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Isolation and Characterization of a cDNA Encoding an Orthologue of *ROUGH SHEATH2 (OsRS2)* from Rice

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Abstract

Understanding the molecular mechanisms of making leaves from the shoot apical meristem (SAM) is one of the important issues in plant developmental biology. In this paper, we report on the isolation and characterization of the *OsRS2* gene, which corresponds to the maize *ROUGH SHEATH2 (RS2)*. In situ mRNA localization of *OsRS2* has revealed that *OsRS2* is preferentially expressed at future vascular regions and the abaxial side of lateral organ primordia around SAM. Rice has several advantages in studying on the molecular basis of the phyllotaxis because of its relatively simple phyllotactic pattern. Thus, *OsRS2* will be useful for analyzing the mechanism of the leaf initiation in SAM as a molecular marker of primordium of lateral organs in SAM.

Accession numbers: OsRS2 cDNA (AB064519), OsRS2 genome (AB071600), RS2 (AF126489), AS1 (AF175996), PHAN (AJ005586), AtMYB52 (AF062888), OsMYB (AF172282), AtMYB101 (AC004681), PhMYB3 (Z13998), OsGAMYB (X98355), AtMYB13 (Z50869), PhMYB2 (Z13997), AtMYBGL1 (M79448), AtWER (AC391149), OsMYBC1 (Y15219), ZmMYBC1 (AF320614).

Keywords: in situ hybridization, rice, rough sheath2, shoot apical meristem.

Abbreviations

SAM, shoot apical meristem; *RS2*, *rough sheath2*; *KNOX* genes, *KNOTTED1*-like homeobox genes.

Introduction

The entire ground portion of a plant body is an assembly of shoot units termed phytomeres, which consist of an axillary bud, a stem and a leaf. The shoot apical meristem (SAM) continuously produces these units, at the same time maintaining itself as a collection of indeterminate stem cells (Steeves *et al.*, 1989). The mechanisms, in which indeterminate stem cells are maintained and cells destined to lateral organs are differentiated in SAM, are largely unknown. However, several *KNOTTED1*-like homeobox genes (*KNOX* genes) are likely to be involved in these processes.

KNOX homeodomain proteins encoded by *KNOX* genes are preferentially accumulated in the indeterminate stem cells around SAM, but not in the determinate lateral organs (Jackson *et al.*, 1994; Sentoku *et al.*, 1999). Based on these expression patterns, *KNOX* genes are thought to be involved in the process of making lateral organs or the maintenance of stem cells in SAM. In *Arabidopsis*, loss-of-function mutations in the one of the *KNOX* genes, *SHOOTMERISTEMLESS*, result in the embryo with no shoot apical meristem (Long *et al.*, 1996). Similar mutations are reported in maize *knotted1*, although the limited shoot phenotype, in which the embryonic shoots stop making leaves due to the abortion of SAM, is highly depending on the genetic background (Vollbrecht *et al.*, 2000). In these mutants, these phenotypes are interpreted that cells in SAM are consumed for the differentiation of lateral organs because of the defect in maintaining indeterminate cells. In contrast, gain-of-function mutations that result in ectopic expression of

KNOX genes in maize cause overgrowth of leaf tissues due to the ectopic presence of cells with indeterminate characters in the leaves with determinate cells (Freeling, 1992; Sinha *et al.*, 1994). Similarly, overexpression of *KNOX* genes in transgenic rice, tobacco and *Arabidopsis* causes abnormal leaf development (Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Nishimura *et al.*, 2000; Sentoku *et al.*, 2000). Thus, *KNOX* genes are likely to work as switches to change indeterminate and determinate state.

Recessive mutants such as named *phantastica* (*phan*) in *Antirrhinum* (Waites *et al.*, 1998), *asymmetric leaves1* (*as1*) in Arabidopsis (Byrne *et al.*, 2000), and *rough sheath2* (*rs2*) in maize (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999) show leaf phenotypes similar to either dominant mutants for the *KNOX* gene loci or transgenic plants in which *KNOX* genes are overexpressed. In addition, mRNA or protein from some of the KNOX genes accumulated ectopically in the leaves of these mutants. These findings suggest that *PHAN*, *AS1* and *RS2* are

(A)

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        10        20        30        40        50        60        70        80        90       100
CTCGTGCTCCTTGTCCCCCACCTCCTTTACTTGAGCTTGCTCACCACTGCCTCCATATACAGAGAGTCTTCTCTGTATTTCTCTCTCTCAGTGGGAGGCT
       110       120       130       140       150       160       170       180       190       200
CACAAGAATCTTTCAGCTCACATCTCACTGTCCTTGCCTTTCTCTAGCTAGGGAGGTCCAAGAACAGAGCAGCAGGATGGTGCAGTGGCGTTTCTagtac
       210       220       230       240       250       260       270       280       290       300
ttttgggggtttatggattgtttcctcctactcaagagaggGAGAGCAGCATGAGTACTCGCACCACCGAGCTGGGATGGTCGGCGATGGCGAACACCGAT
       310       320       330       340       350       360       370       380       390       400
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          M  R  E  R  Q  R  W  R  P  E  E  D  A  I  L  L  A  Y  V  R  Q  Y  G  P  R  E  W  S  L
       410       420       430       440       450       460       470       480       490       500
GTGTCCCAGCGGATGAACCGCCCCCTCCACCGCGACGCCAAGTCCTGCCTCGAGCGCTGGAAGAACTACCTCCGCCCGGGGATCAAGAAAGGTTCGCTCA
V  S  Q  R  M  N  R  P  L  H  R  D  A  K  S  C  L  E  R  W  K  N  Y  L  R  P  G  I  K  K  G  S  L  T
       510       520       530       540       550       560       570       580       590       600
CCGACGACGAGCAGCGCCTCGTCATCCGCCTCCAGGCCAAGCACGGCAACAAGTGGAAGAAGATCGCCGCCGAGGTCCCCGGCCGGACGGCGAAGCGGCT
  D  D  E  Q  R  L  V  I  R  L  Q  A  K  H  G  N  K  W  K  K  I  A  A  E  V  P  G  R  T  A  K  R  L
       610       620       630       640       650       660       670       680       690       700
GGGCAAGTGGTGGGAGGTGTTCAAGGAGAAGCAGCAGCGGGAGCTCCGGGATCGGGATCGCCGGCGACTCCCGCCGCCGCTGGACGGCGACGAGCGCGGC
 G  K  W  W  E  V  F  K  E  K  Q  Q  R  E  L  R  D  R  D  R  R  R  L  P  P  P  L  D  G  D  E  R  G
       710       720       730       740       750       760       770       780       790       800
TGCGCCGGCGGGCGATACGACTGGCTCCTCGAGGACTTCGCCGACAAGCTCGTCAACGACCACCACCGCCGAATGATGGCTGCCCCGATCCTCCCGCCGT
C  A  G  G  R  Y  D  W  L  L  E  D  F  A  D  K  L  V  N  D  H  H  R  R  M  M  A  A  P  I  L  P  P  W
       810       820       830       840       850       860       870       880       890       900
GGATGTCGTCGTCGCCGTCGTCTTCTTCCTCGCCGTCGGTGACGCTTAGCCTCGCGTCGGCGGCGGTGGCGCCGGCGCCTGCGGCGCCGCCGCCGACGTG
  M  S  S  S  P  S  S  S  S  S  S  P  S  V  T  L  S  L  A  S  A  A  V  A  P  A  P  A  A  P  P  P  T  W
       910       920       930       940       950       960       970       980       990      1000
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 G  G  G  G  G  G  E  V  V  V  A  E  L  M  E  C  C  R  E  M  E  E  G  Q  R  A  W  A  A  H  R  K  E
      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
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A  A  W  R  M  K  R  V  E  M  Q  L  E  T  E  R  A  C  R  R  R  E  A  T  E  E  F  E  A  K  M  R  A  L
      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TGAGGGAGGAGCAGGCAGCTGCGGTGGAGCGGGTGGAGGCGGAGTACCGGGAGAAGATGGCCGGCCTCCGCCGCGACGCGGAGGCCAAGGAGCAGAAGAT
  R  E  E  Q  A  A  A  V  E  R  V  E  A  E  Y  R  E  K  M  A  G  L  R  R  D  A  E  A  K  E  Q  K  M
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
GGCGGAGCAGTGGGCGGCCAAGCACGCCCGCCTCGCCAAGTTCCTCGACCAGGTCGCCGCCTGCCGCCGCTGGCCGCCCGTAGAGATCAACGGCGGCGGC
A  E  Q  W  A  A  K  H  A  R  L  A  K  F  L  D  Q  V  A  A  C  R  R  W  P  P  V  E  I  N  G  G  G
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
GGCGGCGGCCCCGGCGGCGGCCGGTGAGCCGCCGGACGGTGGCGCTGCATGCATGCGTAGTTGGACACCTCGCTTTCTTGCTTCTTCTTCTCGTGTCCTT
G  G  G  P  G  G  G  R  *
      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
TTTACAACTGTTGGAATTCACTTCTCTCTAGAAATATGTAATCTTTTGAGTCTCTTTTGATCATTCCAAGTTTCCTACTAGTAGCTCTATTGAATAATAA
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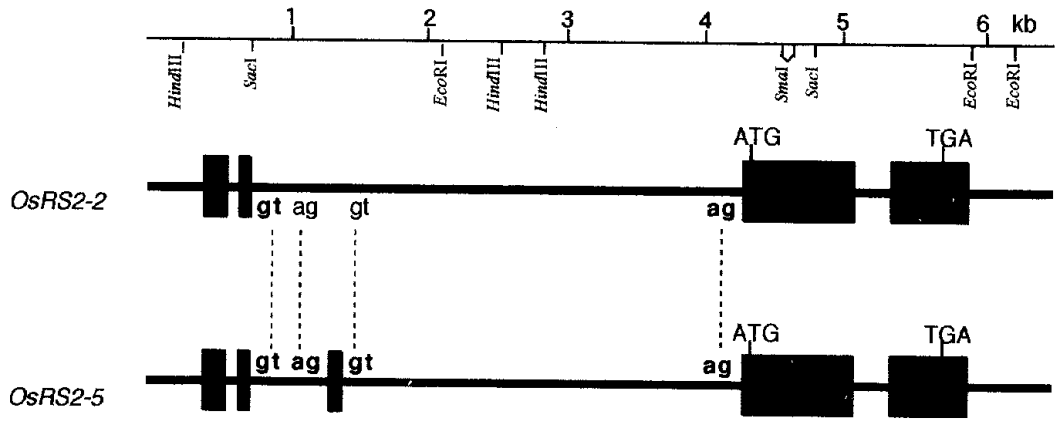
(B)



involved in the negative regulation of *KNOX* genes in leaves (Waites *et al.*, 1998; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999; Byrne *et al.*, 2000; Ori *et al.*, 2000). It is now revealed that *PHAN*, *AS1* and *RS2* encodes highly homologous proteins each other and they all have conserved MYB domain in their N termini. Expression patterns of these genes are similar to each other, and they are expressed in the primordia of lateral organs in SAM. Considering the high degrees of similarities of their primary structures and their expression patters, all of them are thought to be orthologous genes each other and engaged in developmental processes common to three species.

Rice has several advantages to analyze the process of the lateral organ initiation from SAM. First, alternate phyllotaxis in rice makes easier to predict the position of the initiation of new lateral organs in SAM. Second, molecular and genetic approaches can be applied to analyze the function of genes in rice. Therefore, cloning of *PHAN*, *AS1* and *RS2* orthologue from rice will give us clues to understand the molecular mechanisms regulating the balance between the maintenance of stem cells and differentiation of lateral organs in SAM. In this paper, we describe the cloning and characterization of *OsRS2*, which encodes a MYB protein with the highest similarities to *PHAN*, *AS1* and *RS2* group of the MYB gene family. *OsRS2* is the most similar to *RS2*. Mapping of *OsRS2* revealed that *OsRS2* and RS2 are mapped to the chromosomal regions where the synteny is observed between rice and maize (Ahn *et al.*, 1993; Wilson *et al.*, 1999). In situ mRNA accumulation pattern of *OsRS2* around SAM was also similar to that of *RS2*. For above reasons, we have concluded that *OsRS2* is the rice orthologue of *RS2* and it can be used as a molecular marker of the position of lateral organ initiation in SAM.

Materials and Methods

Plant material

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

Cloning of cDNAs and genomic clones

An EST clone (S5465) was given from Rice Genome Project (Tsukuba). The fragments (ca. 800 bp) digested by *Sal*I were labeled by random priming with ^{32}P-dCTP. A rice cDNA library was constructed using poly(A)$^+$ RNA prepared from rice seedling with a Lambda ZAP II XR library construction kit (Stratagene, La Jolla, CA, U.S.A.). Approximately 5 x 10^6 phage recombinants derived from the rice seedling cDNA library were screened with the fragments as a probe. We obtained six positive clones by screening, and partially sequenced their 5' ends. Two clones that had the longest 5' sequence were chosen for further analysis.

Rice genomic library constructed with Lambda gt11 was screened with the same probe as used for cDNA screening. One of the positive clones with the strongest signal was subcloned to pBluescriptSK and the sequence covering entire *OsRS2* gene was determined. The accession number for the genomic sequence is AB071600.

RNA extraction and blot analysis

Total RNA was isolated from the tissues by SDS-phenol extraction, followed by LiCl precipitation. Formaldehyde agarose gel electrophoresis of total RNA was performed using standard procedures (Sambrook *et al.*, 1989). The RNAs were blotted onto a Hybond N+ membrane (Amersham Pharmacia) and hybridized with ^{32}P-labeled cDNA inserts. Hybridization was carried out in 0.25 M

Fig. 1 Rice *OsRS2* gene structure.

(A) Nucleotide sequence and deduced amino acid sequence of rice *OsRS2* cDNA. Capital letters indicate the nucleotide sequence of the cDNA clone *OsRS2-2*, and small letters indicate the extra exon present in the cDNA clone *OsRS2-5*. Box indicates the predicted MYB domain. Triangles show the insertion sites of introns. The nucleotide sequences of *OsRS2* have been submitted to the DDBJ nucleotide sequence databases with the accession number AB064519.

(B) Genomic structure of rice *OsRS2*. The scale bar and some restriction enzyme sites are presented at the top line. Boxes and lines indicate exons and introns, respectively. ATG and TGA indicate the location of the initiation and termination codons of the gene, respectively. Bold "gt" and "ag" indicate the donor and acceptor sequences for RNA splicing, which should be used in splicing mRNAs for each cDNA clone. Plain "gt" and "ag" correspond to the donor and acceptor sequences for splicing of *OsRS2-5*, which are not used for splicing of *OsRS2-2*.

NaH_2PO_4 buffer (pH 7.2), 0.25 M NaCl, 7% (w/v) SDS, 10% (w/v) dextran sulfate (Amersham Pharmacia), and 1% (w/v) polyvinylpyrrolidone K30 at 65 ℃ for more over 16 h. The membrane was washed in 2 X SSPE (1 x SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) and 0.1% SDS for 30 min at room temperature, and then 2 X SSPE and 0.1% SDS at 65 ℃ twice for 30 min each. A Fuji Imaging Plate was exposed to the filters for 4 h. The image was visualized with a BAS2000 Imaging Analyzer (Fuji Photo Film Co., Japan).

DNA sequencing and phylogenetic analysis

The cDNA and genomic clones were sequenced by the dideoxynucleotide chain-termination method using an automatic DNA sequencer (model 377, Applied Biosystems, Inc., Foster City, CA, U.S.A.) according to the manufacturer's protocol. Both strands were entirely sequenced. Phylogenetic anayses were performed using PAUP*, version 4.0 (Swofford, 1999). A tree was constructed based on the neighbor joining anaysis, with genes in subgroup C as an outgroup. Node support is assessed with 1000 bootstrap replicates of the data.

Mapping

Ninety eight BC_1F_5 lines (backcross inbred lines) derived from the cross a japonica variety, Nipponbare, and an *indica* variety, Kasalath were used for mapping of *OsRS2* clone (Lin *et al.*, 1998). For making a CAPS marker, we produced primers located at the 3'-end portion of intron 2 (5'-side primer; 5'-aacttgtagaagtcgaattt-3') and in the exon3 region (3'-side primer; 5'-tgctgcttctccttg-aacac-3'). The size of PCR products from Nipponbare and Kasalath corresponded to 620 bp, while the product from Nipponbare contained two *Acc*II sites, while the product from Kasalath had one site. Linkage analysis of *OsRS2* and 245 RFLP markers was performed using MAPMAKER Version 3.0 (Lander *et al.*, 1987).

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 ℃, 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, U.S.A.). 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 *et al.* (1993). Digoxigenin-labeled RNA was produced from the coding region of *OsRS2* gene.

Results and Discussion

To isolate the orthologue of maize *RS2* which is known to be expressed in the leaf primordium in SAM, cDNA library constructed from mRNA extracted from rice seedling was screened with a rice EST clone (S5465) which shares high similarity with *RS2* and six positive clones were obtained. We determined the entire sequences of two independent clones with the longest insert size, and found that one clone (*OsRS2-2*) contains the longest 5' end of cDNA and contains an ORF with 342 amino acids (**Fig. 1A**). The other clone (*OsRS2-5*) contained a shorter 5' end of cDNA but had an additional 45-bp sequence in the 5' untranslated region (UTR). By comparing the cDNA sequences of the both cDNA clones and the genomic sequence of *OsRS2*, we found that *OsRS2-2* is consisted of four exons whereas *OsRS2-5* is consisted of five exons (**Fig. 1B**). The extra 45-bp exon in *OsRS2-5* locates in 5' UTR. Thus, a part of the second intron in *OsRS2-2* is transcribed as an exon in mRNA for *OsRS2-5* by alternative splicing. These alternative RNA products are probably produced by the use of the different combinations of intron donors and acceptors (**Fig. 1B**). Alternative RNA products from a gene sometimes encode proteins with different function or localization (Tamaoki *et al.*, 1995; Mano *et al.*, 1999). In this case, however, the difference in two RNA products resides in the 5' UTR. Considering that there is no difference in the deduced amino acid sequences of both cDNA clones, it is possible that the presence or absence of the 45-bp exon in the 5' UTR affects the stability of RNA. The difference in the use of intron donor and acceptor may be involved in the post transcriptional regulation of *OsRS2* gene expression through the regulation of RNA stability of the gene.

OsRS2 encodes a protein with a highly conserved MYB domain in its N terminus. The amino acid identity between OsRS2 and maize RS2 is 90.0% in the MYB domain and 70.5% in the entire protein (**Fig. 2A**). MYB domain in OsRS2 is classified to the R2R3 type. MYB domain with R2R3 is subdivided to four subgroups named A, B, C, and MYBPHAN type (Jin *et al.*, 1999). MYB domain of OsRS2 is classified into the MYBPHAN subgroup, to which RS2, AS1 and PHAN belong, and most similar to RS2 (**Fig. 2B**). This suggests that OsRS2 may be an orthologue of RS2, AS1 and PHAN and could have functions equivalent to those.

It is often difficult to find orthologous rela-

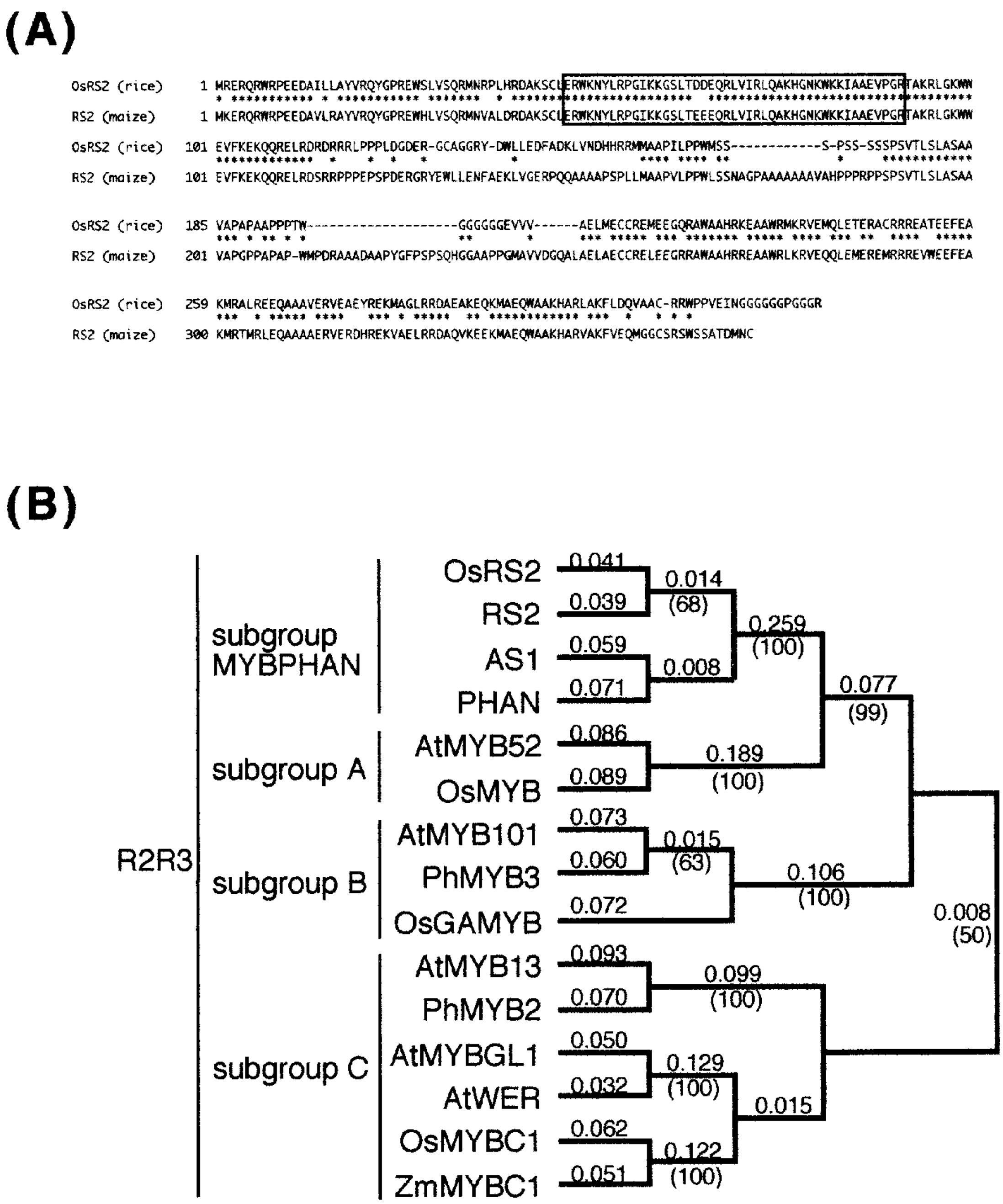


Fig. 2 Structural relationship of OsRS2 from rice and RS2 from maize, and other plant MYB proteins.

(A) Alignment of RS2 clones from rice (AB064519), and maize (AF126489). Dashes indicate gaps introduced to maximize alignment. Identical amino acid residues are represented by asterisk and the predicted MYB domain was boxed.

(B) Phylogenetic relationships of plant R2R3 MYB family. A tree was constructed based on the neighbor joining analysis, with genes in subgroup C as an outgroup. Decimal numbers represent the length of nodes and integers in parentheses indicate percentage of the bootstrap support of nodes, which are present 50% or more of the bootstrap replicate analyses. Node support is assessed with 1000 bootstrap replicates of the data. The phylogram shown was constructed with R2R3 MYB domains. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ph, *Petunia hybrida*; Zm, *Zea mays*.

tionship of genes from monocot and dicot plants. On the other hand, it is more reliable to discuss orthologous relationships among the grass families such as rice and maize, because a precise synteny map between these two species is constructed (Ahn *et al.*, 1993; Sentoku *et al.*, 1999; Wilson *et al.*, 1999). In order to confirm that *OsRS2* is an orthologue of *RS2*, we determined the chromosomal location of *OsRS2*. *OsRS2* was mapped to the long arm of chromosome 12 (**Fig. 3**). According to the synteny

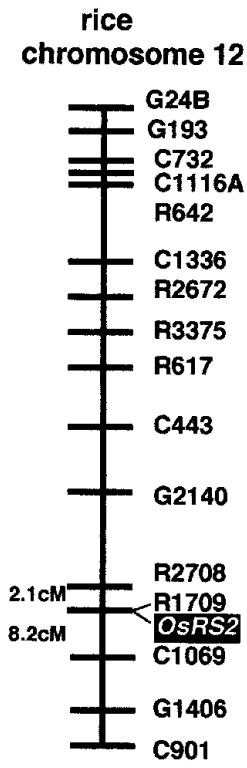


Fig. 3 Chromosome mapping of rice *OsRS2*.
OsRS2 is mapped on the long arm of the rice chromosome 12 where corresponds to the centromere region of the maize chromosome 1 according to the synteny relationship between rice and maize chromosome proposed by Ahn *et al.* (1993).

map by Ahn *et al.* (1993), this region corresponds to the centromere region of maize chromosome 1, where *RS2* locates (**Fig. 3**). This strongly support the idea that *OsRS2* is an orthologue of *RS2*.

mRNA accumulation pattern of *OsRS2* in various rice organs was tested by RNA gel blot analysis (**Fig. 4**). The *OsRS2* transcript was detected in all organs tested. Considering that the RNA samples we used for the analysis are all from lateral organs in which *RS2* is supposed to be expressed, the result seems reasonable. The size of the transcript was about 1.5 kb and was approximately the same as the longest cDNA clone. This indicates that our cDNA clone covers entire or almost entire sequence of the *OsRS2* transcript. We also tested the in situ mRNA accumulation pattern of *OsRS2* around SAM to confirm that the tissue specific localization of *OsRS2* transcript is similar to that of *RS2*.

In the longitudinal section of shoot apex of the vegetative stage, no *OsRS2* expression was detected in the center of SAM, but was faintly detected at the plastochron1 (P1) (**Fig. 5A**). Relatively strong signal was found at vascular region of the P2 leaf (arrowhead in **Fig. 5A**). Plastochron denotes the interval between initiation of leaves such that the

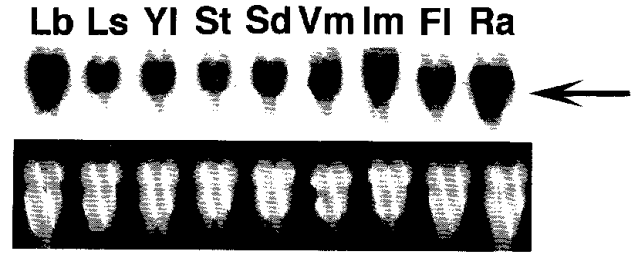


Fig. 4 Accumulation levels of the rice *OsRS2* mRNAs in various organs.
Total RNA was extracted from various tissues from rice. Total RNA (5 μg) was electrophoresed on 1% agarose gels, and blotted onto Hybond N+ membrane. The membrane was probed with a part of *OsRS2* cDNA. This probe does not contain MYB domain and is supposed to be gene specific. The arrow indicates the approximate position of the signal for *OsRS2* mRNA. The bottom panel shows the ethidium bromide (EtBr) stained RNA gel. Lb, leaf blade; Ls, leaf sheath; Yl, young leaf; St, stem; Sd, seedling; Vm, vegetative meristem enriched tissue; Im, inflorescence meristem enriched tissue; Fl, flower buds; Ra, rachis.

primordium closest to the meristem is P1, the next one out from the meristem is P2, and so on. In the cross section of a vegetative shoot around the arrow at the left side of **Fig. 5A**, expression of *OsRS2* was observed at the abaxial side of the central or lateral region of the P1 leaf. The abaxially localized expression was also seen in the P2 leaf. In this leaf, the expression was not spread around whole abaxial region but located at specific region as a stripe pattern where would form bundle sheath extensions in the future (arrows in **Fig. 5B**). Bundle sheath extension is the tissue specific for grass leaves, which vertically runs from vascular tissues to the both adaxial and abaxial sides. In the rice leaf development, the formation of bundle sheath extension is firstly observed around the midvein region at the P5 stage (Kaufman, 1959), therefore the *OsRS2* expression at the abaxial bundle sheath extension occurs about three plastochron earlier than its morphological development. The *OsRS2* expression was also seen at the vascular regions as seen in the longitudinal section (**Fig. 5A** and **B**). Faint but certain signal was also observed at the leaf margin of the P2 leaf (arrowheads in **Fig. 5B**). The localized expression of *OsRS2* in vascular tissues and leaf margin was similar to that of maize *RS2* while the expression in bundle sheath extension was not seen in the case of maize *RS2*. This difference in expression pattern depends on the difference in the leaf structure of these plants, indeed rice plants form aerenchyma in the leaves, which is the tissue con-

taining large gas-filled intercellular spaces, while maize does not develop such tissue. We also tested the expression pattern of *OsRS2* around SAM during embryogenesis. In rice, three foliage leaves are formed from SAM during embryogenesis. In the embryo at five days after pollination (5 DAP), the first foliage leaf is formed and the second starts to appear (**Fig. 5C**). At this stage, no *OsRS2* expression was observed in SAM, but was observed in the abaxial sides of P1 and coleoptile, which is a leaf-like organ formed early in the embryogenesis (**Fig. 5C**). This observation demonstrates the abaxial-specific expression of *OsRS2* not only in true leaves but also in a leaf-like organ, coleoptile.

The vascular and bundle sheath extension specific expression in leaf primordia is also observed in the case of a rice gene, which is an orthologous gene to Arabidopsis *ZWILL/Pinhead* gene (*OsPNH*), but interestingly, the *OsPNH* expression is specifically localized in the adaxial but not abaxial side in contrast to the abaxial specific expression of *OsRS2* (Nishimura *et al.*, unpublished observation). This contrastive expression pattern of *OsRS2* and *OsPNH* in leaf primordia leads us to speculate that the both genes may involve in the establishment of dorsoventrality of leaves in the leaf developmental process. Based on the phenotypic analysis of loss-of-function mutants of *PHAN*, which is the *Antirrherium* gene orthologue to maize *RS2* and also to rice *OsRS2*, indeed, Waites and Hudson (1995) speculated that *PHAN* is required for establishing the dorsoventral identity in the *Antirrheniun* leaf development. Similarly, *Arabidopsis pnh* mutant defects in the formation of axially buds, which is the typical adaxial character of leaves, and consequently, *PNH* may commit to the establishment of the adaxial identity (Lynn *et al.*, 1999). Although there are some differences in the leaf structure between grasses and these dicot plants, possibility of the commitment of the same genes in the leaf developmental process suggests the similarity in the mechanism of the establishment of the dorsoventral identity between monocot and dicot plants.

Most class1 type *KNOX* genes are expressed in SAM with indeterminate cells and not expressed in the determinate lateral organs and their initials in SAM (Jackson *et al.*, 1994; Sentoku *et al.*, 1999). In contrast, *OsRS2*, *RS2*, *AS1* and *PHAN* are only expressed in cells destined to determinate lateral organs. Thus, class1 type *KNOX* genes and *OsRS2*, *RS2*, *AS1* and *PHAN* are expressed mutually in an exclusive way around SAM. These results look quite reasonable since *RS2*, *AS1* and *PHAN* act to suppress the expression of *KNOX* genes in leaves. However, the fact is not so simple, because the expression of class1 type *KNOX* genes in the founder cells of lateral organs are still down regulated even in *rs2* and *as1* mutant (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999; Byrne *et al.*, 2000). This means that the *RS2* and *AS1* are not required for the down regulation of *KNOX* gene expression in leaf founder cells in SAM but rather they are involved in keeping *KNOX* gene expression be silenced in leaves. The molecular mechanism for the *KNOX* gene expression to keep silenced in leaves is unknown. Dominant mutations in *KNOX* gene loci in maize, barley, wheat and tomato invoke us the existence of common mechanism to keep suppressing *KNOX* gene expression in leaves (Smith *et al.*, 1992; Müller *et al.*, 1995; Parnis *et al.*, 1997; Takumi *et al.*, 2000). Cloning of *OsRS2* will help us to understand these processes by virtue of advantages of both molecular and genetic approaches in rice (Izawa *et al.*, 1996). Furthermore, *OsRS2* will be useful as a molecular marker for leaf founder cells in SAM. Due to the relatively simple pattern of leaf initiation in rice (i.e. alternate phyllotaxis), analysis of the mechanism of leaf initiation in SAM with using *OsRS2* as a marker will help understanding the molecular basis of this process.

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