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Makorin RING zinc finger protein gene expresses during leaf vascular pattern development in rice (*Oryza sativum* L. var. Nipponbare)

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Abstract

The molecular mechanisms of plant vascular tissues development havefascinated biologists for centuries. Vascular patterns in leaf are created de novo during development of each primordium and thus, it represents an attractive system to study the dynamics underlying pattern formation. Using non-radioactive in situ hybridization technique, we studied the makorin RING zinc finger protein gene (MKRN) expression pattern during embryonic and post-embryonic leaf vascular pattern development in rice (Oryza sativa L. var. Nipponbare). MKRN was ubiquitously expressed in shoot apical meristem and embryonic leaves in the embryo 10-11 and 15 days after pollination (DAP). In the embryo 15 DAP, expression of MKRN was concentrated in the developing provascular tissues whereas, it was gradually reduced in the surrounding mesophyll cells. It indicates that MKRN expression remains continuous in the cells of shoot apical meristem which later converted into provascular cells or procambium. During maturation of embryo 45 DAP, MKRN expression was restricted only to the developing provascular tissues and was found to be relatively reduced than the earlier growth stages of embryo. However, MKRN expression was again found to beincreased in the provascular bundle cellsof 1 dayimbibed embryo than in the dry mature embryo. This expression pattern was also found similar in coleoptile collected from imbibed seeds for six days. The expression pattern of MKRN was observed during the initiation of provascular cells, its differentiation, cell elongation, thus suggesting its important role in vascular pattern development in rice.

Keywords: Embryo, leaf, *MKRN*, rice, vascular bundle, Zinc finger.

Introduction

Plant vascular tissues are the organized network of interconnected cell files, which enables transport of water, nutrient, small molecules and also provide mechanical support[1]. Its pattern establishment initiates during early embryo development and continues in post-embryonically developed organs by retaining its ability to multiply[2,3]. In newly formed shoots and roots, the procambium or provascular tissue is the apical meristem that gives rise to primary vascular tissues i.e. xylem; the water conducting tissues and phloem through which photosynthetic compounds and signaling molecules are transported[4]. Xylem is composed of conducting tracheary elements and non-conducting elements such as xylary parenchyma cells and xylary fibers. Phloem is composed of conducting sieve elements and non-conducting cells such as parenchyma cells and fibers. The sites for procambial cell initiation determine the pattern of vascular organization and that the activity of procambial cells controls the differentiation of vascular tissues[5].

The primary vascular tissue forms continuous apico-basally connected structure from the root tips to the vein endings of the leaves, flowers, and fruits[6]. In rice, leaf venations patterns are usually characterized as parallel or striate i.e. veins typically diverge at base of the lamina and converge toward apex[7]. Vascular bundles in rice maintain polarity during development where, xylem formed on the adaxial side, and phloem on the abaxial side and these tissues are enclosed by bundle sheath cells[8,9]. Procambium formation to its maturation follows various cell processes like, cell division, initiation of differentiation, cell elongation, secondary cell thickening and cell death[5]. Genetic studies have identified many genes viz., *Oshox1, OsPNH1*, *DROOPING LEAF, etc.* to be involved in various stages of leaf vascular development in rice[10-13].

Makorin RING zinc finger protein gene (*MKRN*) family encodes a distinct protein with characteristic arrays of zinc finger motifs including C3H motifs, a novel Cys-His motif and a RING finger[14]. *MKRN* orthologs are conserved in fungi, plants and animals.The gene expression of *MKRN* is found to be present in various vital organs of human, mouse and yellowtail fish[14-16]. In plants, *MKRN* expression was observed in embryo, shoot and root meristem, leaves, shoot and root vascular bundles and lateral root primordia[17-21]. *MKRN* acts as an E3 ligase for human telomere reverse transcriptase enzyme during terminal differentiation[22,23]. It also inhibits RNA polymerase-II dependent transcription of androgen and retinoic acid receptors[24]. *MKRN2* plays a role in PI3K/Akt-mediated neurogenesis during embryonic development of Xenopus[25]. MKRN1 mediates p-53 dependent cell cycle arrest and apoptosis by differentially co-regulating transcription through ubiquitination of p53 and p21[26]. In mammalian neuron, a MKRN1 isoform modulates translation of dendritic *mRNA* at

synapses[27].

In the present study, we studied the gene expression pattern of *MKRN* during the initiation of procambium in early embryonic development to differentiation of vascular bundle in post-embryonically developed leaves in rice (*Oryza sativa* L. var. Nipponbare).

Material and Methods

Plant material: Rice (*Oryza sativa* L. var. Nipponbare) seeds were obtained from Ehime Research Institute of Agriculture, Forestry and Fisheries, Matsuyama, Japan. Seeds were imbibed in water for 24 h at 22 ˚C in dark and then seeds were sown in vermiculite to germinate at 22˚C under 16 h light: 8 h dark conditions.

Selection of growth stages and sectioning: Tissues were harvested at various growth stages (table 1). The dissected tissue samples were fixed in fresh 4% paraformaldehyde [prepared in 0.1 M Phosphate Buffer Saline (PBS)] and vacuum infiltrated for 1 h. The tissue samples were stored in fresh 4% paraformaldehyde solution for overnight. The fixed tissues were washed in 1X PBS and embedded in 5% carboxymethyl cellulose on the stage of cryomicrotome (Yamato ROM-380, Japan) at -15 ˚C and tissues were sectioned at appropriate thickness (table 1).

Table-1
Growth stages selected for experiment

Name of the stage	Tissue sample harvested for experiment	Thickness of tissue sections
Embryogenesis	Rice embryo 11-12 Days after Pollination (DAP)	9 µm
	Rice embryo 15-16 DAP	
	Rice embryo 45 DAP	
Imbibition	Rice embryo from 1 day imbibed seeds	
	Coleoptile (obtained from 6 days imbibed seeds)	20 µm

Non-radioactive in situ hybridization: The tissue sections were first treated with 100% ethanol for 10 min followed by 70% ethanol for 5 min. The sections were then washed in sterile RNase free water for 2 min followed by 1X PBS for 5 min, then again fixed with 4% paraformaldehyde for 15 min and finally washed twice in 1X PBS for 5 min. Hybridization of these sections was performed at 22˚C for 24-36 h with either biotin labeled sense or antisense probes diluted to a concentration of 200 ng of probes per ml of mRNA in situ hybridization solution (Sigma, USA). The biotin-labeled, synthetic oligonucleotide probes, specific for rice MKRN gene (GenBank accession no: AK120250) were purchased from (http://www.genedetect.com/). The post-hybridization washes were performed with high stringency washing conditions according to the manufacturer's instructions (http://www.genedetect.com/protocols.htm).

For detection of hybridization signal, the sections were washed three times in Buffer 1 (100 mM Tris HCl pH 7.5, 150 mM NaCl) for 5 min and then immersed in Buffer1 containing 0.5% blocking reagent (Roche, USA), followed by incubation for 5 h with the streptavidin-alkaline phosphatase conjugate (Amersham, UK), diluted to 1:500 with Buffer 1 containing 0.1% triton X and 1% blocking reagent. The excess conjugates were removed by washing the sections four times with Buffer1 containing 0.1 % triton-X for 20 min. The sections were then equilibrated with Buffer2 (100 mM Tris HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 5 min, followed by staining with NBT/BCIP solution [1 tablet NBT/BCIP (Roche, USA) in 10 ml of distilled water] for 5-15 minutes. Tissue sections were washed in distilled water and observed under bright field view with an inverted microscope (Nikon Microphot-FXA, Japan) and tissue images were captured using a Pixera cooled CCD digital camera (Pixera Penguin 600CL). Three independent in situ determination assays on the same developmental stage were performed and 15 tissue sections of embryos/shoots for each treatment were analyzed.

Results and Discussion

MKRN expression was investigated using non-radioactive in situ hybridization, where tissue sections of various growth stages of rice were hybridized with biotin labeled sense and antisense probes. The tissue sections hybridized with antisense probes appeared to be pale to dark purple whereas, sense probes were off white or with no color development.

***MKRN* expression pattern during embryonic shoot development:** Figure 1 shows *MKRN* transcripts expressions on the longitudinal sections of embryo 10-11 DAP (panels a-c) and transverse sections of embryo 15 DAP (panels d-f), using non-radioactive in situ hybridization. Panels a, d and b, c,e, f show sections hybridized withsense probes and antisense probe, respectively. Panel c and f shows the magnified view of shoot shown in panel b and e, respectively. The biotin labeled antisense probes hybridized with the *MKRN* transcripts appeared pale to dark purple in the shoot apical meristem (SAM; panel c), coleoptile and emerging embryonic leaves P1 and P2 (panel c) whereas, the sense probes were of pale off-white color (panel a). When the cross sections of an embryo 15 DAP were hybridized with the antisense probes (panels e, f) and compared with those with the sense probes (panel d), expression of *MKRN* transcripts was obvious in the central shoot apical meristematic cells of the embryo (panel f), where the cell division takes place. *MKRN* expression was also very prominent and ubiquitous in surrounding leaves of SAM and vascular bundle of coleoptile (panel f). The outer leaves of shoot (P3; panel f) showed concentration of *MKRN* expression in the provascular tissues while, surrounding mesophyll cell showed gradual reduction in *MKRN* expression.

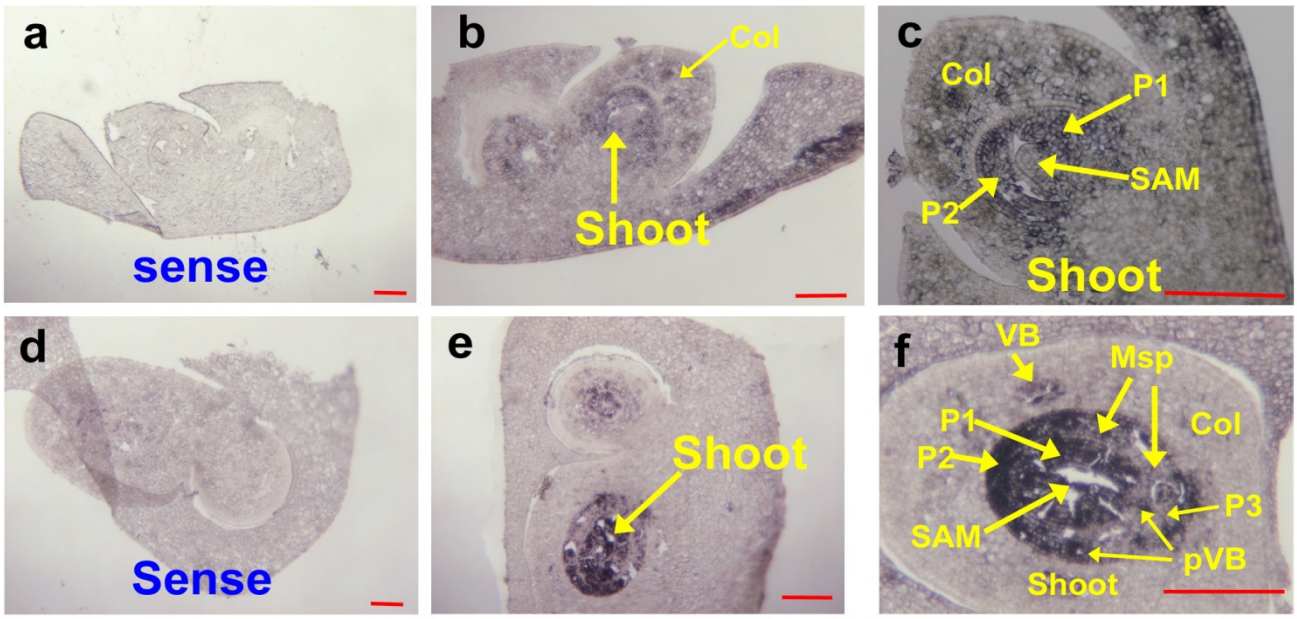

Figure-1

Localization of *MKRN* transcripts in embryo 10-11 DAP (panels a-c) and 15-16 DAP (panels d-f). The median longitudinal sections (panel a-c) and transverse sections (panelsd-f) were hybridized with biotin labeled sense (panel a, d) and antisense probe (panel b-c, d-f). Panel c and f are the enlarged view of shoot shown in panel b and e, respectively. Notations are coleoptile, Col; shoot apical meristem, SAM; provascular bundle, pVB;youngest leaf primordia and so on…, P1, P2...; vascular bundle, VB; mesophyll cells, Msp. Bar represents scale of 40 µm (a, d, e), 80 µm (b), 100 µm (f) and 150 µm (c).

Figure 2 shows expressions of *MKRN* transcripts on the longitudinal (panels a-c) and transverse sections (panels d-f) of embryo 45 DAP, using non-radioactive in situ hybridization. Panels a andd are the views of section hybridized with sense probes; panels b-c and e-f are sections which were hybridized with antisense probes. Panelsc and f are the magnified views of shoot shown in panels b and e, respectively. When the longitudinal sections with the antisense probes (panels b and c) were compared with those with sense probe (panels a), expression of *MKRN* transcripts was obvious in SAM, parallel vascular veins of embryonic leaves, the first emerging leaf (P1; panel c), vascular bundle of coleoptile (panels b and c). *MKRN* expression was also found in the vascular bundle which is connecting the shoot with root and the base of striate rice embryonic leaves (LB; panel b) which are attached to the base of the shoot (shown in series of arrows in panel b). In transverse sections (panels d-f), *MKRN* expression was very prominent in the SAM, developing provascular bundle cells in the emerging embryonic leaves, but it was absent in the mesophyll cells surrounding the provascular cells (panels e and f). Interestingly, *MKRN* expression was ubiquitous in the SAM and first leaf (P1), but it was found to be gradually decreased in other developing embryonic leaves. Expression only continued in provascular cells (panels e and f). It was also found that *MKRN* expression was comparatively reduced in the embryo 45 DAP than embryos 15 DAP (figures 1e-f and 2d-f). In the large vascular bundle of coleoptile, *MKRN* expression was found more prominent in phloem than xylem (panel f).

During early embryonic events of organ formation, complex networks of procambial cells emerge from homogeneous sub-epidermal tissues in the early leaf primordia[28]. These procambial cells become apparent as narrow, cytoplasm-dense cells emerging from procambium in ground meristem[2,6]. Procambium is a characteristically arranged in continuous strands and acquire their narrow shape through coordinated, oriented divisions, parallel to the axis of the emerging strand[29].During the course of embryogenesis, thevascular pattern is established, but the differentiation ofvascular cell types arrest at the procambial stage[30]. In rice embryo, establishment of organs complete by 10-11 DAP, followed by elongation of organ systems up to 20 DAP[9]. In our study, we found the ubiquitous expression of *MKRN* in the SAM and emerging P1 leaf in the embryo 10-11 DAP (figure 1b, c). However, in the embryo 15 DAP, *MKRN* expression remain concentrated in provascular tissues while it was gradually disappear from mesophyll cell (figure 1e, f). *MKRN* expression was found quite different to some of provascular markers which does not express in SAM but expresses only in sub-epidermal cells which will become vascular tissues[10]. This expression pattern suggests that *MKRN* maintains its activity in meristematic cells or the meristem like cells (procambium; figure 1c, f). During maturation of embryo, though vascular growth has been arrested at procambial stage, *MKRN* expression was continuously observed in the procambium (figure 2). It suggests the fundamental role of *MKRN* in cell activities which will configure the future growth of vascular tissues in both

embryonic and post-embryonic development. This expression pattern was analogous with the *MKRN* expression pattern in human, mouse and yellowtail at embryo and adult stage, where its expression was found in various vital cell organelles[14, 16]. Similar expression pattern was also observed in pea dry mature embryo and during embryonic root development in rice root[19-21]. Du *et al.*[31] found*MKRN1* acts downstream of *OCT4* transcriptional factor which is responsible for maintaining totipotency or pluripotency of embryonic and undifferentiated stem cells in mice. Thus, emphasizing the possible role of *MKRN* in growth meristematic tissue.

***MKRN* expression pattern in post-embryonic shoot:** *MKRN* expression pattern was studied in the dry mature rice embryo after imbibition of one and six day, to understand the trend of expression after entry of embryo into post-embryonic developments. Maturation of embryo and dormancy period may have impact on the expression pattern of genes at one or various development processes of particular organ or whole embryo/seedling. Also, the vascular bundle cells are not fully differentiated during embryonic growth[30]. The full differentiation of primary tissues of vascular system occurs during post-embryonic development. Figure 3 shows expression pattern of *MKRN* in the embryo and coleoptile after one and six days of imbibition, respectively. Panels a and e represent the sections hybridized with sense probe whereas, panels b-d and f-h represent the sections hybridized with antisense probes. Panel b is the section of dry mature embryo and panels c-d are the sections of one day imbibed embryo. In the sections shown in panels b and c, *MKRN* expression was concentrated in the provascular cells. Interestingly, *MKRN* expression was more prominent in the provascular tissues of the imbibed embryo than in the dry mature embryo (panels b and c). Panel d represents the section taken just below the SAM where, expression of *MKRN* was little or absent in the central tissues but it was prominent in the leaf base region (figures 2b and 3d). Panels e-f show *MKRN* expression in the coleoptile. Under anaerobic condition (due to 6 days of imbibition), coleoptile grows prior to radicle[32]. We studied *MKRN* expression in the dome shaped coleorhiza, where the central hollow is occupied by growing SAM and immature leaves. *MKRN* expression was present in the mesophyll cells and provascular and vascular tissues of coleoptile (panels f-h). In figure 3h, the expression was found in differentiating xylem and phloem of vascular bundle of coleoptile.

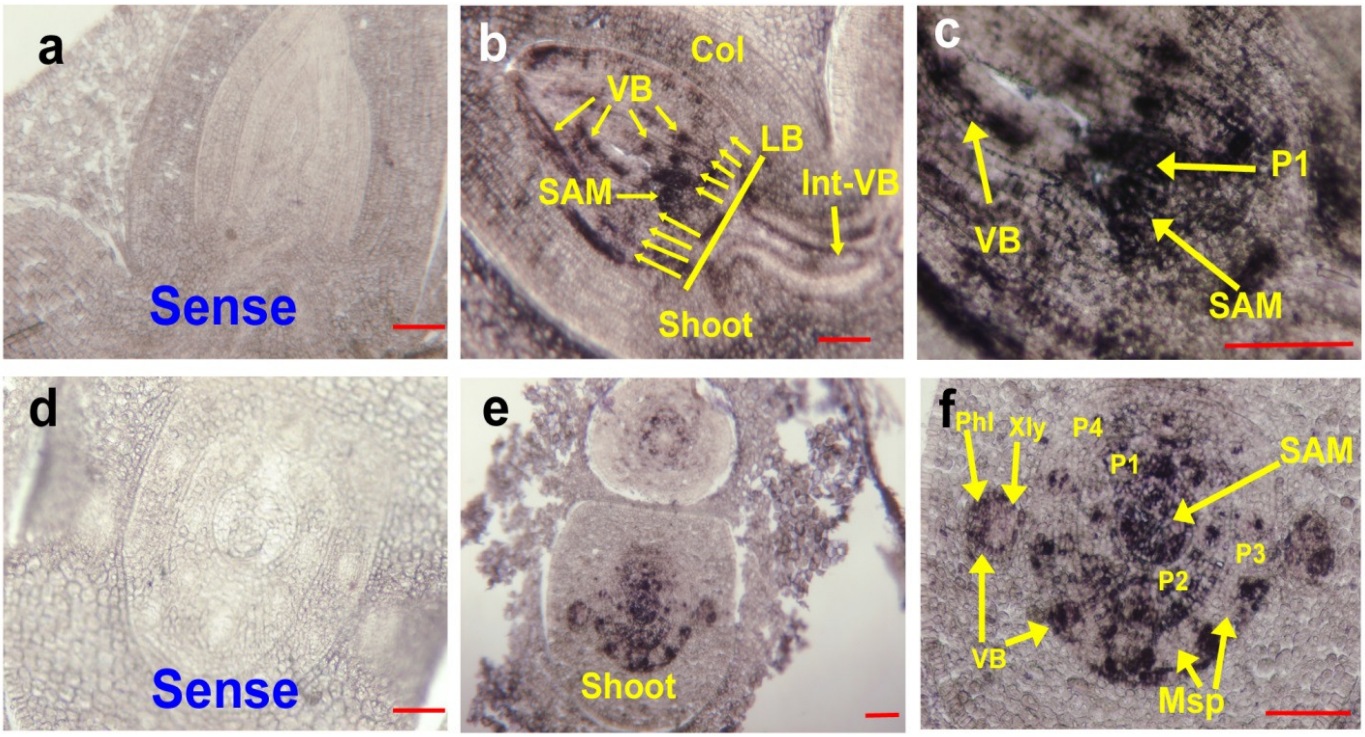

Figure-2

Localization of *MKRN* transcripts in embryo 45 DAP. The median longitudinal sections (panel a-c) and transverse sections (panelsd-f) were hybridized with biotin labeled sense (panel a, d) and antisense probe (panel b-c, d-f). Panel c and f are the enlarged view of shoot shown in panel b and e, respectively. Notations are coleoptile, Col; interconnecting vascular bundle between shoot and root, Int-VB; shoot apical meristem, SAM; provascular bundle, pVB;youngest leaf primordia and so on.., P1,P2…; phloem, Phl; xylem, Xly; leaf base, LB; vascular bundle, VB; mesophyll cells, Msp. Bar represents scale of 40 µm (e), 100 µm (a, b, d), 150 µm (f) and 200 µm (c)

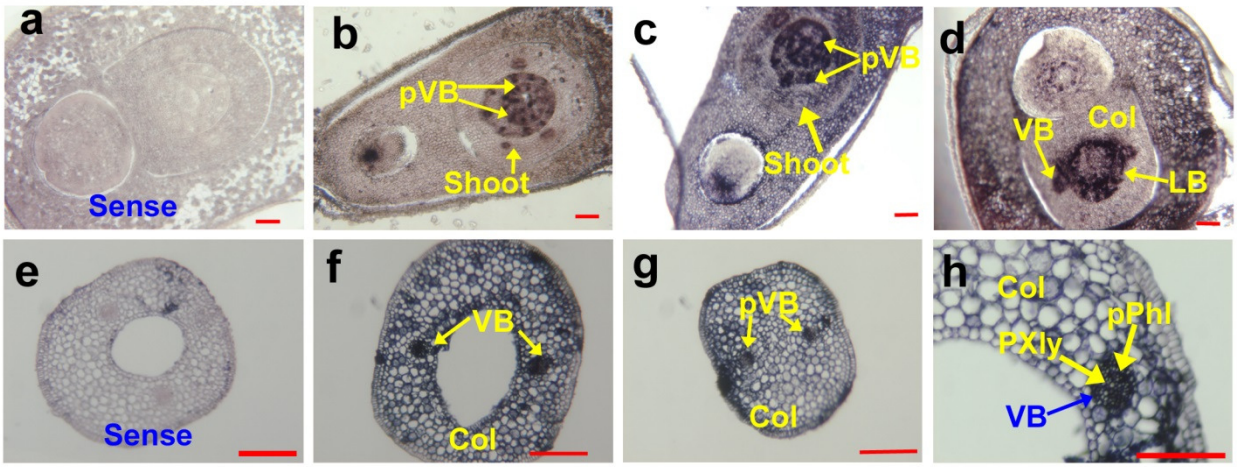

Figure-3
Localization of *MKRN* transcripts in dry mature embryo (panel b), one day imbibed embryo (panels c, d) and coleoptile after six days of imbibition (panels e-h). The transverse sections were hybridized with biotin labeled sense (panel a, e) and antisense probe (panel b-d, f-h). Panel his the enlarged view of vascular bundle shown in panel f. Notations are coleoptile, Col; shoot apical meristem, SAM; provascular bundle, pVB;leaf base, LB; vascular bundle, VB. protophloem, pPhl; protoxylem, pXly. Bar represents scale of 40 µm (a-d), 100 µm (e-g) and 150 µm (h).

Mature embryos contain procambial cells that will differentiate into xylem and phloem following germination[33]. In embryos however, further differentiation of the procambium is halted during embryo maturation and only proceeds when growth resumes following seed germination[34]. In most non-embryonic tissue, vascular development is a continuous process in which procambium development is followed by differentiation of the xylem and phloem[34]. Here, we observed that the *MKRN* expression was concentrated in the provascular tissues in the dry mature embryo. Its expression further increased in the provascular tissues of the imbibed seeds (figure 3b-d). In the coleoptile, expression was observed in differentiating phloem and xylem (figure 3f-h). It suggests that *MKRN* may have a role in cell division, differentiation and cell elongation of vascular tissues of rice leaf. Gray *et al.*[14] speculated that the presence of multiple classes and numbers of zinc finger present in MKRN enables its interaction with diverse molecules including other proteins, RNA, and possibly DNA.Arumugam *et al.*[18] reported the increase in expression of *MKRN* during imbibition and germination. Expression of *MKRN* in vascular bundle of pea and rice was compared with expression *MKRN1* in mouse embryo, which suggested its evolutionary role in development of organisms[20]. Also various functional studies have suggested the role of MKRN as E3 ligase for ubiquitination of important protein of cell cycle regulation, transcriptional regulator of various receptors and in modulation of translation in nerve cells[22-27].

Conclusion

We found that the expression of *MKRN*begins with initiation of provascular tissues in ground meristem and itcontinued during differentiation and elongation of rice embryonic and post-embryonic vascular pattern formation. These expression patterns suggest the involvement of *MKRN* in basic cell activities like, cell division, elongation and differentiation. The presence of various zinc motifs in MKRN may be a possible reason for its broad activity in plant development. To understand the distinct role of *MKRN* in different aspects of vascular pattern formation like, vein pattern orientation, polarity of primary vascular tissues, etc. needs further empirical studies with mutants.

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