloroplast precursor|
| AK072719       | Os10g0574800   | 5 0 0 0 0                 | Similar to ARF GAP-like zinc finger-containing protein ZIGA2 |
| AK064995       | Os12g0197500   | 5 0 0 0 0                 | Putative zinc finger, XS, and XH domain-containing protein|
| Os01t0267600-01| Os01g0267600   | 1 10 0 0 0               | Sad1/UNC-like, C-terminal domain-containing protein       |
| AK071495       | Os11g0255300   | 0 9 0 0 0                 | Cysteine endopeptidase                                   |
| AK071561       | Os05g0163700   | 0 7 0 0 0                 | Similar to acyl-coenzyme A oxidase 4, peroxisomal        |
| AK065231       | Os01g0323100   | 0 7 0 0 0                 | Similar to Pto kinase interactor 1                       |
| AK107034       | Os02g0185200   | 0 6 0 0 0                 | Cytochrome P450 family protein                           |
| AK065311       | Os06g0174400   | 0 6 0 1 0                 | Similar to vesicle-associated membrane protein 712      |
| Os040611200-00  | Os04g0611200   | 0 6 0 1 0                 | Similar to OSGBa0152L12.11 protein                        |
| AK069025       | Os04g0569000   | 1 6 0 0 0                 | Similar to activator 1 40-kDa subunit                    |

(continued)
| cDNA accession | Gene locus     | Number of matched peptide | Annotations                                           |
|---------------|---------------|---------------------------|-------------------------------------------------------|
| AK066587      | Os03g0220100  | 0 5 0 0 0                | Similar to very long chain fatty acid-condensing enzyme CUT1 |
| AK099178      | Os02g0726000  | 0 5 0 0 0                | FAS1 domain-containing protein                         |
| AK105867      | Os02g0608900  | 0 5 0 0 0                | Epstein–Barr virus U2-IR2 domain-encoding nuclear protein |
| AB087745      | Os05g0595100  | 0 5 1 0 0                | Similar to UDP-glucose-4-epimerase                      |
| AK069984      | Os02g0775200  | 1 5 0 0 0                | Similar to activator 1 36-kDa subunit                   |

Matched peptides indicate the number of MS-identified tryptic peptides of the protein
Annotations are referred from The Rice Annotation Project Database (RAPDB)
E egg cell, S sperm cell, C callus, L seedling, P pollen grain
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Fig. 30.2 Rice (a) and Arabidopsis (b) mutants showing defects in seed set or seed development. (a) Fertility of a rice TOS17 transposon insertional line (ND8460) for Os11g0143400, a sperm cell-enriched gene. In panicles of wild-type (Nipponbare), more than 95% of seeds developed fully with light brown color. In the mutants, undeveloped seeds were often observed. Two typical undeveloped seeds are indicated by arrowheads in each panel. (b) Dissected siliques of wild-type (Colombia 0) and T-DNA insertional lines (SALK_095847) for At4g02060, a gene orthologous to the rice gene (Os12g0560700) encoding a sperm-specific protein. Failed ovules or seeds arrested at early immature stages are visible in the mutant siliques.
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