Cloning of a Gene That is Specifically Expressed during Somatic and Zygotic Embryogenesis in Rice

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Summary
cDNA libraries were constructed from poly(A)+-RNAs that had been isolated from calli 7 days after induction of regeneration on two different media, namely, embryogenesis-inducing medium and organogenesis-inducing medium. Differential screening of the libraries with cDNA probes prepared from unorganized calli and from calli that had been induced to undergo somatic embryogenesis and organogenesis (formation of adventitious shoots) allowed us to identify a cDNA clone, pRSEMI, that was specific to regeneration. Analysis of the temporal accumulation of the mRNA that corresponded to pRSEMI during embryogenesis and organogenesis indicated the specific accumulation of the mRNA in calli that had been induced to undergo somatic embryogenesis. In plants, RSEMI mRNA was detectable in zygotic embryos but not in shoots, roots or flowers. Thus, RSEMI mRNA accumulated specifically in somatic embryos in culture and in zygotic embryos in the intact plant.

Key Words: Oryza sativa, cDNA cloning, differential screening, somatic embryogenesis, regeneration, zygotic embryo.

Introduction

Somatic embryogenesis provides an attractive system for studies of the developmental processes that occur in the zygotic embryos of plants. Embryogenic cell culture systems have been established in several plant species, including alfalfa (Dudits et al. 1991), carrot (Nomura and Komamine 1985), eggplant (Saito and Nishimura 1994), and rice (Ozawa and Komamine 1989). In particular, the carrot system is especially well suited for analysis of somatic embryogenesis because such embryogenesis can be induced efficiently and in a synchronized manner by removal of auxin from the medium. Several genes that are expressed preferentially in somatic embryos of carrot have been isolated (Aleith and Richter 1990, Choi et al. 1987, Kawahara et al. 1992, Wilde et al. 1988, Wurtele et al. 1993), and some of them have been shown to encode a class of proteins called LEA (late embryogenesis abundant) proteins (Goupil et al. 1992, Kiyosue et al. 1992, 1993, Ulrich et al. 1990, Wurtele et al. 1993).

In contrast to the ease with which cell culture systems can be established in dicotyledonous plants, it is generally difficult to establish cell culture systems in monocotyledonous plants when there is a requirement for the maintenance of a high capacity for propagation and regeneration (Ozawa and Komamine 1989). The rice culture system that we established recently (Yoshida et al. 1994a) is useful because regeneration can be achieved at high frequency. In addition, this culture system is unique because we can separate the morphogenic pathways into the embryogenic pathway, which leads to the formation of somatic embryos, and the organogenetic pathway, which leads to the formation of adventitious shoots, by transferring unorganized calli onto two different regeneration media, namely, embryogenesis-inducing medium and organogenesis-inducing medium, respectively. Therefore, this culture system should potentially allow us to isolate novel genes that are essential for embryogenesis if we compare calli that have been induced to undergo embryogenesis (designated embryogenic calli) not only with unorganized calli but also with calli that have been induced to undergo organogenesis (designated organogenetic calli).

We previously reported the detection of several proteins that appeared after induction of embryogenesis and organogenesis by two-dimensional polyacrylamide gel electrophoresis of total proteins from the three types of callus mentioned above (Yoshida et al. 1992). In order to isolate genes associated with embryogenesis, we employed the differential screening method among the three different types of callus, and we successfully isolated a cDNA clone that was specific to embryogenic calli. We also analyzed the temporal accumulation of the corresponding mRNA during embryogenesis in culture and in intact plants.

Materials and Methods

Plant materials

Rice (Oryza sativa, var. japonica, cv. Kamenoo) plants were grown in a paddy field under standard conditions. Mature seeds were harvested and used to initiate suspension cultures. The culture conditions for induction of callus, maintenance of suspension cells and induction of

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regeneration (embryogenesis and organogenesis) have been described earlier (Yoshida et al. 1994a).

Adventitious shoots appeared after about 14 days of culture on the organogenesis-inducing medium, whereas different structures composed of shoots and radicles appeared after about 40 days on the embryogenesis-inducing medium. The embryogenic and organogenetic potentials of the calli used in this study were routinely monitored by checking 40-day-old calli on the embryogenesis-inducing medium and the organogenesis-inducing medium, respectively. Over 90% of the calli used in this study retained their characteristic features, as described above.

Rice cultivar Taichung 65 was used for preparing total RNAs from flowers.

Construction of cDNA libraries and differential screening
Total RNA was extracted by the SDS-phenol method and precipitated with lithium chloride (Watanabe and Price 1982). Poly(A)$^\text{+}$-RNAs were prepared using Oligotex-dT30 (Takara Shuzo, Kyoto, Japan). The poly(A)$^\text{+}$-RNAs prepared from calli that had been grown for 7 days on the embryogenesis-inducing medium and the organogenesis-inducing medium were separately used for construction of cDNA libraries with ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA).

Differential screening was performed separately for each of the cDNA libraries. Three types of $^{32}$P-labeled first strand cDNA probe were generated by reverse transcription of poly(A)$^\text{+}$-RNAs that had been isolated from the unorganized calli, the 7-day-old embryogenic calli, and the 7-day-old organogenetic calli. Synthesis of cDNA probes by random primer labeling, plaque hybridization and selection were carried out according to the standard procedures (Sambrook et al. 1989). Three replicate membranes were made and they were hybridized to the three types of cDNA probe. Plaques that gave a signal only with the cDNA probes synthesized from the calli on regeneration media were recovered. Inserts from the recombinant clones were subcloned into the Bluescript SK$^+$ vector (Stratagene) and colony hybridization was carried out by the standard procedures (Sambrook et al. 1989).

Northern blot analysis
Total RNAs were prepared using the ISOGEN system (Nippon Gene, Tokyo, Japan) from shoots and roots of 7-day-old seedlings, from flowers just before heading, from immature embryos at 14 days after flowering and from calli harvested at various times after the induction of regeneration. The samples of total RNA were then fractionated by electrophoresis on 1.2% agarose gels that contained 0.73 M formaldehyde. The RNAs were then transferred from gels to nylon membranes (Hybond-N$^+$; Amersham, Buckinghamshire, UK). $^{32}$P-labeled probes were prepared using a Random Primed DNA Labeling Kit (Takara Shuzo). Hybridization was performed in accordance with the manufacturer's recom-

Results

Isolation of cDNAs specific to embryogenic calli
A striking feature of the present culture system is that the calli can be directed towards either somatic embryogenesis or organogenesis and production of adventitious shoots by incubation on different regeneration media. The timing of the expression of the genes associated with embryogenesis or organogenesis is unknown. However, within 7 days after transfer to the embryogenesis- or organogenesis-inducing medium, we were able to observe specific morphological changes in the calli under the light microscope. Since we are interested in the genes that are expressed during the early stages of regeneration (embryogenesis and organogenesis), we carefully selected 7-day-old calli on the embryogenesis-inducing medium that had a smooth nodular surface, which we called embryogenic calli, and those on the organogenesis-inducing medium that had turned green, which we called organogenetic calli (Fig. 1). cDNA libraries were constructed from poly(A)$^\text{+}$-RNAs that had been isolated from these two types of callus. The two cDNA libraries were screened independently.

In the case of the cDNA library constructed from the

![Fig. 1. Morphological aspects of calli 7 days after the induction of regeneration. A: Embryogenic calli (EC) with a smooth nodular surface. B: Organogenetic calli with greening spots (GS). Scale bars indicate 200 μm.](image-url)
embryogenic calli, 15,000 plaques were screened with the three probes and two cDNA clones, designated pRSEM1 and pRSEM2, which were specific to the embryogenic calli, were identified. The cDNA library constructed from the organogenetic calli (approx. 9,000 plaques) was also differentially screened in the same way. However, no clones specific to the organogenetic calli were isolated. RSEM1 was analyzed further.

Expression of RSEM1 mRNA during regeneration

The accumulation of the mRNA that corresponded to pRSEM1 was analyzed by Northern blot analysis of total RNAs isolated from the unorganized calli, the embryogenic calli and the organogenetic calli. The mRNA corresponding to pRSEM1 was detected only in the embryogenic calli. pRSEM1 hybridized to a single species of poly(A)^+^-RNA of about 900 bases in length (Fig. 2). RSEM1 mRNA was hardly detectable in the unorganized calli.

To analyze the temporal accumulation of the mRNAs that corresponded to pRSEM1 during embryogenesis and organogenesis, total RNAs were prepared from calli that had been cultured for various periods of time (2, 4, 7 and 20 days) after the induction of either embryogenesis or organogenesis. RSEM1 mRNA was detected 2 days after the induction of embryogenesis (Fig. 2). The level of this mRNA gradually increased until 7 days after induction of embryogenesis and then it decreased, reaching a low level by 20 days after induction. The RSEM 1 mRNA was not detectable during organogenesis (Fig. 2).

Zygotic embryo-specific accumulation of RSEM1 mRNA

To investigate the spatial patterns of expression of RSEM1 mRNA in rice plants, Northern blot analysis was performed using total RNAs isolated from various organs of rice. The mRNA corresponding to pRSEM1 accumulated at high levels in zygotic embryos but not in shoots, roots or flowers (Fig. 2). Thus, the RSEM1 mRNA accumulated specifically in zygotic embryos, as well as in somatic embryos.

Discussion

We have isolated a cDNA clone, pRSEM1, that is specific to embryogenic calli by differential screening of the unorganized calli, embryogenic calli and organogenetic calli. RSEM1 mRNA was detected in the embryogenic calli but was not detectable in the organogenetic calli. The mRNA was hardly detectable in the unorganized calli (Fig. 2). In plants, the mRNA was detected only in the zygotic embryos (Fig. 2). Thus, the specific accumulation of the RSEM1 mRNA was demonstrated in zygotic embryos, as well as in somatic embryos. The fact that RSEM1 mRNA accumulated in the embryogenic calli but not in the organogenetic calli indicates that the accumulation of this mRNA was not stimulated by the transfer of the calli from liquid to solid medium but was induced during somatic embryogenesis.

We recently reported a novel method for differential screening (Yoshida et al. 1994b), whereby cDNAs are compared that have been randomly amplified by the polymerase chain reaction with a single primer for detection of randomly amplified polymorphic DNA (RAPD). When we applied this method to our rice culture system, a number of differentially expressed transcripts were detected and several clones that appeared to be specific to regeneration were obtained. All three types of regeneration-specific cDNA, namely, embryogenesis-specific cDNAs, organogenesis-specific cDNAs and cDNAs common to embryogenesis and organogenesis, were detected. From their sizes and patterns of expression, the transcript that correspond to pRSEM1 appears to be different from those that we obtained in our previous study (Yoshida et al. 1994b).

Only 21 genes that are differentially expressed during somatic embryogenesis in carrot have been isolated to date even somatic embryogenesis in carrot has been extensively studied (for review, see Zimmerman 1993). The mRNAs corresponding to 6 cDNA clones out of the 21 have been shown to be expressed in carrot zygotic embryos. We have now isolated a total of 6 clones (Yoshida et al. 1994 b). Our results indicate that our culture system has a high potential for isolation of various types of gene that have not previously been isolated from culture systems of dicotyledonous plants, such as carrot. Although the functions of the gene that correspond to pRSEM1 are unknown, the clone can be exploited as useful markers for the identification of specific cells during embryogenesis. Identification of genes essential for both somatic and zygotic embryogenesis will lead to an understanding of the unique feature of embryogenesis.

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