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Protocols

Single-Embryo RT-PCR Assay to Study Gene Expression Dynamics During Embryogenesis in *Arabidopsis thaliana*

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Abstract. We have developed a single-embryo RT-PCR protocol for studying gene expression during plant embryogenesis. Four genes, *glyceraldhyde-3-phosphate dehydrogenase* (*GAPC*), *shoot-meristemless* (*STM*), *monopteros* (*MP*), and *shaggy-like kinase etha* (*ASKη*), from *Arabidopsis thaliana* were used to test the sensitivity and reliability of this method by analyzing the differential signal intensities of their RT-PCR products. The method could detect genes expressed during embryogenesis at a single-embryo level and, therefore, can be used to identify phenotypes. When in vitro, embryogenesis also is used to control the time course of zygote development exactly. The single-embryo RT-PCR protocol becomes a powerful method to survey the dynamics of specific gene expression.

Key words: *Arabidopsis thaliana*, embryogenesis, gene expression, RT-PCR, single embryo

Abbreviations: *ASKη*, *shaggy-like kinase etha*; DAP, days after pollination; DEPC, diethylpyrocarbonate; *GAPC*, *glyceraldhyde-3-phosphate dehydrogenase*; *MP*, *monopteros*; RT-PCR, reverse transcriptase–polymerase chain reaction; RVC, ribonucleoside vanadyl complexes; *STM*, *shoot-meristemless*.

Introduction

In flowering plants, all tissues undergo dramatic shifts in gene expression patterns during early development, particularly after fertilization when the zygotic program is initiated (Preuss, 1999). The formation of the zygotic embryo has been described in some detail, but the molecular mechanisms controlling initiation and cell differentiation are not well understood. In recent years, plant molecular and genetic studies have led to the identification and characterization of genes controlling the establishment of polarity, tissue differentiation, and the elaboration of patterns during embryo development (Long and Barton, 1996; Hardtke and Berleth, 1998). Analysis of gene expression has become increasingly important for understanding how genetic profiles affect cell phenotype and function. In animals and plants, recent attention has been paid to gene expression in specific single cells

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and embryos at certain developmental stages or with specific phenotypes (Karrer et al., 1995; Adjaye et al., 1999; Kawasaki et al., 1999).

Gene expression has been surveyed routinely using northern blotting and RNase protection assay, but these techniques require relatively large amounts of starting RNA. Radioactive in situ hybridization provides important information regarding anatomical distribution and density patterns, but it is limited by its detection sensitivity and resolution at the cellular level. Although in situ RT-PCR and nonradioactive probes can improve the sensitivity and resolution, a major restriction of the approach still is the number of mRNA species that can be identified at any time (Jacqueline and Lipski, 2000).

A highly sensitive and relatively simple technique is single-cell RT-PCR. However, to our knowledge, the technique is only possible in plants when the cell wall is removed or the cytoplasm is extracted from the cell by micromanipulation, allowing release of RNA during lysis (Richert et al., 1996). With in vitro or in vivo techniques for embryogenesis, it is difficult to obtain enough synchronously developed young embryos for classical methods. Protoplasts can be isolated from embryos; however, the gene expression pattern may change under the stressful and lengthy isolation conditions, and the transcripts in the cell may not be representative of the natural process of embryogenesis. Therefore, we developed an RT-PCR technique for detection of target gene expression in a fresh single embryo without the extraction of RNA.

Materials and Methods

Plant materials

Plants of *Arabidopsis thaliana* were grown in a culture chamber under standard conditions. Developing siliques at 2, 4, and 6 DAP were chosen for the isolation of embryos.

Gene-specific primers

Four genes, *GAPC, MP, STM,* and *ASKη,* were chosen to test the efficiency and sensitivity of this RT-PCR detection method. Specific primers of the 4 selected genes were designed based on their published sequences, as follows: The sense primer of *GAPC* (5'-CACTTGAAGGGTGGTGCCAAG-3') flanked the boundary of intron 5 and exon 5, the antisense primer (5'-CCTGTTGTCGCCAACGAAGTC-3') was in exon 9, and the band amplified from cDNA was 543 bp. The sense primer of *Mp* (5'-TGTCCAGTCGCAGATCACAT-3') was in exon 11, the antisense primer (5'-TTATGCACCTTACGCATCCC-3') was in exon 13, and the bands amplified from cDNA and genomic DNA were 393 bp and 601 bp, respectively. The sense primer of *ASKη* (5'-CTGTGGATCAGCTCGTGGAAAT-3') was in exon 7, the antisense primer (5'-GACCTTGTGGCTCGTGTAATGG-3') was in exon 9, and the bands amplified from cDNA and genomic DNA were 475 bp and 709 bp, respectively. Because only the mRNA sequence of *STM* has been reported, the specific primers for *STM* (5'-GAGCCTCAAGCAAGAGTTCA-3' and 5'-CAAAGCATGGTGGAGGAGAT-3') were designed based on the mRNA sequence. We also could see a band of about 1 kb after amplification of the genomic

DNA with the *STM* primers, indicating amplification of the corresponding products from genomic DNA.

Solutions and regents

- DEPC-treated and autoclaved distilled water
- 2% Sodium hypochloride
- RVC solution: 20 mM RVC (Sigma) in DEPC-treated distilled water
- Lysis buffer: 0.02 M DTT (Gibco BRL), 8 units/μL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Gibco BRL)
- First strand cDNA synthesis mix: 2.5 units/μL Superscript™ II RNase H⁻Reverse Transcriptase (Gibco BRL); 0.025 g/L of Oligo dT (12-18 mer, Gibco BRL); 500 μM of each dNTP (Clontech); 2 units/μL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Gibco BRL); 37.5 mM Tris-HCl (pH 8.3); 56.25 mM KCl; 5 mM $MgCl_2$; 0.01M DTT
- 25 mM EDTA (Gibco BRL)
- PCR master mix: 200 μM of each dNTP (TaKaRa); 0.2 μM of respective 3' and 5' gene-specific primers; 2 mM $MgCl_2$; 5 μL 10x *EX Taq* Buffer (TaKaRa); 2.5 units *EX Taq* polymerase (TaKaRa) with 2 μL of product from reverse transcription

Protocol

- The developing siliques of *Arabidopsis thaliana* at various developmental stages were cut off and surface-sterilized with 2% sodium hypochloride for 10 min at 22°C, rinsed with sterile water, and then placed on a glass slide.
- With a scalpel, the ovules were isolated from the siliques and transferred immediately into 50 μL RVC solution.
- Under an inverted microscope, the embryos were isolated from the ovules and transferred as soon as possible into a droplet of 5 μL lysis buffer on a 3.5-cm Petri dish by a handmade glass micropipette.
- Under an inverted microscope, each embryo was held by a glass micropipette to fix its position in the same droplet of lysis buffer. A stainless steel needle was used for crushing and abrading the embryo several times on the bottom of the glass Petri dish so that it became shattered and invisible.
- The lysis buffer containing the shattered embryo was transferred into a thin-walled tube, and the place left by the embryo was washed by 2-3 μL DEPC-treated distilled water, which was transferred into the same tube.
- The mixture was frozen in liquid nitrogen, thawed, vortexed briefly, and then centrifuged gently.
- DNase I treatment was performed using 1 unit of deoxyribonuclease I, (Amplification Grade, Gibco BRL) at 25°C for 1 h. The DNase I was inactivated by adding 1 μL of 25 mM EDTA to the reaction mixture and heating for 10 min at 65°C. The lysate was ready to use for reverse transcription before amplification.
- Reverse transcription was performed in a thermocycler (Biometra UNOII) at 42°C for 70 min. The final volume for the reaction was 20 μL. The enzyme was

inactivated by heating for 15 min at 70°C, and the RNA digested by adding RNase H and incubating for 20 min at 37°C.

- The PCR reaction was performed in a Biometra UNOII thermocycler using a final volume of 50 µL, including the 2-µL template from the reverse transcription reaction. After a first denaturation step of 5 min at 95°C, 30 cycles were performed (30 s at 94°C, 30 s at respective annealing temperature, 45 s at 72°C), followed by a final extension step of 10 min at 72°C. The first PCR amplification products (2 µL) were used to perform the second amplification using the above conditions.
- PCR with the genome sequence was performed using 100 ng total genomic DNA as a template instead of the 2-µL reverse transcription reaction mixture, and the template was amplified during 30 cycles under the above reaction conditions.
- After RT-PCR, 8 µL of the second PCR reaction mixtures was loaded into a 1% agarose gel containing ethidium bromide. For PCR experiments with genomic DNA, 8 µL of the PCR reactions was loaded into the same gels. The gels were viewed and photographed under UV light.

Notes

1. Einmal-Micropippettes (50 µL, IntraMARK) with aperture diameters of about 25 and 200 µm were used as holders and for embryo transfer, respectively. The micropipette tips were beveled at an angle of about 30°, which facilitated manipulation and protected the fragile borosilicate glass tips.
2. The tip of the stainless steel needle (diameter = 150 µm), which was fixed in the end of a glass tube, was abraded to a bevel at an angle of 45° for efficient grinding.
3. All equipment was kept as clean as possible, and gloves were worn at all times during the process because skin is a major source of RNase and other contaminating genetic materials. Before the experiment, all solutions, containers, and tubes were autoclaved, and solutions were made from water previously treated with diethylpyrocarbonate (DEPC), a compound that inactivates RNase. All manipulations were carried out under sterile conditions.

Results and Discussion

Tiny embryos of *A. thaliana* were isolated by dissection, transferred to a lysis buffer containing RVC and RNase inhibitors, and then subjected to mechanical grinding. Embryo isolation and cell lysis were finished within 10 min, minimizing changes in gene expression during manipulation. Speed is critical for study of gene expression during embryogenesis. The method for crushing the embryo is illustrated in Figure 1. With proper pressure, most embryo cells were shattered into invisibility and released most of their RNA. Abrading an embryo against the bottom of a glass Petri dish is important for crushing its cells. Also, the liquid nitrogen freezing followed by thawing releases most of the RNA from the cells (Richert et al., 1996).

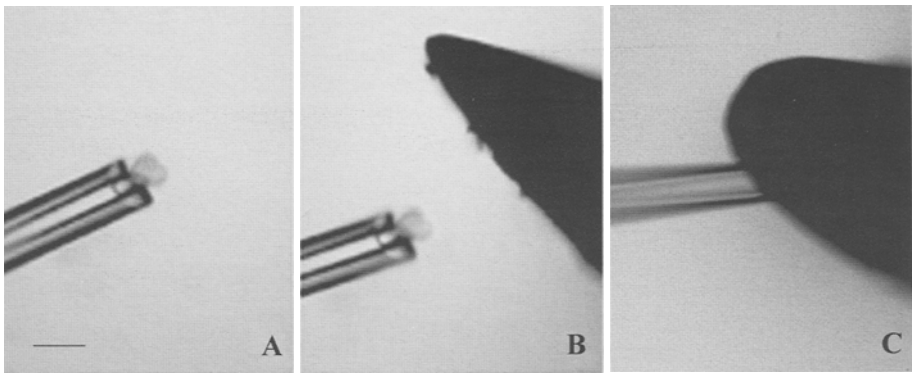

Figure 1. Grating of single embryos under an inverted microscope. (A) Holding the embryo by the glass micropipette. (B) Approaching a needle to the embryo. (C) Grating the embryo by pressing and rotating the needle on it. Bar = 25 μm

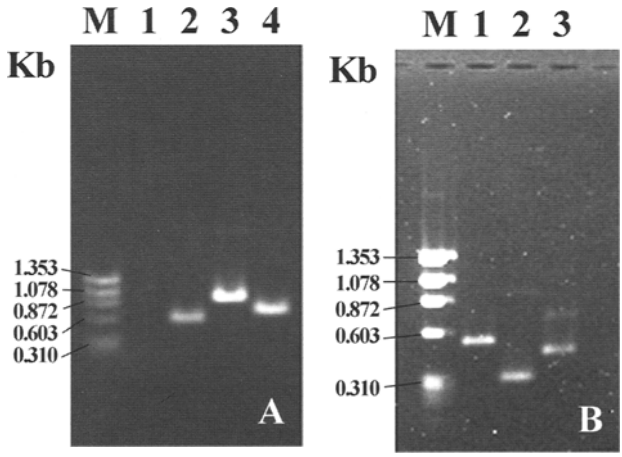

Figure 2. Amplified fragments from genomic DNA (A) and from the template without being treated with RNase-free DNase I (B). M: Molecular weight marker. ϕX174DNA/*Hae* III. (A) 1, *GAPC;* 2, *MP;* 3, *STM;* 4, *ASK*η. (B) 1, *GAPC;* 2, *STM;* 3, *ASK*η.

Table 1. Effect of DNase I treatment on genomic DNA amplification.

	0 min		45 min		60 min	
	Genomic DNA	mRNA	Genomic DNA	mRNA	Genomic DNA	mRNA
First amplification	Yes	Yes	No	No	No	No
Second amplification	—	—	Yes	Yes	No	Yes

In general, genomic DNA has negligible effects on single-cell RT-PCR, so pretreatment with DNase is not needed (Johansen et al., 1995). However, we observed signals corresponding to expected genomic *MP, STM,* and *ASK*η products

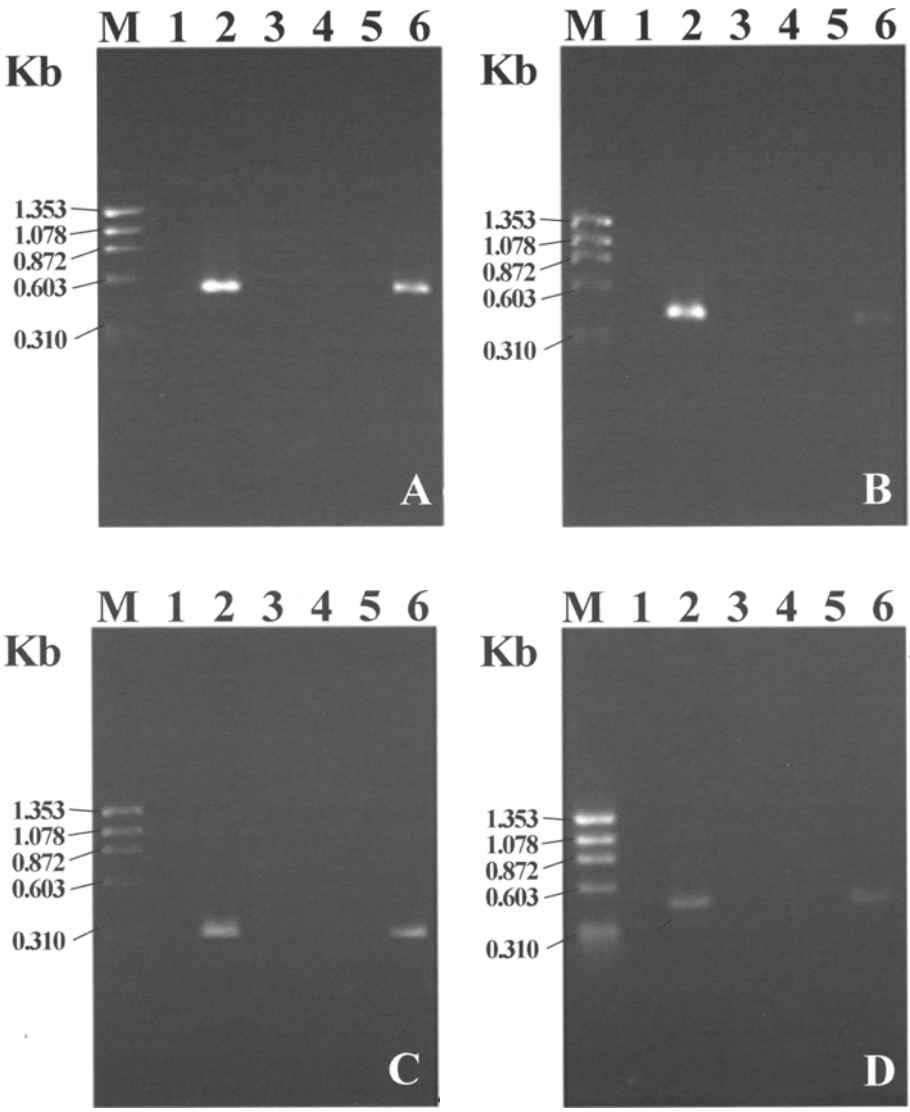

Figure 3. Amplified fragments from the embryo at 2 DAP. (A) *PC,* (B) *MP,* (C) *STM,* and (D) *ASK*η. M: Molecular weight marker. ϕX174DNA/*Hae* III. (1) Negative control for PCR (reverse transcription mix is replaced by water); (2) positive control (addition of diluted total RNA from *Arabidopsis* stem or leaf); (3) negative control for RT-PCR (without template); (4) negative control for RT-PCR (without RT enzyme); (5) products of the first amplification; and (6) products of the second amplification.

and a *STM* product of about 1 kb after amplification of genomic DNA. With direct RT-PCR on lysed whole embryos, we obtained the desired bands from mRNA of *GAPC*, *STM,* and *ASK*η, as well as the undesired signals from genomic DNA of *STM* and *ASK*η (Figure 2). Thus, we found it necessary to pretreat the template in the lysis buffer by RNase-free DNase I. Pretreatment for 60 min was

sufficient (Table 1). This procedure is not necessary with primers designed to amplify mRNA from a genomic region containing an intron.

To test if our method can rupture the entire embryo and release most of the mRNA into the lysis buffer, we chose oligo dT for the reverse transcription step. We also chose the *GAPC, MP, STM,* and *ASKη* genes because of their diverse spatiotemporal expression during *A. thaliana* embryogenesis (Shih and Goodman, 1991; Long and Barton, 1996; Dornelas and Kreis, 1997; Hardtke and Berleth, 1998). After RT-PCR, the expected signals of the 4 genes could be detected (Figure 3), which were consistent with early in situ hybridization studies (Shih and Goodman, 1991; Long and Barton, 1996; Dornelas and Kreis, 1997; Hardtke and Berleth, 1998). Because the *ASKη* gene is only expressed in the suspensor cells, the lysis procedure released most RNAs from a single embryo. This high sensitivity allows study of the expression dynamics of very weakly expressed genes. Repeated PCR experiments with embryos at various developmental stages were consistent with the early in situ hybridization work (data not shown).

Because in vitro fertilization and zygotic embryogenesis are now possible (Kranz et al., 1991; Faure et al., 1994; Sun et al., 2000), specific phenotypes can be created under controlled conditions. Single-embryo mutants can be identified by following the exact time course for development. The single-embryo RT-PCR assay will be especially useful for underlining specific phenotypes or mutants. Furthermore, this technique also provides a basis for the construction of a single-embryo cDNA library in higher plants.

Acknowledgments

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