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A molecular insight into Darwin’s ‘plant brain hypothesis’ through expression pattern study of the MKRN gene in plant embryo compared with mouse embryo

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Introduction

Development and evolutionary biologists seek to understand the logic behind evolutionary development through inter kingdom comparative studies of various molecular, cellular and physiological processes. Studies pertaining to plant and animal analogies date back to the time of Aristotle, Father of biology.1 Aristotle in his work “De Partibus Animalium” and “De Incessu Animalium” is reported to have said that the anterior and posterior poles of plants are like animals upside down.2 In modern times, it was Charles Darwin who for the first time suggested that the root apices may be the regions similar to brains in animal.3 A comparative study of various physiological and molecular processes across kingdoms will give us a better understanding of evolution and development. Spatial and temporal gene expression pattern studies have made it easy to characterize the developmental process during embryogenesis and germination in plants.4,5 Such Advances in the field of molecular techniques has given impetus to understand the concepts pertaining plant and animal development.

MKRN, a zinc ring finger protein characterized by a unique array of C3H, RING and a characteristic cys-his motif, is encoded by makorin gene family in eukaryotes. Nine MKRN family loci have been found to be scattered throughout the human genome and MKRN1 was characterized in number of organisms, which include human, mouse, wallaby, chicken, fruit fly and nematode.6 MKRN2, another gene of makorin family, resulted from gene duplication of MKRN1 some 450 million years ago, and it was characterized by Gray et al. (2001)7 in human, mouse and fish. In plant systems, there has been substantial work since initial characterization of a MKRN in Arabidopsis by Gray et al. (2000).6 Abe et al. (2007)8 reported the expression of MKRN in pea which provides the first experimental evidence that hints at the functional role of MKRN in plants followed by the characterization of MKRN in rice.9 They also investigated the genomic organization of rice, pea, Arabidopsis and other metazoans, and showed phylogenetic relationships between them.

MKRN1 is one of those genes which act downstream of OCT 4, a transcriptional factor which has a role in establishment of...
and maintenance of totipotency or pluripotency of embryonic and undifferentiated stem cells. MKRN1 has also been reported to act as an E3 ubiquitin ligase for p21 and p23 cycles.

Expression pattern of MKRN1 in murine embryonic nervous system and testis suggests that MKRN1 may have an important role in embryonic development and neurogenesis. Yang et al. (2008) for the first time characterized the functional role of MKRN2 in Xenopus laevis and reported that MKRN2 plays a role in PI3 K/Akt mediated neurogenesis during embryonic development of Xenopus. Expression studies of makorin genes (MKRN 1 and MKRN 2) in yellowtail fish, mouse and humans and Xenopus have paved the way for understanding of this gene family in animal systems. In plants, expression of MKRN transcripts was reported in differentiating tissues as well as differentiated tissues during germination and early vegetative stages of rice and pea.

In the present study we studied the MKRN expression pattern in rice and pea seeds, using non radioactive in situ hybridization technique, and compared it with the MKRN expression pattern data of a developing mouse embryo, obtained from the Mouse Genome Database (MGD).

Results

Embryos from mature rice and pea seeds were compared and their position shown in Figure 1.

In silico analysis. On aligning the amino acid sequences of rice MKRN (accession number Q5ZA07), pea MKRN (accession number BAC81564.1) and MKRN1 of mouse (accession number NP_061280.2), we found all the six hallmark motifs found in makorin (Fig. 2A) conserved and sequentially arranged. Based on the genomic sequence we deduced the structure of makorin proteins in rice, pea and mouse which shows the exon/intron positions and the motifs (Fig. 2B).

Non radioactive in situ hybridization of rice seed. BIOTIN labeled antisense probes hybridized with the MKRN transcripts on sections of rice embryo, to give a purple color and those hybridized with sense probes gave a light purple color (Fig. 3). Figure 3A shows the rice seed section, without probe, Figure 3B shows the section hybridized with sense probe and Figure 3C and D show sections hybridized with antisense probes. Sections hybridized with antisense probes (Fig. 3D) clearly show the expression of MKRN transcripts, in shoot apical meristem (SAM), root apical meristem (RAM) and the vascular region, when compared with sections hybridized with sense probe (Fig. 3B). There was no expression of MKRN in the endosperm of rice seed (Fig. 3C).

Non radioactive in situ hybridization of pea seed. The DIG labeled antisense probes hybridized with the MKRN transcripts on the section to give a purple color and the sections hybridized with sense probes turned out to be off white with a faint tinge of purple. In Figure 4C, F and I show sections hybridized with antisense probes and Figure 4B, E and H show sections hybridized with sense probes. On comparing the sections hybridized with sense probes (Fig. 4B, E and H) with those hybridized with antisense probes (Fig. 4C, F and I) we found that the expression of MKRN was obvious in the SAM, region of vascular bundle and the RAM. Figure 4A, D and G are sections without any hybridization.

Non radioactive in situ hybridization of mouse embryo. The image of mouse embryo (MGI:3501182) retrieved from the Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine, World Wide Web (www.informatics.jax.org), shows expression of MKRN1 in the brain and possible expression in the regions of spinal cord and the genital ridge.

Discussion

With a number of plant and animal genome sequences available, it has become easier to understand their similarities and differences. One of the ways by which plant and animal comparisons can be made is through pattern formation. In Plants, the basic body plan and stem cell populations for generation of organs, in later stages, is established during embryogenesis. In the present study we compared the expression pattern of MKRN in mature seeds of pea and rice with the expression pattern of MKRN1 gene in mouse embryo.

Makorin in animals was characterized by Gray et al. (2000) for the first time and MKRN in plants was first characterized in pea followed by rice. It is known that plant and animal makorins share a common ancestor and that plant MKRN is more similar to MKRN1 of animals. On aligning the rice MKRN, pea MKRN and mouse MKRN1 amino acid sequences, we found the six hallmark motifs of makorin to be conserved.

We found the expression of MKRN in mature pea embryo and rice embryo to be similar. In both, MKRN transcripts were expressed prominently in the radicle, though not in the root cap. The expression was prominent in shoot apical meristem and also in the region of vascular bundle. Our spatial expression results are consistent with the previous reports on the temporal expression of MKRN in dry rice and pea seeds. Studies have shown the spatial expression of MKRN in the meristematic tissues, lateral root primordia and the vascular regions of young germinating rice. Also, expression of MKRN has been reported in the shoot...
apical meristem, vascular region and the root tissues during early germination stages of pea.\textsuperscript{15} Our plant \textit{MKRN} results when compared with the expression of \textit{MKRN1} in the brain of mouse embryo (Fig. 5), shows a similar pattern of expression based on the polar axes. The spatial annotation of \textit{MKRN1} expression in mouse embryo shows expression in brain, nervous tissues and genital ridge (Fig. 5C). This is consistent with previous studies on expression of murine \textit{MKRN1} in the embryonic brain, nervous tissues and the testis.\textsuperscript{6,19} Expression of \textit{MKRN1} has also been reported in brain and gonads of humans, chicken, fishes and amphibians.\textsuperscript{6,19-21}

Analogous expression pattern of \textit{MKRN} and \textit{MKRN1} in plants and mouse, suggests to the importance of this gene in inter kingdom studies. Drawing an analogy between RAM and brain,

**Figure 2.** (A) Alignment of amino acid sequences of rice \textit{MKRN} (\textit{O. sativa}), pea \textit{MKRN} (\textit{P. sativum}) and mouse \textit{MKRN1} (\textit{M. musculus}). The characteristic six motifs of makorin family are shaded. The asterisk marks below the sequences denotes complete conservation, two dots shows highly similar amino acids and one dot shows moderately similar amino acids. (B) A comparison of genomic structure of mouse \textit{MKRN1} with rice and pea \textit{MKRN}. The characteristic motifs of Makorin gene family are shown in red (C3H), green (cys-his) and blue (RING). The exons are numbered and the intron sizes are given in base pairs (kb) below the line.
one might wonder as to why the plants have their brain like tissues underground. To this, Baluška et al. (2004) have suggested three reasons: first they suggest that the soil environment is more stable than the air environment. Second, it is protected from animals and other sources of physical destruction. Lastly, they suggest that since the posterior end of plant (stem) bears the organs of reproduction (flowers), the anterior end (root), logically becomes the brain.

The roots and shoots of a plant are interconnected by the vascular system, which is composed of continuous strands of elongated cells. Auxin, which is in abundance in the polar regions of the plant maybe referred as plant neurotransmitter. The fact that RAM is rich in auxin related activities further supports the root brain theory. They have further suggested that the RAM represents sink of oxygen, just like the brain. The supporting parenchyma cells of the vascular tissues (xylem and phloem) are anatomically similar to the glial cells which support the neurons. Plant homologues of ionotropic glutamate receptors have been reported to be expressed in the vascular tissues. Recent advances in plant signaling and physiological studies have revealed similarities between plant cells and neurons. Evidences that neurotransmitters like acetylcholine and serotonin activate ion pumps in xylem parenchyma further suggests to the functional similarity between vascular tissues and the neurons.

Similar expression pattern of MKRN in RAM of plants and of MKRN1 in mouse brain, gives an impetus to Darwin’s “root brain theory.” Expression patterns of MKRN in the roots tissues and lateral root primordia of rice and pea and MKRN1 in brain of humans, mouse, yellowtail fish, zebrafish, medaka, clawed frog are analogous. Similarly based on the expression of MKRN in vascular regions and the nervous tissues of mouse, an analogy can be drawn between the vascular tissues and the nervous tissues. Lastly, the expression of MKRN in SAM of pea and rice is similar to the expression of MKRN1 in gonads of animals.

Based on the comparative study of the expression pattern of MKRN in plants and MKRN1 in animals, it is quite possible that the analogy between the plant and animal, as suggested by Aristotle, Darwin and many recent scientists, may find strong support.

Materials and Methods

In silico analysis. Protein sequences of pea and rice MKRN and mouse MKRN1 from NCBI database were taken for analysis. Using T-COFFEE, Version 8.99 multiple sequence alignment was performed (http://tcoffee.crg.cat/apps/tcoffee/play?name=regular).

mRNA in situ hybridization of pea seed. Pea seeds (Pisum sativum L. var Alaska) were fixed in freshly prepared 4%
Paraformaldehyde solution overnight and sections of 30 μm were obtained using Yamato cryomicrotome.\(^{15}\) Pre-hybridization washes\(^{4}\) were followed by hybridization of the tissue samples with DIG labeled sense and antisense probe. After the probes (2 μl) were mixed with Dako mRNA in situ hybridization solution in a plastic container, tissue sections were soaked in plastic container and incubated at 37°C, for 15 hrs. Post-hybridization was carried by subjecting the hybridized sections with buffers according to the protocol provided by the company (www.genedetect.com). After the post-hybridization washes the sections were washed three times in Buffer1 (100 mM TRIS-HCl pH 7.5, 150 mM NaCl) for 5 min each. The washed tissues were then immersed in Buffer1 containing 0.5% Blocking reagent (Roche), followed by incubation for two hours with the anti-DIG antibody, diluted to 1: 500 with buffer1 containing 0.1% triton X smf 1% Blocking Reagent. The antibody was removed by washing the sections four times with buffer1 containing 0.1% triton for 20 min. The sections were then equilibrated with Buffer 2 for 5 min, followed by staining with NBT/BCIP solution (1 tablet NBT/BCIP in 10 ml of distilled water) in a plastic box overnight. In order to confirm the presence of mRNA before hybridization of sections with sense and antisense probes, we used polydT. We also prepared a section from without any probes, as a control. The sections stained were washed in tap water and observed under microscope and photographs were taken using digital cameras Nikon Coolpix for stereo microscope and pixera for inverted microscope.

**mRNA in situ hybridization of rice seed.** Rice seeds (*Oryza sativa* L. var Nipponbare) were fixed in freshly prepared 4% paraformaldehyde solution overnight and sections of 30 μm
were obtained using Yamato cryomicrotome. Pre-hybridization washes of the tissue samples followed by hybridization with BIOTIN labeled sense or antisense probes was done according to the method described by Arumugam et al. (2007). Post-hybridization was carried by subjecting the hybridized sections with buffers according to the protocol provided by the company (www.genedetect.com). After the post-hybridization washes the sections were washed three times in Buffer1 (100mM TRIS-HCl pH 7.5, 150mM NaCl) for 5 min. The washed tissues were then immersed in Buffer1 containing 0.5% Blocking reagent (Roche), followed by incubation for two hours with the streptavidin, diluted to 1: 500 with buffer1 containing 0.1% triton X smf 1% Blocking Reagent. The antibody was removed by washing the sections four times with buffer1 containing 0.1% triton for 20 min. The sections were then equilibrated with Buffer 2 for 5 min, followed by staining with NBT/BCIP solution (1 tablet NBT/BCIP in 10 ml of distilled water) in a plastic box overnight.

In order to confirm the presence of mRNA before we started hybridization of sections with sense and antisense probes, we used polydT. The sections stained were washed in tap water and observed under microscope and photographs were taken using digital cameras Nikon Coolpix for stereo microscope and pixera for inverted microscope.

**Non radioactive in situ hybridization of mouse embryo.** We obtained the image (EMAGE: 1588) of mouse embryo (10.5dpc) hybridized with DIG labeled MKRN1 probes from EMAGE gene expression database (www.emouseatlas.org/emage/).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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