Original Paper Plant Biotechnology, 15(2), 77-81(1998)

Isolation and Analysis of New Molecular Markers for Early Embryogenesis in Rice (*Oryza sativa* L.)

Kazuhiro KIKUCHI*, Chan Sun CHUNG** and Kaoru T. YOSHIDA*†

*Grad. Sch. Agric. Life Sci., University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
**Faculty of Agric., Sangji University, Wonju 220-702, Korea

Received 20 August 1997; accepted 20 November 1997

Abstract

Two cDNA fragments that preferentially accumulate in very young rice embryos at 4 days after anthesis (DAA) were isolated using simplified differential display. The first morphological change is observed in the embryos at 4 DAA and most of the embryo tissue has already differentiated by 7 DAA. RT-PCR was used to quantitate the expression levels of the genes corresponding to the two cDNA clones, REE5 and REE8. They were expressed at higher levels in the embryo at 4 DAA than in the embryo at 7 and 14 DAA as well as in the pistil 1 day before anthesis. The consistent result was obtained by *in situ* hybridization experiments using REE5 antisense probe. REE5 gene was expressed in the whole embryo at 4 DAA and 5 DAA. The lower expression levels were observed in the embryos at 3 and 7 DAA. RT-PCR analysis revealed that both the REE5 and REE8 transcripts also accumulated in inflorescence meristems prior to the emergence of floral organs, but not in roots, leaves or flowers. The common feature of the young embryos and the inflorescence meristems is the onset of differentiation of various tissues. These may be useful molecular markers for analysis of the events of differentiation during early embryogenesis.

1. Introduction

Information about the early stages of plant embryogenesis is important for the understanding of plant morphogenesis, as the embryo axis is established and the formation of major plant tissues is initiated, during these stages. Despite the importance of the early stages of embryogenesis, little is known about the molecular steps specific to these stages. This is because the developing zygotic embryos are very difficult to handle and because standard approaches including differential screening are not suited to the isolation of genes expressed at low levels. Those genes that play key roles in plant development are usually expressed only at low levels. Differential display is an effective method for isolating genes that are expressed specifically but at low levels [1]. Simplified differential display, which we developed previously [2], is also useful for cloning genes that are expressed at low levels. In this study, simplified differential display was used to isolate cDNAs that preferentially accumulate in young rice embryos, in order to extend our knowledge of the molecular events that occur during early embryogenesis. Two cDNA fragments isolated using this method were characterized and shown to be expressed in a temporally and spatially regulated manner during the early stages of embryogenesis in rice.

2. Materials and Methods

Rice plants (*Oryza sativa* L., var. japonica cv. Kamenoo) were grown in a pot in a greenhouse under standard conditions. For histological studies, ovaries at 3 to 6 days after anthesis (DAA) were fixed for 14 hours in CRAF solution. They were dehydrated using an ethanol-tertiary butyl alcohol series, embedded in Paraplast Plus (Oxford Labware, St. Louis, MO), and cut into 10 μm sections. The sections were stained with the method of Sharman [3].

For simplified differential display, mRNAs from embryos at 4 DAA, embryos at 14 DAA, ovaries at 1 DAA and pistils 1 day before anthesis were prepared using Oligotex-dT30 (Takara Shuzo, Kyoto, Japan) from total RNA isolated using an ISOGEN kit (Nippon Gene, Tokyo, Japan). The conditions for simplified differential display have been described earlier [2]. Nineteen primers of 10 bases each were used (Operon Technologies, Alameda, CA). PCR products were fractionated in a 1.6% agarose gel. Differential bands identified by ethidium bromide staining were recovered from the gel and cloned into a pCRII vector (Invitrogen, San Diego, CA).

For RT-PCR analysis, mRNA was prepared from embryos at 4 DAA, roots and leaves of seedlings at 7

† To whom correspondence should be addressed.

days after germination, young panicles, and flowers just prior to heading as described above. An actin gene was used as control in the RT-PCR analysis. Internal primer sets of eighteen bases each were designed for amplification of REE5, REE8, and actin gene fragments. The conditions for RT-PCR analysis were as follows: 94°C denaturation for 1 min, annealing for 1 min, 72°C extension for 2 min. The annealing temperatures for REE5, REE8 and actin were 52°C, 58°C, and 56°C, respectively. The number of PCR cycles for amplification of REE5, REE8 and actin was 26, 28 and 28, respectively.

Both strands of the cDNA inserts of the clones for REE5 and REE8 were sequenced using a PRISM Dye Primer Cycle Sequencing kit and a 377 automatic DNA sequencer (Applied Biosystems Inc, Foster City, CA). Nucleotide and amino acid searches of EMBL, SWISS-PROT, PIR, PRF, and GENBANK databases for sequences similar to REE5 and REE8 were made using the BLAST program.

Embryos were processed for *in situ* hybridization as described by Kouchi and Hata [4]. Ovaries at different stages after anthesis were fixed for 20 hours in 100 mM phosphate buffer, pH 7.2, 3% paraformaldehyde, and 0.25% glutaraldehyde. The ovaries were rinsed in phosphate buffer and dehydrated using a graded series of ethanol solutions with concentrations ranging from 30-100%. The ovaries were embedded in Paraplast Plus and cut into 10 μm sections. The sections were hybridized to digoxigenin-labeled RNA probes synthesized from the REE5 clone. Antisense and sense transcripts of REE5 were transcribed using SP6 or T7 polymerase. Prehybridization, hybridization, washings, RNase treatment and immunological detection were performed using a digoxigenin nucleic acid detection system (Boehringer Mannheim, GmbH, Germany), according to the manufacturer's instructions.

3. Results

When isolating morphogenesis-associated early embryo-specific genes, it is important to choose appropriate combinations of mRNA populations for comparison, in order to eliminate genes necessary for maintenance of rapid cell division in the developing embryos [5], as well as genes abundant in late stage embryos such as LEA. Embryos at 4 DAA were used as the source of mRNA for cDNA cloning, as the embryo is globular before 3 DAA and the first morphologically organized structure develops by 4 DAA. The events that occur at this stage include the emergence of the coleoptile, the formation of the shoot apical meristem beneath the coleoptile, and the establishment of the radicle meristem (**Fig. 1**). To eliminate genes that are specifically expressed during late embryogenesis (such as LEA protein-encoding cDNAs), mRNA from embryos at 14 DAA was compared with that from embryos at 4 DAA. All morphological events are complete by 14 DAA. To eliminate genes expressed during rapid cell division (*i.e.*, chromatin metabolism- and cell wall synthesis-related genes), and to identify the genes unique to embryogenesis, we compared mRNA from embryos at 4 DAA with that from ovaries at 1 DAA, as well as with that from pistils 1 day before anthesis. Simplified differential display was used to compare the cDNAs prepared from these four types of tissues using a single arbitrary primer (10-mer). The PCR products were separated using 1.6 % agarose gels and visualized by ethidium bromide staining. Individual display experiments typically yielded 10-20 cDNA fragments. Of the 19 different primers tested, 6 produced amplified bands expected for early embryogenesis-specific cDNA fragments. A representative example of the simplified differential display patterns is shown in **Fig. 2**. Sixteen candidates for genes related to early embryogenesis were detected, and of these, 11 cDNA fragments were recovered from the gel and cloned into pCRII vectors for further

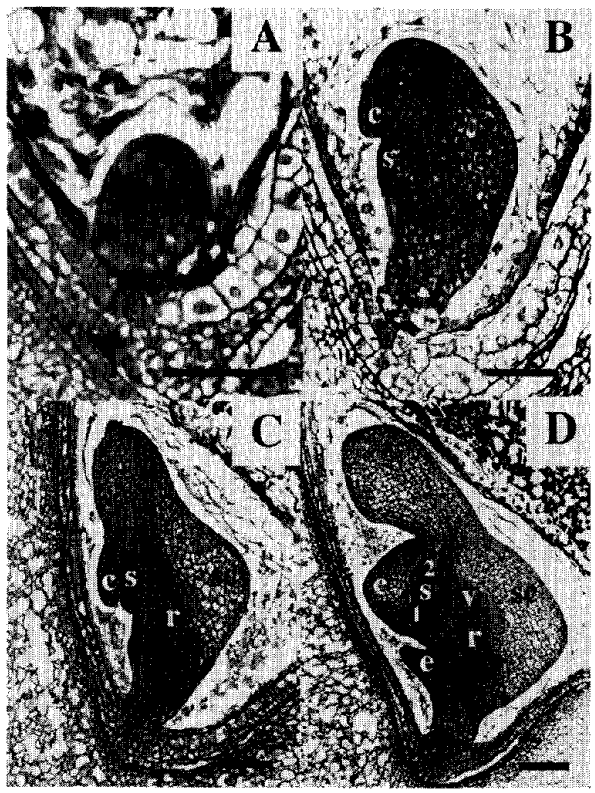

Fig. 1 Histological observations of embryos at 3 DAA (A), 4 DAA (B), 5 DAA (C) and 6 DAA (D). c, coleoptile; e, epiblast; r, radicle; s, shoot apical meristem; sc, scutellum; v, vascular bundle; 1, 1st leaf; 2, 2nd leaf. Scale bars indicate 50 μm.

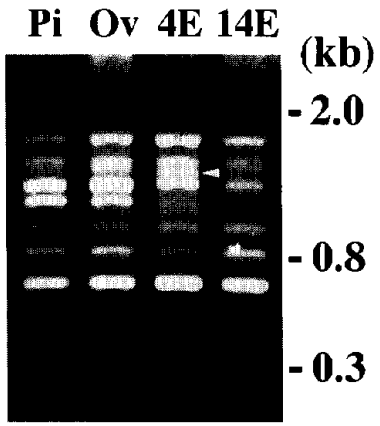

Fig. 2 A representative result of simplified differential display of mRNA from pistils 1 day before anthesis (Pi), ovaries at 1 DAA (Ov) and embryos at 4 DAA (4E) and 14 DAA (14E). A PCR fragment that is specific to embryos at 4 DAA is indicated by an arrow head.

analysis.

The expression pattern of these clones was analyzed by RT-PCR, because genes identified using the differential display method may be expressed at very low levels and are not readily detected by Northern blot analysis. Moreover, it is difficult to prepare a sufficient number of zygotic embryos for Northern blot analysis. Partial sequences of these clones were determined and used to design internal primers for RT-PCR analysis. Preliminary experiments indicated that 2 clones were not specific to embryogenesis and 3 clones showed multiple RT-PCR fragments. Six clones out of the 11 were thought to exhibit an embryogenesis-associated pattern (data not shown). Among the 6 clones, 2 clones (designated as REE5 and REE8) exhibited the expected early embryogenesis-specific expression patterns. Therefore, further analysis was focused on these 2 clones. The primer sets of REE5 and REE8 gave a single cDNA fragment. REE5 and REE8 were expressed at specific stages of embryonic development (**Fig. 3**). Compared to the expression pattern of the actin gene, both of the genes corresponding to REE5 and REE8 were expressed at higher levels in the embryo at 4 DAA than in the embryo at 7 and 14 DAA; as well as in the pistil 1 day before anthesis. In particular, a PCR fragment corresponding to REE8 could be detected only in the embryo at 4 DAA. In the embryo at 7 DAA the scutellum, coleoptile, plumule with 1st and 2nd leaves, radicle, vascular bundle and epiblast have already differentiated, as in a mature embryo (**Fig. 1, 5**). The higher expression levels of REE5 and REE8 in the embryo at 4 DAA compared to the later stages suggested that these genes are associated with differentiation processes in the young embryo. To

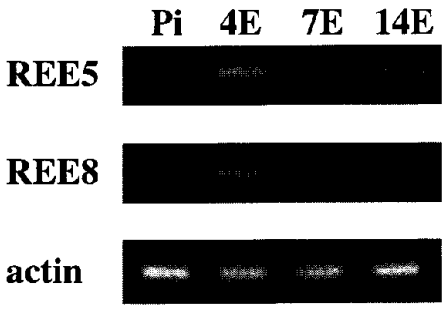

Fig. 3 RT-PCR analysis of REE5, REE8 and actin with cDNA templates prepared from pistils 1 day before anthesis (Pi), embryos at 4 DAA (4E), 7 DAA (7E) and 14 DAA (14E).

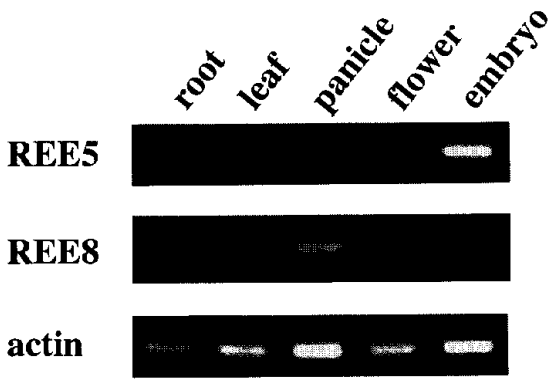

Fig. 4 RT-PCR analysis of REE5, REE8 and actin with cDNA templates from roots and leaves 7 days after germination, young panicles, mature flowers just prior to heading and embryos at 4 DAA (E).

examine the organ specificity of the transcripts corresponding to REE5 and REE8, RT-PCR analysis was performed using mRNA isolated from roots and leaves at 7 days after germination, young panicles, flowers just before heading, and embryos at 4 DAA (**Fig. 4**). Strikingly, transcripts corresponding to REE5 and REE8 were expressed in young panicles as well as in immature embryos. In other tissues, namely roots, leaves and flowers, the transcripts could not be detected. The young panicles used in this study was an inflorescence meristem about 700 μm long and was prior to the emergence of floral organs. Both the embryos at 4 DAA and young panicles at this stage are in the process of differentiating various types of tissues.

Sequence analysis determined that the REE5 and REE8 clones were 254 and 461 nucleotides long, respectively [6]. The sequences were used to search the DNA and protein databases using the BLAST program, in order to investigate the possible identities of these clones. The cDNAs did not share significant homology with any sequences currently reported in

the databases.

In order to know if these clones could be used for *in situ* hybridization analysis for definition of the spatial expression patterns during embryogenesis, genomic Southern blot analysis was performed. REE5 probe detected a single band, but REE8 probe detected multiple bands (data not shown). *In situ* RNA hybridization experiments were, therefore, performed using REE5-specific digoxigenin-labeled antisense RNAs as probes. **Fig. 5** shows the specific accumulation of REE5 mRNA in zygotic embryos. The transcript levels were higher in the embryos at 4 and 5 DAA than in the embryos at 3 and 7 DAA. The temporal pattern observed in the *in situ* hybridization experiments was consistent with the results of RT-PCR analysis (**Fig. 3**). The transcript corresponding to REE5 was expressed in the whole embryo but not in the endosperm or the aleurone layer. Therefore, REE5 was expressed in a tissue (*i.e.* embryo)-specific, manner. The embryo at 4 to 5 DAA is the critical phase of rice embryogenesis, as initial tissue differentiation including coleoptile, shoot apical meristem, radicle meristem, vascular systems and scutellum has just begun (**Fig. 1**). The specific expression of the REE5 transcript during early embryogenesis may therefore be correlated with early embryo specification.

4. Discussion

Extensive efforts have been made to identify genes that are preferentially expressed in early embryogenesis using somatic embryos. Most of the genes that have been isolated, however, are expressed in very high abundance and accumulate relatively late in embryo development [7]. This is because genes that are expressed during early somatic embryogenesis overlap extensively with those expressed during callus proliferation [7, 8]. The present study shows that simplified differential display using zygotic embryos is an effective experimental approach for the isolation of genes specifically involved in early embryogenesis. We were able to identify two transcripts that accumulate at very early stages of embryogenesis (**Fig. 3**). Recently, Heck *et al.* isolated a gene (AGL15) encoding a new member of the MADS domain gene family, using differential display to identify genes preferentially expressed in young embryos at the transition and heart stages in *Brassica napus* [9]. Their results also suggest that differential display is a useful method for the isolation of low-abundance mRNAs such as the putative transcription factor expressed in developing embryos.

Homeobox genes are expected to play an important role in plant morphogenesis as in animals. Recently, the expression of a rice homeobox gene, OSH1, during embryogenesis was investigated [10]. OSH1 is expressed in a specific ventral region of early embryos. The spatially and temporally different expression pattern of REE5 gene was observed in this study (**Fig. 5**). This indicates that REE5 and OSH1 genes are controlled by different gene-cascades. Differential display methods may allow the isolation of various types of differentially expressed genes.

Both mRNAs corresponding to REE5 and REE8 accumulate in immature embryos and inflorescence meristems, but not in roots, leaves or flowers (**Fig. 4**). The feature common to both the immature embryos and the inflorescence meristems is the onset of differentiation of various types of tissues. Therefore, the two genes corresponding to REE5 and REE8 appear to be associated with differentiation and morphogenesis. The transcript corresponding to REE5 is present in all of the cells of the embryo proper, but not in other tissues of the seed, such as endosperm and aleurone (**Fig. 5**). It is unlikely that the gene plays a role in specification of particular cells or determination of polarity in the plant body. The pattern of expression may indicate a more general regulatory role such as maintenance of embryo-specific programs. Recently, several investigators have reported the isolation of genes that are specifically expressed in early somatic or zygotic embryogenesis [9-13]. These genes, as well as the genes that we have isolated, may be useful as molecular markers for analysis of the earliest events of embryogenesis.

Acknowledgements

The authors are grateful to Prof. S. Naito of Hokkaido University for valuable discussion and critical reading of the manuscript. We also thank Dr. K. Nemoto of University of Tokyo for technical instruction in carrying out the histological studies and *in situ* hybridization analysis.

References

[1] Liang, P., Pardee, A. B., 1992. Science, **257**: 967-971.

[2] Yoshida, K. T., Naito, S., Takeda, G., 1994. Plant Cell Physiol., **35**: 1003-1009.

[3] Sharman, B. C., 1943. Stain Technol., **18**: 105-111.

[4] Kouchi, H., Hata, S., 1993. Mol. Gen. Genet., **238**: 106-119.

[5] Fujimura, T., Komamine, A., Matsumoto, H., 1980. Physiol. Plant., **49**: 255-260.

[6] The nucleotide sequences were submitted to DDBJ, EMBL, GenBank nucleotide sequence databases. The accession numbers of REE5

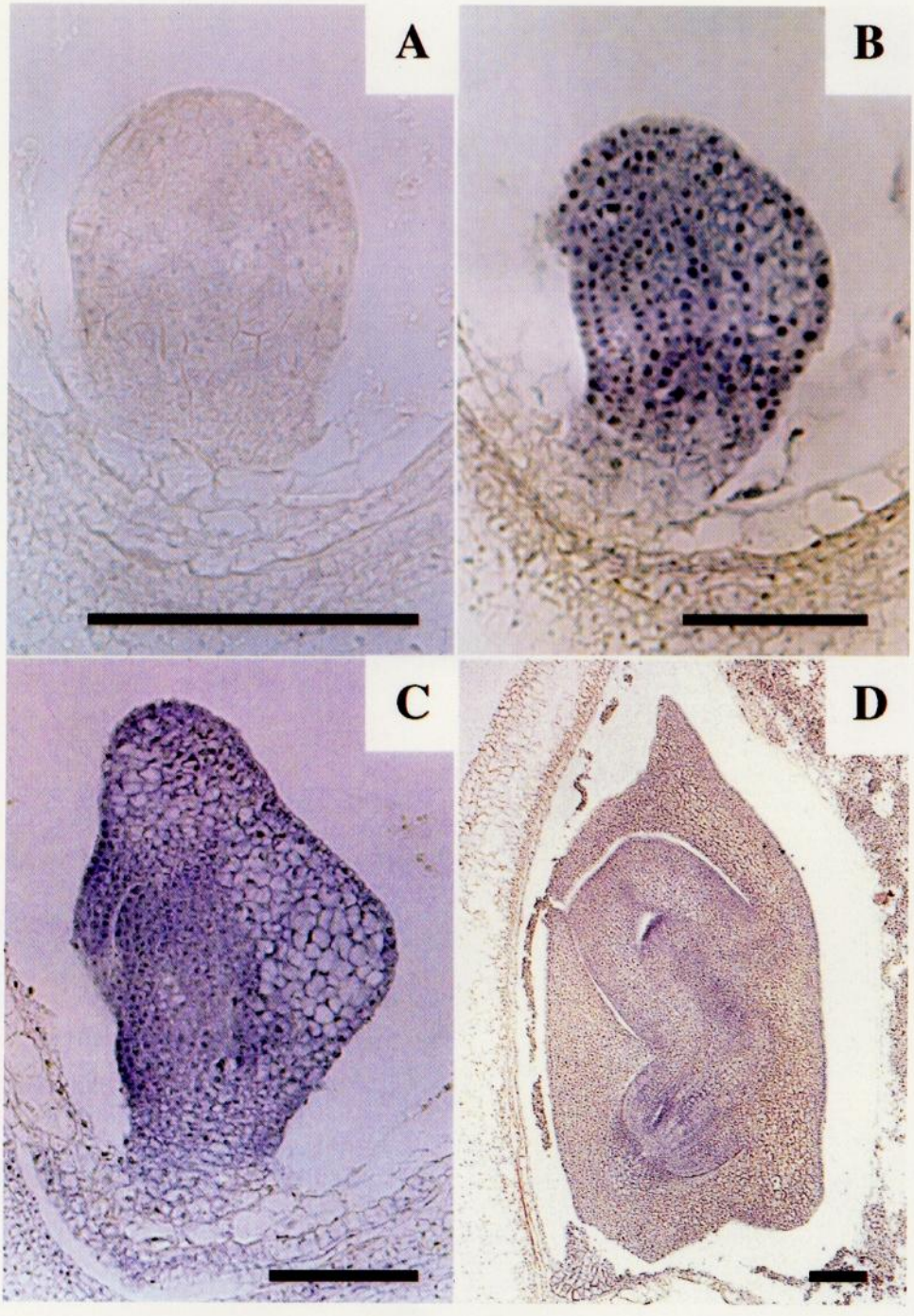

Fig. 5. *In situ* localization of REE5 mRNA in developing embryos. Longitudinal sections of embryos at 3 DAA (A), 4 DAA (B), 5 DAA (C) and 7 DAA (D) were hybridized with digoxigenin-labeled RNA probe. The hybridization signal is visualized as purple color. Scale bars indicate 50 μm.

and REE8 are AB006325 and AB006326, respectively.

[7] Zimmerman, J. L., 1993. Plant Cell, **5**: 1411-1423.

[8] Choi, J. H., Sung, Z. R., 1984. Plant Mol. Biol. Rep., **2**: 19-25.

[9] Heck, G. R., Perry, S. E., Nichols, K. W., Fernandez, D. E., 1995. Plant Cell, **7**: 1271-1282.

[10] Sato, Y., Hong, S.-K., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y., Matsuoka, M., 1996. Proc. Natl. Acad. Sci., **93**: 8117-8122.

[11] Momiyama, T., Afele, J. C., Saito, T., Kayano, T., Tabei, Y., Takaiwa, F., Takayanagi, K., Nishimura, S., 1995. Biochem. Biophys. Res. Commun., **213**: 376-382.

[12] Sato, S., Toya, T., Kawahara, R., Whittier, R. F., Fukuda, H., Komamine, A., 1995. Plant Mol. Biol., **28**: 39-46.

[13] Lin, X., Hwang, G.-J. H., Zimmerman, J. L., 1996. Plant Physiol., **112**: 1365-1374.