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Identification and tissue specific expression analysis of *MKRN* gene in rice

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Abstract : The makorin *(MKRN)* RING finger protein gene family encodes proteins (makorins) with a characteristic array of zinc-finger motifs and which are present in a wide array of eukaryotes. In the present study, the structure and expression of a putative makorin RING finger protein gene were analyzed in rice *(Oryza sativa* L. ssp. *japonica* cv. Nipponbare). From the analysis of the genomic (AP003543), mRNA (AK120250) and deduced protein (BAD61603) sequences of the putative *MKRN* gene of rice obtained from GenBank, it was found that it was indeed a *bona fide* member of the *MKRN* gene family. The rice *MKRN* cDNA encoded a protein with four C3H zinc-finger-motifs, one putative Cys-His zinc-finger motif, and one RING zinc-finger motif. The presence of this distinct motif organization and overall amino acid identity clearly indicated that this gene was indeed a true *MKRN* ortholog. Isolated RNA from embryonic axes of rice seeds at various stages of imbibition and germination and were studied for the temporal expression profile of *MKRN* by RT-PCR. This analysis revealed that *MKRN* transcripts were present at all the time points studied. It was at very low levels in dry seeds, increased slowly during imbibition and germination, and slightly declined in the seedling growth stage. After 6 days of germination, an organ-dependent expression pattern of *MKRN* was observed: highest in roots and moderate in leaves. Similarly to *MKRN* transcripts, transcripts of cytoskeletal actin and tubulin were also detected in dry embryos, steadily increased during imbibition and germination and leveled off after 24 hours of germination. The presence of *MKRN* transcripts in dry seeds, its early induction during germination and its continued spatiotemporal expression during early vegetative growth suggest that *MKRN* has an important role in germination, leaf and lateral root morphogenesis and overall development in rice.

Abbreviations: *MKRN*, makorin RING finger protein gene; TAE, tris-(hydroxymethyl)-amino-methane acetate ethylenediaminetetraacetic acid; KOD, DNA polymerase obtained from *Thermococcus kodakaraensis;* BLAST, Basic Local Alignment Search Tool; RT-PCR, reverse transcriptase polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid.

Key words: Gene expression, germination, makorin, rice, RT-PCR.

Introduction

Makorin *(MKRN)* gene family encodes distinct proteins with a unique composition and organization of zinc-finger motifs, including several C3H motifs, a RING motif and a Cys-His motif playing a major role in proteolytic degradation of proteins. Gray *et al.* (2000) characterized a new gene family, makorin *(MKRN),* by identifying and characterizing a *MKRN1* gene from human, mouse, wallaby, chicken, fruitfly, and nematode. A second gene, *MKRN2,* encodes a protein that retains all the hallmarks of zinc-finger motifs characteristic of the makorin family and is thought to originate from an ancestral *MKRN1* by a gene duplication event early in vertebrate evolution, over 450 million years ago (Gray *et al.,* 2001). The discovery and characterization of the *MKRN2* locus in yellowtail fish in laboratory greatly enhanced studies of the makorin gene family and characterization *of MKRN2* orthologs from human, mouse and zebra fish (Gray *et al.,* 2001). *MKRN1* is one of the putative genes that acts downstream of *OCT-4,* a transcriptional factor suggested to play an essential role in the establishment and maintenance of the toti/pluripotency of embryonic and undifferentiated embryonic stem cells, embryonal carcinoma cells, and embryonic germ cells *in vitro* (Du *et al.,* 2001). The expression patterns *of MKRN 1* and *MKRN2,* the two major vertebrate paralogs of *MKRN,* have been studied in several tissues of mouse and human (Gray *et al.,* 2000; Gray *et al.,* 2001). The elucidation of the genomic organization *of MKRN 1* and expression profiles of *MKRN1, MKRN2* in yellowtail fish in lab has greatly enhanced the understanding of expression of *MKRN* in animals (Chamnan *et al.,* 2003).

A plant MKRN was putatively identified bioinformatically from the *Arabidopsis* genome project in earlier work (Gray *et al.,* 2000). In the most recent study on makorins (Abe *et al.,* 2006), using germinating pea *(Pisum sativum* L. var. Alaska) seeds, was the first experimental evidence that hints at the function of a plant *MKRN* provided. In this study (Abe *et al.,* 2006), the genomic organization and temporal expression profile of pea *MKRN* were showed and reported the presence of *MKRN* transcripts in dry seeds and the very early induction of *MKRN* in germinating peas, and it was suggested the a developmental role was there for makorin in pea germination. Since *MKRN* is expressed during embryogenesis and differentiation in mouse (Gray *et al.,* 2000) and during germination in peas (Abe *et al.,* 2006), it was anticipated that *MKRN* genes might also function during early stages in plant morphogenesis.

Rice is a good model plant for developmental, evolutionary and agronomical studies of cereals (Itoh *et. al.,* 2005), where its developmental program is separable into three phases: embryogenesis, vegetative growth, and reproductive growth, with seed dormancy, germination, and the onset of inflorescence development respectively typically delimit these three phases. Further the availability of whole genome sequence information and other molecular tools like microarrays made rice as a choice of plant molecular biologists. In the present study, the genomic organization and phylogenetic relations of *MKRN cDNA* was examined in rice, which encoded a predicted makorin protein with shared characteristics of makorins from pea, *Arabidopsis* and metazoans. Then, the changes in transcript abundance and the spatiotemporal expression patterns of the rice *MKRN* mRNA in dry and germinating seeds were shown.

Materials and Methods

Plant material and tissue samples used

Dry mature seeds of rice *(Oryza sativa* L. ssp. *japonica* cv. Nipponbare) were imbibed in water for 24 hours in the dark at 25 °C and designated zero hours imbibition (0H1) to 24 HI. At 24 HI, seeds were sown in vermiculite to begin germination (0HG) in a growth chamber at 25 °C under 16 hours light and 8 hours dark cycle up to 12 days. Tissues were harvested from dry seeds and at various times during imbibition and germination.

Cloning and sequencing of rice MKRN cDNA

Makorin gene sequences obtained from the previous experiments on pea (Abe *et al.,* 2006) were used to search against the NCBI database to identify the rice homolog. A set of gene specific primers, OS-MKRNIB-F and OS-MKRNIB-R, (Table 2) designed based on the rice *MKRN* cDNA sequence (AK 120250) obtained from GenBank were used to clone and confirm rice MATWcDNA sequence.

Phylogenetic analysis of makorins from divergent classes of organisms

In order to determine the evolutionary relationship between makorin protein sequences from multiple species representing invertebrates, vertebrates and plants, the deduced amino acid sequences encoded by *MKRN* from rice, *Arabidopsis,* pea, human, mouse, wallaby, chicken, zebra fish, yellowtail fish, sea squirt, fruit fly, and nematode, were aligned using ClustalW program with the BLOSUM series protein weight matrix, an open gap penalty of 10.0, a gap extension penalty of 0.2, and a gap separation distance of 8. A tree was generated from this alignment using the bootstrap neighbor joining method, excluding gap positions and correcting for multiple substitutions, running 1,000 bootstrap trials. The PHYL1P output data with the nodal bootstrap values were displayed as an un-rooted tree using the Tree View program.

Isolation of total RNA

Embryonic tissues were dissected from rice seeds at various stages of germination and total RNA isolated using an RNA extraction kit (RNeasy Plant Mini Kit, QIAGEN). To check the integrity of RNA, total RNA (100 ng) isolated from each tissue was separated in a 1% agarose gel containing 1 μg ml^{-1} ethidium bromide in 0.5xTAE buffer. Bands of undegraded 28S and 18S rRNAs were confirmed to check the intactness of total RNA.

Analysis of gene expression by RT-PCR

Expression analysis of makorin *(MKRN),* actin *(ACT)* and tubulin *(TUB)* was carried out by semi-quantittaive RT-PCR technique. The XL-PCR kit (Applied Biosystems) was used for carrying out all RT-PCR reactions. RT-PCR for *MKRN* and *ACT was* carried out as follows: an initial denaturation for 15 seconds at 94°C, then 30 cycles of: 15 seconds at 94°C, 1 minute at 50°C, 2 minutes at 72°C, and then a final extension for 10 minutes at 72°C, while PCR for *TUB* was the same except only 25 cycles were used. MKRN-Forward and MKRN-Reverse primers (Table 2) were used for expression analysis of MKRN transcripts. ACTIN- Forward and ACTIN- Reverse primers (Table 2) were used for analysis of actin transcripts. TUBULIN-Forward and TUBULIN-Reverse primers (Table 2) were used for analysis of tubulin transcripts. The PCR products were electrophoresed in 0.5xTAE composed of 20mM Tris (pH 8.0), 9.5mM acetic acid and 0.5mM Na_2EDTA, the gel irradiated at 310 nm and photographed using a gel documentation system (Pharmacia Biotech).

Table 1. List of organisms and accession numbers of MKRN

Common Name	Scientific Name	GenBank Accession Number	Name of the Gene
Rice	*Oryza sativa*	AP003543	*MKRN*
Thale cress	*Arabidopsis thaliana*	BT000988	*MKRN*
Pea	*Pisum sativum*	AB116263	*MKRN*
Human	*Homo sapiens*	AF192784	*MKRN1*
		AF302084	*MKRN2*
Mouse	*Mus muscullus*	AF192785	*MKRN1*
Wallaby	*Macropus eugenii*	AF192786	*MKRNI*
Chicken	*Gallus gallus*	AF192787	*MKRN1*
Zebra fish	*Danio rerio*	AF277173	*MKRN1*
		AAG27597	*MKRN2*
Yellowtail fish	*Seriola quinqueradiata*	AB073985	*MKRN1*
		AB078011	*MKRN2*
Sea squirt	*Ciona intestinalis*	CI0100145491 (in Ciona database)	*MKRN*
Fruit fly	*Drosophila melanogaster*	AF192788	*MKRNI*
Nematode	*Caenorhabditis elegans*	AC024826	*MKRN*

Table 2. List of primers used

Primer	Sequence (from 5' to 3')	Source sequence
OS-MKRN1B-F	5'-ACGGGATCCATGTCGACCAAGAGGGTTCTTTGC-3'	AK120250
OS-MKRN1B-R	5 -GATCTGCAGCTAAAGATGTAACCGACTGAGG-3'	AK120250
MKRN-Forward	AAAGGTTCATGCTCGTATGG	AK120250
MKRN-Reverse	AAGCCACCACAAATAGGCAG	AK120250
ACTIN-Forward	GTGTGTGACAATGGAACTGG	AY212324
ACTIN-Reverse	TTGATCTTCATGCTGCTTGG	AY212324
TUBULIN-Forward	AGTTCTGGGAGGTGATCTGC	D30717
TUBULIN-Reverse	TAACACAAGGGAGCACATCC	D30717

Results and Discussion

Structure of rice makorin and phylogenetic analysis

The rice *MKRN* cDNA was identified in the NCBl-rice database by using pea MKRN as the query sequence. The sequence analysis of the rice makorin gene (AK120250; Sasaki *et al.*, 2001) revealed that it encodes for a protein containing 368 amino acids. The coding sequence of the putative makorin RING finger protein (Fig. 1) shows the presence of initiation codon (ATG) at 224^{th} bp and the stop codon (TAG) at 1328^{th} bp. The predicted molecular weight of the protein is 41.67 kD. The putatively encoded polypeptide was highly homologous to those encoded by

other members of the *MKRN* gene family with six zinc-finger motifs (Fig. 1) characteristic of makorin proteins in other species (Gray *et al.,* 2000) Three C3H zinc-finger motifs, in the form Cys-X7-Cys-X5-Cys-X3-His, occurred at amino acid residues 8-26, 36-54 and 153-171. A Cys-His zinc-finger motif (C2H2CH) thought to be unique to makorins (Gray *et al.,* 2001), occurred at amino acid residues 175-202. A highly conserved RING (C3HC4) zinc-finger motif in the form of Cys-X2- Cys-X20-Cys-X-His-X2-Cys-X2-Cys-X24-Cys- X2-Cys occurred at amino acid residues 216-273. A fourth C3H zinc-finger motif, in the form Cys-X9-Cys-X5-Cys-X3-His, was found at amino acid residues 309-329.

The makorin gene family was characterized first in mammals and other animal models (Gray *et al.,* 2000). Studies of the makorin gene family were greatly enhanced by the discovery of a second locus (MKRN2) in yellowtail fish, a gene duplication event occurring early in the evolution of vertebrates (Gray *et al.,* 2001). In addition, a plant *MKRN* was putatively identified from the *Arabidopsis* genome project (Gray *et al.,* 2000) and the first experimental investigation of plant makorins in plants to elucidate the genomic organization and expression profile of *MKRN* in germinating peas (Abe *et al.,* 2006). The encoded rice makorin protein possesses the typical arrangement of all the hallmarks for the makorin RING finger protein, *i.e.* four C3H zinc-finger motifs, a CysHis zinc-finger motif, and a well-conserved RING zinc-finger motif and furnishing evidence that it is indeed a true member of *MKRN* gene family and therefore is a genuine rice *MKRN* gene ((Fig. 1). In vertebrates, the genomic duplication took place 4 to 5 million years ago and produced the two major *MKRNI* and *MKRN2* paralogs in this lineage, from a single progenitor locus similar to *MKRN1*.

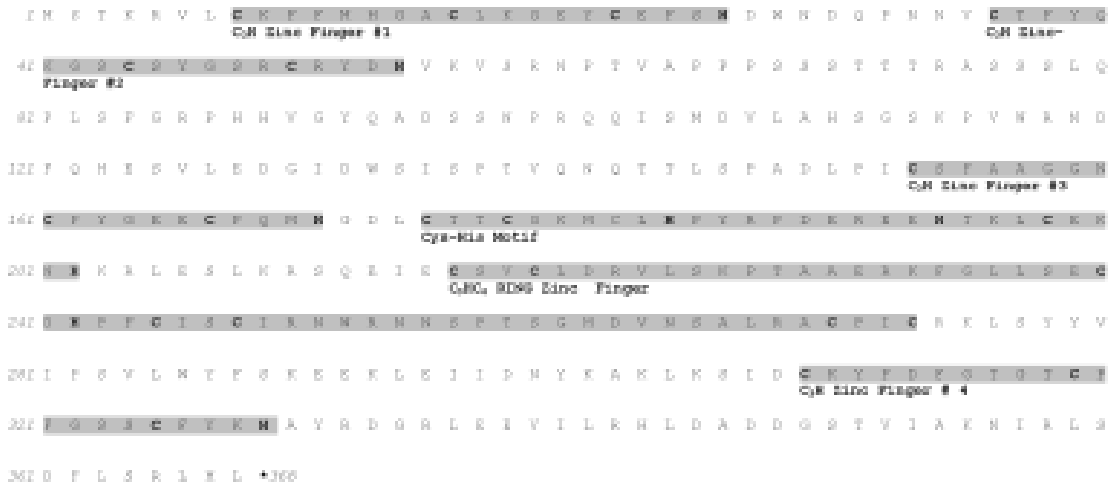

Fig. 1. Coding sequence of the cDNA and encoded amino acid sequence of rice *MKRN*. Shown is the coding sequence of rice *MKRN* cDNA (lower case letters) and the deduced translation (upper case letters) represented by one letter codes below the codons of the open reading frame. Numerals to the left of each nucleotide row indicate nucleotide number and the italicized numerals to the left of each amino acid row indicate amino acid number. The initiation codon (ATG) is underlined and the stop codon (TAG) is indicated with an asterisk. The shaded regions in the amino acid sequence indicate the zinc-finger motifs.

Phylogenetic analyses in previous studies suggest that the *MKRN* locus present in invertebrates and plants has arisen from a single ancient progenitor *MKRN* locus. Therefore the deduced makorin protein in rice is more similar to makorin-1 (42%) but less to makorin-2 (34%) in vertebrates. This is consistent with the idea that plant MKRN was generated from the common ancestor of animal MKRNs.

The phylogenetic tree was generated from the alignment of putative makorin sequences from different organisms and displayed as an un-rooted tree using the Tree View program (Fig. 2). The nucleotide accession numbers of these makorins are given in Table 1. This phylogenetic tree shows that the makorin homologs are separated into the clades of *MKRNI* and *MKRN2* in vertebrates and those in the urochordate, *Ciona intestinalis,* insect, nematode and plants. The phylogenetic relations of the makorin homologs shown in Figure 2 were consistent with those expected from the evolutionary trends of these species. However, *Arabidopsis* makorin was closer to rice makorin than to pea makorin, which was not expected from the phylogeny between a dicot (pea and *Arabidopsis)* and a monocot (rice). Much of the divergence of pea makorin from rice and *Arabidopsis* was attributable to its extended C-terminal region.

Analysis of temporal expression of MKRN by RT-PCR at various stages of imbibition, germination and growth

The relative abundance of transcripts for makorin *(MKRN)* and of the reference genes, actin *(ACT)* and tubulin *(TUB)* during early developmental stages of rice seedlings was examined by RT-PCR, and the results are shown in Figure 3. Low, but detectable, levels of *MKRN* transcripts were observed in dry seeds (OHI, lane 1) and increased throughout imbibition (lanes 2-5) and germination until 24HG (lanes 5-9), with the largest increase at 24 hours (lane 9), before declining at 30HG (lane 10). When the radicle and shoot were emerging at 36HGR (lane 11) and 36HGS (lane 12), respectively, there was an obvious increase in the expression *of MKRN* transcripts. After 6 days, rice *MKRN* was highly expressed in the primary roots (6DGR, lane 13) and moderately in the primary leaves (6DGL, lane 14). Tubulin transcripts were present in dry seeds in trace amount, increased gradually to 18 hours imbibition (lanes 2-4), increased significantly at the end of imbibition at 24HI (lane 5), kept increasing throughout the early stages of germination until 24HG (lanes 6-9), and leveled off afterwards (lanes 10-14). Actin transcripts were already present in moderate amounts in dry seeds (OHI, lane 1), increased to a significant amount at 6 HI (lane 2), declined somewhat at 12 HI and 18 HI (lanes 3-4) before increasing significantly at the end of imbibition at 24H1 (lane 5), kept increasing throughout the early stages of germination until 24HG (lanes 6-9), before leveling off afterwards (lanes 10-14). These experiments were done three times and the results obtained from those experiments were consistent.

Plant morphogenesis is divided into three major phases: embryogenesis, vegetative growth, and reproductive growth and these phases are delimited by seed dormancy, seed germination, and the onset of inflorescence development, respectively. In rice, embryogenesis (*i.e.,* the period from fertilization to seed maturation and seed dormancy) is divided into 10 stages using a number of criteria (Itoh *et al.,* 2005). When the embryo matures, it accumulates storage products and desiccates to produce a dry seed. The mRNAs stored in this dry

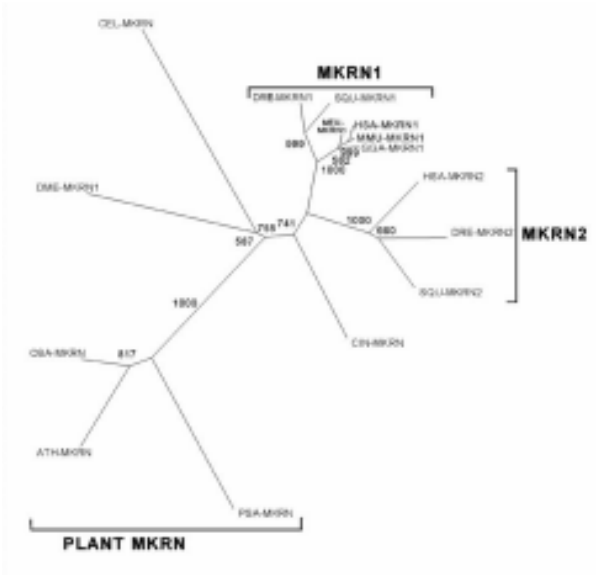

Fig. 2. An unrooted tree illustrating the evolutionary relationships among makorins in various species. The abbreviations denote the following organisms: Osa: *Oryza sativa,* Ath: *Arabidopsis thaliana,* Psa: *Pisum sativum,* Hsp: *Homo sapiens,* Mmu: *Mus muscullus,* Meu: *Macropus eugenii,* Gga: *Gallus gallus,* Dre: *Danio rerio,* Squ: *Seriola quinqueradiata,* Cin: *Ciona intestinalis,* Dme: *Drosophila melanogater,* Cel: *Caenorhabditis elegans.* The scale bar indicates the branch length corresponding to the mean number of differences (0.1) per residue along each branch. Bootstrap values supporting the branches connecting the subgroups are indicated at the corresponding nodes.

seed are likely to encode proteins with roles in germination (Rajjou *et al.,* 2004. Nakabayashi *et al.,* 2005). Accordingly, findings from temporal expression studies (Fig. 3) that rice *MKRN* transcripts were present in dry seeds suggested a role for makorin in the early stages of germination. Moreover, our observation of a pronounced accumulation of *MKRN* transcripts early in germination (Fig. 3) was consistent with this role. This finding is consistent with previous findings that pea *MKRN* transcripts are present in dry seeds and their very early induction during germination suggests a developmental role for makorin in pea during germination (Abe *et al.,* 2006).

Conclusion

The presence of *MKRN* transcripts in dry seed, its early induction during germination and its continued temporal expression during the early vegetative phase suggest that *MKRN* has a particular role in germination and a general role in the development of rice. However, since this study was done only during initial stages of vegetative growth, studies on later developmental stages are needed to determine its morphogenetic role during the entire life cycle. Future work will seek to characterize the function of makorin and determine its functional contribution to plant, vertebrate and invertebrate development.

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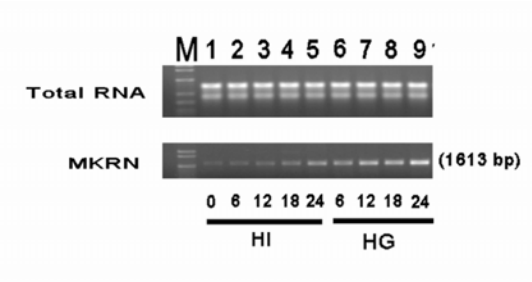

Fig. 3. RT-PCR analysis of transcript accumulation patterns *of MKRN, TUB* and *ACT* genes during germination in rice. (A) Ethidium bromide stained agarose gels of total RNA or RT-PCR products of *MKJRN* cDNA (30 cycles), *TUB* cDNA (25 cycles), and *ACT* cDNA (30 cycles) isolated from different tissues. Lane numbers are indicated on top of each lane and the tissue samples analyzed are indicated in the bottom of each lane. 'M' refers to molecular markers *(Hind III* digest of lamda DNA).

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