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Transcriptome Analysis

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Whole Genome Expression Analysis

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Edited by Günter Kahl and Khalid Meksem

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Single Cell Expression Profiling: Transcript and Protein Analyses in Isolated Higher Plant Gametes and Zygotes

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Abstract

In recent years the interest in analyses of single cells has increased continually in the biological and medical sciences. Knowledge regarding the behavior of the single cell as the basic unit of an organism is important for our understanding of the whole organism. Thus, a broad and detailed knowledge of the processes of, for example, signal transduction, cellular communication, cell division, differentiation and development of cells is therefore important in basic and applied sciences. In this chapter we will describe techniques of micromanipulation, including micro-dissection of tissues, isolation and handling of individual cells, cell fusion and analyses of transcript and protein expression of single or a small number of cells. This chapter reviews these methodologies and applications, focuses in particular on gamete isolation, *in vitro* fertilization (IVF) and studies on gamete identity and early seed development in higher plants. The potential of single cell expression profiling for plant genome analysis are discussed.

1.1
Introduction

Gametes and zygotes are special cells that are worthy of study at the single cell level, and *in vitro* fertilization of such isolated cells provides a powerful system for developmental studies. Therefore, methods were developed to isolate single gametes from higher plants in order to study methods of gamete characterization, and techniques for gamete fusion and single cell culture systems for the exploration of the first steps in zygote and embryo formation. Double fertilization is the fusion of one sperm with the egg to create the embryo and the fusion of the other sperm with the central cell to form the endosperm [1]. Gamete fusion occurs *in vivo* deep within the ovule tissues in the embryo sac, and generally with the help of one of the two synergids. In contrast to animals and lower plants, *in vitro* fertilization in higher plant

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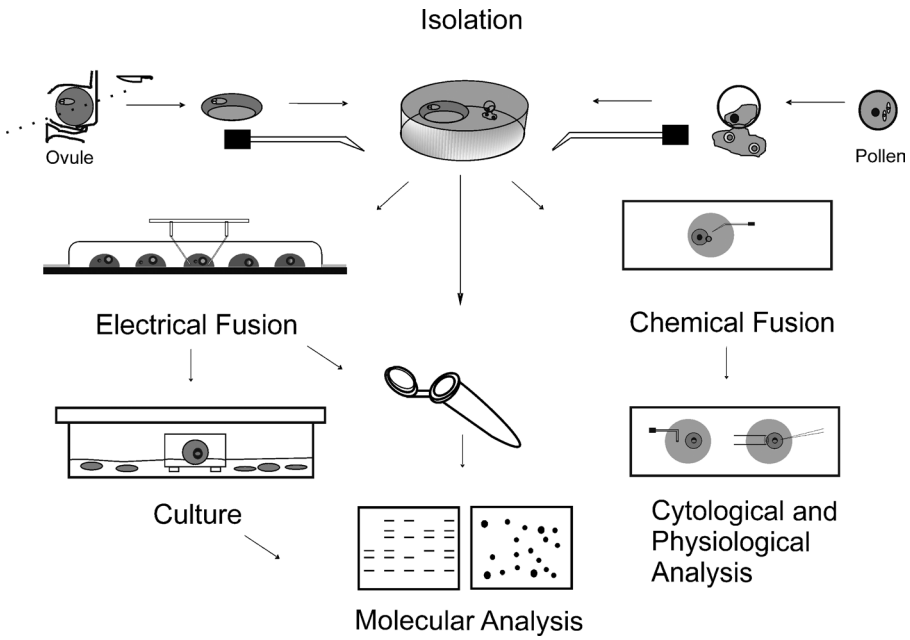

Figure 1.1 *In vitro* fertilization of single gametes of maize. Isolation: ear spikelets are cut as indicated (dotted lines, left). Cells from the embryo sac are manually isolated from nucellar tissue pieces in a plastic dish using a needle, following transfer with a capillary into microdroplets on a coverslip for gamete fusion. Subsequently, sperm cells are selected in the isolation chamber after release from pollen grains by osmotic shock (right), and a sperm is transferred into a microdroplet containing an egg cell. The figure shows manipulations in microdroplets placed on a coverslip for electrofusion (left), calcium-mediated cell fusion (right), cytological and physiological analyses, for example, microinjection and ion measurements, staining and immunochemistry on a coverslip (right, below), and culture in 'Millicell'-dishes for growth and developmental analyses (left, below) as well as molecular analyses (in the middle, below) ([46] modified).

gametes presupposes their isolation. The egg and central cells need to be isolated from an embryo sac which is generally embedded in the nucellar tissue of the ovule and normally contains two synergids and some antipodal cells. Moreover, sperm cells should be isolated from pollen grains or tubes.

With modified microtechniques, originally developed for somatic protoplast fusion [2,3], defined gamete fusion is possible (Figures 1.1 and 1.2). Because they are protoplasts, individual isolated gametes have been fused electrically, for example, [4–12] and chemically using calcium [13–15] or by polyethylene glycol [16–18]. Zygotes and primary endosperm cells can be cultured in tiny droplets of culture medium which are covered by mineral oil [5,7,10,19]. Thus, very early steps in zygote and endosperm development can be analyzed without feeder cells. Sustained growth of zygotes and endosperm can be achieved by co-cultivation with feeder cells [4,7–9,20,21]. Embryogenesis and plant formation from isolated male and female gametes fused *in vitro* have been achieved in maize and rice using electrofusion techniques

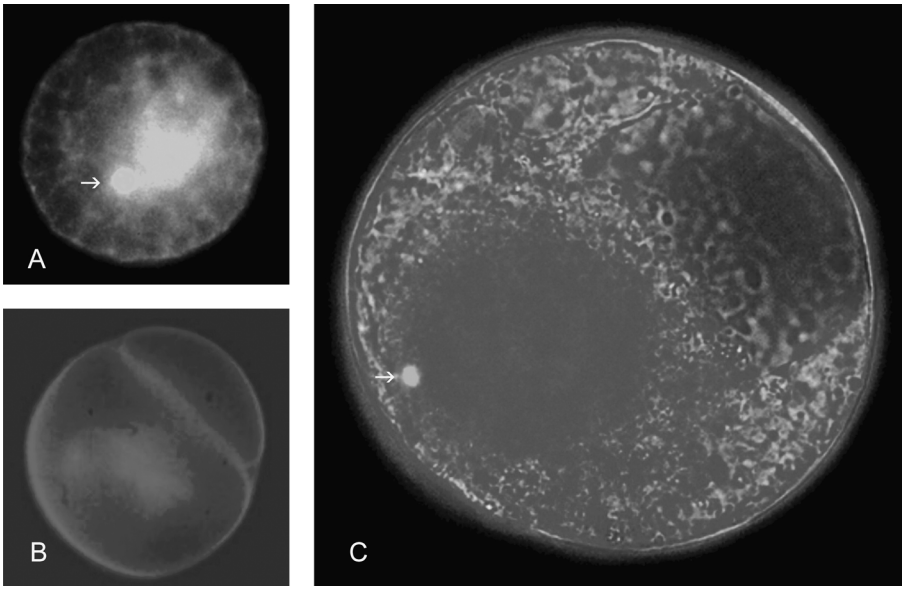

Figure 1.2 *In vitro* fertilization in maize. Epifluorescence micrographs of a fertilized egg cell, stained with DAPI (A; [7]), two-celled embryo, cell wall stained with calcofluor white (B; [7]) and fertilized central cell, stained with DAPI (C; [8]). Arrows indicate integrated sperm nuclei.

and nurse culture [4,12]. In maize, endosperm development has also been triggered after *in vitro* fusion of the sperm-central cell [8]. In these systems, zygote, embryo, plant and endosperm development take place in the absence of mother tissue, as is the case with endosperm formation without an embryo, and embryo development without endosperm.

By using single-cell micromanipulation techniques, the possibility of selection, transfer and handling of single gametes, zygotes and primary endosperm cells together with a high frequency of fusion and cell divisions enables physiological and molecular studies at the single cell level. Even using only a relatively minute amount of material, sufficient numbers of such cells can be obtained to allow the study of gene and protein expression especially of those genes involved in early events in zygote formation, early embryogenesis and endosperm development [21–26]. For example, we developed an immunocytochemical procedure to examine subcellular protein localization in isolated and cultured single cells [27]. This method is described in this chapter. Using RT-PCR (reverse transcription-polymerase chain reaction) methods, cDNA-libraries have been generated from egg cells [28] and *in vitro* zygotes [29] to isolate egg- and central cell-specific [30] and fertilization-induced genes (e.g. [24]). Further, we describe the use of micromanipulation and IVF techniques for example, to separate apical and basal cells from the two-celled embryo. Additionally, specific gene expression has been introduced into these cells in order to elucidate mechanisms of early embryonic patterning in higher plants [24,25]. Moreover, a

description of an adapted method for the analyses of lysates from a few egg cells and zygotes by polyacrylamide gel electrophoresis and subsequent mass spectrometry-based proteomics technology is presented to identify major protein components expressed in these cells [23,26,31]. The techniques are described for maize (*Zea mays*) unless otherwise mentioned. IVF systems offer the potential to (1) analyze zygotes, primary endosperm cells, very young embryos and endosperm at stages characterized by an exactly defined time after fertilization, (2) study cellular events which take place immediately after fertilization and (3) produce zygotes from gametes of different cultivars. The potential of IVF to elucidate mechanisms of fertilization and early development have been reviewed, for example [32–35]. Here, we describe the application of molecular tools to studies aimed at producing a more detailed characterization of gametes and early post-fertilization events which are based upon microdissection and *in vitro* fertilization techniques.

1.2 Microdissection, Cell Isolation

In contrast to animals and lower plants, female angiosperm gametes are deeply embedded within the maternal tissues. Sperm cells need to be isolated from pollen grains or tubes, and female gametes from an embryo sac which is generally embedded in the nucellar tissue of the ovule (Figure 1.1). Double fertilization which is frequently assisted by one of the two synergids, generally occurs within the ovular tissue of the embryo sac. Sperm cells are isolated by osmotic burst, squashing or grinding of the pollen grains or tubes. Female gametes can be obtained by mechanical means using, for example, thin glass needles [6,7,20,36–38], but also by using mixtures of cell wall-degrading enzymes in combination with a manual isolation procedure. In maize, for example, treatment of the nucellar tissue with such a mixture of enzymes for a short period of time prior to the manual isolation step is often useful for softening this tissue to avoid rupture of the gametic protoplasts [5].

1.3 *In Vitro* Fertilization

Three basic microtechniques are involved in *in vitro* fertilization (IVF): (1) the isolation, handling and selection of male and female gametes, (2) the fusion of pairs of gametes, and (3) the single cell culture. The first *in vitro* fusion of isolated, single female and male angiosperm gametes was developed nearly 20 years ago as a result of the application and development of several new micromanipulation techniques. Some of these were originally developed for somatic protoplasts. Additionally, it became necessary to develop an efficient single cell culture system to ensure the sustained growth of single or small numbers of zygotes and primary endosperm cells.

Fusion of individual isolated plant gametes can be achieved by three different methods: (1) electrically (for example [5,6,8,9,11,12]), (2) chemically, using calcium [8,13–15,39–41] or (3) polyethylene glycol (for example [16–18,42]). Cell fusion using electrical pulses is a well-established and efficient method for producing sufficient numbers of zygotes for use in growth studies and molecular analyses. It is conceivable that media including calcium may be used to determine the conditions and factors which promote adhesion, *in vivo* membrane fusion, and possibly recognition events taking place during the fertilization process. However, the efficiency of this method has still to be optimized [13,15]. To date, this method has been used to study the differential contribution of cytoplasmic Ca^{2+} and Ca^{2+} influx during gamete fusion and egg activation [34,39–41]. Although early development of zygotes can be initiated after Ca^{2+}-mediated gamete fusion [13], there are no reports of sustained development of embryos or plant regeneration resulting from this type of fusion. One reason for this might be that the number of zygotes obtained by this method is insufficient for use in growth experiments.

Development after *in vitro* gamete fusion was achieved in monocots (e.g. in maize, wheat and rice), but not in dicots. The cell size of the gametes of various dicotyledonous plants (e.g. *Arabidopsis*) is small compared to that of cereals, a characteristic which hampers their manipulation and handling. In addition, a particularly critical factor for egg activation might be that the stages of the cell cycle in the isolated male and female gametes are not synchronous and therefore cannot activate the nuclear and cell division processes in the zygote [43,44]. Micromanipulation, microdissection and single gamete fusion techniques have been described in detail elsewhere [45,46].

1.4
Techniques for Molecular Analyses of Single Cell Types

1.4.1
Sampling of Single, Living Cells

The collection and storage of single, living cells is an essential step which must be carried out prior to molecular analysis. This can be achieved in various ways depending on the approach, and the number of cells required at one time. Generally, it is important to freeze or lyse the cells as fast as possible after isolation and to minimize the number of handling steps in order to avoid degradation and loss of the limited material. Mannitol is usually used to adjust the osmolality during cell isolation and does not interfere with any of the approaches described here. Long-term storage of single cells or groups of cells in mannitol solution droplets is feasible after snap-freezing in liquid nitrogen at -70 to $-80\,^{\circ}C$ for periods of more than 1 year. Transient storage in this way can accumulate sufficient cells for use in experiments which require a specific number of cells. An alternative procedure is the direct lysis of cells after isolation in sodium dodecyl sulfate- (SDS) or lithium dodecyl sulfate (LiDS)-containing buffer at concentrations of at least 1%. These buffers inhibit all enzymatic activity and therefore preserve the integrity of the nucleic acids

and proteins. For that reason, it is practical to collect a number of cells during the isolation procedure in lysis buffer and freeze them collectively for storage. However, the following approach should either be SDS insensitive, or it should be possible to remove the SDS quantitatively during the procedure preceding enzymatic reactions. For analysis of single cells the individual cells should be directly transferred into and stored in the tube which is to be used in subsequent steps. For single cell reverse transcription-polymerase chain reaction (RT-PCR) it is essential that the cell isolation buffer is compatible with the reverse transcriptase.

1.4.2 Analyses of Gene Expression

The analysis of gene expression at the transcript level in specific cell types always requires a step in which the message is amplified to detectable amounts. Selected combinations of techniques for transcript analyses using limited amounts of sample which were all successfully carried out with single plant gametes or small numbers of single cell types from the female or male gametophyte are described in the following sections.

1.4.2.1 Single Cell Gene-by-Gene Analysis

The simplest method of obtaining information relating to gene expression in a single cell is direct RT-PCR with the single cell as template. This method is useful if, for example, the segregation of a heterozygous locus is to be tested in gametes. Obvious disadvantages of this method are the limited number of genes which can be analyzed in one reaction, and the lack of quantitative expression data. The first disadvantage may be overcome, at least to some extent, by multiplex reactions.

Both RT and PCR reactions are carried out in the same tube in a thermocycler. Several one-tube RT-PCR kits are available from different suppliers and may be used for the analysis of single cells. However, the approach described in [22] was adapted for the analysis of plant gametes. It uses standard reagents in a two-step protocol and is modifiable to specific requirements depending on the genes of interest. The first step consists of the RT reaction. Most important is the rapid addition of the RT master-mix either before or at the time the cells are thawing, which avoids any RNA degradation. Richert *et al.* [22] did not use an initial step at 70 °C, but elevated reaction temperatures to melt secondary structures of the RNA template. Nevertheless, in some cases where the RNA templates have more stable secondary structures, higher temperatures may be necessary during some steps in the procedure. If a higher temperature step is necessary, the reaction mix protocol should be divided up. The primers alone should first be added to the cell, followed by the addition of the remainder of the RT reaction mixture in a second step preceding RT. It is reasonable to use gene-specific reverse primers for the RT reaction because this decreases the generation of unspecific products at elevated temperatures. PCR is the second step of the procedure, whereby the highest sensitivity is obtained when the PCR master mix is added to the complete RT reaction. After 40 cycles of PCR, highly abundant transcripts can be detected by agarose gel electrophoresis. If higher sensitivity is required, the gels may be blotted

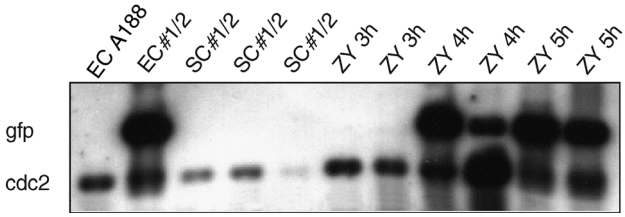

Figure 1.3 Multiplex transcript analysis in single cells. Transcripts of the GFP gene and the CDC2 gene were simultaneously detected in single egg cells (EC), groups of five sperm cells (SC) and single zygotes (ZY) at the indicated time points after fertilization. Egg cells from wild-type and transgenic plants line #1/2 were used as negative and positive control, respectively. GFP transcripts were not detectable in transgenic sperm cells. Between 3 and 4 h after fertilization of wild-type egg cells with transgenic sperm cells the paternal expression of the transgene was initiated. To enhance the signal, the PCR products were transferred to membranes and hybridized with probes against GFP and CDC2 sequences at the same time ([21], modified).

and hybridized with gene-specific probes. Further details of the procedure and some of the modifications mentioned here can be found in [21,47].

To analyze more than one gene simultaneously, a multiplex reaction can be performed. This is a simple way of reducing the time spent on isolating the cells and, importantly, provides amplification of a positive control in the same reaction, that is, by using the same cell. Designing primers for multiplex reactions follows similar principles to those used in normal PCR reactions. The primers should not fold into a hairpin, and none of the primers used in one reaction should form dimers. Primers should anneal to only one transcript sequence of the multiplex reaction. The performance of specific primer combinations should always be evaluated empirically. Figure 1.3 shows a multiplex reaction after blotting and detection by hybridization with two gene-specific probes in parallel. These experiments were performed in the context of paternal genome activation in maize zygotes and used the second gene of the multiplex RT-PCR, which is known to be constitutively expressed, as the positive control within the same cell.

1.4.2.2 **Amplification of Whole cDNA Populations**

Synthesis and amplification of cDNA populations representing all transcripts within specific cell types constitutes the basis for a number of approaches to the analysis of gene expression. Some of these approaches such as real time PCR, cDNA library construction, cDNA subtraction and microarray analyses are introduced in the following sections. Here we discuss important aspects of a cDNA synthesis and amplification protocol which has been successfully applied to a limited number of cells.

The first consideration is to decide how many cells should be used in a single cDNA synthesis reaction. Certainly, a larger number of cells will provide a better representation of low abundance transcripts. To achieve a representative cDNA population, we recommend using no less than 20 cells, because material is lost at each step in the procedure. However, some approaches have employed far fewer cells. For example, 10 cells were used to generate cDNA libraries of wheat egg cells and two-celled

embryos [48], and five cells were sufficient to explore the differential gene expression in the apical and basal cell after the first zygotic division [24].

An important prerequisite for representative cDNA populations is highly efficient reverse transcription. Because reverse transcriptases are strongly inhibited by any impurities, the RNA preparations should be as clean as possible. An approved method for mRNA isolation which gives highly reproducible results in cDNA synthesis and amplification is the use of oligo $dT_{(25)}$-coated magnetic beads (Dynal, Invitrogen). The washing steps applied to the bead-bound mRNA are highly effective and result in pure mRNA. Another advantage is that the volume of the elution buffer can be reduced without the loss of any mRNA or the need to precipitate the sample. However, column-based kits or alternative protocols might also be suitable or adaptable for RNA isolation from small samples. With oligo $dT_{(25)}$-coated magnetic beads, only an analysis of transcripts with a poly(A) tail is possible. This, of course, represents a drawback if RNA species other than mRNAs are to be analyzed but offers a great advantage in transcript analyses, since it greatly reduces sample complexity and therefore the background. We always used SMART cDNA synthesis and amplification (Clontech) with our maize gamete samples. This method utilizes the ability of the Moloney murine leukemia virus reverse transcriptase (MMLV RT) to add a few non-template deoxynucleotides (mostly cytosines) to the 3′ end of a newly synthesized cDNA strand upon reaching the 5′ end of the RNA template. An oligonucleotide containing an oligo(rG) sequence at the 3′ end is added to the RT reaction together with base pairs containing the deoxycytidine stretch produced by MMLV RT. The reverse transcriptase then switches the templates, and continues replicating using the oligonucleotide as template. In this way, a unique and known sequence complementary to the added oligonucleotide is attached to the 3′ terminus of the first strand of the cDNA synthesized, which can serve as a universal forward primer-binding site to amplify the whole cDNA population. A universal reverse primer-binding site is also introduced during the RT reaction with a sequence at the 5′ end of the poly (T) primer. Although this method is less efficient than 'conventional' first and second strand synthesis followed by adapter ligation for the generation of universal primer-binding sites for the amplification of the whole cDNA population, it is simpler and quicker. The reduced handling requirements may outweigh these disadvantages, because loss of material may occur during each step. For a more detailed description and comprehensive discussion of both methods the reader is referred to [49]. Control of the amplification reaction is essential in both these cDNA synthesis methods. To avoid distortion of the cDNA samples and to preserve the original relative abundance of the transcript, the cycle number must be adjusted for each individual sample. The optimal cycle number required to produce the maximum concentration of non-distorted cDNA can easily be determined by running a test reaction and taking samples every three cycles. It is therefore important that the whole reaction mixture (usually 5 times 100 µl) is prepared immediately and cycled together for a specific number of cycles. After taking a sample for gel electrophoresis, an aliquot of the reaction mixture is cycled for another three cycles, with samples being taken between cycles. Meanwhile the remainder of the reaction is stored on ice. Once the optimal cycle number is determined, the PCR reaction is continued with

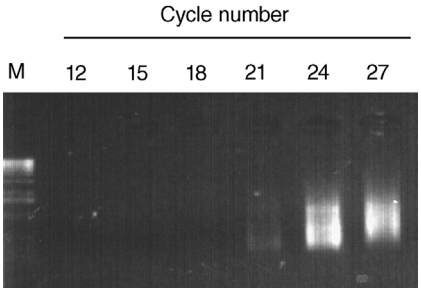

Figure 1.4 Determination of an optimal cycle number for global cDNA amplifications. Test reactions were withdrawn at the number of PCR cycles indicated, and the quantity of amplification product is shown. mRNA was isolated from 25 primary endosperm cells using SMART RT-PCR. The products first became visible after 21 PCR cycles. The concentration of PCR product leveled off between 24 and 27 cycles. Therefore, the optimal cycle number was determined to be 23 which was used for the rest of the reaction.

the appropriate cycle number. A typical cycle number optimization is shown in Figure 1.4. The cycle number for cDNA amplification should always be well below the plateau of the reaction (i.e. one or two cycles less than the cycle number after which no further increase is observed). In case of doubt we recommend a conservative approach in choosing the lower cycle number. Preservation of the original relative abundance of the transcript is the most important prerequisite for the generation of reliable data in downstream approaches using amplified cDNAs.

1.4.2.3 **Quantification of Transcript Levels**

Devising approaches to measure relative transcript levels quantitatively in single cells is challenging. A two step RT-PCR-based method with pre-amplification of several transcripts in a multiplex reaction followed by real-time PCR quantification of single gene transcripts in aliquots of the first reaction has been developed [50]. Interestingly, this approach revealed considerable cell-to-cell variations in an apparently homogenous Tcell population. These findings highlight the individual transcriptional state of each single cell, and means that sampling a number of cells for analysis implies averaging transcriptional states. However, the number of genes which can be analyzed using this approach is limited. To obtain information about the expression levels of a large number of genes in specific cell types or to compare the expression level between two different cell types, sampling of several cells followed by cDNA synthesis and global amplification, as described above, provides a basis for generating a significant amount of expression data. By global amplification of all expressed genes within a cell type, a permanent cDNA archive can be generated and may serve as a template for hundreds of quantitative reactions. Direct comparisons of the relative levels of transcripts between first strand cDNA and amplified cDNA populations revealed that both methods produced comparable results [51]. In our hands, the comparison of quantitative RT-PCR results from amplified and non-amplified cDNA by quantification of the actin gene with two different template concentrations for each cDNA, revealed a high correlation coefficient of 0.96 [52].

1.4.2.4 Library Construction and EST Sequencing

To identify and clone new genes, the generation of cDNA libraries is a reasonable step especially if the work is being carried out with non-model species. In addition, cDNA libraries may serve as templates to produce arrays of individual, unknown cDNA fragments which can be used to characterize the expression pattern of the corresponding genes by hybridization methods. This procedure is useful for selecting genes which are potentially involved in a process of interest. To obtain information about the expression profile of single cell types and identification of new genes or transcripts within a cell, the generation of ESTs is an effective method. Several examples show that ESTs of specific cell types uncover previously unknown sequences, even in model species, where several hundred thousand ESTs already exist [48,53].

The preparation of cDNA libraries from small samples starts with the global amplification of cDNAs as described. It is important to implement restriction enzyme sites in the primer or adaptor sequences to simplify the cloning procedure and to increase its efficiency. Because the cDNA is not limited after the global amplification procedure a size fractionation step before cloning may enhance the fraction of full-length cDNAs, or at least long cDNA fragments. Various standard procedures can be followed to generate cDNA libraries with amplified cDNA populations. The SMART cDNA library construction (kit manufactured by Clontech) is a convenient method for cDNA library construction which we used successfully with samples of 25 maize central cells in combination with mRNA isolation on oligo $dT_{(25)}$-coated magnetic beads.

1.4.2.5 Targeted Approaches Using cDNA Subtraction

Specific approaches aimed at identifying differentially expressed genes between various cell types involved in plant reproduction by applying randomly amplified polymorphic DNA (RAPD) primer-driven PCR or suppression subtractive hybridization (SSH) were successful [24,54]. To identify highly abundant, differentially expressed transcripts in subtracted cDNA populations, differential screening using microarray hybridizations is highly effective [30]. The basis for all these approaches is a global cDNA amplification procedure. A disadvantage of the SSH technique is that the cDNAs must be restricted before hybridization to equalize the hybridization efficiencies of the diverse cDNAs within the population. Full-length cDNAs of interesting clones need to be reconstituted in a second step using other methods such as rapid amplification of cDNA ends (RACE). Control PCR reactions after SSH are shown in Figure 1.5. These controls are important in indicating whether the subtraction was successful before cloning and screening of the subtracted cDNA populations. Targeted approaches with gametes and fertilization products demonstrate that these types of experiments provide valuable insights into reproduction-related gene expression differences and, moreover, identify candidate genes with a high potential for important roles during plant reproduction and early seed development for further characterization.

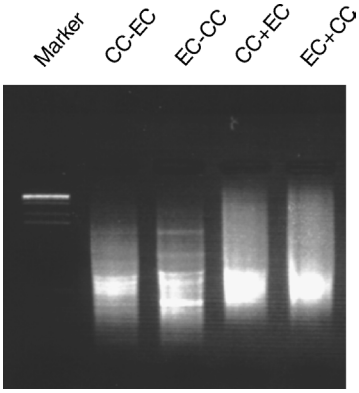

Figure 1.5 Control of suppression subtractive hybridization reactions. Subtracted cDNA populations and mixes of both cDNAs used for subtraction were amplified by PCR and separated using agarose gel electrophoresis. In this example, cDNAs of egg cells (EC) and central cells (CC) were used for subtraction in both directions. Subtracted cDNAs are indicated by '−,' corresponding control mixes are indicated by '+'. The different appearances of subtracted and non-subtracted cDNA populations indicate successful subtraction reactions. Differences between the two subtractions using the same cell types but different directions of subtraction indicate the diverse transcriptional profiles of the two cell types.

1.4.2.6 **Microarray Analyses**

Various types of amplification techniques have been developed to enable microarray gene expression analysis when the starting material is limited. The two main strategies are linear amplification, using *in vitro* transcription, and exponential amplification, based on PCR. If the reactions are well controlled, both methods preserve the relative abundance of the transcripts to a comparable extent (see, e.g. [55]).

Aspects of cDNA amplification methods have already been described in detail above. T7-based amplification of copy RNA (cRNA) by *in vitro* transcription, a method originally developed in the laboratory of James Eberwine [56], was recently evaluated in our laboratory as an alternative to produce targets for oligonucleotide microarray hybridizations. The protocol starts with mRNA isolation using oligo $dT_{(25)}$-coated magnetic beads. The mRNA of 25 egg cells and zygotic cells was then amplified using the Amino Allyl MessageAMP II aRNA Amplification kit (Ambion). After reverse transcription, second strand synthesis, cDNA purification and *in vitro* transcription for 14 hours (the maximum time period recommended), the reactions yielded around 1 μg of cRNA. This cRNA was subjected to a second round of the procedure combined with the incorporation of aminoallyl-modified nucleotides during *in vitro* transcription. After coupling fluorescent dyes to the labeled cRNAs hybridization of 70mer oligonucleotide microarrays (www.maizearray.org) high quality hybridization results were obtained. An example of these hybridizations is shown in Figure 1.6. These initial experiments demonstrated that only a few cells involved in the reproduction of higher plants are needed to obtain data on

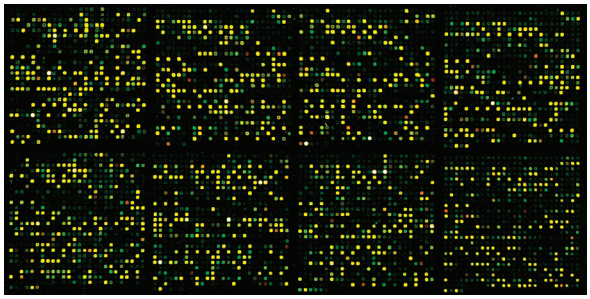

Figure 1.6 Microarray hybridization with cRNA targets from single cell types. The hybridization of maize 57 k oligonucleotide arrays (www.maizearray.org) with cRNA generated in two rounds from 25 egg cells to 25 zygotes resulted in high-quality hybridization signals. The various red and green spots indicate highly differential gene expression.

global expression related to the fertilization event and specific tissue formation in very early seed development.

1.5 Analyses of Protein Expression

Traceable quantities of proteins can be detected in a small volume of single cells by minimizing the gel size for one- and two-dimensional polyacrylamide gel electrophoresis. Protein components can be identified by highly sensitive liquid chromatography coupled in tandem with mass spectrometry (LC-MS/MS) [23,31]. Protein patterns of differentiated cells reflect the biological function of these cells. Egg cells are such highly specialized cells which are fertilized by sperm to undergo early embryogenesis. Thus, the identification of proteins in gametes and zygotes will provide important data for understanding the mechanisms of gametogenesis, fertilization and early embryogenesis of higher plants.

Proteomics is an area of research that evaluates protein expression by resolving, identifying, quantifying, and characterizing proteins. Techniques for such studies include two-dimensional polyacrylamide gel electrophoresis, and tandem mass spectrometry and computer analysis [57]. These technologies now make it possible to identify the proteins in a relatively low concentration of cells. Such analyses were initiated to reveal which proteins are present in abundance in plant egg cells [23,31]. By minimizing the gel size in polyacrylamide gel electrophoresis, proteins of only a few egg cells can be detected. Fifteen or 45 cells respectively, are sufficient to produce detectable silver-stained protein bands or spots in SDS- or 2D-PAGEs using small sized gel molds ($50 \times 60 \times 1$ mm). Egg cell lysates from 75 to 180 cells respectively, were used for both SDS- and 2D-PAGEs for in-gel tryptic digestion and subsequent

highly sensitive LC-MS/MS analyses. Three cytosolic enzymes in the glycolytic pathway, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and triosephosphate isomerase, two mitochondrial proteins, an ATPase β-subunit and adenine nucleotide transporter, and annexin p35 were identified as major proteins in maize egg cells using tandem mass spectrometric analysis and amino acid microsequencing. Thus, five of the six major egg proteins identified are thought to be involved in energy production pathways, suggesting that the egg cell has sufficient enzymes and transporters to produce and transport an energy source. The amount of protein in a maize egg cell was estimated to be 100–200 pg [23].

It is reasonable to assume, that energy-consuming serial zygotic events, such as migration of cytoplasmic organelles, the formation of a new cell wall around the zygote and nuclear division, explain why egg cells contain an abundance of energy-producing proteins.

In addition to the initial data concerning the protein composition of higher plant egg cells, these protein analyses also provide an indication of the sensitivity of and number of cells required to achieve comprehensive protein profiles of single cell types.

1.6 Prospects

These days, micromanipulation methods are routinely used to isolate gametes from higher plants and to fertilize them *in vitro*. From some higher plants, zygotes and embryos, and fertile plants and endosperm can be obtained by *in vitro* fusion of pairs of sperm and egg cells, and of pairs of sperm and central cells, respectively. This makes it possible to examine the earliest developmental processes precisely timed after fertilization. Furthermore, single zygotes, young embryos and endosperm can be isolated from *in vivo* material. Obviously only a small amount of such material can be obtained, especially if it is produced *in vitro*. In addition, micromanipulation techniques are not restricted to reproductive cells, but may be used to isolate and select various other specific cell types. The adaptation of highly sensitive molecular methods to specific cell types or even single cells, as described in this chapter, significantly expands our insight into gene expression. These methods provide a high degree of sensitivity and specificity which is necessary to understand the role of genes in differentiation, and especially in reproductive processes. With this information the genes involved in developmental processes can be defined and reverse genetic approaches to characterize their function can be initiated. Because 'whole transcriptome' arrays for various species are currently available, the exploitation of these arrays to analyze expression information in specific cell types will provide comprehensive and conclusive genetic information. Together with the emerging technologies for the analysis of proteins in the same cell type, this repertoire of methods will greatly enhance our understanding of developmental and reproductive plant biology.

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