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Differential gene expression in egg cells and zygotes suggests that the transcriptome is restructed before the first zygotic division in tobacco

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Abstract **We applied suppression subtractive hybridization and mirror orientation selection to compare gene expression profiles of isolated *Nicotiana tabacum* cv SR1 zygotes and egg cells. Our results revealed that many differentially expressed genes in zygotes were transcribed de novo after fertilization. Some of these genes are critical to zygote polarity and pattern formation during early embryogenesis. This suggests that the transcriptome is restructed in zygote and that the maternal-to-zygotic transition happens before the first zygotic division, which is much earlier in higher plants than in animals. The expressed sequence tags used in this study provide a valuable resource for future research on fertilization and early embryogenesis.**

Keywords: Egg cell; Fertilization; Gene expression; Mirror orientation selection; Suppression subtractive hybridization; Zygote; Zygotic gene activation

1. Introduction

Fertilization occurs when a sperm penetrates an egg cell. The fertilized egg cell, or zygote (one-cell embryo), then undergoes a series of complex morphological and physiological changes. Among these changes, a critical event for the onset of early embryogenesis is zygotic gene activation (ZGA), which denotes the de novo transcription at the beginning of embryogenesis. In animals, the timing of ZGA is regulated by a ''zygotic clock'' and is somewhat species-dependent. The established theory is that the unfertilized egg cells of animals store abundant mRNAs encoding the proteins that sustain early embryo development, and the onset of global ZGA occurs much later after fertilization, even up to the 16-cell embryo stage [1–4].

In contrast to animals, fertilization and embryogenesis in higher plants occur deep within the maternal tissues of the ovule. Although several laboratories have worked with a variety of plant species, little is known about the timing of ZGA and maternal-to-zygotic transition during fertilization and early embryogenesis because of the difficulty in accessing female gametes, zygotes, and early embryos. Microarray analyses of precocious embryonic development in apomictic hybrids between maize and its wild relative *Tripsacum* have shown that early embryo development occurs without significant changes in the transcript population in the unfertilized ovule of sexual maize, suggesting that the maternal-to-zygotic transition does not occur until at least 3 days after fertilization when proembryos have already formed [5].

Different evidence has also been presented. Fertilization of wild-type egg cells with transgenic pollen results in detectable GUS activity during early embryogenesis in *Arabidopsis* [6,7]. In maize, a transgene driven by a 35S promoter in the paternal genome is almost immediately transcribed and translated in the zygote [8]. In *Arabidopsis*, an investigation of the timing of transgene activation after fertilization has also shown de novo GUS activity in zygotes [9]. An analysis of individual gene expression in in vitro fertilized egg cells of maize indicated that ZGA occurs earlier in plants than in animal systems [10,11]. Genes upregulated in the apical or basal cells after in vitro fertilization are already expressed in the early zygotes, but not in egg cells, implying that expression is initiated in maize zygotes [12]. As the only example, a global study of the transcript profile of wheat egg cells and two-celled proembryos has shown that the transcription composition of two-celled proembryos is distinct from that of egg cells [13]. To date, most available data have been from various studies of individual genes. These distinct data do not present an overview of differentially expressed genes in fertilized egg cells. Also, to our knowledge, there is no direct evidence showing that endogenous genes are actively and widely transcribed in zygotes of dicotyledonous plants.

To better understand the molecular regulation of fertilization, we have developed reliable techniques to isolate healthy eggs and zygotes from tobacco to investigate gene expression in egg cells before and after in vivo fertilization. In this study, we constructed tobacco egg and zygote cDNA libraries and contrasted their mRNA expression profiles to reveal genes that are differentially expressed using suppression subtractive hybridization (SSH) [14,15]. To identify truly differentially expressed genes, we used mirror orientation selection (MOS), which is efficient for eliminating background molecules in the subtracted library [16].

2. Materials and methods

2.1. Isolation of eggs, zygotes and sperm cells

Egg cells were isolated from tobacco (*Nicotiana tabacum* cv SR1) ovules 40 h after pollination (HAP) as previously described [17], and zygotes were isolated from ovules 96 HAP using a method of enzymatic maceration combined with grinding [18]. Sperm cells were isolated as described [19]. To inhibit the modulation of gene expression in response to possible stresses during physical isolation and enzymatic

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digestion, two transcription inhibitors, actinomycin D (50 mg/L) and cordycepin (100 mg/L), were applied to all solutions in the isolation process. Single cells were transferred to 2× lysis/binding buffer in 0.2-ml tubes and frozen immediately in liquid nitrogen for later use.

2.2. mRNA isolation and cDNA synthesis

Sixty-one egg cells and zygotes, respectively, to be used for SSH were lysed, and mRNA was isolated using a Dynalbeads mRNA DIRECT Micro kit (Dynal Biotech, Oslo, Norway), following the manufacturer's instructions (although the annealing volume was reduced to 50 μl for semiquantitative RT-PCR). A SMART cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA) was used to reverse-transcribe mRNA isolated from eggs and zygotes and amplify cDNA. Each individual amplification required 23 PCR cycles.

2.3. Suppression subtractive hybridization and mirror orientation selection

SSH was performed with a PCR-Select cDNA Subtraction kit (Clontech) according to the manufacturer's recommendations but with modifications for MOS [16]. MOS cDNA was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into DH5α for differential screening.

2.4. Reverse cDNA dot-blot analysis

Clones obtained by SSH and MOS were further analyzed by dot-blot analysis. Randomly selected clones were arrayed in 96-well microtiter dishes with 200 μl of Luria–Bertani broth containing ampicillin and cultured overnight on a shaker. We used 1 μl of the culture for PCR with the primer NP2Rs (5′-GGTCGCGGCCGAGGT-3′). PCR products were spotted on positively charged nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany). DIG-labeled DNA probes were generated by a DIG DNA Labeling Kit (Roche), following the manufacturer's instructions but using a random nonamer mix instead of hexanucleotides. Hybridization and screening were conducted as described previously [15]. Putative differential clones were selected for DNA sequencing.

2.5. DNA sequence analysis

Insert-DNA sequencing was performed on ABI3730 machines (PE–Applied Biosystems, Foster City, CA, USA). Vector and adaptor sequences were trimmed using Vector NTI Suite 8.0 (Informax, North Bethesda, MD, USA) prior to further analysis. Chimeric cDNA sequences containing transcripts from different genes [20] were eliminated. The sequences were clustered using CAP3 [21]. Groups that contained only one sequence were classified as singletons. To assign functions, the assembled consensus sequences and valid expressed sequence tags (ESTs) were used as a query in BLASTN (non-redundant database and EST_others database) and BLASTX (non-redundant database and SwissProt databases) searches [22–24] of the National Center for Biotechnology Information (NCBI) Web Service Sequence (http://www.ncbi.nlm.nih.gov).

2.6. Semiquantitative RT-PCR

Putative differentially expressed cDNA clones that yielded strong positive signals in differential screening analysis were selected for semi-quantitative RT-PCR. The mRNA of sperm cells (400 cells), egg cells and zygotes (20 cells each) were isolated using a Dynalbeads mRNA DIRECT Micro kit as described above, and first-strand cDNA synthesis was performed directly with mRNA bound to the magnetic beads, as recommended by the manufacturer. First-strand cDNA was synthesized in a 10-μl reaction volume containing 100 U SuperScriptIIReverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality and quantity of cDNAs were tested using PCR with primers for the tobacco *G3PDH* gene (Accession No. AJ133422 G3PDHS: 5′-GGCTGTTACTGTTTTTGGCTTTA-3′, G3PDHAS1: 5′-TCGGGCTTGTATTCTTTCTCATT-3′) and *actin* gene (Accession No. X63603, ACTINS: 5′-CAAGGCAGGGTTTGCTGGAGATG-3′, ACTINA: 5′-GTCGAACCGCCACTGAGTACAAT-3′). Specific oligonucleotides were designed for each chosen cDNA using Primer Premier (Premier Biosoft International, Palo Alto, CA, USA) and used in sets for PCR. The PCR conditions were 30–39 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. Each reaction was repeated with another batch of cDNA. After separation on 2% (w/v) agarose gels, the intensities of the same DNA products were compared between reactions from different templates.

3. Results and discussion

3.1. Isolation of egg cells and zygotes from the female gametophyte of tobacco

In tobacco, the time course of fertilization and the first division are variable. Fertilization takes place around 45–48 HAP, and the first division of in vivo zygotes occurs around 120 HAP [18]. The long time interval between fertilization and the first cell division makes tobacco an ideal model plant to investigate spatial and temporal patterns of ZGA. For this study, we chose unfertilized mature egg cells from ovules 40 HAP and zygotes from ovules 96 HAP. A well established method based on enzymatic maceration combined with brief grinding [17,18] allowed us to isolate enough egg cells and zygotes for cDNA synthesis. To inhibit the modulation of gene expression in response to possible stresses during physical isolation and enzymatic digestions for egg cells and zygotes, two transcription inhibitors, actinomycin D and cordycepin, which have been proven effective in suppressing the induction of stress-inducible genes [25], were applied during all steps of cell isolation. After the treatment and isolation procedure, the egg cells and zygotes were viable and healthy (Fig. 1).

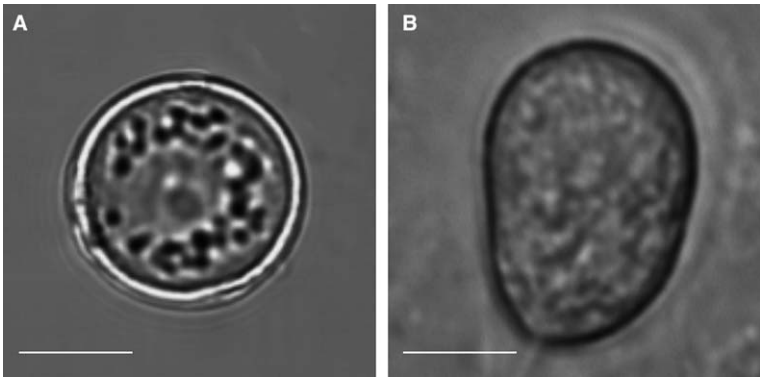

Fig. 1. Isolated egg cell and zygote from a female gametophyte of tobacco. (A) Isolated egg cell from an ovule 40 HAP. (B) Isolated zygote from an ovule 96 HAP. Scale bar = 10 μM.