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

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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 fusogens 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 fusogens, 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).

Fig. 30.1 Isolation of gamete from rice flowers (a–g), in vitro fusion of gametes (h–k), early development of zygote produced by in vitro fertilization into a globular embryo (l–p), and development and regeneration of globular embryos (q–u). (a) Rice plants. (b) Dissected rice flower. (c) Isolated ovary. Red line indicates incision line for egg isolation. (d) Cut ovary. (e) Rice egg cell being released from basal portion of dissected ovary. (f) Isolated egg cell. (g) Two sperm cells released from a pollen grain. (h–k) Serial images for electro-fusion of a sperm cell with an egg cell. Gametes were aligned on one of the electrodes under an alternating current field, and the aligned egg and sperm cells are fused by a negative direct current pulse. Arrowheads indicate sperm cell or fusion point. (l) Zygote 1 h after fusion. (m) Zygote 4 h after fusion. Two nucleoli are indicated by arrowheads. (n) Asymmetrical two-celled embryo. (o, p) Nuclear staining of globular embryo, visualized by bright-field and fluorescence microscopy, respectively. (q) Cell mass developed from globular-like embryo. (r) White cell colony from cell mass in q. (s) Developed cell colony after transferring white cell colony into regeneration medium. Green spots are visible on the cell colony. (t) Regenerated shoots from green spots in s. (u) A plantlet after subculturing a regenerated shoot

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

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 protein	2.28

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

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*, is 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

Table 30.2 Genes enriched in rice sperm cells

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
Os01g0853600	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

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

Table 30.3 Fifteen genes whose expression levels were most putatively up-regured in zygotes after fertilization

Gene locus	Annotations	Fold change (zygote/egg)
Os01g0840300	Similar to WUSCHEL-related homeobox 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

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-regured 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-regured 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; Haecker 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

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 3×10^4 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 Orbitrap 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
		E	S	C	L	P	
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	Tetratricopeptide-like helical domain-containing protein
AK067215	Os01g0698000	8	0	0	0	0	Conserved hypothetical protein
AK121612	Os02g0717400	8	0	0	0	1	Tetratricopeptide-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
Os04t0611200-00	Os04g0611200	0	6	0	1	0	Similar to OSIGBa0152L12.11 protein
AK069025	Os04g0569000	1	6	0	0	0	Similar to activator 1 40-kDa subunit

(continued)

Table 30.4 (continued)

cDNA accession	Gene locus	Number of matched peptide					Annotations
		E	S	C	L	P	
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

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 (Columbia 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 silique

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