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Stem cell lineage in body layer specialization and vascular patterning of rice root and leaf

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Abstract Since the first appearance of vascular plants during evolution, the plant body has become specialized for adaption to land conditions. Much of our knowledge of plant body specialization and the origins of tissues from stem cells have been obtained from studies on the dicot *Arabidopsis thaliana*. However, less is known about plant body specialization in monocots, another important branch of angiosperms. In this study, we analyzed stem cell lineage and differentiation during development of the root and leaf of the monocot model plant rice (*Oryza sativa*). Our results showed that three body layers of rice are established from stem cells accompanied by progressively reduced pluripotency. Layer 1 (L1) is a single-cell layer of epidermis; L2 is the cortex/endodermis in the root and the mesophyll in the leaf; and L3 is the site of vascular initiation. At least two common steps in vascular development are shared between rice root and leaf. The preprocambium divides to form the procambium and root pericycle or leaf outer sheath. The procambium further differentiates into the xylem, phloem and circumambient cells. We found that the outer sheath of leaf vascular bundles originates not only from the preprocambium of L3, but also from the mesophyll precursor cells of L2. In addition, *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes are expressed in not only the stem cell niche but also metaxylem precursor in rice. This pattern differs from that of homologs in *Arabidopsis*, suggesting that *WOX* functions have been recruited in different stem cells in dicots and monocots.

Keywords *Oryza sativa* · Stem cell · Body layer · Preprocambium · Vascular development · *WOX*

1 Introduction

More than 400 million years ago, the colonization of land by plants had a great impact on the evolution of both plants and animals [1–3]. Plant bodies became functionally specialized to adapt to life on land. The vasculature, which is located in the inner center of all organs, is one of those specialized structures. Vascular tissues contain not only the xylem and phloem but also adult stem cells. Together, these tissues and cells function in the long-distance transport of water, nutrients and other substances, in the physical support of the plant body, and in the initiation of de novo organogenesis [1, 4–7].

Specialized tissues originate from stem cells, which are characterized by their ability to both self-renew and differentiate into functional cells [8–11]. Different from

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those in animals, stem cells in plants are able to maintain their activity throughout the whole life of the plant to continuously produce organs at the post-embryo stage, allowing some plant species to have almost interminable lives [8, 9]. Stem cells are usually located in the meristem, which comprises the stem cell niche and neighboring transit-amplifying cells [8]. The stem cell niche usually consists of an organizer and its surrounding initial cells. The initial cells usually undergo cell division to form two daughter cells; one daughter cell adjacent to the organizer retains its initial cell identity and the other gives rise to transit-amplifying cells. The primary function of the organizer is to send signals to the initial cells to maintain their undifferentiated state. Transit-amplifying cells can undergo rapid cell division and begin to differentiate into specialized tissues.

The maintenance and differentiation of plant stem cells are controlled by a complex molecular network, in which *WUSCHEL-RELATED HOMEOBOX* (*WOX*) family genes have an essential role [12]. *WOX* genes encode homeodomain transcription factors and are found in a wide range of plant species from green algae to angiosperms [12–17].

A recent study suggested that the origin of angiosperms, the most highly evolved group of vascular plants, was traced back to 225–240 million years ago in the Late to Middle Triassic. The origin of monocots, a subgroup of angiosperms, was estimated to be 154–191 million years ago in the Jurassic [18]. To date, most studies on the mechanism controlling stem cells have been conducted in dicots using the model plant *Arabidopsis thaliana*, and little is known about this mechanism in monocots. However, the organization of the tissue structures differs between monocots and dicots [19–21]. Unraveling the common and diverse mechanisms controlling tissue formation from stem cells in dicots and monocots will improve our understanding of how plant body structures have evolved in angiosperms. In this study, we performed detailed histological and molecular analyses of stem cell lineage in body layer formation and vascular patterning in the monocot model plant rice (*Oryza sativa*).

2 Materials and methods

2.1 Plant materials and growth conditions

Oryza sativa L. *japonica*. cv. Nipponbare was used as the wild-type rice, and Columbia-0 (Col-0) was used as the wild-type *A. thaliana*. Rice plants were grown at 29 °C with a 12-h light (~10,000 lux)/12-h dark photoperiod in a greenhouse or plant chamber.

2.2 Sectioning and microscopy observation

For paraffin sectioning, samples were fixed in FAA solution (v/v: 50 % ethanol, 5 % acetic acid, 3.7 % formaldehyde) at 4°C for 24 h. Samples were dehydrated in a graded ethanol series followed by a graded ethanol/Histo-Clear series with safranin O staining. Then, tissues were embedded in paraffin and cut into 9-μm sections onto poly-L-lysine coated slides. The sections were de-paraffinized in Histo-Clear.

For thin sectioning [22], samples were fixed in FAA solution at 4 °C for more than 24 h. Samples were then dehydrated with a graded ethanol series and acetone, infiltrated in a series of resin and acetone solutions, immersed in resin for 24 h and embedded in Epon 812 resin. After polymerization at 35 °C for 6 h and 60 °C for around 2 days, 3-μm-thick sections were cut using a Leica 2265 microtome (Leica Microsystems GmbH, Wetzlar, Germany). The sections were stained with toluidine blue.

Differential interference contrast (DIC) microscopy observations were performed as previously described [23, 24]. Samples were observed under a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

2.3 In situ hybridization and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

In situ hybridization was performed according to our previous method [25, 26], and the probes were subcloned into the pGEM-T Easy vector (Promega, USA) using the following primers: 5′-ATGCCTCAGACCCCTTCGAC-3′ and 5′-TTAATTGGTGGAGGTGGAGC-3′ for *OsNAL2*, 5′-ATGAGGCTTCACCATCTGCATG-3′ and 5′-TTAAG CTTTTCCCTGGGGATG-3′ for *OsWOX4*, and 5′-ATG GAGGCTCTTAGCGGGCGAG-3′ and 5′-ACTAGGAC TAGGCACAGCGACA-3′ for *OsWOX5*.

RNA extraction and qRT-PCR were performed as previously described [22, 27], using the following gene specific primers: 5′-CCCGTCGGCGGAGCAGATAAA G-3′ and 5′-AGCGTGCTGAGGGTGAGGAGGG-3′ for *OsWOX4*; and 5′-GGTATTGTTAGCAACTGGGATG-3′ and 5′-GATGAAAGAGGGCTGGAAGA-3′ for *OsAC TIN*. The qRT-PCR results are shown as the relative expression levels, which were normalized against those produced by the primers for *OsACTIN*.

2.4 Accession numbers

Sequence data of rice *WOX* genes can be found in the Rice Genome Annotation Project under the following accession numbers: *OsWUS* (LOC_Os04g56780), *OsWOX2* (LOC_Os01g62310), *OsWOX3B* (LOC_Os05g02730),

OsNAL3 (LOC_Os12g01120), *OsNAL2* (LOC_Os11g01130), *OsWOX4* (LOC_Os04g55590), *OsWOX5* (LOC_Os01g63510), *OsWOX9A* (LOC_Os01g47710), *OsWOX9B* (LOC_Os07g34880), *OsWOX9C* (LOC_Os05g48990), *OsWOX11* (LOC_Os07g48560), *OsWOX12A* (LOC_Os08g14400), *OsWOX12B* (LOC_Os03g20910) and *OsWOX13* (LOC_Os01g60270).

3 Results

3.1 Cell lineage in the body layer specialization of rice and *Arabidopsis* roots

To analyze the cell lineage and tissue structure in rice root, we sectioned the root apical meristem (RAM), using the primary root (radicle) of 5-day-old rice plants. Within the RAM, the stem cell niche was composed of quiescent center (QC, the organizer) cells and surrounding initial cells (Fig. 1a) [8, 28]. Three body layers could be traced according to their initial cells (Fig. 1a). Layer 1 (L1) was composed of only one layer of epidermal cells, which were derived from the epidermal initial cell. The endodermis–cortical initial cell in L2 produced the endodermis and cortex. The vascular initial cell in L3 functioned as the preprocambium, which gave rise to two kinds of transit-amplifying cells; the procambium and pericycle cells. In the primary root of rice, procambium and pericycle cells together formed a group of five sublayers of cells (L3-1 to L3-5), and differentiated to form the vascular cylinder (also called the stele) (Fig. 1a). The rice root cap, which was initiated from the columella initial cell and lateral root cap initial cell [29], was independent from the three layers (Fig. 1a).

In the stem cell niche of *Arabidopsis* root, the endodermis–cortical initial cell in L2 was also adjacent to the QC (Fig. 1b). However, the initiations of the epidermis in L1 and the vascular cylinder in L3 differed between *Arabidopsis* and rice (Fig. 1b) [9, 30]. In *Arabidopsis*, the epidermis and the lateral root cap had a common initial cell, known as the epidermal/lateral root cap initial cell (Fig. 1b) [9, 30], while the lateral root cap initial cell and the epidermal initial cell were separate in rice (Fig. 1a) [29]. Therefore, the lateral root cap in *Arabidopsis* seems to be a derivative of L1 structure.

During vascular formation, the preprocambium cell of L3 was not retained in the post-embryo growth of the primary root in *Arabidopsis* [31] (Fig. 1b). The preprocambium of *Arabidopsis* appears at the embryo stage and differentiates into a group of procambium initial cells and the pericycle initial cell during embryo development [31]. At the post-embryo stage, the *Arabidopsis* root stem cell niche retained the procambium initial cells and the pericycle initial cell in L3, which together served as the vascular initial cells (Fig. 1b). However, the preprocambium was retained throughout the post-embryo stage in rice root (Fig. 1a).

3.2 Vascular patterning in rice root

To trace the cell lineage in vascular patterning, we prepared a series of sections from the primary root of 5-day-old rice (Fig. 2a, b). The procambium underwent rapid cell division, leading to a radial symmetry structure with four sublayers. The four sublayers (L3-1 to L3-4) of procambium cells and one sublayer (L3-5) of pericycle cells constituted the immature vascular cylinder (Fig. 2c). At this stage, the procambium was composed of parenchyma cells that were indistinguishable from each other, except for the very large cell in the center (Fig. 2c).

The procambium then differentiated to form vascular tissue precursors (Fig. 2d) [20, 32]. Phloem and protoxylem precursors were identified in the L3-4 sublayer. Six cells in L3-4 divided to form phloem precursors. Each phloem precursor was composed of four cells that originated from a single cell (Fig. 2d). Six protoxylem precursors were also identified in L3-4 (Fig. 2d). The protoxylem precursors and phloem precursors showed an alternate and radially symmetrical distribution pattern (Fig. 2d). In the center of the vascular cylinder, the single L3-1 cell was the metaxylem precursor (Fig. 2d). Because phloem and protoxylem precursors could be identified, the pericycle cells were classified as the phloem-pole and xylem-pole pericycles (Fig. 2d).

The vascular tissue precursors further differentiated to form mature vascular tissues (Fig. 2e, f) [20, 32]. The first maturation was observed in the four-cell structure of the phloem precursor, as the cell toward the pericycle became the protophloem sieve-tube element (also called protophloem sieve-tube member) (Fig. 2e). Mature phloem and xylem structures formed in the maturation zone of the root and consisted of six phloem and six protoxylem vessel elements (also called protoxylem vessel members) alternately distributed around the large metaxylem vessel element in the center (Fig. 2f). Each phloem comprised a protophloem sieve-tube element, a metaphloem sieve-tube element and two companion cells (Fig. 2f). Pericycle cells also underwent fate transition at this stage. Some xylem-pole pericycle cells underwent cell division and became thick-walled sclerenchyma (Fig. 2f), while the phloem-pole pericycle cells gave rise to the lateral root primordium (Fig. 2f). Some studies have indicated that the phloem-pole endodermis also gives rise to outer cells of lateral root primordia in rice [20, 33–36].

In the very mature root from 20-day-old rice plants, the vascular cylinder was fully differentiated (Fig. 2g). A

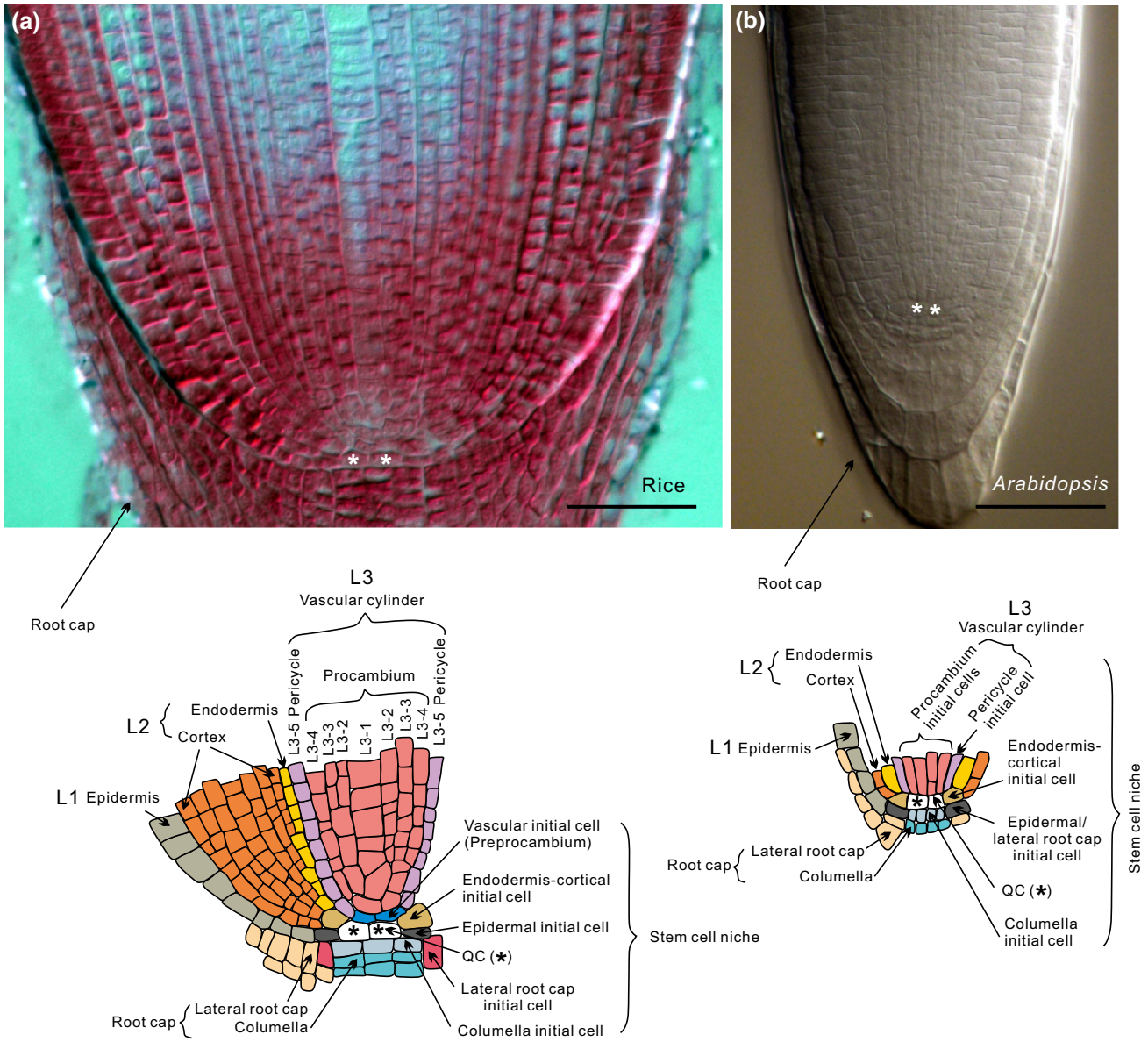

Fig. 1 Stem cell niche and body layer specification in RAM. Stem cell niche in RAM of primary root from 5-day-old rice plant (**a**) or 10-day-old *Arabidopsis* plant (**b**). Asterisks indicate QC cells. Lower panel of each figure shows schematic of section in upper panel. Paraffin sections and DIC microscopy images are shown in (**a**) and (**b**), respectively. Scale bars, 50 μm in (**a**) and (**b**)

typical feature at this stage was that the remaining procambium cells formed one or more circle(s) of cells (referred to as circumambient cells in this study for convenience) around each xylem vessel element (indicated in blue in Fig. 2g). The circumambient cells around protoxylem vessel elements came not only from the procambium cells, but also from the xylem-pole pericycle cells (Fig. 2f). At this stage, the procambium and pericycle cells had fully differentiated into vascular tissues and lateral roots.

There was diverse vascular patterning in different types of rice roots. The primary root had a single metaxylem in the center (Fig. 2g), while there could be several metaxylems in the adventitious root (Fig. 2h). In the lateral root, the vascular cylinder was very simple, without differentiation of complex structures of vascular tissues (Fig. 2i).

3.3 Body layer formation and vascular patterning in rice leaf

To analyze cell lineage in the formation of body layers and the vascular bundle (vascular cylinder in the leaf), we prepared a series of sections from leaves at different stages of development. Because tissues in the leaf become mature

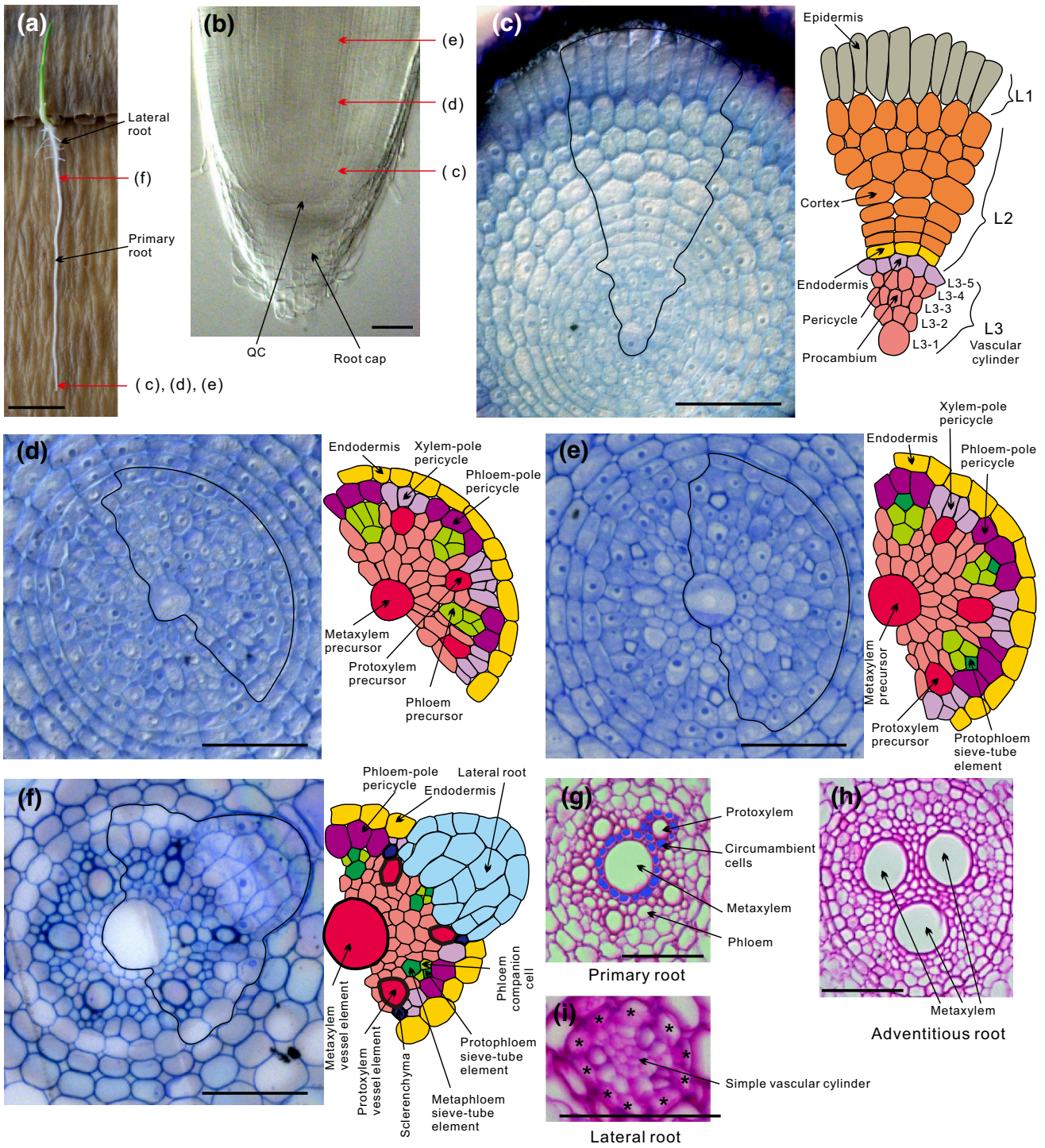

Fig. 2 Vascular patterning in rice root. Root of 5-day-old rice plant (**a**) and its RAM (**b**). Red arrows show sectioning positions in **c**–**f**. **c**–**f** Series of thin sections in different positions (indicated in **a** and **b**) of primary root from 5-day-old rice plant, showing vascular patterning and lateral root formation. **g** Paraffin section of primary root from 20-day-old rice plant. Circumambient cells surrounding xylem vessel elements are arbitrarily labeled in blue. Paraffin sections showing vascular cylinders in mature adventitious (**h**) and lateral (**i**) roots. Asterisks in (**i**) indicate pericycle. Right panel of each figure in **c**–**f** is schematic of section in left panel. Scale bars, 1 mm in **a**, and 50 μm in **b**–**i**

from the distal end (leaf tip), we prepared sections to observe immature regions at the proximal end (leaf base) of the leaf primordium (Fig. 3a), the 2-mm young leaf (Fig. 3b) and the 8-mm young leaf (Fig. 3c, d). To observe mature regions, we sectioned the middle part of a 10-cm leaf (Fig. 3e–h) and a 30-cm leaf (Fig. 3i). Vascular

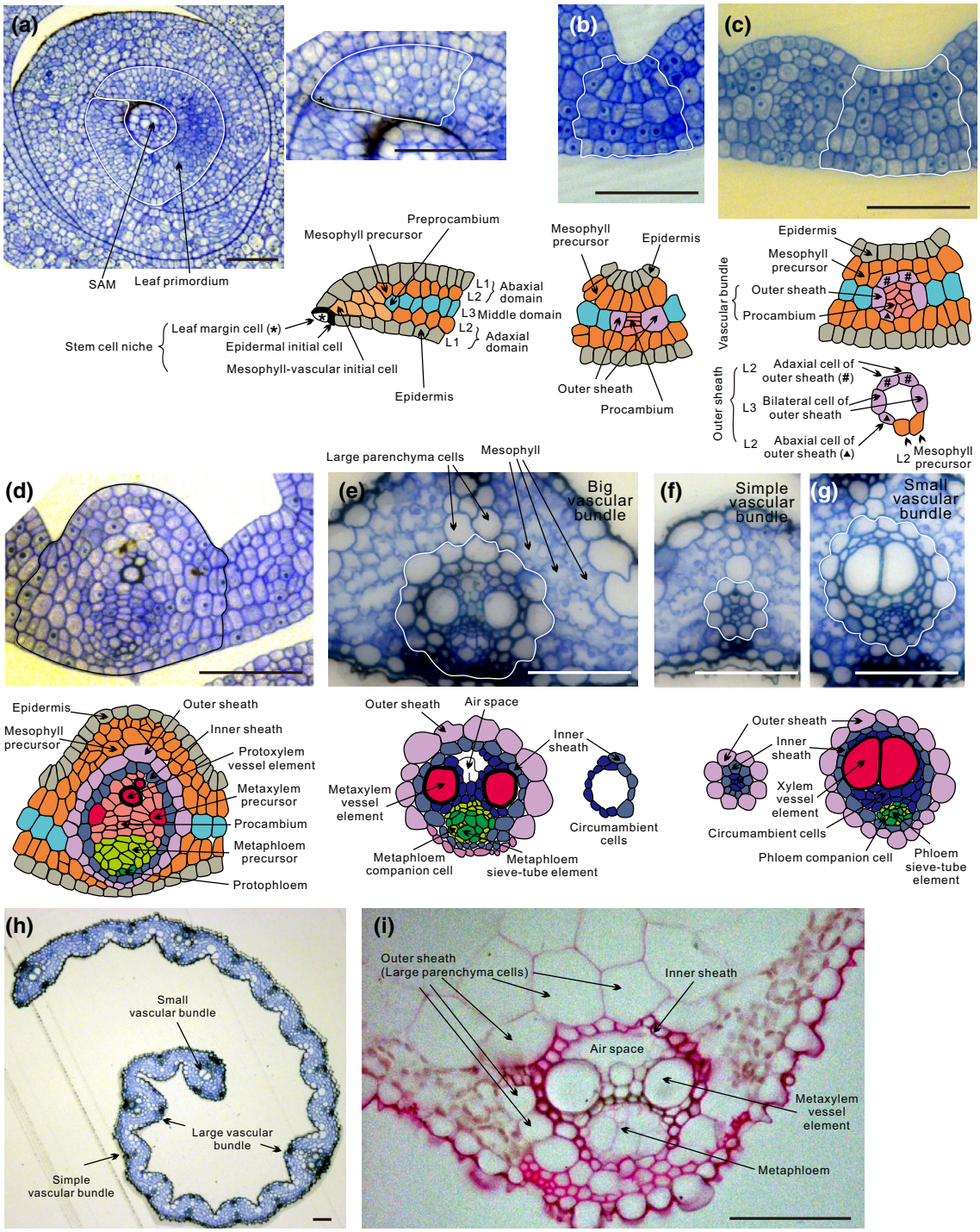

Fig. 3 Stem cell niche, body layer specification and vascular patterning in rice leaf. **a** Thin section of SAM and leaf primordia, showing formation of three body layers from leaf margin region. The right panel is an enlarged leaf primordium. Asterisk indicates the leaf margin cell, which probably serves as organizer of stem cell niche. **b** Thin section at base of 2-mm leaf, showing three cells from L3 preprocambium that initiate vascular bundle. Note that inner cell of the vascular bundle is dividing to form procambium, whereas left and right cells form bilateral cells of outer sheath. **c.** Thin section at base of 8-mm leaf, showing formation of outer sheath. Outer sheath consists of bilateral, adaxial (hash symbol), and abaxial (triangle symbol) cells. Bilateral cells are derived from L3, while the adaxial and abaxial cells are derived from L2. Note that abaxial part of outer sheath is incomplete at this stage, and mesophyll precursor (arrowheads) will form abaxial other sheath cell via one round of cell division. **d** Thin section at base of 8-mm leaf showing immature large vascular bundle. Thin section at middle region of 10-cm leaf (**h**), showing large (**e**), simple (**f**), and small (**g**) vascular bundles. **i** Paraffin section of 30-cm leaf, showing midrib. Note that outer sheath differentiates into large parenchyma cells at this stage. Lower panel of each figure in **a**–**g** is schematic of section in upper panel. Scale bars, 50 μm in **a**–**i**

bundles in rice leaves were classified as large, small or simple (Fig. 3h).

Previous studies have suggested that a meristem exists at the leaf margin [37]. In addition, leaf margins in monocots may recruit all layers of cells from the shoot apical meristem (SAM) [38, 39]. In the rice leaf primordium, we observed the formation of three body layers at the leaf margin region (Fig. 3a). There was a single cell at the leaf margin, which we called the leaf margin cell (probably serving as the organizer, see "Discussion" section). Adjacent to the leaf margin cell were two epidermal initial cells (L1) at both the adaxial and abaxial sides, and one or several mesophyll–vascular initial cell(s) (L2 and L3) beneath the epidermis (Fig. 3a). Adjacent to the mesophyll–vascular initial cell, there were transit-amplifying cells, i.e., the mesophyll precursor cells at both the adaxial and abaxial sides to form L2 and the preprocambium at the middle domain to form L3 (Fig. 3a).

When all of the body layers had been established, L1 and L2 each had two cell layers: L1 consisted of adaxial and abaxial epidermal cells; L2 formed adaxial and abaxial mesophyll precursors. L3 was a single layer at the juxtaposition of the adaxial and abaxial domains, i.e., the middle domain (Fig. 3a) [40] and served as the preprocambium, which gave rise to vascular bundles.

Three or more neighboring preprocambium cells of L3 initiated vascular tissues (Fig. 3b). The middle preprocambium cell(s) divided to form the procambium, while the bilateral preprocambium cells formed the bilateral cells of the outer sheath (Fig. 3b).

The outer sheath and procambium underwent further differentiation (Fig. 3c). The outer sheath could be subdivided into bilateral, adaxial, and abaxial cells. The bilateral cells of the outer sheath were derived from the L3 preprocambium, while mesophyll precursor cells in L2 formed the adaxial and abaxial cells of the outer sheath (Fig. 3c). At this stage, the procambium was composed of parenchyma cells that were still dividing (Fig. 3c).

The first tissues to reach maturity in the large vascular bundle were the protophloem and the protoxylem (Fig. 3d). The inner sheath, which was beneath the outer sheath, formed from the procambium cells (Fig. 3d). Therefore, the outer sheath and inner sheath have partially different origins [41]. At this stage, the metaphloem precursor and the metaxylem precursor could be identified (Fig. 3d).

All tissues in the large vascular bundle were further differentiated (Fig. 3e). The protophloem was extruded and disappeared when the metaphloem (i.e., the metaphloem sieve-tube element and companion cells) reached maturity. The protoxylem also degraded and became the air space when two metaxylem vessel elements formed (Fig. 3e). The remaining procambium, together with partial inner sheath, formed circumambient cells around xylem vessel elements (Fig. 3e), like its pattern in the root. At this stage, most of the mesophyll precursor cells had differentiated into mesophyll or large parenchyma cells (Fig. 3e). In addition, the L3 cells that had not developed into vascular bundles differentiated into mesophyll cells (Fig. 3e).

The simple and small vascular bundles had similar outer and inner sheaths (Fig. 3f, g). However, the simple vascular bundle had no complex structures, only some small cells inside the inner sheath (Fig. 3f), similar to that in the lateral roots (Fig. 2i). Small vascular bundles had a differentiated xylem and phloem, but harbored only protoxylem, and no metaxylem (Fig. 3g).

In an old leaf, the outer sheath had differentiated into large parenchyma cells (Fig. 3i).

3.4 *WOX* genes in stem cells of rice root and leaf

The WOX family genes encode important transcription factors controlling stem cells. These transcription factors can be classified into three clades (ancient, intermediate, and WUS clades) according to their presence during the evolution of plants [12, 13, 42]. The three clades of *Arabidopsis* and rice WOX proteins can be further classified into subclades (Fig. 4a) [14, 42].

Members of the WOX family contain a conserved homeodomain, which consists of three helixes [16] (Fig. 4a). Alignment of the homeodomains of 14 *WOX* genes in *Arabidopsis* and 14 *WOX* genes in rice revealed a peptide sequence in helix 3 that can distinguish the three clades of WOX proteins (Fig. 4a): YNWFQNR in the ancient clade, FYWFQNR in the intermediate clade, and FYWFQNH in the WUS clade. The "YN" to "FY" change in the sequence occurred during the evolution from the ancient to the intermediate clade [15], and the "R" to "H" change may have occurred during the evolution from the intermediate clade to the WUS clade. Interestingly, the WUS clade ancestor CrWUL in the fern *Ceratopteris richardii* does not harbor this FYWFQNH sequence; instead, its sequence is FYWFQNQ [15]. This indicates that the evolution of the WUS clade in fern is not complete, although CrWUL contains the WUS-clade-specific WUS-box and the WUS/WOX5-specific EAR-like domain [15].

To study the role of *WOX* genes in root and leaf stem cells of rice, we analyzed the expression patterns of several candidates from the WUS clade. *NARROW LEAF2* (*OsNAL2*) and *OsNAL3* genes (also called *OsWOX3A*) were shown to be involved in leaf margin identity and leaf expansion [45, 46], similar to their homologs in maize and *Arabidopsis* [38, 47]. We analyzed *OsNAL2* expression in the rice leaf primordium (Fig. 4b–d). In most cases, *OsNAL2* was specifically expressed in the single leaf margin cell (Fig. 4b, d) and occasionally also in the epidermal initial cell and mesophyll–vascular initial cell

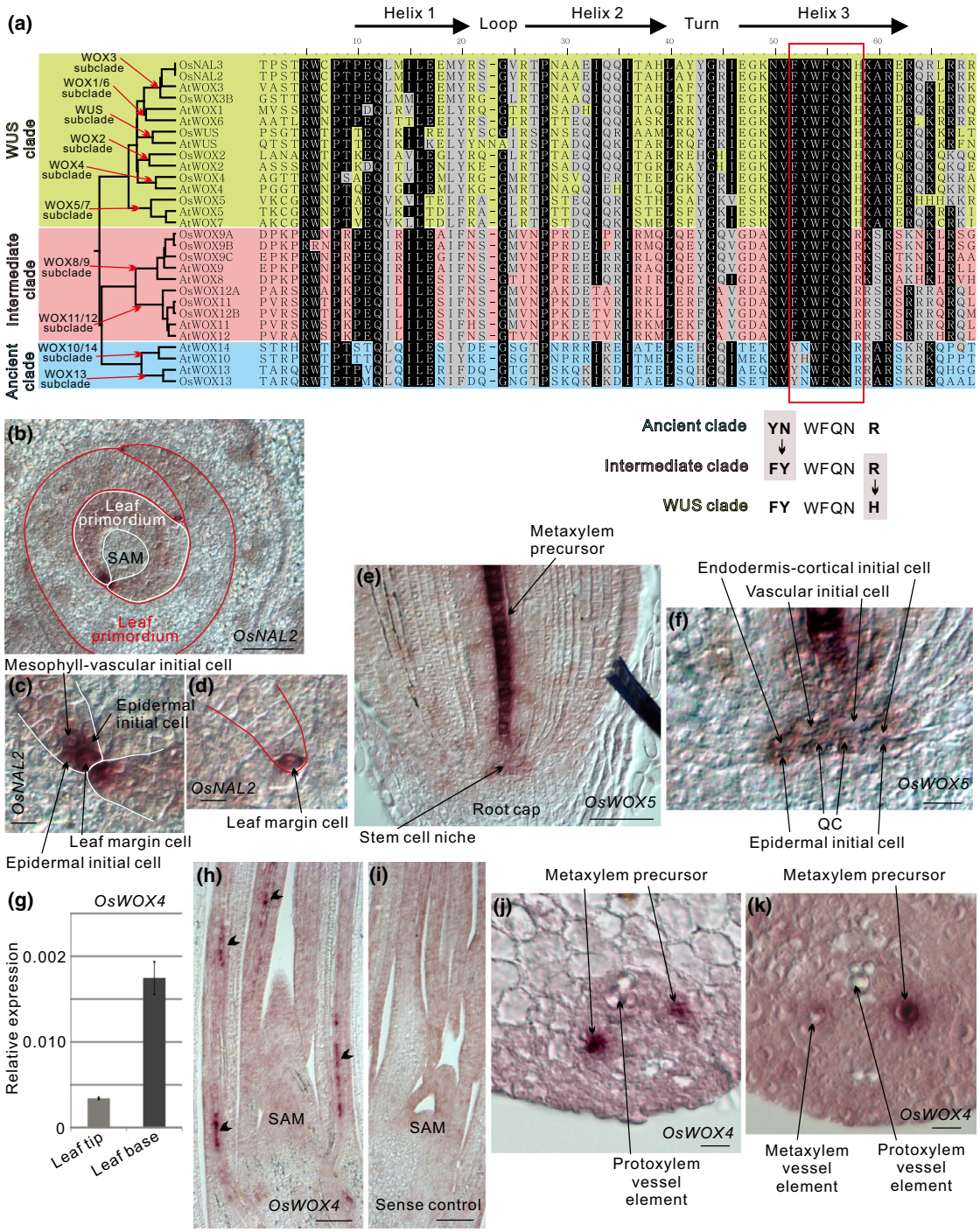

Fig. 4 Expression patterns of *WOX* genes in stem cells. **a** Alignment of WOX homeodomains from *Arabidopsis* and rice. Red box indicates sequence used to classify *WOX* genes into three evolutionary clades. Note that *AtWOX10* was indicated to be pseudogene [43]. Homeodomain sequences were included in the phylogenic analysis using MEGA3.0 [44]. **b** Transverse section of shoot apical meristem and leaf primordium from 3-day-old rice plant, showing *OsNAL2* expression at leaf margin. **c, d** Magnifications of leaf margin in (**b**). **e** Longitudinal section of primary root tip from 3-day-old rice plant, showing in situ hybridization of *OsWOX5* gene. Note that *OsWOX5* is expressed in metaxylem precursor and stem cell niche. **f** Magnification of stem cell niche in **e**. **g** qRT-PCR analysis of *OsWOX4* expression at leaf base and leaf tip of 8-mm leaf. Bars show standard error from three technical repeats from one experiment. We performed three biological repeats, and the results were consistent. **h** Longitudinal section of shoot apical meristem, leaf primordia and young leaves from 4-day-old rice plant, showing in situ hybridization of *OsWOX4*. Note that *OsWOX4* is expressed in vascular bundles of young leaves (arrowheads). **i** Sense control of the data in **h**. **j**, **k** Transverse section of young leaf from 4-day-old rice plant, showing in situ hybridization of *OsWOX4*. Note the presence of *OsWOX4* signal in metaxylem precursor (**j**) and the absence of *OsWOX4* signal from mature metaxylem vessel element (**k**). Scale bars, 50 μm in (**b**), 10 μm in **c**, **d**, **f**, **j**, **k**, and 100 μm in **e**, **h**, **i**

(Fig. 4b, c). This expression pattern suggested that the leaf margin cell together with the initial cells may serve as the stem cell niche.

AtWOX5 in *Arabidopsis* was shown to be specifically expressed in root QC cells [48]. Interestingly, our in situ hybridization results showed that *OsWOX5* has a more extensive expression pattern in rice. First, *OsWOX5* was strongly expressed in the metaxylem precursor cells in the primary root (Fig. 4e), consistent with the results of a recent study [49]. Second, *OsWOX5* was expressed at relatively low levels in the stem cell niche of the root tip, including the QC as well as the epidermal initial cell, endodermis–cortical initial cell, and vascular initial cell (Fig. 4f). However, it was not expressed in the columella initial cell and lateral root cap initial cell (Fig. 4f). Rice has a single *OsWOX5* gene in the WOX5/7 subclade, while *Arabidopsis* has two (*AtWOX5* and *AtWOX7*) (Fig. 4a). *AtWOX7* was shown to be expressed in the endodermis–cortical initial cells [50]. Therefore, it is possible that the single *OsWOX5* in rice has the functions of both *AtWOX5* and *AtWOX7* in *Arabidopsis*.

OsWOX4, like its homolog *AtWOX4* in *Arabidopsis* [51, 52], was shown to be involved in procambium development in rice [53]. Our qRT-PCR data showed that *OsWOX4* was strongly expressed at the leaf base, but weakly expressed at the leaf tip (Fig. 4g), suggesting that *OsWOX4* expression decreases as the leaf matures. Next, we performed in situ hybridization to analyze the spatial expression pattern of *OsWOX4* in leaves. The hybridization signal was enriched in the vascular bundles of young leaves (Fig. 4h, i). Further analysis showed that *OsWOX4* was specifically expressed in the metaxylem precursors of large vascular bundles (Fig. 4j), and its expression ceased when the metaxylem precursor differentiated into the metaxylem vessel element (Fig. 4k), consistent with the qRT-PCR results (Fig. 4g). Therefore, we suppose that *OsWOX4* may be involved in metaxylem precursor specification in the procambium.

4 Discussion

4.1 Body layer specialization in rice

The typical structure of the rice body can be divided into three body layers (see model in Fig. 5). Layer formation is an old theory. In 1868, Hanstein [54] proposed the histogen theory, which suggested that all tissues of higher plants originate from three layers of cells, i.e., dermatogen, periblem, and plerome. The three body layers in plants seem to be similar to the three germ layers in animals [55]. However, there is a typical difference in the formation of the three layers between plants and animals. In animals, the germ layers form at the embryo stage. In higher plants, however, the three body layers can continuously form during the post-embryo stage because most plant organs are formed post-embryonically.

4.2 Organization of stem cell niche and meristem in rice root and leaf

Plant tissues and organs differentiate from various stem cells [8, 9, 56]. According to their differentiation abilities, stem cells can be classified as totipotent, pluripotent, or unipotent [10, 11]. In higher plants, a stem cell usually cannot differentiate into a mature cell via a single step; therefore, stem cells are organized in a complex environment—the meristem, which consists of the stem cell niche surrounded by transit-amplifying cells (Fig. 5a) [8]. Here, we summarize the cell lineage in rice root and leaf (Fig. 5a–c).

In rice root (Fig. 5b), the stem cell niche is composed of the QC and its surrounding initial cells. The currently accepted concept is that only initial cells serve as stem cells in the RAM. However, in *Arabidopsis*, the QC might act not only as the organizer of the stem cell niche, but probably also as the primary stem cell with the highest pluripotency, because initial cells could be replenished by a low proliferation rate of the QC [30, 57]. This could also be the case in rice, although such a cell lineage is still unclear (question marks in Fig. 5b). On the other hand, transit-amplifying cells serve as the transitional cells from the initial cells (stem cells) to differentiated cells. This suggests that transit-amplifying cells might also keep partial features of stem cells. For example, pericycle cells serve as "adult stem cells" in de novo organogenesis in *Arabidopsis* [7, 58]. Therefore, we suppose that the organizer, initial cells, and transit-amplifying cells have the lineage with gradually decreasing pluripotency and increasing cell division activity.

In rice leaf (Fig. 5c), the leaf margin was thought to maintain the meristem feature [37, 59]. In the leaf of monocots, the margin recruits leaf cells from the shoot apical meristem [38, 39]. Interestingly, our results showed that *OsNAL1* and *2* are specifically expressed in the leaf margin cell (Fig. 4b–d). This suggests that the leaf margin cell (probably as an organizer) together with its adjacent initial cells could function as a stem cell niche in rice (Fig. 5a, c). However, it is currently unclear whether the leaf margin cell itself serves as the organizer and the primary stem cell that replenishes all three layers of cells in the whole leaf. It is also unclear whether all three layers of cells originate from the initial cells adjacent to the leaf margin cell (question marks in Fig. 5c). Revealing the cell lineage from the leaf margin and illustration of the function of *OsNAL*s in the initiation of the three

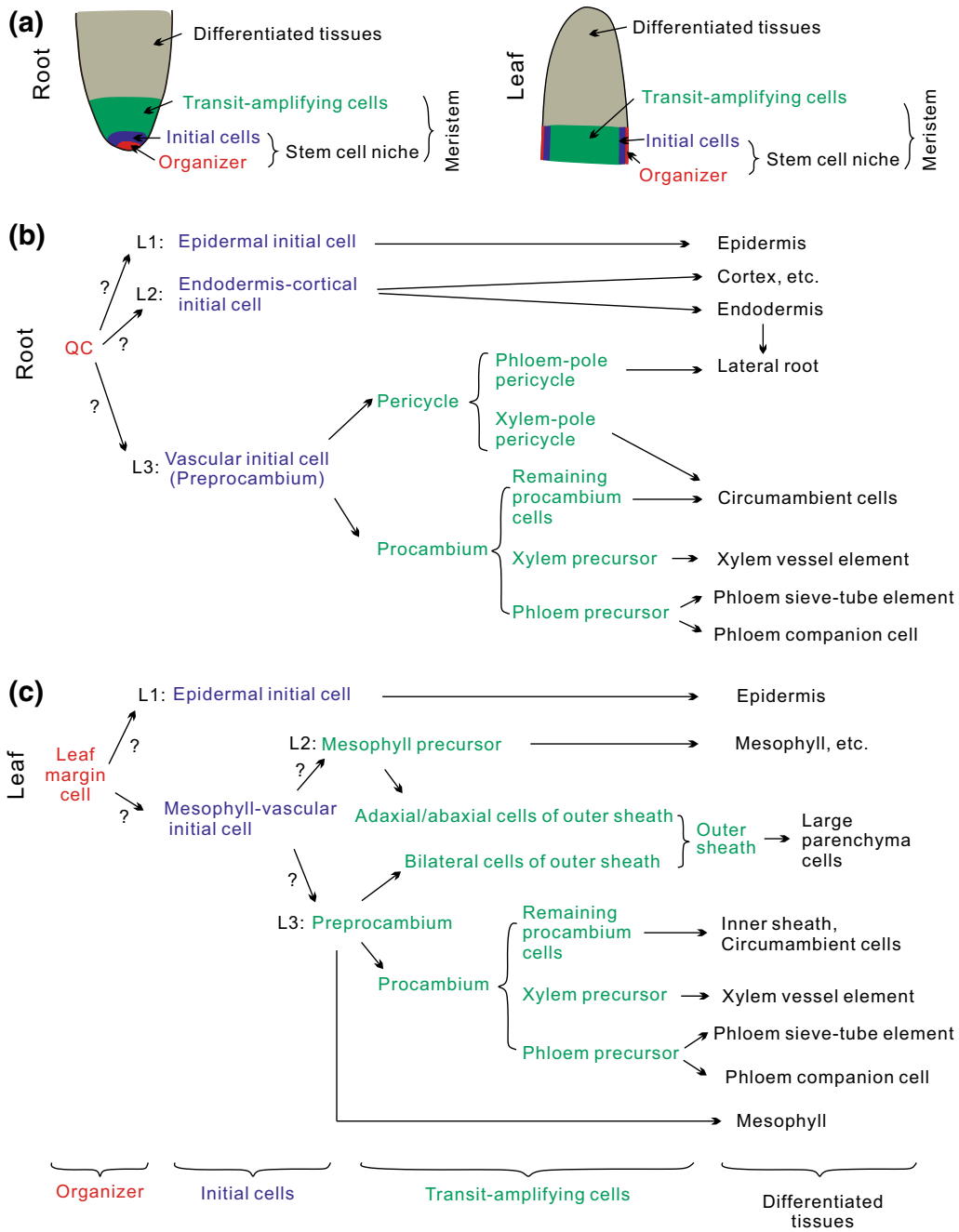

Fig. 5 Model of stem cell lineage in rice root and leaf. **a** Organization of stem cell niche and meristem in rice root and leaf. Root cap is not shown. Cell lineage in rice root (**b**) and leaf (**c**). Note that cell lineage in root cap formation is not shown. Question marks indicate unclear cell lineage

layers may shed light on the role of the stem cell niche in the leaf margin.

The preprocambium and procambium in the rice leaf are probably transit-amplifying cells. The preprocambium retains a large number of cells in the middle domain of the leaf and can produce many vascular bundles, and this is different from that in the root. In addition, the adaxial, abaxial, and bilateral cells of the outer sheath are transit-amplifying cells similar to the pericycle in the root, and they finally differentiate into large parenchyma cells.

An interesting discovery in this study is that the formation of the outer sheath in rice leaf requires the cooperation of both L2 and L3 cells. The vascular bundle is not only produced from the L3 preprocambium, but also from

the mesophyll precursor cells (L2). Therefore, the mesophyll precursor cells may also serve as transit-amplifying cells that can differentiate into adaxial/abaxial cells of the outer sheath and mesophyll (Fig. 5c).

4.3 Role of *WOX* genes in rice stem cells

WOX family genes are key regulators of stem cells in plants. The primary role of *WOX* genes may be to retain the identity of undifferentiated stem cells, i.e., to prevent the differentiation of stem cells. For example, the roles of *OsWOX5* and *OsNAL2/3* in the stem cell niches of root and leaf, respectively, could be to retain their pluripotency.

An interesting discovery in this study is that *OsWOX4* is specifically expressed in the leaf metaxylem precursor cells, a special type of cell in the procambium. This expression pattern differs from that of its *Arabidopsis* homolog *AtWOX4*, which is expressed in nearly all of the procambium cells [51, 52]. This may be because of the different vascular structures in *Arabidopsis* and rice. In *Arabidopsis*, procambium cells are retained for the continuous production of xylem and phloem in leaves during the whole life of the plant. In contrast, in rice, the leaf vascular bundles do not retain the procambium after maturation, and all of the cells in the rice procambium become specialized and differentiated. The metaxylem in the large vascular bundle is a special structure, because its differentiation is quite late, and occurs after the differentiation of protoxylem. Therefore, *OsWOX4* might serve to maintain the undifferentiated state of metaxylem precursor cells to prevent their early maturation. This can explain why *OsWOX4* expression was not observed in small and simple vascular bundles, because they lack metaxylem.

Recent studies have shown that the expression pattern of *OsWOX5* is not fully equivalent to that of *AtWOX5* in *Arabidopsis* [48, 49]. *OsWOX5* is expressed in root metaxylem precursor cells as well as in the root tip stem cell niche ([49] and this study). We did not observe *OsWOX5* expression in root protoxylem precursor cells. The role of *OsWOX5* in the root metaxylem precursor might be similar to the proposed role of *OsWOX4* in the leaf metaxylem precursor, i.e., to prevent the early maturation of the metaxylem before the differentiation of the protoxylem. Further analysis of all the *WOX* functions in stem cells of dicots and monocots will improve our understanding of stem cell evolution in angiosperms.

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