Regional Expression of the Rice KN1-Type Homeobox Gene Family during Embryo, Shoot, and Flower Development

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We report the isolation, sequence, and pattern of gene expression of members of the KNOTTED1 (KN1)-type class 1 homeobox gene family from rice. Phylogenetic analysis and mapping of the rice genome revealed that all of the rice homeobox genes that we have isolated have one or two direct homologs in maize. Of the homeobox genes that we tested, all exhibited expression in a restricted region of the embryo that defines the position at which the shoot apical meristem (SAM) would eventually develop, prior to visible organ formation. Several distinct spatial and temporal expression patterns were observed for the different genes in this region. After shoot formation, the expression patterns of these homeobox genes were variable in the region of the SAM. These results suggest that the rice KN1-type class 1 homeobox genes function cooperatively to establish the SAM before shoot formation and that after shoot formation, their functions differ.

INTRODUCTION

In animal embryogenesis, homeobox-containing genes work as spatial cues at all levels of the developmental hierarchy. For example, in Drosophila embryogenesis, bicoid is maternally transcribed, and its mRNA is specifically localized at the anterior tip of the early embryo. Here, the bicoid gene product establishes a pattern in the anterior half of the embryo in a concentration-dependent manner (Driever and Nüsslein-Volhard, 1988). A gap gene, orthodenticle, is expressed in a circumferential stripe in the presumptive head region of the blastoderm embryo and is necessary for the formation of anterior head structures (Finkelstein and Perrimon, 1990). The Distalless transcript is expressed in the limb primordia of the embryo and is required for the development of all limb structures in the larva and the adult (Cohen, 1990). eyeless is transcribed in the embryonic primordia of the eye and is the master control gene for eye morphogenesis (Quiring et al., 1994). In each case study reported thus far, Drosophila homeobox genes are involved in determining segmental identity, regionalization, or cell identity.

In addition, it is known that the product of one homeobox gene can regulate the expression of other homeobox genes to create a more detailed positional information. The pair rule genes, even-skipped (eve) and fushi-tarazu (ftz), for example, are complementarily expressed in adjacent regions of the blastoderm embryo (Lawrence et al., 1987). The position and width of the regions in which eve and ftz are expressed coincide with the odd- and even-numbered parasegmental anlagen, respectively, indicating that the two genes mutually but competitively define the parasegmental regions. In this case, each homeobox gene mutually regulates the expression of the other to determine the more detailed regionalization after the broader regionalization has been defined.

The involvement of plant homeobox genes in embryogenesis was first demonstrated by the analysis of an Arabidopsis embryogenesis-defective mutant, shoot meristemless (stm). Embryos of stm mutants lack shoot meristems, whereas other embryonic organs, such as cotyledons, hypocotyls, and radicles, develop normally (Barton and Poethig, 1993). STM encodes a KNOTTED1 (KN1)-type homeodomain protein and is expressed in the region of the shoot apical meristem (SAM) during embryogenesis (Long et al., 1996). Localized expression of other KN1-type homeobox genes has also been observed around the region in which the SAM develops early in embryogenesis (Smith et al., 1995; Sato et al., 1996, 1998). These findings demonstrate the involvement of this class of homeobox genes in SAM formation and maintenance during embryogenesis.

In a previous study, we found that expression of two rice homeobox genes, OSH1 and OSH15, during embryogenesis overlaps in the region in which the SAM develops, prior to shoot formation (Sato et al., 1998). After shoot formation, the expression patterns of the two genes become distinct.

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from one another. This observation suggests the possibility that homeobox genes in this class may act cooperatively in SAM formation, after which they may act independently or competitively to maintain the SAM or to form lateral organs in a fashion analogous to the Drosophila homeobox genes.

To test this possibility, we attempted to isolate all of the members of the KN1-type class 1 homeobox gene family from rice and to examine their expression patterns during embryogenesis and in vegetative and floral shoots. In situ mRNA localization analyses revealed that the rice KN1-type class 1 homeobox genes distinguish in some manner the region in which the SAM develops from other regions prior to shoot formation. Based on these observations, we discuss the involvement of KN1-type homeobox genes in shoot formation during rice embryogenesis.

RESULTS

Cloning of Five Novel KN1-Type Genes from Rice

In previous studies, we isolated two rice clones, OSH1 and OSH15, which are members of the class 1 family of KN1-type homeobox genes (Matsuoka et al., 1993; Sato et al., 1998). In this study, we attempted to isolate as many of the remaining KN1-type class 1 homeobox genes as possible from rice. For this purpose, we constructed two cDNA libraries using poly(A)⁺ mRNA extracted from the shoot apical region and rachis, because most KN1-type class 1 homeobox genes are expressed mainly in these tissues (Kerstetter et al., 1994; M. Matsuoka, unpublished results). The cDNA libraries were screened with the homeobox regions of OSH1 and OSH15 under low-stringency conditions, leading to the isolation of >40 clones that hybridized with the probes. To confirm that these clones represented KN1-type class 1 genes, we amplified their homeobox regions using polymerase chain reactions (PCRs) with primers flanking conserved regions of KN1-type class 1 homeobox genes. Approximately 90% of the clones produced DNA fragments of the expected size, and all of the amplified DNA fragments were cloned and sequenced. Sequence analysis revealed that the clones represented six different KN1-type class 1 homeobox genes. Two of these were OSH1 and OSH15 (Matsuoka et al., 1993; Sato et al., 1998). The remaining four were novel KN1-type class 1 homeobox genes that we named OSH3, OSH6, OSH43, and OSH71.

We also tried to isolate novel homeobox genes by using rice genomic DNA and PCR with primers flanking a conserved region of the previously reported KN1-type class 1 homeobox genes. Because this conserved region included parts of exons 4 and 5 in the OSH1 and OSH15 genes (see Figure 1A), the sizes of the resulting PCR products were expected to vary, depending on the length of intron 4 (assuming that other rice KN1-type class 1 homeobox genes contain an intron at the same position or no intron). Seven fragments of different sizes were amplified from rice genomic DNA. All of the PCR products were cloned and sequenced, revealing that six of the fragments corresponded to the previously identified OSH clones (OSH1, OSH3, OSH6, OSH15, OSH43, and OSH71).

In the second approach, we screened a rice genomic library using the homeobox sequences of OSH1 and OSH15 as probes under low-stringency conditions. More than 100 clones were characterized by using PCR with various primers flanking the conserved ELK (glutamate, leucine, and lysine) homeodomain sequences present in the previously reported KN1-type class 1 genes (Kerstetter et al., 1994). Fragments of various sizes were amplified from all of the clones. Based on the sizes of the PCR products, we could classify the clones as corresponding to specific OSH genes because each OSH gene gives a band of unique size, which is determined by the length of the intron sequence flanked by the primers. Among these clones, we identified one novel KN1-type class 1 gene, OSH10.

We analyzed the entire nucleotide sequences of the OSH3, OSH6, OSH43, and OSH71 cDNA clones. These cDNA clones contained open reading frames encoding 367, 301, 341, and 311 amino acids, respectively. We also analyzed the partial sequence of OSH10 around the homeobox region. All of these clones encoded a 64-amino-acid homeodomain sequence that is well conserved throughout its entire length (Figure 1A). In particular, the third helix, which is predicted to be a recognition helix, is totally conserved in all OSH homeodomain proteins. The flanking ELK domain, which has been found in all other plant KN1-type homeodomain proteins (Kerstetter et al., 1994; Ito et al., 1999; Postma-Haarsma et al., 1999), was also conserved. Moreover, there was another conserved region on the N-terminal side of the ELK homeodomain that has often been seen in the KN1-type homeodomain proteins; this domain is referred to as the KNOX domain.

To elucidate the structural relationship of the OSH genes to the maize KN1-type homeodomain proteins, we performed a phylogenetic analysis of the rice and maize homeodomain sequences by using the unweighted pair group method with arithmetic mean (UPGMA). Figure 1B demonstrates that each OSH protein corresponds to one or two counterpart homeodomain protein(s) in maize. For example, OSH1 is paired with KN1, and OSH15 is paired with RS1 and KNOX4. Similar pairing was also seen between OSH3 and KNOX3, OSH6 and LG3, OSH43 and KNOX8, OSH10 and KNOX10, and OSH71 and KNOX5 or KNOX11.

We also mapped the OSH homeobox genes on the rice genome by using restriction fragment length polymorphism (RFLP) analysis. RFLPs of OSH1, OSH3, OSH6, OSH10, OSH15, OSH43, and OSH71 were present between Asominori (a Japonica rice) and IR24 (an Indica rice) DNA digested with DraI, Apal, Apal, BgIII, EcoRV, DraI, and KpnI, respectively. Linkage analysis was performed with digested genomic DNA from recombinant inbred lines of crosses
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between Asominori and IR24. Chromosomes 1, 5, and 7 contained single OSH genes OSH6, OSH71, and OSH15, respectively. Chromosome 3 contained a small cluster of four linked OSH genes, OSH1, OSH3, OSH10, and OSH43 (Figure 2A).

According to the comparative linkage between the rice and maize genomes (Ahn and Tanksley, 1993), we were able to compare the locations of the OSH homeobox genes on the rice chromosomes with those of the maize homeobox genes (Figure 2A; Kerstetter et al., 1994). This comparative analysis demonstrated that the paired homeobox genes in the phylogenetic tree (Figure 1B) mapped at analogous positions on the rice and maize chromosomes (see Discussion).

Because OSH1 and OSH3 were found to be closely linked (Figure 2A), we investigated the relationship between these genes in more detail by hybridization of the genes to yeast...
artificial chromosomes. We found one yeast artificial chromosome clone, Y4583, that contained both genes. The physical map around the genes demonstrated that OSH1 and OSH3 lie within a 37-kb stretch of DNA. The two genes are oriented in the same direction, with OSH3 lying upstream of OSH1 (Figure 2B).

In Situ Localization of the OSH mRNAs during Rice Embryogenesis

Rice embryos complete all morphogenetic events within 9 days under normal conditions. The globular stage lasts until almost 3 days after pollination (DAP) (Sato et al., 1996). The first morphological differentiation is recognized as a ventral protrusion of the coleoptile primordium at the late stage of 3 DAP embryos 100 μm in size (late 3 DAP) or the early stage of 4 DAP embryos 150 μm in size (early 4 DAP) (Figure 3W). At 4 DAP, when the embryo reaches ~200 μm long and comprises 800 to 900 cells, shoot and radicle apices are first observed (Figure 3X). The first through third foliage leaves are formed successively from the SAM at 5, 7, and 9 DAP, respectively, in an alternate phyllotaxis.

To define the spatial expression pattern of the OSH homeobox genes during embryogenesis, we conducted mRNA in situ hybridization experiments using digoxigenin-labeled
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OSH3 is present in the central part of the SAM (Figure 3J). As the globular embryos grew to 100 μm long at the 3 DAP globular stage, just before coleoptile differentiation, OSH3 expression was downregulated in the central region and in the center of the ventral region of the embryo (Figure 3B), where the shoot would subsequently form. OSH3 expression was still observed in other regions but was weaker than in early globular stages. Before this stage, no organ differentiation was observed. An expression pattern similar to that seen at the late globular stage persisted to the coleoptilar stage (early 4 DAP; Figure 3C). OSH3 expression became weaker as embryogenesis progressed, and no signal was seen after late 4 DAP (Figure 3D).

OSH6 expression was uniformly detected in early globular stage embryos at 2 DAP, similar to the case for OSH3, but no expression was seen in the endosperm (Figure 3F). After the 3 DAP globular stage, OSH6 expression was restricted to the region around or just below the center of the ventral side of the embryo, which includes the region in which the shoot apex would subsequently arise (Figure 3G). This localized expression around the shoot apex persisted in the coleoptile stage (Figure 3H). During the transition from the coleoptile stage to the shoot apex differentiation stage (late 4 DAP), OSH6 expression was divided between the upper and basal regions of the shoot area, which corresponded to the upper part of the SAM and the notch between the first leaf primordium and epiblast, respectively (Figure 3I). At a still later stage (6 DAP), when the first leaf primordia were evident, OSH6 mRNA was localized to the notches between the SAM and the first leaf primordium (Figure 3J) and the putative second leaf primordium (Figure 3J) but was not present in the central part of the SAM (Figure 3J).

The expression pattern of OSH43 differed from those of OSH3 and OSH6 and was similar to that of OSH1 (Figures 3U to 3Y; Sato et al., 1996). At the early globular stage (2 DAP), no signal was detected in any cells within the embryo (Figure 3K). Expression was first detected in globular embryos at 3 DAP, when it was restricted to a small region just below the center of the ventral portion of the embryo (Figure 3L). At the late globular through coleoptile stages, OSH43 was mainly expressed in the basal and central parts of the ventral side of the embryo (Figure 3M). The signal was localized in cells just below the coleoptile protrusion extending to the basal and inner region of the embryo and corresponding to the epiblast and part of the shoot region. At the shoot apex differentiation stage (late 4 DAP), OSH43 was expressed in the SAM (Figure 3N), the epiblast, and tissues lying between them but not in the region between the SAM and epiblast where the first leaf primordium would subsequently form (Figure 3N). Suppression of OSH43 expression in the L1 of the shoot apex area was also seen in the case of OSH1 (cf. Figures 3N and 3X). Expression was also observed in the cells surrounding the ventral side of the root apical meristem but not in the meristem itself. This spatial pattern of OSH43 expression was essentially maintained in subsequent stages after the second leaf primordium was formed (Figure 3O).

OSH71 was expressed at the globular embryo stage in a pattern similar to that of OSH43. Expression of OSH71 was not observed in the early globular stage at 2 DAP (Figure 3P). In globular stage embryos (3 DAP), the hybridization signal of OSH71 was detected around or just below the center of the ventral region of the embryo (Figure 3Q). The signal observed occasionally in the maternal tissue (see Figures 3L and 3Q) is likely to be an artifact. In coleoptile-stage embryos (early 4 DAP), OSH71 was expressed in the corresponding region of the epiblast and the central part of the embryo, whereas a weaker signal was detected in the SAM (Figure 3R). When the provascular tissue and radicle had differentiated at the shoot apex differentiation stage (late 4 DAP), localization of OSH71 expression was better defined, transcripts being detected in the cells surrounding the provascular tissue and radicle primordia but not in the provascular tissue or radicle themselves; weaker hybridization signals were observed in the SAM (Figure 3S). In nearly mature embryos (6 DAP), OSH71 expression was still observed around the basal part of the provascular tissue and radicle, whereas expression in the SAM was suppressed (Figure 3T). Expression around the shoot region was restricted to the base of the first leaf primordium and the notch between the SAM and the second leaf primordium (Figure 3T).

Double Staining in Situ Hybridization of OSH1 and OSH3 mRNAs

To compare the onset of OSH1 expression and the down-regulation of OSH3 expression at the ventral side of the embryo, we performed double staining in situ hybridization. At the early globular stage, localized expression of OSH1 was seen in the center to lower part of the ventral side (violet or black color in Figure 4B), whereas localized suppression of OSH3 had not occurred (red color in Figure 4A). At a later stage by which coleoptile differentiation had begun, suppression of OSH3 expression was observed in the region of subsequent SAM formation (Figure 4C), whereas high-level expression of OSH1 was seen at the ventral side of the embryo (Sato et al., 1996), covering the region of OSH3 suppression and the area in which the epiblast would be formed (Figure 4D). These results demonstrate that downregulation of OSH3 follows the increase in OSH1 expression, and the regions in which these events occur partially overlap in the area of SAM formation.
Figure 3. Localization of OSH3, OSH6, OSH43, OSH71, and OSH1 Transcripts during Rice Embryogenesis.

Longitudinal sections through rice embryos (2 to 6 DAP) were hybridized with OSH3, OSH6, OSH43, OSH71, and OSH1 antisense and OSH3 sense RNA probes labeled with digoxigenin-UTP. The transcript-specific hybridization signal is visualized as blue to violet color.

(A), (E), (F), (K), (P), and (U) Embryos at 2 DAP.
(B), (G), (L), (Q), and (V) Embryos at 3 DAP.
(C), (H), (M), (R), and (W) Embryos at early 4 DAP.
(D), (I), (N), (S), and (X) Embryos at late 4 DAP.
(J), (O), (T), and (Y) Embryos at 6 DAP.

In (A) to (D), sections were hybridized to a digoxigenin-labeled OSH3 antisense probes; in (E), the section was hybridized to an OSH3 sense probe; in (F) to (J), sections were hybridized to an OSH6 antisense probe; in (K) to (O), sections were hybridized to an OSH43 antisense probe; in (P) to (T), sections were hybridized to an OSH71 antisense probe; and in (U) to (Y), sections were hybridized to an OSH1 antisense probe. Arrowheads indicate the SAM, filled arrows indicate the notches between the SAM and leaf primordium, and open arrows indicate leaf primordia. c, coleoptile; lp1, first leaf primordium; s, shoot meristem. Bars in (A) to (Y) = 40 μm.
In Situ Localization of the OSH mRNAs in Vegetative Tissues

The localization of the OSH mRNAs in vegetative tissues was determined by using in situ hybridization. The expression of five OSH genes, OSH3, OSH6, OSH43, OSH71, and OSH1, was examined in near-median longitudinal sections through the shoot apex of 1-month-old plants (Figures 5A, 5D, 5H, 5L, and 5P, respectively). At this stage, the meristem had initiated approximately six to seven leaves.

The spatial expression patterns of four OSH genes, OSH6, OSH43, OSH71, and OSH1, around the shoot apex region were essentially the same as those observed in the nearly mature embryo stage (cf. Figures 3J, 3O, 3T, and 3Y). Expression of OSH43 was observed throughout the corpus but not in the tunica layer of the SAM or in leaf primordia. Such expression is very similar to that of OSH1 in vegetative shoot apices (Figures 5H and 5P).

OSH6 and OSH71 showed more precisely localized expression patterns around the vegetative SAM. Preferential expression of OSH6 and OSH71 mRNA was observed with lower intensity in the periphery of the SAM but not in the central zone of the corpus or the tunica. Relatively strong signals of both mRNAs were observed below the P0 leaf primordium (Figures 5D and 5L). Weaker signals were also observed at the opposite side of the SAM, between the SAM and the P1 leaf primordium (Figures 5D and 5L) and the upper region of the P0 leaf primordium (Figures 5D and 5L). No expression was observed in leaf primordia themselves. The expression patterns of OSH6 and OSH71 in the basal region of leaves were similar to that of OSH15 in rice (Sato et al., 1998) or rs1 in maize (Jackson et al., 1994). No OSH3 expression was seen in sections of the vegetative shoot apex (Figure 5A).

In Situ Localization of OSH mRNAs in Inflorescence and Floral Tissues

Expression of the OSH genes was also examined in inflorescence and floral tissues. In longitudinal sections of inflorescence shoots at the rachis primordium differentiation stage, we observed two expression patterns throughout the axillary inflorescence meristem, whereas expression patterns of all four genes changed with the transition between the inflorescence and floral phases. OSH3 was expressed in the inflorescence meristems but not in the floral meristem (Figures 5B and 5C, respectively). OSH6 and OSH71 were uniformly expressed in the inflorescence meristem (Figures 5E and 5M), but after the transition from inflorescence to the floral phase, expression of these genes was located specifically in the notches between the floral meristem and glume primordia, similar to the expression pattern in vegetative meristems (Figures 5F and 5N, respectively). At later stages of flower development, OSH6 and OSH71 were uniformly expressed throughout the corpus of the meristem (Figures 5G and 5O). Expression in the notches between glume primordia persisted but was less well defined than in the previous stage (Figures 5G and 5O, respectively).

Similarity of the expression patterns of OSH1 and OSH43 was also observed during these stages. OSH1 and OSH43 mRNA was detected only in the corpus of the rachis primordium but not in the tunica layer (L1) (Figures 5Q and 5I, respectively). After floral induction, OSH1 and OSH43 mRNAs were detected in both tunica and corpus (Figures 5R and 5J) but were not expressed in floral organ primordia. Differences in the expression patterns of OSH1 and OSH43 were first observed later in flower development: expression of OSH1 continued in the corpus of the floral meristem (Figure 5S), whereas OSH43 expression became undetectable (Figure 5K).

DISCUSSION

OSH Genes Are Members of the KN1-Type Class 1 Homeobox Gene Family

In this study, we have attempted to isolate as many novel KN1-type class 1 homeobox genes from rice as possible.
Figure 5. Localization of OSH6, OSH43, OSH71, and OSH1 Transcripts in Vegetative, Inflorescence, and Floral Shoots.

Longitudinal sections were hybridized with OSH3, OSH6, OSH43, OSH71, and OSH1 antisense RNA probes labeled with digoxigenin-UTP.

(A), (D), (H), (L), and (P) Vegetative shoots.
(B), (E), (I), (M), and (Q) Inflorescence shoots.
(C), (F), (J), (N), and (R) Young floral shoots.
(G), (K), (O), and (S) Developed floral shoots.

Sections in (A) to (C) were hybridized to a digoxigenin-labeled OSH3 antisense probe. Those in (D) to (G) were hybridized to an OSH6 probe, those in (H) to (K) were hybridized to an OSH43 probe, those in (L) to (O) were hybridized to an OSH71 probe, and those in (P) to (S) were hybridized to an OSH1 probe. Filled arrows indicate the notch between the SAM and leaf primordium, and open arrows indicate leaf primordia. gl, glume; lm, lemma; lp0, leaf primordium 0; lp1, leaf primordium 1; pl, palea; pr, primary rachis; r.gl, rudimentary glume; s, shoot meristem; st, stamen. Bars in (A) to (S) = 100 μm.
For this purpose, we screened two cDNA libraries from shoot apices and rachis and a genomic library. Five novel KN1-like homeobox genes, OSH3, OSH6, OSH10, OSH43, and OSH71, were isolated. We also attempted to isolate class 1 homeobox genes by PCR with primers flanking conserved sequences of the previously reported KN1-type homeobox genes. This approach also yielded five novel KN1-like homeobox genes that corresponded exactly to those isolated from the libraries. Interestingly, we were unable to isolate an OSH10 clone from the cDNA libraries. A similar situation has been seen with knox10, the putative maize ortholog of OSH10 (S. Hake, personal communication), suggesting that the knox10 and OSH10 genes might be pseudogenes.

The products of the five novel genes and the two previously isolated ones, OSH1 (Matsuoka et al., 1993) and OSH15 (Sato et al., 1998), shared a highly conserved homeodomain with an invariant third helix. This invariant sequence, which includes 16 residues of the third helix, is conserved without exception in KN1-type homeodomain proteins from other plants, including dicots, demonstrating that it is a characteristic feature of the KN1-type homeodomain proteins (Kerstetter et al., 1994; Lincolin et al., 1994; Hareven et al., 1996; Tamaoki et al., 1997).

Invariant amino acid stretches were also observed in the regions between helix 1 and helix 2 and between helix 2 and helix 3. These invariant stretches are conserved not only in the KN1-type class 1 genes but also in the class 2 genes (Tamaoki et al., 1995), indicating that they are characteristics of both class 1 and class 2 homeobox genes. Recently, Bürglin (1997) has shown that the invariant amino acid stretch (proline-tyrosine-proline) between helix 1 and helix 2 can be found not only in the plant KN1-type genes but also in homeobox genes from other organisms. These three amino acids appear as unique extra residues when these homeodomain proteins are compared with other homeodomain proteins such as Antennapedia. Based on this unique feature, Bertolino et al. (1995) coined the term TALE (three-amino acid loop extension) to refer to this particular homeobox group. Bürglin (1997) has pointed out that some TALE homeodomain proteins share a conserved amino acid sequence at the C-terminal side of the homeodomain that is referred to as the KNOX domain. All OSH proteins also shared the KNOX domain with some exchanges (Figure 1A).

It has been reported that the position of the intron in the homeodomain is conserved in KN1-type homeobox genes. All of the OSH genes also contained an intron at the expected position, with the exception of OSH3, which contained no introns in its homeodomain (Figure 2B). To the best of our knowledge, this is a novel example of a KN1-type homeobox gene lacking an intron at this position.

The phylogenetic analysis based on the degree of similarity between deduced amino acid sequences from the rice and maize KN1-type class 1 homeobox genes demonstrated that each rice gene shares the highest degree of sequence similarity with one or two corresponding maize genes (Figure 1B). The relationship between these pairs of rice and maize genes was confirmed by the map positions of the genes on the rice and maize genomes (Figure 2A). According to the comparative linkage map of the rice and maize genomes (Ahn and Tanksley, 1993), we can deduce which rice homeobox genes are likely to correspond to maize homeobox genes. For example, OSH1 and OSH3 were mapped near a phytochrome (phy) gene on the short arm of chromosome 3 (Figure 2A; Matsuoka et al., 1993). The comparative linkage maps reported by Ahn and Tanksley (1993) indicate that the position near the phy gene on the short arm of rice chromosome 3 corresponds either to a position near the phy A1 gene on the long arm of maize chromosome 1 or to a position near the phy A2 gene on the short arm of chromosome 5. At these positions, we find KN1 and KNOX3 on chromosome 1 and KNOX10 on chromosome 5 in the maize genome, but we can deduce that OSH1 and OSH3 correspond to KN1 and KNOX10 on chromosome 5 in the maize genome, and we can deduce that OSH1 and OSH3 are clustered within <40 kb, and OSH1 and KNOX3 are closely linked on the maize genome (Kerstetter et al., 1994). In support of this deduction, the primary structures of OSH1 and OSH3 show the highest similarity to KN1 and KNOX3, respectively. The comparative analysis of other OSH genes showed that the maize paired genes in the phylogenetic tree (Figure 1B) mapped at analogous positions on the rice and maize chromosomes (Figure 2A), suggesting that the pairs of rice and maize genes may have orthologous relationships.

**Involvement of OSH Homeobox Genes in SAM Formation**

Molecular studies of Drosophila embryogenesis have revealed that homeobox genes are involved in morphogenetic processes, such as formation of the embryonic axis, segmentation, determination of segmental and cell identity, and cell differentiation. Before the morphogenetic events are visible, homeobox genes are expressed in the presumptive region in which the corresponding structures later arise.

By analogy to the involvement of Drosophila homeobox genes in developmental events in that species, we tested the possibility that KN1-type homeobox genes in rice would show localized expression in the rice embryo. All of the homeobox genes that we tested were expressed in limited areas of the embryo but in different patterns. We have categorized the homeobox genes into three groups based on their comparative expression patterns in the globular stage (Figure 6A).

The first group includes four genes, OSH1 (Sato et al., 1996), OSH15 (Sato et al., 1998), OSH43, and OSH71, whose expression was not detected in any area in the early globular stage at 2 DAP (<100 cells) but was detected at 3 DAP (200 cells) in the limited area at which the SAM and epiblast would subsequently develop. The second and third
groups consist of single genes, namely, OSH6 and OSH3. Expression of these genes was uniformly observed in the early globular stage at 2 DAP, but in the late globular stage at 3 DAP, expression of OSH6 became restricted to a region predicted to give rise to the SAM and epiblast, whereas expression of OSH3 was downregulated in the ventral region.

It should be emphasized that we can visualize the specific region of the globular embryo that later gives rise to the shoot by using different homeobox probes. Such regionalization of homeobox gene expression suggests that this class of homeobox genes may contribute to the establishment of positional information in the presumptive shoot region and/or to shoot formation itself. The involvement of homeobox genes in these processes could occur in two ways. The genes of the first and second groups, OSH1, OSH6, OSH15, OSH43, and OSH71, whose expression is localized in the area of subsequent shoot development, may be positively involved in shoot establishment. By contrast, OSH3, which represents a third group, could be involved in establishment of positional information for the shoot region but not in shoot formation because its expression is specifically suppressed in this area.

It is an interesting question whether localized expression of the genes precedes the determination of the position of shoot formation. Our intensive morphological investigations indicate that the earliest expression of OSH1, OSH15, OSH43, and OSH71 occurs in embryos with 100 to 200 cells, and localized expression of OSH6 begins at approximately the same stage (data not shown). It is hardly possible to find any differences in cell morphology in globular embryos at this stage, suggesting that the earliest expression of OSH1, OSH15, OSH43, and OSH71 and the localized expression of OSH6 precede the morphological development of the shoot. In contrast to these genes, downregulation of OSH3 in the region of the SAM was observed at a later stage than that of the first group. These observations indicate that downregulation of OSH3 is a later event than the localized expression of the first group of genes and may be closely linked to the onset of shoot formation. However, it is still possible that downregulation of OSH3 gives a positional cue for shoot formation because it occurs before formation of the SAM (Figure 3B).

It is also interesting to speculate on the functional relationships among the homeobox genes in the process of shoot establishment. It may be relatively easy to infer the function(s) of the first group of genes, OSH1, OSH15, OSH43, and OSH71. The expression of these genes begins during the middle or late globular stages in the region of presumptive shoot formation but before actual shoot formation. Thus, these genes may act cooperatively in shoot formation during embryogenesis. Cooperative involvement of these homeobox genes with similar expression patterns and sequences leads us to speculate that this group of genes may be redundant for shoot formation during embryogenesis. Indeed, an OSH15 loss-of-function mutant did not show loss of shoot formation or abnormal shoot development in rice embryogenesis but instead showed a defect in internode elongation (Sato et al., 1999). Thus, loss-of-function of OSH15 alone does not affect shoot formation. A similar observation has been reported for the maize KN1 gene, whose pattern of expression in maize embryogenesis resembles that of the first group of OSH genes. Loss of function of KN1 did not cause loss of shoot formation or abnormal shoot de-
velopment but instead resulted in defective growth of lateral buds in vegetative and reproductive stages (Kerstetter et al., 1997).

In contrast to the first group, the second and third groups, consisting of OSH6 and OSH3, respectively, may not be functionally redundant because no other homeobox genes had similar expression patterns, at least among the genes that we investigated. Convergence of OSH6 expression to the specific region in which the shoot later develops may suggest that cells in the very early stage of embryo development possess the ability to become shoot primordia, but as embryogenesis progresses, such cells become restricted to a specific region through the transition to localized expression of OSH6. Continuous expression of OSH6 in these cells may induce the expression of members of the first group of homeobox genes, which may then act cooperatively to promote shoot formation. Thus, an epistatic relationship may exist between OSH6 and the homeobox genes in the first group.

As previously mentioned, the disappearance of OSH3 mRNA in the region of SAM formation occurred after the appearance of expression of the first group of homeobox genes. This indicates that suppression of OSH3 does not cause the induction of expression of the other genes. The close map positions and opposing expression patterns of OSH1 and OSH3 led us to speculate that these genes may be cooperatively involved in shoot formation in a manner similar to the specification of segment identity by the animal homeobox genes (McGinnis and Krumlauf, 1992). However, in situ hybridization analyses revealed that the regions of OSH3 suppression and OSH1 expression only partially overlapped (Figure 4). This observation suggests that OSH1 and OSH3 may not act as mutually competitive factors in the establishment of the shoot region.

Separable Functions of OSH Genes after Shoot Formation

After shoot formation, the expression patterns of the six OSH genes can be categorized into three groups. Expression of the first group, which includes OSH1 and OSH43, is maintained in the SAM (Figure 6B). Expression of OSH1 and OSH43 within the SAM is observed after seed germination and continues until development of the inflorescence meristem. This suggests that homeobox genes of the first group are involved in maintaining the indeterminate state of the SAM in vegetative and inflorescence stages, as is the case for KN1 in maize (Kerstetter et al., 1997).

In contrast to the first group, the expression of genes in the second group, which includes OSH6, OSH71, and OSH15, is downregulated in the SAM and in turn is localized at the boundaries of the shoot lateral organs. Thus, the expression pattern of the second group changes markedly before and after formation of the SAM during embryogenesis. This change suggests that the second group of homeobox genes may have different functions before and after SAM formation. The homeobox genes in this group may first function in SAM formation, cooperating with genes in the first group. However, after shoot formation, these homeobox genes are not directly involved in maintenance of the SAM, whereas the first group is continuously involved in this process. After shoot formation, the patchlike expression of the second group is always observed between the SAM and newly formed determinate lateral organs, such as leaves and floral organs, of the vegetative and floral meristem. Such localized expression between lateral organs and the SAM indicates that the homeobox genes in the second group may be involved in or respond to an early patterning event that defines the segmental units of the plant body designated phytomers, as proposed by Jackson et al. (1994) and Schneeberger et al. (1995) for maize r1. Alternatively, the expression of these homeobox genes may mark the future internodes in the postembryonic stages of development and the mesocotyl during embryogenesis and may be involved in their differentiation.

In contrast to the patchlike expression in the vegetative and floral meristem, the homeobox genes of the second group are expressed uniformly in the inflorescence meristem. The uniform expression of these genes in the inflorescence meristem is consistent with the hypothesis that they are involved in internode differentiation, because the products of the inflorescence meristem, rachis-branch primordia, are indeterminate organs that form indeterminate lateral buds and inflorescence shoot internodes. In the floral meristem, in turn, lateral organs formed from the meristem are determinate, and so the homeobox genes in this group are downregulated in the meristem, and the patchlike expression is once again observed between the meristem and lateral organ. A third group consists of only one homeobox gene, OSH3. The expression of this gene is observed in the inflorescence meristem but was not detected in the vegetative or floral meristem by in situ hybridization.

In light of the collective data on the expression patterns presented here, all or some of the rice OSH homeobox genes may be involved in regionalization of the shoot area and/or the establishment of the SAM itself before shoot formation early in embryogenesis. After shoot formation, however, the functions of the homeobox genes appear to differ. Some of these genes may maintain SAM activity in an indeterminate condition through continuous expression in the SAM, whereas others may be involved in pattern formation of the segmental units of the plant body and/or internode development. Prepatterning of specific cells by the expression of homeobox genes before morphological organ formation is the same as in organ formation during animal embryogenesis. Even though the body structures of plants and animals are quite different, there may be some common mechanisms in organ establishment and development in terms of homebox function. The collection and analysis of loss-of-function mutants of the OSH genes are essential to clarify the functions of these genes during embryogenesis and after seed germination.
METHODS

Plant Material

Wild-type rice plants (Oryza sativa) grown in fields in Nagoya were used for construction of cDNA and genomic libraries and for in situ hybridization experiments.

Isolation of Homeobox Genes from Rice cDNA and Genomic Libraries

cDNA libraries were constructed from shoot apices and rachis. Total RNA was extracted from these tissues, and poly(A)+-enriched RNA was purified by two passages through an oligo d(T) cellulose column. The poly(A)+ RNA was used to synthesize double-stranded cDNA, which was cloned into the EcoRI site of λgt11 (Stratagene, La Jolla, CA).

Nuclear genomic DNA was isolated from 2-week-old seedlings. The DNA was partially digested with Sau3AI and enriched for fragments of ~20 kb on a sucrose gradient. The fragments were cloned into the BamHI site of EMBL3 (Stratagene).

Screening by hybridization was performed in 30% formamide, 6× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt’s solution (1× Denhardt’s solution: 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.5% SDS, and 0.1 mg mL⁻¹ salmon sperm DNA at 42°C for 14 hr, using the homeobox sequence of OSH1 or OSH15 (150 bp) as a probe (Matsuoka et al., 1993; Sato et al., 1998).

For polymerase chain reaction (PCR) screening, three oligonucleotide primers corresponding to the conserved amino acids of the ELK (glutamate, leucine, and lysine) homeodomain were used (5'-AA[AG][TA][AG][TG][TA][CA][TG][CC]-3' or 5'-CA[TC][TT][AC][CC][CA][TG][GG][CC][CA][GT][TA][CT][CC][CG]-3' and 5'-TG[TT][GA][AC][CG][TT][GT]-3'; the locations of the primers are indicated in Figure 1A). Amplified fragments were cloned into pCRII (Invitrogen, Leek, The Netherlands) and sequenced.

Sequence Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an automated DNA sequencing system (ABI 373A; Applied Biosystems, Inc., Foster City, CA), according to the manufacturer’s protocol (Perkin-Elmer). cDNA clones were completely sequenced on both strands. Analyses of DNA and amino acid sequences were performed using GENETYX computer software (Software Kairatsu Co., Tokyo, J apan).

Mapping of the OSH Genes in Rice Recombinant Inbred Lines

To map the OSH genes, 71 recombinant inbred lines from a cross between two rice cultivars, Asominori (a Japonica rice) and IR24 (an Indica rice), were used. Restriction fragment length polymorphism (RFLP) analyses were performed with probes specific for each OSH gene. The linkage analysis was calculated using the MAPMAKER program (Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research, Wilmington, NC).

In Situ Hybridization

Plant materials were fixed in 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, dehydrated through a graded ethanol series and then a t-butanol series (Sass, 1958), and finally embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO). Microtome sections (8 to 10 μm thick) were mounted on glass slides treated with silane.

Hybridization and immunological detection of the hybridized probes were performed according to the method of Kouchi and Hata (1993). Digoxigenin-labeled RNA was produced from the coding region of each gene without the ELK homeodomain region to exclude cross-hybridization among the OSH genes. Double in situ hybridization stained with fluorescein-labeled OSH1 and digoxigenin-labeled OSH3 probes was performed as described by Kouchi et al. (1995).

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REFERENCES


Expression Patterns of Rice Homeobox Genes


Regional Expression of the Rice KN1-Type Homeobox Gene Family during Embryo, Shoot, and Flower Development
Naoki Sentoku, Yutaka Sato, Nori Kurata, Yukihiro Ito, Hidemi Kitano and Makoto Matsuoka
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