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Homeobox Gene (*OSH1*) Expression in Embryonic Mutants of Rice (*Oryza sativa* L.)

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Recent identification and characterization of plant homeobox genes suggest that they play important roles in morphogenetic events. *OSH1*, one of the rice homeobox genes, is thought to be related to organ development since the changes of *OSH1* gene expression cause morphological abnormalities of leaves by the ectopic expression and is expressed during early embryogenesis. In this experiment, the expression pattern of *OSH1* was analyzed in embryo mutants by *in situ* hybridization, and *OSH1's* potential as a molecular marker was explored. Region-specific expression of *OSH1* during early embryogenesis shows that *OSH1* could be used as a molecular marker for characterizing embryo mutants. Although several organless and shootless mutants showed normal expression of *OSH1*, some mutants exhibited abnormal expression patterns. In a minute organless *cle1-1* embryo whose epidermis resembled morphologically the epithelium of scutellum, *OSH1* expression was limited to a small basal region. This expression pattern suggests the gross deletion of the basal part. In a radicleless mutant, *odm*115, *OSH1* expression was detected in a basal region instead of subcentral region of the ventral side. Together with other characteristics (short embryo and normal adventitious roots), *odm*115 was estimated to be derived from the deletion of basal region. Among five shootless mutants, three showed normal expression of *OSH1*. In the *shl2* embryo, no expression of *OSH1* was observed. In the *shl1* embryo, however, *OSH1* expression was extended to a dorsal side, indicating that *SHL2* might be related to dorsoventral patterning. The above results of *in situ* hybrydization clearly indicate that *OSH1* can be utilized as a marker for characterizing gene functions of embryo mutants.

One of the approaches for understanding the regulatory mechanisms involved in plant embryogenesis is to identify molecular markers which enable researchers to monitor cell specification events during early embryogenesis (Goldberg et al., 1994). Along with this approach, it has been demonstrated that some genes are expressed in specific cell types, regions or organs of embryo (Goldberg et al., 1989, Jufuku et al., 1989).

In *Drosophila*, the principles of the genetic control during embryogenesis have been unraveled through combined genetic and molecular approaches (for review, see Ingham, 1988). These approaches demonstrate that the homeobox genes, which encode evolutionarily conserved 61-amino-acid domains called homeodomains, play important roles in cellular or regional differentiation of *Drosophila* embryos. Homeodomain proteins function as transcriptional regulators, and the homeodomain is responsible for sequence-specific binding to DNA via a helix-turn-helix motif (Gehring, 1987; Laughon, 1991; Scott et al., 1989). In plants, homeobox genes have been isolated from several species such as maize (Vollbrecht et al., 1991; Bellmann and Werr, 1992; Hake, 1992), rice (Matsuoka et al., 1993; Tamaoki et al., 1995), *Arabidopsis* (Ruberti et al., 1991, Schena and Davis, 1992), soybean (Ma et al., 1994) and carrot (Kawahara et al., 1995). On the analogy of the functional roles of animal homeobox genes, plant homeobox genes are expected to encode transcriptional regulators that mediate important developmental processes during embryogenesis (Schena and Davis, 1992). It has not been demonstrated, however, that the plant homeobox genes are involved in embryogenesis, while ectopic expressions of the homeobox genes cause the abnormal leaf development in the transgenic plants in vegetative phase (Kano-Murakami et al., 1993; Matsuoka et al., 1993; Schenan et al., 1993; Sinha et al., 1993; Lincoln et al., 1994; Matsuoka et al., 1995). Only recently, homeobox genes have been suggested to be associated with the formation of shoot apical meristem (Klinge and Werr, 1995; Smith et al., 1995).

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To elucidate the possibility that plant homeobox genes are concerned with embryo development, the temporal and spatial expression of a rice homeobox gene, *OSH1*, during rice embryogenesis (Sato et al., 1996) was examined in the wild type and various embryonic mutants which showed abnormalities at the early stage. The results described here suggest that *OSH1* is not directly associated with shoot development, but functions to specify cell identity and provides a regional information for the formation of shoot and adjacent tissues, and that embryonic mutants would be well characterized using *OSH1* as a molecular marker.

Materials and Methods

Plant materials

To observe *OSH1* expression in wild type embryo, a rice cultivar Taichung 65 was used. The following embryo mutants were used: globular embryo 1 (*gle1*), globular embryo 2-2 (*gle2-2*), club-shaped embryo 1-1 (*cle1-1*), shootless 1 (*shl1*), shootless 2 (*shl2*), shootless 3 (*shl3*), *odm*66, *odm*67 and *odm*115. Of them, *orl1* is characterized as bearing organless but relatively large embryo. On the other hand, *gle1* and *gle2-2* produce organless but very small embryos, and *cle1-1* also produces an organless small embryo with club-like shape. The five mutants, *shl1*, *shl2*, *shl3*, *odm*66 and *odm*67, show shootless phenotype. The *odm*115 is radicleless mutant, although adventitious roots are normally differentiated.

Preparation of rice embryo sections

The developmental course of wild-type rice embryos was examined by the standard paraffin method. For paraffin sectioning, seeds at various developmental stages were fixed in FAA (formalin/glacial acetic acid/50% ethanol, 5:5:90), dehydrated in graded ethanol series, and embedded in paraffin. Samples were sectioned at 10 μm and stained with hematoxylin.

In situ hybridization

Embryos at 2 days after pollination (2 DAP) through 7 DAP of the wild type and *orl1* were used. In other mutants, embryos at 5 DAP were mainly used. Digoxigenin-labeled RNA was produced from the *OSH1* coding region of 1.1 kbp without poly(A) which was kindly provided by Prof. M. Matsuoka (Nagoya University, Japan). *OSH1* (*Oryza sativa homeobox* 1) contains a 1086 bp open reading frame, capable of encoding a polypeptide of 361 amino acid residues, with a 216 bp 5′ noncoding region and a 240 bp 3′ noncoding region (Matsuoka et al., 1993). *In situ* hybridization was conducted as described by Kouchi and Hata (1993). Embryos were fixed with 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer and embedded in Paraplast Plus (Fisher Scientific). Microtome sections of 7-10 μm thick were placed onto slide glass treated with Vectabond (Vector Lab.). Hybridization and immunological detection of the hybridized probe were performed by the method of Kouchi and Hata (1993).

Results

OSH1 expression in organless embryo mutants

Sections revealed that *OSH1* was expressed in a specific region from the early globular stage, and was

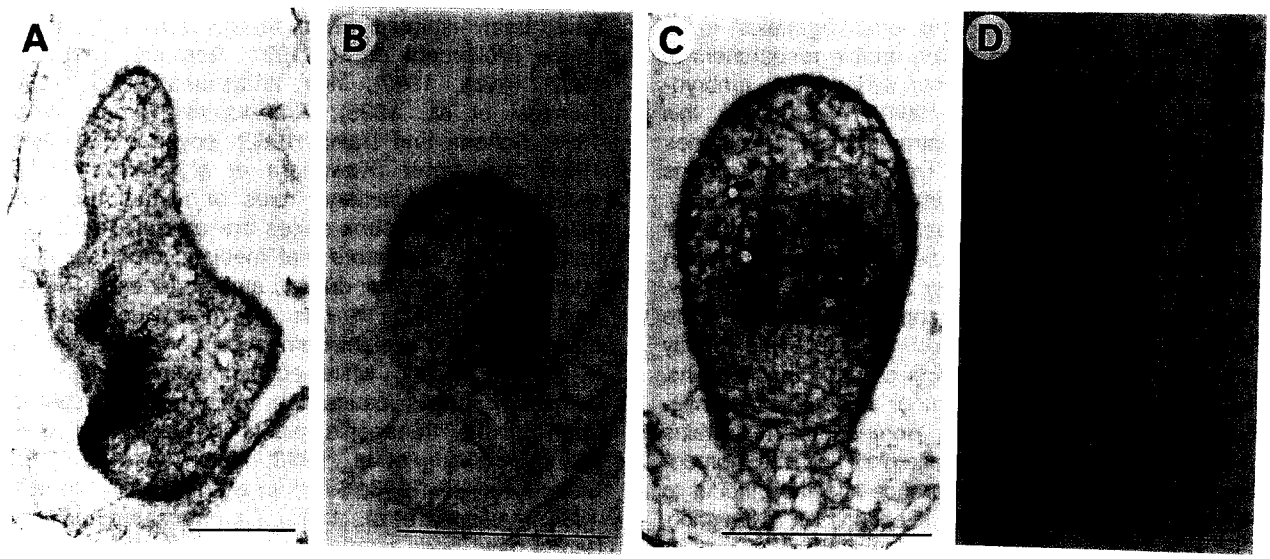

Fig. 1. *OSH1* expression in organless embryo mutants at 5 DAP. A, Wild-type embryo. B, *gle1* embryo showing normal *OSH1* expression. C, *gle2-2* embryo showing *OSH1* expression extended to the dosal region. D, *cle1-1* embryo in which *OSH1* expression is limited in a small basal region. Scale bars=0.1 mm.

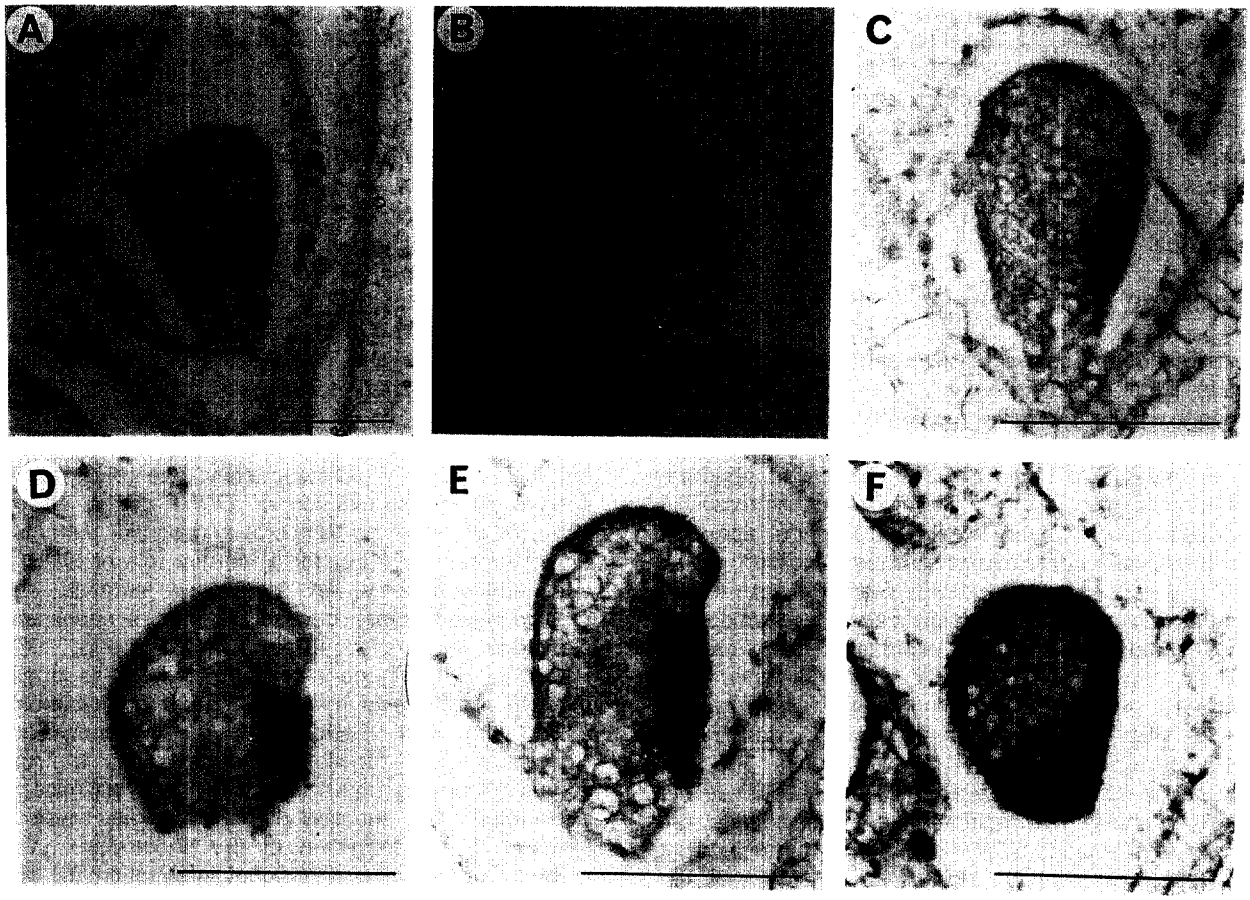

Fig. 2. *OSH1* expression in shootless and radicleless mutants at 5 DAP. A, *shl1* embryo showing *OSH1* expression extended to the dosal region. B, *shl2* embryo showing no expression of *OSH1*. C-E, Normal expression of *OSH1* in *shl3*, *odm*66 and *odm*67 embryos, respectively. F, *odm*115 embryo where *OSH1* expression is observed in the basal region. Scale bars=0.1 mm.

not directly associated with shoot differentiation. These temporal and spatial specificities of *OSH1* expression indicate that *OSH1* can be used as a region-specific molecular marker for characterizing embryo mutants. Then, the *OSH1* expression was examined in several kinds of organ-deficient embryo mutants by *in situ* hybridization.

First, the *OSH1* expression was analyzed in three organless mutants, *gle1*, *gle2-2* and *cle1-1*. The expression pattern in *gle1* was quite normal (Fig. 1B), indicating that *gle1* mutation caused abnormal development in the downstream of *OSH1*-related process or in a process independent of *OSH1*. In contrast, another globular embryo mutant, *gle2-2*, showed a different pattern of *OSH1* expression (Fig. 1C). Hybridization signals were extended to an internal dorsal region from the normal ventral region. Therefore, *gle2-2* would be a mutation of gene functioning in the upstream of *OSH1*-related process. Since the abnormal expression pattern was detected mainly in the dorsoventral direction, *gle2-2* might be related to dorsoventral pattern formation. In *cle1-1* embryo, hybridization signals were limited to a narrow region at the basal end (Fig. 1D). If *OSH1* is expressed region-specifically, this pattern suggests a gross deletion of basal region in *cle1-1* embryo.

OSH1 expression in shootless and radicleless embryo mutants

Several shootless and radicleless mutants were examined. Among shootless mutants, *shl3*, *odm*66 and *odm*67 showed a normal pattern of *OSH1* expression (Fig. 2C-E). Accordingly, these mutations would affect a process downstream of *OSH1*-related process. Two shootless mutants, *shl1* and *shl2*, exhibited abnormal *OSH1* expression. In *shl1* embryo (Fig. 2A), *OSH1* was expressed in a wider region extending to dorsal half as in *gle2-2*. Accordingly, *shl1* is expected to be a dorsoventral pattern mutation. Interestingly, no hybridization signal for *OSH1* was detected in *shl2* embryos at 5 DAP (Fig. 2B). This suggests that *SHL2* functions epistatically to *OSH1* in the same cascade, or is identical with *OSH1*.

A radicleless mutant, *odm*115, also showed an abnormal pattern of *OSH1* expression (Fig. 2F). Signals

were detected in a basal region larger than that of *cle1-1*, suggesting a deletion of basal region in *odm*115 embryo.

Thus, *in situ* hybridization analysis has clearly indicated that *OSH1* expression is modified in several embryo mutants. Therefore, embryo mutants would be sucessfully characterized using *OSH1* as a molecular marker, together with conventional morphological and anatomical markers.

Discussion

Plant homeobox genes act to determine regional and cellular identities during embryogenesis. Plant homeobox genes have been already confirmed to be expressed in the vegetative phase. Although ectopic expressions of *KN1* of maize and *OSH1* of rice cause abnormal leaf morphologies, both of them are not expressed in wild-type leaves but are expressed in shoot apex and the derivative indeterminate tissues (Jackson et al., 1994; Matsuoka et al., 1995; Smith et al., 1995). Evaluating their functional significance in development requires monitoring of their expression going back to the embryonic phase. In this experiment, we have examined the spatial and temporal expression pattern of the rice homeobox gene, *OSH1*, during rice embryogenesis and in embryogenic mutants. The main function of *OSH1* in embryo is to establish cellular identity in the ventral region at the globular stage, and at the later stages, *OSH1* is required for maintaining the identity (Sato et al., 1996).

The expression pattern of *OSH1* is almost the same as that of *KN1*, a maize counterpart gene for *OSH1*. *KN1* is expressed in the vegetative shoot apex, except in the L1 layer and leaf primordia (Jackson et al., 1994). From the expression pattern of *KN1* in the wild-type and dominant *KN1* mutant plants, Hake and her colleagues proposed a hypothesis that *KN1* functions to promote indeterminate growth and its down regulation is important for the entry of leaf founder cells into a determinate developmental pathway (Smith et al., 1992). Recently, they examined the expression of *KN1* during maize embryogenesis and showed that the *KN1* expression is closely associated with the shoot apex differentiation, since the *KN1* expression is first detected when and where shoot apex primordium is anatomically visible (Smith et al., 1995). Although the spatial expression pattern of *KN1* in embryo coincided with that of *OSH1*, the onset of *OSH1* expression seems much earlier than that of *KN1*. This indicates that there may be a functional difference between *OSH1* and *KN1* during embryogenesis.

Analyzing many rice embryonic mutants, Kitano et al. (1993) presented a model for regulatory process active during rice embryogenesis. In this scheme, they have proposed several regulatory processes before the onset of morphogenetic events, such as pattern formation (apical-basal, dorsal-ventral), determination of organ differentiation, positional regulation, and size regulation. The present data concerning the *OSH1* expression in specific cells of ventral region suggest that *OSH1* is related to the embryonic pattern, especially dorsoventral pattern. Embryonic pattern is estimated to be determined quite early in embryogenesis, because *Arabidopsis* pattern genes such as *GNOM* and *MONOPTEROS* function at the very early stage of globular embryo (Berleth and Jurgens, 1993; Mayer et al., 1993). As *OSH1* is expressed from the middle globular stage (ca. 100-cell stage), it would be not directly involved in the pattern formation, but would function in specifying cell identity or in the detailed regionalization after embryonic pattern is roughly established.

To date, a loss of function mutation of *OSH1* has not been isolated. Consequently, we can not yet specify the exact function of *OSH1* during embryogenesis, although the temporal and spatial patterns of *OSH1* expression imply the important role of *OSH1* on early embryogenesis. An *Arabidopsis* mutation, *shoot meristemless1* (*stm1*), caused the loss of shoot apical meristem in embryo (Barton and Poetig, 1993). Recently, *STM1* gene has been characterized as a putative *Arabidopsis* homeobox gene homologous to *OSH1* and *KN1* (Barton, personal communication). Considering that functions of Hox genes are often conserved among organisms as different as mice and *Drosophila*, the function of *OSH1* may also be involved in the formation of the shoot apex in the embryo by providing a regional information for shoot differentiation.

In situ hybridization analysis of organless mutants using *OSH1* as a probe showed that mutations may be categorized into two groups: one functioning in the upstream of *OSH1* and the other in the downstream. Mutants showing normal expression of *OSH1* such as *gle1*, *shl3* and odm66 would be defective in regulatory processes after *OSH1*-related process (pattern formation or regionalization) has been normally accomplished.

On the other hand, mutations showing unusual *OSH1* expression are interesting to discuss. Plausible interpretations on gene functions can be made on two mutants, *cle1-1* and *odm*115 (Fig. 3). Anatomically, epidermis of *cle1-1* resembles the epithelium of scutellum (Fig. 3E, F), suggesting that *cle1-1* embryo is mostly composed of scutellum. In *cle1-1* embryo, *OSH1* expression was limited to a small basal region (Fig. 3G). If *OSH1* is expressed in a ventral and central region, this expression pattern implies that *cle1-1* embryo has lost most of the basal region (Fig. 3H). A similar interpretation can be made for *odm*115 embryo. Morphologically, the *odm*115 embryo is shorter than the wild type embryo, and its shoot is differentiated in the central part of the embryo more basally compared to the wild type embryo (Fig. 3I and J). In addition, the ability of producing adventitious roots is not impaired in *odm*115. These characteristics

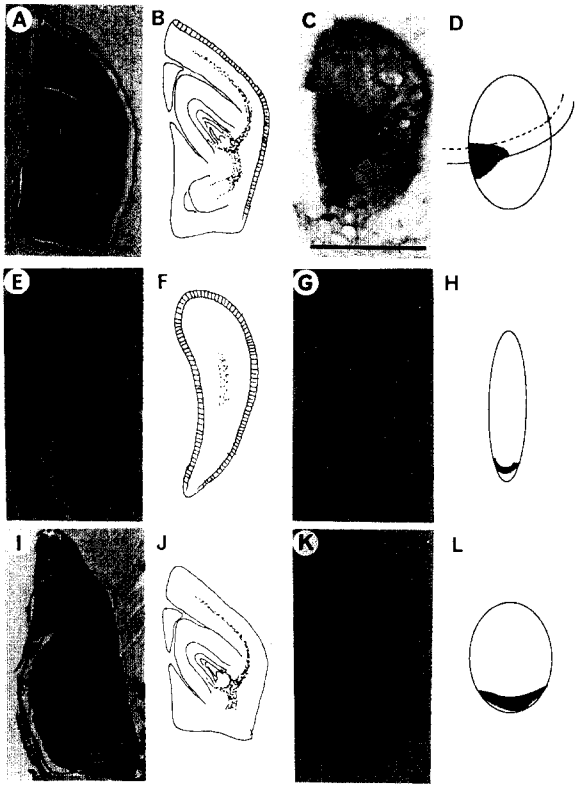

Fig. 3. Estimation of gene functions in *cle1-1* and *odm*115. A-D, Wild-type embryo, E-H, *cle1-1* embryo and I-L, *odm*115 embryo. A, E, and I, Median longitudinal sections of mature embryos. B, F, and J, Schematic presentation of mature embryo, C, G, and K, *OSH1* expression pattern. D, H, and L, Schematic presentation of regionalization. Epidermal layer of *cle1-1* embryo (E, F) is more morphologically similar to the epithelium of scutellum. Region below the dotted curve is estimated to be lost in *cle1-1* (H) and that below the solid curve *odm*115 (L). Arrows, shoot apex; arrowheads, radicle. Scale bars=0.1 mm.

suggest a deletion of the basal region where radicle is to be formed. Together with the *OSH1* expression in the basal region (Fig. 3K), *odm*115 would be a basal-deletion mutant (Fig. 3L).

These results strongly indicate that *OSH1* is a powerful region-specific marker. Thus, by examining the expression pattern of *OSH1*, we can characterize embryo mutants more precisely in relation to pattern formation which, in turn, enriches our understanding on plant homeobox genes.

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