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Expression of a Rice Homeobox Gene Causes Altered Morphology of Transgenic Plants

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We have isolated a cDNA clone encoding a homeobox sequence from rice. DNA sequence analysis of this clone, which was designated as *Oryza sativa* homeobox 1 (*OSH1*), and a genomic clone encoding the *OSH1* sequence have shown that the *OSH1* gene consists of five exons and encodes a polypeptide of 361 amino acid residues. Restriction fragment length polymorphism analysis has shown that *OSH1* is a single-copy gene located near the phytochrome gene on chromosome 3. Introduction of the cloned *OSH1* gene into rice resulted in altered leaf morphology, which was similar to that of the maize morphological mutant *Knotted-1* (*Kn1*), indicating that *OSH1* is a rice gene homologous to the maize *Kn1* gene. RNA gel blot analysis has shown that the gene is primarily expressed in the shoot apices of young rice seedlings. This finding is supported by results of transformation experiments in which the 5′ flanking region of the gene directed expression of a reporter gene in the shoot apex, particularly in stipules, of transgenic Arabidopsis. To elucidate the biological function of the *OSH1* gene product, the coding region was introduced into Arabidopsis under the control of the cauliflower mosaic virus 35S promoter. Almost all transformants showed abnormal morphology. The typical phenotype was the formation of clumps of abundant vegetative and reproductive shoot apices containing meristems and leaf primordia, which did not form elongated shoots. Some transformants with a less severe phenotype formed elongated shoots but had abnormally shaped leaves and flowers with stunted sepals, petals, and stamens. The abnormal phenotypes were inherited, and the level of expression of the introduced *OSH1* correlates with the severity of the phenotype. These findings indicate that the abnormal morphologies of the transgenic plants are caused by the expression of the *OSH1* gene product and, therefore, that *OSH1* is related to the plant development process.

INTRODUCTION

Homeobox genes were first found as regulatory genes that control the morphogenesis of a fruit fly, Drosophila (for review, see Gehring, 1987). Products of these genes share a unique structure, the homeodomain. This homeodomain's structure is similar to the helix-turn-helix motif, and it recognizes and binds specific DNA sequences (Hayashi and Scott, 1990). Through the DNA binding property of the homeodomain, the products of these genes are believed to regulate the expression of batteries of target genes as transcriptional factors (Affolter et al., 1990). Recently, homeobox genes have been cloned from two plant species, maize and Arabidopsis. The Arabidopsis genes have the conserved homeobox sequences, but unlike the animal genes, they have a leucine zipper motif adjacent to the carboxy terminus of the homeodomain (Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992). In contrast, the maize gene, *Knotted-1* (*Kn1*), does not contain a leucine zipper motif or the typical homeodomain. Its homeobox more closely resembles the helix-turn-helix structures of the yeast cell type–specific gene *MAT*Pi and the human pre-B cell lymphoblastic leukemia gene *Prl* (Vollbrecht et al., 1991). These findings suggest that there are at least two different types of homeobox genes in the plant kingdom.

Homeobox genes in plants, by analogy to the functional roles of similar animal genes, are thought to encode transcriptional regulators and to mediate important developmental processes (Schena and Davis, 1992). This has been supported by genetic analysis of the maize *Kn1* gene, duplication of which disorders leaf development (Veit et al., 1990). To address their biological function in plants, we have isolated a homeobox gene from rice and expressed the gene product in transgenic rice and Arabidopsis. Here, we describe transgenic plants expressing the homeoprotein and the accompanying phenotypic changes. We have also investigated the tissue specificity of *OSH1* gene expression by in situ histochemical staining and RNA gel blot analysis.

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RESULTS

Isolation and Characterization of a Rice Homeobox Gene

We screened a cDNA library constructed from poly(A)-rich RNA extracted from young rice seedlings using the homeobox sequence of *Kn1* (Vollbrecht et al., 1991) and obtained seven positive clones. Judging from their restriction maps, these clones are all independent. We focused on one of these clones, which we designated *OSH1* for *Oryza sativa homeobox* 1, because the expression of the clone resulted in the altered morphology of transgenic rice and Arabidopsis plants (see below). *OSH1* 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, as shown in Figure 1. The deduced amino acid sequence was compared with that of the *Kn1* gene in maize, as shown in Figure 2. When an alignment that permits maximum similarity is introduced, 78.4% of the amino acids were found to be identical. The rice and maize sequences are essentially identical, particularly in the carboxy-terminal region, which contains the homeodomain and a few conservative amino acid exchanges. Such high homology supports the conclusion that *OSH1* is a rice gene homologous to maize *Kn1*.

We also cloned and characterized the genomic DNA encoding the *OSH1* transcript. Comparison of the cDNA and genomic sequences revealed four introns in *OSH1* (Figure 1B). The exon–intron relationship of *OSH1* is identical to that of the *Kn1* gene (Vollbrecht et al., 1991), indicating that *OSH1* is homologous to *Kn1*. To confirm whether the biological function of *OSH1* is homologous to that of *Kn1*, the entire cloned fragment of *OSH1*, from the ApaI site to the EcoRI site at the 3′ end position shown in Figure 1B, was introduced into rice with a plasmid containing a gene encoding hygromycin phosphotransferase under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The plasmid was transferred into protoplasts and 10 independently regenerated plants were obtained by selection for hygromycin resistance. As shown in Figure 3, seven plants had abnormal leaves with a morphology similar to that of the maize *Kn1* mutant, which is caused by duplication of the *Kn1* gene (Veit et al., 1990). DNA gel blot analyses indicate that these rice plants with altered morphology also contained one or two extra copies of *OSH1* in their genomes as a result of introducing the cloned gene (data not shown). These findings confirmed that *OSH1* is related to leaf development in a manner similar to that of *Kn1*.

Primer extension analysis indicates that there are several transcriptional initiation sites (data not shown). The strongest signal was located 218 nucleotides upstream of the first nucleotide of the translation initiation codon. This putative initiation site is just three nucleotides upstream of the 5′ end of the cDNA clone (Figure 1). The location of this initiation site was confirmed by polymerase chain reaction (PCR) using RNA from young seedlings as a template (data not shown). In the region

Figure 1. Structure of the *OSH1* Gene.

(A) Nucleotide sequence of the exons and flanking regions of *OSH1*. The deduced amino acid sequence is shown below the nucleotide sequence in single-letter code. Sites for introns 1 to 4 are marked by triangles. The first nucleotide of the transcription start site and the first amino acid of the OSH1 protein are numbered 1. Hooked arrows indicate transcription start and polyadenylation sites. A vertical arrow indicates the start of the cDNA. Possible TATA boxes are underlined with thick lines. Direct repeats and the sequence homologous to the *MAT*a1/α2 binding site are indicated by horizontal arrows and a thin line, respectively. Sequence data has been submitted to DDBJ as accession number D16507.

(B) Restriction map of the *OSH1* gene is presented at the top. Exons and introns are indicated by boxes and lines, respectively. ATG and TAG indicate the locations of the initiation and the termination codons of the gene, respectively.

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OSH1   MEEISHHFGVVGAS--GVHGG-HQHQHHHHPWGSSLSAIVAPPPPPQLQQQQTQAGGMAH  57
       ****::**** ::*    **  *:*:******:*****:** * ***   ::::: ::
Kn1    MEEITQHFGVGASSHGHGHGQHHHHHHHHHPWASSLSAVVA-PLPPQ---PPSAGLPLTL  56

OSH1   TPLTLNTAAAAVGNPVLQLAN-GSLLDACGKAKEASASASYAADVEAIKAKIISHPHYSS 116
       :::: :::::: ********* *:***** ****:*:*::**:*************** *
Kn1    NTVAATGNSGGSGNPVLQLANGGGLLDACVKAKEPSSSSPYAGDVEAIKAKIISHPHYYS 116

OSH1   LLAAYLDCQKVGAPPEVAARLTAVAQDLELRQRTALGVLGAATEPELDQFMEAYHEMLVK 176
       **:***:*:********:****:::**:* ******* *:********************
Kn1    LLTAYLECNKVGAPPEVSARLTEIAQEVEARQRTALGGLAAATEPELDQFMEAYHEMLVK 176

OSH1   YREELTRPLQEAMEFLRRVETQLNTLSISGRSLRNILSSGSSEEDQEGSGGETELPEIDA 236
       :**************:****:***:*********************************:**
Kn1    FREELTRPLQEAMEFMRRVESQLNSLSISGRSLRNILSSGSSEEDQEGSGGETELPEVDA 236

OSH1   HGVDQELKHHLLKKYSGYLSSLKQELSKKKKKGKLPKDARQQLLNWWEIHYKWPYPSESQ 296
       ****************************************:******:**: *********:*
Kn1    HGVDQELKHHLLKKYSGYLSSLKQELSKKKKKGKLPKEARQQLLSWWDQHYKWPYPSETQ 296

OSH1   KVALAESTGLDLKQINNWFINQRKRHWKPSDEMQFVMMDGYHPTNAAAFYMDGHFINDGG 356
       ****************************:**: :******:**  **************
Kn1    KVALAESTGLDLKQINNWFINQRKRHWKPSEEMHHLMMDGYHTTN--AFYMDGHFINDGG 354

OSH1   LYRLG 361
       *****
Kn1    LYRLG 359
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Figure 2. Comparison of the Deduced Amino Acid Sequences of *OSH1* and *Kn1*.

Hyphens indicate gaps introduced to facilitate alignment. The homeodomain sequence reported by Vollbrecht et al. (1991) is boxed. The proline/glutamine–rich region of the OSH1 protein and the histidine-rich region of the KN1 protein are underlined with solid and dashed lines, respectively. Identical residues and conserved exchanges of amino acids are indicated by asterisks and colons, respectively.

upstream of the transcription initiation site, a possible TATA box is located at positions −38 to −41. Another TATA box–like sequence is present at positions −60 to −65. Further upstream, the promoter region contains a 13-bp direct repeat, TATAGCT(A/G)ACTAC (indicated by horizontal arrows in Figure 1A). The significance of this repeat in the transcriptional regulation of the gene is not known at this time. It is interesting that a sequence in this promoter, TACATCA, corresponds to the 3′ end of the cell-type gene *MAT*α1/α2 consensus binding sequences in yeast (Miller et al., 1985) (see Discussion). No consensus sequences found in other transcriptional factors, such as a CCAAT box, an S1 binding site (Kadonaga et al., 1986), an octamer sequence (Falkner and Zachau, 1984), or animal homeobox protein binding sites (Hayashi and Scott, 1990), were found in this region.

DNA hybridization was performed to investigate the genomic organization of *OSH1*. High molecular weight genomic DNA was digested with BamHI, BglII, EcoRV, and HindIII and hybridized with the cDNA clone. Only one fragment hybridized with the probe in each case, and the hybridization intensity corresponded to that of one copy of the gene (data not shown), suggesting that the gene is present in a single copy in the rice genome. To determine the chromosomal location of the gene, an analysis of restriction fragment length polymorphisms (RFLPs) was used. RFLPs are present between FL134 (a Japonica rice) and Kasalath (an Indica rice) DNA digested with BamHI, BglII, EcoRV, and HindIII (data not shown). RFLP mapping was performed with EcoRV-digested genomic DNA from an F_2 population of FL134 and Kasalath, as shown in Figure 4, because this enzyme produced the clearest polymorphism between these rice lines. Cosegregation analysis of the RFLPs with other markers revealed linkage to three DNA markers, 249, 66, and a phytochrome gene, which have been mapped to chromosome 3 (Figure 4). Thus, *OSH1* is near a phytochrome gene on chromosome 3. It is noteworthy that *Kn1* and a phytochrome gene are also linked on chromosome 1 in the maize genome (Coe et al., 1990). This suggests that the genome organization around *OSH1* in rice is similar to that of *Kn1* in maize.

Organ and Spatial Expression of the *OSH1* Gene

Figure 5 shows RNA gel blot analysis of total RNA extracted from various organs of rice plants. Clear signals were only detected in tissues consisting primarily of meristematic shoots. The size of the RNA is ~1.6 kb, corresponding to that of the cDNA clone. Faint signals of the same size were obtained in glume tissue, whereas broad faint bands of different sizes were found in other organs. These may be due to nonspecific hybridization of the probe with rRNAs.

We also analyzed the temporal and spatial regulation of the *OSH1* promoter in transgenic Arabidopsis. The 5′ flanking region (−846 to +182) was fused to the coding sequence of β-glucuronidase (*GUS*) to create *OSH1–GUS*. The *OSH1–GUS* construct was introduced into Arabidopsis. Approximately 10 independent transformants were obtained by selection for kanamycin resistance. The organ specificity of *OSH1–GUS* expression was determined by in situ histochemical staining for GUS activity. GUS activity, as revealed by blue color, was detected specifically in limited areas of vegetative shoot apices

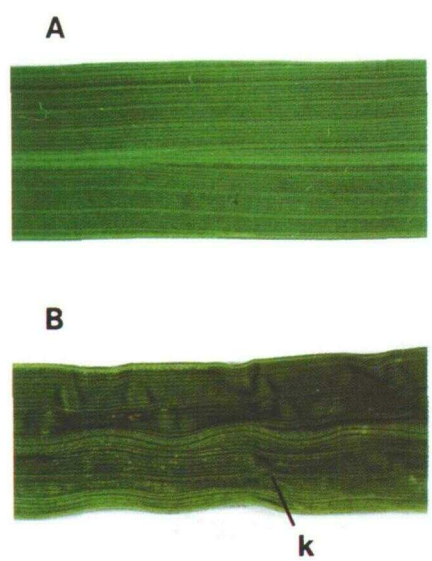

Figure 3. Morphology of Nontransgenic and Transgenic Rice Leaves.

(A) The veins of a nontransformed leaf that run in straight, parallel lines. **(B)** The veins of a transformed leaf that are distorted. The knots (k) occur sporadically along the lateral veins.

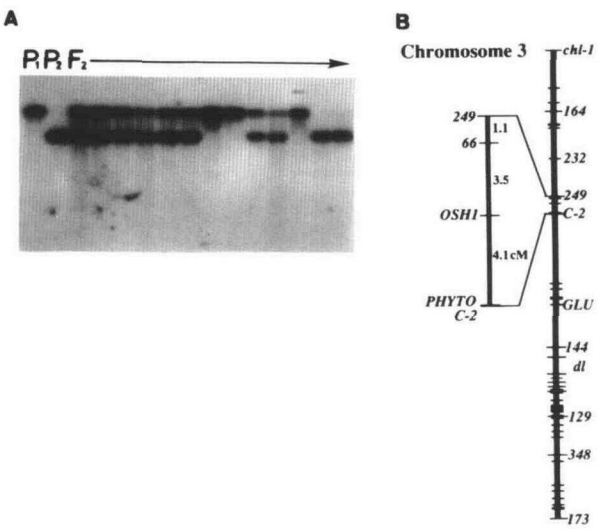

Figure 4. RFLP Mapping of *OSH1*.

(A) Linkage analysis of F_2 populations from a cross between Kasalath (P_1) and FL134 (P_2). Genomic DNA of P_1, P_2, and F_2 plants were digested with EcoRV and used in DNA hybridization experiments after electrophoresis on 0.8% agarose gels.

(B) The locations of *OSH1* and some RFLP markers are shown on rice chromosome 3. *chl-1*, chlorina-1; *PHYTO*, phytochrome; *C-2*, unidentified cDNA; *GLU*, glutelin; *dl*, drooping leaf; cM, centimorgan.

of T_2 transgenic seedlings, as shown in Figures 6A to 6C. Although there was some variation in staining intensity between different transformants, essentially the same patterns were obtained. The stipules of rosette leaves were the most strongly stained tissue in all transgenic plants (Figures 6B and 6C). After bolting, GUS activity was observed in shoot meristems (data not shown), in the stipules of cauline leaves on the primary floral apex (Figure 6D), and on the leaf axil (Figure 6E). The expression of *OSH1–GUS* in transgenic Arabidopsis is consistent with the results of RNA gel blot analysis in rice (Figure 5) and indicates that the 850 bp of the 5′ flanking region of *OSH1* confers shoot apex–specific expression upon a distantly related dicot plant, Arabidopsis.

Expression of the *OSH1* Gene Product in Arabidopsis

To study the biological function of *OSH1*, we introduced the coding sequence of *OSH1* under the control of the CaMV 35S promoter into Arabidopsis and studied the expression of the product of *OSH1* in transgenic plants. More than 100 transformants (T_1 generation) were obtained in two transformation experiments. The morphological alterations were found in almost all transgenic plants, as shown in Figure 7. This transformation method has been used to generate hundreds of transgenic Arabidopsis plants, and no previous transformants have had a morphology similar to that produced in the *OSH1* transformants, indicating that the abnormal morphology observed in these transformants is due to the introduced gene rather than to somaclonal variation. Many of the regenerants initially formed clumps of abundant vegetative and reproductive shoot apices containing meristematic tissue and leaf primordia (Figures 7A and 7B). Some transgenic plants with severe phenotypes formed no or very short shoots, and the remaining transformants produced elongated shoots with few or no leaves and much smaller leaves when compared with the wild-type leaf morphology (Figure 7C). Except for a few, the transgenic plants did not develop rosette leaves or roots.

The morphology of mature flowers is also dramatically different in the transformants. In comparison with wild-type floral organs, the sepals of transgenic plants are smaller and have altered morphology (Figures 7D and 7E). Extremely small or no petals were observed in many transgenic lines (Figure 7D), and in some lines with less severe phenotypes, petals with relatively normal morphology were observed (Figure 7E). These ranges of phenotypes probably depend on the physiological condition of the plants or the degree of expression of the *OSH1* gene product. The filaments of the stamens are shorter in length (Figures 7D and 7E), and the number varies from four to six. Anther opening was not seen, although several mature flowers were observed microscopically. The morphology of the pistils seemed to be less affected than in the other floral organs. Very few flowers formed siliques, but some flowers with relatively normal shape occasionally developed malformed siliques (Figure 7F). The development of embryos in these siliques was abnormal, and no mature seed formed.

Abnormal floral and embryo development caused these plants to be sterile, making it difficult to confirm whether the abnormal morphologies were inherited in the next generations.

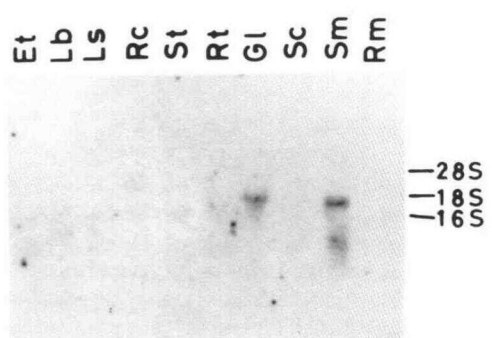

Figure 5. Organ-Specific Expression of *OSH1*.

RNA gel analysis of the *OSH1* transcript in different organs. RNA was isolated from etiolated leaves (Et), leaf blades (Lb), leaf sheaths (Ls), rachis tissue (Rc), stems (St), roots (Rt), glumes (Gl), suspension callus cells (Sc), shoot meristems (Sm), and root meristems (Rm). Ten micrograms of total RNA was used for the RNA gel blot analysis. At right are positions of rRNAs.

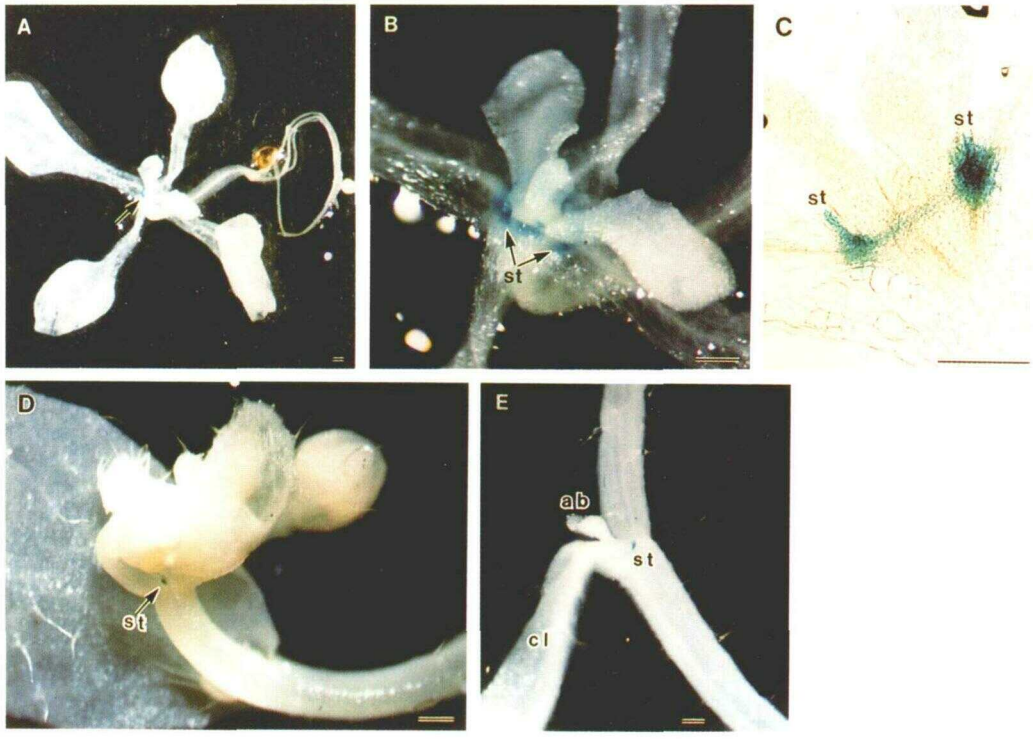

Figure 6. Histochemical Localization of the GUS Activity in T_2 Transgenic Arabidopsis with the *OSH1-GUS* gene.

(A) A whole seedling showing GUS activity in the limited area of the shoot apex (arrow).
(B) Same as shown in **(A)** at higher magnification.
(C) Longitudinal section of the shoot apex.
(D) Developing apical buds showing GUS activity only in the stipules of cauline leaves.
(E) GUS activity in the stipules of a cauline leaf with an axillary bud.
st, stipule; cl, cauline leaf; ab, axillary bud. Bars = 200 μm.

However, a few T_2 seeds were obtained, and T_2 plants that were kanamycin resistant had the same altered morphology as T_1 plants (Figures 7G and 7H).

The expression of *OSH1* in these transformants was tested by RNA gel blot analysis using the *OSH1* coding region as a probe, as shown in Figure 8. Strong signals of 1.6 kb were found in the RNA from clumps producing shoot apices (Figures 7A and 7B), whereas more faint bands of the same size were detected in the RNA of elongated shoots that produced abnormal flowers (Figure 7C). No band was observed in nontransgenic plants. These observations suggest that the expression level of the introduced *OSH1* gene affects the severity of the morphological alteration of transgenic plants.

DISCUSSION

Structure of the Homeobox Gene from Rice

In this study, we have isolated and characterized a homeobox gene, *OSH1*, in rice. DNA sequence analysis of *OSH1* revealed that the deduced amino acid sequence is very similar to that of the *Kn1* gene product in maize. The exon–intron structures of both genes are also identical, and *OSH1* is closely linked to a phytochrome gene, as is *Kn1*. Moreover, increase in dosage of the *OSH1* gene by introduction of the cloned gene resulted in the altered morphology of leaves of transgenic rice, and the morphology was similar to that of a maize *Kn1* mutant (Figure 3). These findings indicate that *OSH1* is the rice homolog of *Kn1*. The similarity between the deduced amino acid sequences around the amino-terminal regions of *Kn1* and *OSH1* is relatively low compared to other regions. Vollbrecht et al. (1991) have reported that *Kn1* contains an unusual polyhistidine block in this region. No such structure is present in *OSH1*. In the corresponding region of the *OSH1* gene, two histidine residues are replaced by glutamine residues and one is deleted (Figure 2). Both histidine and glutamine are encoded by the codon CAX (X is any nucleotide), and the repetition of the CAX codon is often found in developmentally important proteins of Drosophila, as pointed out by Vollbrecht et al. (1991). Therefore, it is possible that histidine residues in this region can be replaced by glutamines without large functional implications. It is noteworthy that the *OSH1* gene product has a proline- and

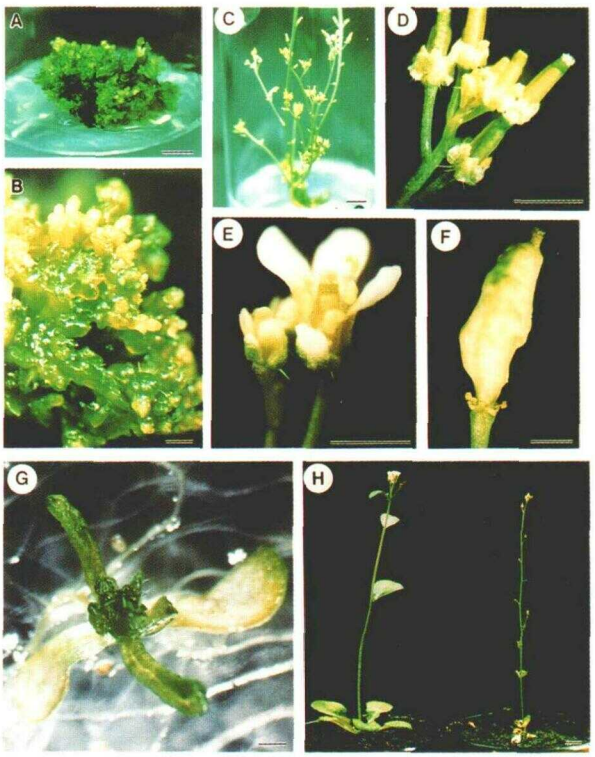

Figure 7. Phenotypes of Transgenic Arabidopsis Expressing the *OSH1* cDNA.

(A) Typical morphology of the transformants (T_1 generation).
(B) Higher magnification of **(A)** showing the formation of multiple shoot and floral apices.
(C) Shoots that developed from a region of multiple shoot apices. Elongated shoots were cut and transferred to kanamycin-containing medium.
(D) Dramatically different morphology of T_1 transgenic flowers. The flowers contain stunted sepals, petals, and filaments.
(E) Less severe morphology of T_1 transgenic flowers with dwarf sepals and filaments. One of the petals is also stunted.
(F) Malformed siliques occasionally developed from flowers with a less severe morphology as shown in **(E)**. Abnormal embryos formed in these siliques.
(G) A T_2 transgenic seedling with multiple shoot apices.
(H) Wild-type plant (left) and the phenotype of a T_2 transgenic plant (right). The transgenic plant developed small rosette leaves, cauline leaves, and abnormal flowers as did the T_1 transformants shown in **(D)**.
In **(A)**, **(C)**, and **(H)**, bars = 5 mm. In **(B)** and **(D)** to **(G)**, bars = 1 mm.

glutamine-rich region at the carboxy end of the histidine/glutamine block, which is not found in the *Kn1* gene product. Since proline- and glutamine-rich regions are often found in transactivation domains of *trans*-acting factors, the amino-terminal region containing these unique regions may act as a transcriptional activation domain.

The putative homeodomain sequence of *OSH1* has very limited similarity (<35%) to previously reported homeodomains, except for that of *Kn1*; nevertheless, the invariant amino acids in this domain are conserved (Scott et al., 1989). It has been demonstrated that the ninth amino acid residue of the third recognition helix is quite important in determining DNA binding specificity (Treisman et al., 1989). *OSH1* encodes isoleucine at this position. The only other homeobox protein known to have isoleucine at this position is the yeast cell type–specific factor *MAT*a1 (Scott et al., 1989). Therefore, it is possible that the *OSH1* gene product can interact with sequences similar to those recognized by the MATa1 protein. Unfortunately, the DNA binding sequence of the *MAT*a1 gene product has not yet been identified, but Miller et al. (1985) reported the binding sequence of heterodimer proteins composed of *MAT*α2 and *MAT*a1. They compared the binding sequences between *MAT*α2 homodimers and *MAT*a1/α2 heterodimers and found that sequences containing a TACATCA motif at the 3′ end are preferentially recognized by *MAT*a1/α2 heterodimers rather than by *MAT*α2 homodimers. They speculate based on this finding that this motif is a possible binding site of the MATa1 protein. Interestingly, this sequence motif is present in the 5′ flanking region of *OSH1*. If the speculation of Miller et al. (1985) is correct, it is possible that the *OSH1* gene product interacts with this sequence and regulates its own expression. Although the possibility of autoregulation of *OSH1* remains to be investigated, it is noteworthy that such regulation has been found in some homeobox genes in Drosophila (Hayashi and Scott, 1990).

Expression and Function of *OSH1* in Arabidopsis

RNA gel blot analysis indicates that *OSH1* is mainly transcribed in shoot meristematic regions. Recently, Smith et al. (1992) reported that the *Kn1* gene product is mainly localized in

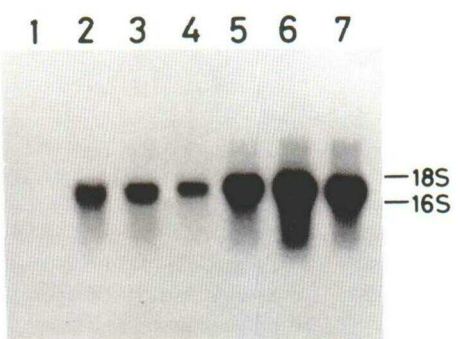

Figure 8. Expression of the *OSH1* Transcript in Arabidopsis Transformed with the 35S–*OSH1* Construct.

Total RNA was isolated from a nontransgenic plant (lane 1), three independent elongated shoots with abnormal flowers, as shown in Figure 6C (lanes 2 to 4), and three independent clumps of shoot apices, as shown in Figure 6A (lanes 5 to 7). Three micrograms of total RNA was used for the RNA gel blot analysis. At right, positions of 18S and 16S rRNAs are shown.

vegetative and floral meristems, indicating that the localization of both proteins is similar. Interestingly, *OSH1* is also expressed in glumes at a relatively high level compared to other organs, except the shoot apex. Because glumes are differentiated reproductive organs, the transcripts observed in glumes are probably not floral meristem transcripts. The expression of *OSH1* in glumes suggests the possibility that it is involved in flower development (see below).

We also investigated the spatial pattern of *OSH1* promoter activity in transgenic plants. In situ analysis of GUS activity revealed that the *OSH1* promoter can direct expression in the shoot apex of Arabidopsis. No other expression of *OSH1* was observed in Arabidopsis. This observation in transgenic Arabidopsis is in agreement with RNA hybridization analysis, even though there are major structural differences between monocot and dicot plants. This is not unusual in that the expression of monocot genes in transgenic dicots mimics expression in the monocot. For example, the promoter of light-regulated genes from rice (Sakamoto et al., 1991; Luan and Bogorad, 1992), wheat (Lamppa et al., 1985), and maize (Matsuoka and Sanada, 1991) remains light responsive in transgenic tobacco. The promoter sequence of the rice proliferating cell nuclear antigen gene, which is mainly expressed in the meristematic regions of rice, permits expression of the *GUS* gene in meristems of transgenic tobacco (Kosugi et al., 1991). These observations indicate that some monocot promoters retain specificity of expression in dicots.

GUS activity in shoot apices was mainly localized in stipules, whereas shoot meristems were stained more faintly. Smith et al. (1992) reported that the *Kn1* gene product was found at a high level in vegetative apical and axillary meristems but not in leaf founder cells on the flank of the apical meristem. The stipule-specific expression of *OSH1–GUS* in Arabidopsis does not coincide with the failure to detect expression of the *Kn1* gene product in leaf founder cells, even though both genes are expressed at the shoot apex. Why such difference in cell localization was observed is not clear at this time, but it is possible that the difference in structures of shoot apices and leaves between monocot and dicot plants may cause the difference in their expression patterns. Indeed, it is not clear which tissue in rice plants corresponds to the Arabidopsis stipule.

The stipule-specific expression of *OSH1* is similar to that of the *GLABROUS1* (*GL1*) gene of Arabidopsis, a *myb*-like oncogene, which is required for leaf trichome differentiation from epidermal tissue (Oppenheimer et al., 1991). It has been reported that knot formation caused by the *Kn1* gene in maize is initiated by abnormal periclinal division of epidermal cells (Gelinas et al., 1969). The biological function of *OSH1* is similar to that of *Kn1* (Figure 3); therefore, the target tissue of the *OSH1* gene product is likely to be the epidermis. In this respect, there may be some similarities in the functional mechanism between *OSH1* and the *GL1* gene, because both genes are primarily expressed in stipules and their target is epidermal tissue.

The expression of the *OSH1* gene product in Arabidopsis affects vegetative and floral meristem differentiation and results in morphologically altered leaves and flowers. The most typical regenerants formed assemblies of many reproductive shoot apices, such as a teratoma. Recently, Smith et al. (1992) reported that the ectopic expression of *Kn1* in developing mutant leaves interferes with the determination of cell fates, and they postulated that the down regulation of the *Kn1* gene product at leaf initiation sites in apical meristems is important for the coordinate changes in cell division, growth, and developmental potential. They also hypothesized that *Kn1* may function to oppose cell differentiation and rapid, longitudinally polarized growth. The phenotypes of transgenic plants correspond well with the model of Smith et al. (1992). Indeed, in transgenic Arabidopsis with high *OSH1* expression, the differentiation process of the leaf from leaf primordium and longitudinally polarized growth were completely inhibited. Under the low level of expression of *OSH1*, longitudinally polarized growth was restored, but differentiation of the leaf did not occur. These findings support the model of Smith et al. (1992), and the *OSH1* gene product may be related to the determination of leaf characteristics in the same manner as the *Kn1* gene product. Ectopic *Kn1* expression within veins of *Kn1* mutant leaf blades causes groups of cells to continue to divide and expand, even after surrounding cells have stopped growing (Hake, 1992); this suggests that the expression of *Kn1* may result in abnormal cell division. The formation of reproductive shoot apices as a teratoma associated with continuous expression of the *OSH1* gene product also supports this suggestion.

The clumps of reproductive shoot apices continuously increase even on phytohormone-free medium. The isopentenyl transferase gene of the Ti plasmid also induces formation of abundant shoot apices in transgenic plants (Smart et al., 1991; Smigocki, 1991; Hagen and Guilfoyle, 1992), as does the *rolC* gene in the Ri plasmid (Estruch et al., 1991) and the rice GTP binding protein gene (Kamada et al., 1992). Overproduction of cytokinins is thought to be the mechanism by which these genes increase the number of shoot apices and reduce apical dominance. Other analyses have shown that increases in numbers of shoot apices also occur when the relative level of auxins is decreased (Romano et al., 1991). Our recent results indicate that the level of auxin is dramatically decreased in reproductive shoot apices relative to that in normal plants, supporting the idea that the morphological alterations are caused by the change in hormonal level (data not shown). It is possible that the *OSH1* gene product, which is expressed in a specific region of shoot apices, regulates the expression of a gene(s) that is involved in hormone metabolism as a *trans*-acting factor, and this changes the hormonal level in a limited number of cells of shoot apices. Further studies must be done to test this possibility.

In addition to the alterations in vegetative tissue development, the expression of the *OSH1* gene product also causes drastic changes in flower development and morphology. The involvement of the *OSH1* gene product in flower development is supported by the observation that some amount of the transcript was detected in rice glumes. Several homeotic genes involved in flower development have been identified in

Arabidopsis (reviewed in Coen and Meyerowitz, 1991), but the morphological changes caused by those genes do not correspond to the alterations induced by *OSH1*. Although the relationship between the *OSH1* gene product and these floral homeotic genes is not clear at this time, it is possible that the expression of *OSH1* in Arabidopsis affects the expression of these homeotic genes controlling flower development directly or via changing the hormone level.

METHODS

Construction and Screening of cDNA and Genomic Libraries

To prepare a probe that covered the *Knotted-1* (*Kn1*) homeodomain for the screening of a cDNA library, we performed the polymerase chain reaction (PCR) using a genomic DNA fragment from maize as a template and synthetic oligonucleotides as primers (5′ primer, GAGACTCAGAAGGTGGC; 3′ primer, CTGGTTGATGAACCAGTTGTT). The PCR product was sequenced to confirm that it encoded the sequence of the *Kn1* homeodomain region (Vollbrecht et al., 1991). A poly(A)-enriched RNA fraction from rice primary seedlings (*Oryza sativa* cv Nipponbare) was used for synthesis of double-stranded cDNA. The cDNA products were cloned into the EcoRI site of λZAPII (Stratagene). Construction of a rice genomic library was performed as described elsewhere (Sakamoto et al., 1991). Screening was conducted with a coding region of the *O. sativa* homeobox 1 (*OSH1*) as a probe.

DNA Sequence Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain termination method using an automated sequencing system (ABI 373A; ABI Japan Co., Tokyo). The cDNA clone was sequenced completely on both strands. All exons and the regions of the first and second intron in the genomic clone were completely sequenced, whereas the sequences of the third and fourth introns were only partially determined around the intron–exon junctions. DNA sequence analysis was performed with GENETYX computer software (Software Kaihatsu Co., Tokyo, Japan).

Restriction Fragment Length Polymorphism Mapping

Linkage analysis of *OSH1* was based on segregation of an F_2 population (144 plants) derived from parental varieties, FL134 (Japonica variety) and Kasalath (Indica variety), which were used for construction of the current restriction fragment length polymorphism (RFLP) map (Saito et al., 1991). RFLP mapping of *OSH1* was performed with BamHI-, BglII-, EcoRV-, and HindIII-digested DNA of the parental lines. DNA hybridization in parental lines and F_2 populations and linkage analysis were performed according to the method of Saito et al. (1991). Digested DNA (5 μg) was separated by electrophoresis on 0.8% agarose gels and transferred to nitrocellulose filters. Hybridization with a labeled cDNA insert was performed in 50% formamide that contained 5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), 6 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS, and 0.1 mg/mL calf thymus DNA at 62°C for 20 hr. Each filter was washed with 0.1 × SSC and 0.1% SDS at 65°C for 3 hr.

RNA Gel Blot Analysis

Total RNA was separately prepared from various organs and tissues for gel blot analysis. Ten micrograms of each RNA preparation was transferred to nitrocellulose membranes and hybridized to the entire *OSH1* insert. The conditions of hybridization and washing were the same as that for DNA gel blot analysis.

Primer Extension Analysis

Primer extension analysis was conducted according to the method of Ishii et al. (1987) using a synthetic oligonucleotide, 5′-GAACTCAAGAGACACGC-3′, which anneals to a sequence just upstream of the *OSH1* coding region.

PCR Analysis

PCR analysis using total RNA preparations as templates was performed for confirmation of the transcription capping site. Synthesis of cDNA from 1 μg of total RNA and PCR amplification were performed under previously described conditions (Frohman et al., 1988). The parameters used for amplification of DNA were 5-min denaturation at 95°C followed by 25 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C. Two oligonucleotides, GAGTGAGAGCTAGCTC and AGCTCAGCTCAAGGTAA, were used as 5′ primers, and GAAGCTCAAGAGACACGC was used as the 3′ primer.

Construction of the *OSH1-GUS* Chimeric Gene and Histochemical Analysis of GUS Activity

A 1.0-kb SphI-PstI fragment (−846 to +182), which contains the 5′ flanking and 5′ noncoding regions of *OSH1*, was inserted into the SphI-SmaI site of pBI221 (Clonetech Co., Palo Alto, CA), which carries the structural gene for β-glucuronidase (*GUS*) and the terminator region of the nopaline synthase (*NOS*) gene. The promoter–*GUS* cassette was cut out with HindIII and EcoRI and ligated into the same site of the binary vector, pLAN421, which contains the right border sequence of the T-DNA and the gene for the selectable marker neomycin phosphotransferase II (Uematsu et al., 1991). Histochemical assays for GUS activity were performed by a previously reported method (Matsuoka and Sanada, 1991).

Construction of the 35S Promoter-*OSH1* Chimeric Gene

The cDNA clone for *OSH1* did not have suitable sites for construction of the cauliflower mosaic virus (CaMV) 35S–*OSH1* gene. Therefore, we amplified the coding region by PCR using the 5′ primer with an XbaI linker (position +194) and the 3′ primer with a SmaI linker (position +1359). The PCR product was cloned into the XbaI-SmaI site of pUC119, and the sequence was analyzed to confirm that nucleotide substitution had not occurred during PCR. The CaMV 35S promoter sequence and the terminator region of the *NOS* gene from pBI221 were then inserted into the HindIII-XbaI and SacI-EcoRI sites, respectively, of the *OSH1* clone in pUC119. Finally, the 35S–*OSH1* cassette was cut with HindIII and EcoRI and inserted into pLAN421.

Transformation and Regeneration of Rice

Protoplasts were isolated from suspension cells of rice according to a previously reported method (Akagi et al., 1989). Rice protoplasts were cotransformed by electroporation with 10 μg of the 35S-hygromycin phosphotransferase plasmid and 20 μg of the *OSH1* gene cloned in pBluescript II SK+ (Figure 1B) (Tada et al., 1990). Clones of cells that developed from transformed protoplasts were cultured and regenerated as reported by Fujimura et al. (1985). Transformed plants were selected from among the regenerants according to the method of Tada et al. (1990).

Transformation and Regeneration of Arabidopsis

The *OSH1–GUS* and 35S–*OSH1* constructs were introduced into *Agrobacterium tumefaciens* EHA101 by electroporation. Transformation was according to the root transformation method of Valvekens et al. (1988). Transformants were selected in medium containing 60 mg/L of kanamycin.

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