Comparative anatomy of embryogenesis in three species of Podostemaceae and evolution of the loss of embryonic shoot and root meristems

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Comparative Anatomy of Embryogenesis in Three Species of Podostemaceae and Evolution of the Loss of Embryonic Shoot and Root Meristems

Running head: The loss of embryonic shoot and root meristems in Podostemaceae

Natsu Katayama,[a,] * Masahiro Kato,[b] Takumi Nishiuchi,[a, c] and Toshihiro Yamada[a]

[a] Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

[b] Department of Botany, National Museum of Nature and Science, Amakubo, Tsukuba 305-0005, Japan

[c] Division of Functional Genomics, Advanced Science Research Center, Kanazawa University, Takaramachi, Kanazawa 920-0934, Japan

* Author for correspondence (email: nuts@stu.kanazawa-u.ac.jp)

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Abstract

During embryogenesis in angiosperms, the embryonic shoot and root meristems are created at opposite poles of the embryo, establishing the vertical body plan. However, the aquatic eudicot family Podostemaceae exhibits an unusual horizontal body plan, which is attributed to the loss of embryonic shoot and root meristems. To infer the embryogenetic changes responsible for the loss of these meristems, we examined the embryogenesis of three podostemads with different meristem characters, i.e., *Terniopsis brevis* with distinct shoot and root meristems, *Zeylanidium lichenoides* with reduced shoot and no root meristems, and *Hydrobryum japonicum* with no shoot and no root meristems. In *T. brevis*, as in other eudicots, the putative organizing center (OC) and L1 layer (= the epidermal cell layer) arose to generate a distinct shoot meristem initial, and the hypophysis formed the putative quiescent center (QC) of a root meristem. *Z. lichenoides* had a morphologically unrecognizable shoot meristem, because a distinct L1 layer did not develop, whereas the putative OC precursor arose normally. In *H. japonicum*, the vertical divisions of the apical cells of 8-cell embryo prevented putative OC initiation. In *Z. lichenoides* and *H. japonicum*, the putative QC failed to initiate because the hypophysis repeated longitudinal divisions during early embryogenesis. Based on their phylogenetic relationships, we infer that the conventional embryonic shoot meristem was lost in Podostemaceae via two steps, i.e., the loss of a distinct L1 layer and the loss of the OC, whereas the loss of the embryonic root meristem occurred once by misspecification of the hypophysis.

INTRODUCTION

Vascular plants grow vertically due to shoot and root apical meristems that almost continuously form new organs throughout their life. As a result, aerial-shoot and underground-root systems develop (Steeves and Sussex, 1989). In angiosperms, this basic body plan becomes apparent during embryogenesis, when the embryonic shoot meristem, cotyledon(s), hypocotyl, primary root, and embryonic root meristem develop along the apical–basal axis of the embryo (Fig. 1A; Esau 1965; Johri et al.1992).

The developmental and genetic aspects of embryogenesis have been most extensively studied in a model eudicot species *Arabidopsis thaliana*. The results suggest that an invariant cell-division pattern during early embryogenesis is required for establishing embryonic shoot and root meristems that mediate growth along the vertical axis (Mansfield and Briarty 1991; J•ü•rgens and Mayer 1994; Laux et al. 2004). By the 8-cell stage, the embryo establishes four regions that have different developmental fates: (1) the apical embryo domain generating the shoot meristem and the cotyledons; (2) the central embryo domain forming the hypocotyl and the primary root; (3) the basal embryo domain (hypophysis) giving rise to the root meristem; and (4) the extra-embryonic suspensor that puts the embryo inside of the ovule and provides a connection to the mother tissue. Subsequently, the organizing center (OC) of the shoot meristem initiates by tangential divisions in apical cells of 8-cell embryos, and the quiescent center (QC) of the root meristem arises by divisions of the hypophyseal cell at the globular embryo stage. In later development, the OC and the QC establish the stem cell niches in each meristem, forming the embryonic shoot and root meristem at apical and basal tips of the embryo, respectively. Many early-embryogenesis mutants can be identified, suggesting that changes in embryonic development can drastically affect

seedling morphology. For example, the seedlings of the strong *shoot meristemless* mutant lack the shoot meristem between the cotyledons because the stem cell layers of the shoot meristem fail to form (Barton and Poethig 1993; Long et al. 1996). The *monopteros* (*mp*) mutants form rootless seedlings because the misspecified hypophysis fails to initiate the QC and the root meristem (Berleth and Jürgens 1993; Hardtke and Berleth 1998; Weijers et al. 2006).

The aquatic eudicot family Podostemaceae, the river weeds, acquire a new body plan by converting the growth direction from vertical to horizontal when they split from the sister group Hypericaceae with a typical eudicot body plan, as represented by St. John's wort (Fig. 1A; Gustafsson et al. 2002; Wurdack and Davis 2009). Podostemaceae grow in rapids and waterfalls in the tropics to subtropics of the world where the water levels change seasonally. The seeds germinate when they get wet at the beginning of the rainy season, and the plants grow by creeping on submerged rock surfaces during the rainy season. As the dry season comes, the plants are exposed to the air, flower, set fruits, and then end their lives. In short, the plants grow exposed to strong current pressure in most of their life beginning as seedlings. Thus, the seedlings of most podostemads cease vertical growth with rudimentary embryonic shoot and root meristems, and instead, an adventitious root arises from the lateral side of the hypocotyl and acts as a lead organ. The adventitious root creeps on the rock surface, forming adventitious shoots on the dorsal or lateral side. As a result, most podostemads develop a horizontal body plan (Rutishauser 1995, 1997; Mohan Ram and Sehgal 1997; Suzuki et al. 2002).

Previous studies on seedling development in three subfamilies of Podostemaceae have suggested that the abortion of vertical growth in Podostemaceae

could be related to loss or reduction of the embryonic shoot and root meristems (Fig. 1; Suzuki et al. 2002; Kita and Kato 2005; Koi and Kato 2007). The seedlings of *Terniopsis*, the basally diverging genus of the subfamily Tristichoideae (Kita and Kato 2001), have a short primary shoot with an embryonic shoot meristem between the cotyledons and a primary root with an embryonic root meristem at the lower tip of the hypocotyl (Fig. 1B; Kita and Kato 2005). *Weddellina squamulosa*, the sole species of the subfamily Weddellinoideae, has a primary shoot with a shoot meristem between the cotyledons, but do not have a primary root (Koi and Kato 2007). In the diversified subfamily Podostemoideae, the seedlings of most members, e.g., *Zeylanidium* and *Cladopus* species, form one to several leaves between the cotyledons in the absence of a distinct shoot meristem (Fig. 1C), while the seedlings of the *Hydrobryum* species examined have neither shoot meristem nor plumular leaves between the cotyledons (Fig. 1D; Suzuki et al. 2002). In contrast to such variations in the seedling shoot apex, a primary root and an embryonic root meristem do not form at the basal tip of the hypocotyl in the Podostemoideae that have been examined (Fig. 1, C and D; Warming 1882; Willis 1902; Philbrick 1984; Mohan Ram and Sehgal 1997; Jager-Zürn 2000; Uniyal and Mohan Ram 2001; Sehgal et al. 2002; Suzuki et al. 2002). Molecular phylogenetic studies show that the subfamily Tristichoideae is basally diverged in the family, followed by the monotypic Weddellinoideae and the most diversified Podostemoideae (Cook 1996; Cook and Rutishauser 2007; Kita and Kato 2001). In the phylogeny of Podostemoideae, the genus *Hydrobryum* form a monophyletic clade with *Cladopus*, and the *Hydrobryum*-*Cladopus* clade is sister to the clade including *Zeylanidium* (Kita and Kato 2001). Thus, phylogenetic relationship in Podostemaceae suggests the following evolutionary scenario for embryonic shoot and root meristems

(Fig.1E; Kita and Kato 2005). A common ancestor of Podostemoideae reduced an embryonic shoot meristem and acquired a novel shoot development mechanism in the absence of a distinct shoot meristem (Kita and Kato 2005; Koi and Kato 2007), and the embryonic shoot meristem was lost completely later in the clade including *Hydrobryum* within Podostemoideae (Suzuki et al. 2002; Kita and Kato 2005; Koi and Kato 2010). In contrast, the embryonic root meristem was lost in the common ancestor of Weddellinoideae and Podostemoideae (Kita and Kato 2005; Koi and Kato 2007).

Although previous studies on seedling morphology predict that a reduction in both apical meristems could play a critical role in the elaboration of the horizontal body plan in Podostemaceae, no information is available on how the reduction is achieved through embryogenesis. Several embryological studies on some Tiristichoideae species have been reported, but cell lineages leading to embryonic shoot and root meristems are not described in detail (e.g., Mukkada 1969; Mukkadda and Chopra 1973; Nagendran et al. 1981). Thus, the embryogenic changes responsible for the loss of the embryonic shoot and root meristems in Podostemaceae remain unclear. In this paper, we describe the embryogenesis with emphasis on the cell lineages of three podostemads with different seedling body plans i.e., *Terniopsis brevis* M. Kato of Trisithichoideae with embryonic shoot and root meristems, *Zeylanidium lichenoides* (Kurz.) Engl. of Podostemoideae with a reduced embryonic meristem and no root meristem, and *Hydrobryum japonicum* Imamura of Podostemoideae with no embryonic shoot meristem and no root meristem. We compare the developmental patterns among the three species to infer the developmental changes in embryogenesis involved in the evolution of the loss of their embryonic shoot and root meristems.

MATERIALS AND METHODS

Plant materials

Wild Podostemaceae plants with flowers and fruits were collected in Thailand (Table 1). Voucher specimens were deposited in the Herbarium of the National Museum of Nature and Science, Japan (TNS). Seeds of St. John's wort (*Hypericum perforatum* L. var. Helos) were purchased from Richters Herbs (Goodwood, Ontario, Canada).

For seedling culture, Podostemaceae seeds were first placed on 3.0% agar medium containing 0.05% (v/v) HYPONeX (Hyponex Japan, Ltd., Tokyo, Japan), and the agar medium was covered with 0.05% (v/v) HYPONeX liquid medium. These plants were incubated at 25ºC under 14 h light/10 h dark (Kita and Kato 2005). St. John's wort seeds were placed on 0.8% agar medium at 25ºC under 16 h light/8 h dark.

For anatomical observation, flowers and fruits were fixed with formalin:acetic acid:50% ethanol at 5:5:90. The ovules and seeds were dissected from the fixed flowers and fruits, dehydrated in an ethanol series, embedded in Thechnovit 7100 (glycol methacrylate; Heraeus Kulzur, Wehrheim, Germany), cut into 3-µm thick sections using a tungsten knife on a microtome, and stained in a solution of safranin, toluidine blue, and Orange G (Jernstedt et al. 1992).

RESULTS

Terniopsis brevis with embryonic shoot and root meristems

The zygote showed a polar organization, with a large vacuole in the basal part and most of the cytoplasm and nucleus in the apical part (Fig. 2A). The zygote was divided

transversely into a small apical cell and a larger basal cell (Fig. 2B). Subsequently, these cells underwent a series of transverse divisions to produce a spherical apical cell and three-celled filamentous suspensor (Fig. 2, B and C). The basal-most cell of the suspensor enlarged to several times its initial size and became a haustrial cell containing several free nuclei at the micropylar region, while a hypophyseal cell arose beneath the embryo proper via transverse division of the uppermost cell of the suspensor (Fig. 2D). The apical cell divided longitudinally, and another vertical division occurred at right angles to the first division, resulting in a four-cell embryo proper (Fig. 2D). Subsequently, a transverse division in the embryo proper yielded an 8-cell embryo with four regions, i.e., the apical embryo domain with upper four cells of embryo proper, the central embryo domain with lower four cells of embryo proper, the basal embryo domain (hypophysis), and the extra-embryonic suspensor (Fig. 2E).

The cells of 8-cell embryo proper underwent tangential divisions, and the protodermis and inner subdermal cells were initiated (Fig. 2F). The anticlinal divisions occurred symmetrically in the protodermal cells of the apical embryo domain, and, subsequently, asymmetric anticlinal divisions occurred in the four protodermal cells above the subdermal cells, resulting in four small protodermal cells at the globular stage (Fig. 2G). From the heart to the torpedo stage, these small protodermal cells and subdermal cells of the apical domain no longer divided and formed an embryonic shoot meristem between the cotyledons (Fig. 2, H–K). Throughout globular embryogenesis, the subdermal cells of the central domain divided longitudinally to generate a layer of ground tissue underlying an epidermal cell layer and central provascular initials (Fig. 2G).

In the transition stage from the globular to heart-shaped embryo, the hypophyseal cell underwent two longitudinal divisions perpendicular to each other to generate four cells (Fig 2H). Then, they divided transversely, forming two layers consisting of four cells derived from hypophysis (Fig. 2I). Four cells of upper layer no longer divided and composed an embryonic root meristem (Fig. 2, I–K).

***Zeylanidium lichenoides* with a reduced shoot meristem and no root meristem**

The zygote had a polar organization and generated an 8-cell embryo comprising an 8-cell embryo proper, a hypophyseal cell, and a three-celled suspensor with a haustrial cell in a series of cell divisions similar to *Terniopsis brevis* (Fig. 3, A–D). Then, two longitudinal divisions of the hypophyseal cell occurred much earlier than in *Terniopsis* (Fig. 3E). The 8-cell embryo proper, as in the *Terniopsis* embryo, underwent tangential divisions to form a protoderm and inner subdermal cells (Fig. 3, E and F). In the globular stage, the protodermal cells of the apical domain divided anticlinally and equally, and four cells initiated above the subdermal cells of the apical domain (Fig. 3G). In contrast to the subsequent asymmetric divisions in the *Terniopsis* embryo, these four protodermal cells no longer divided and did not form four small epidermal cells above subdermal cells (Fig. 2G and 3G). On the other hand, from the heart stage, the subdermal cells of the apical embryo domain underwent an oblique and a transverse division to generate a group of their daughter cells between the cotyledons (Fig. 3, I–K). The cotyledons were tightly appressed to each other at their base, because an epidermal layer that was observed in *T. brevis* did not arise between cotyledons (Fig. 3, J and K).

The subdermal cells of the central domain divided longitudinally to generate a layer of ground tissue underlying an epidermal cell layer and central provascular initials

in globular embryogenesis (Fig. 3G). The daughter cells of the hypophysis that arose by 16-cell embryo stage divided longitudinally to generate a one-cell layer at the basal part of the embryo (Fig. 3, G and H). These derivatives of the hypophysis divided repeatedly during subsequent development (Fig. 3, I–K). In other words, the upper four cells of hypophysis derivatives found in *T. brevis* did not initiate in *Z. lichenoides*.

***Hydrobrym japonicum* with no shoot and no root meristems**

The bipolar-organized zygote underwent the same pattern of cell divisions as seen in *Terniopsis brevis* and *Zeylanidium lichonides* and generated an 8-cell embryo comprised of an 8-cell embryo proper, a hypophysis, and a three-celled suspensor with basalmost haustrial cell (Fig. 4, A–E). Similar to hypophysis development in the *Z. lichenoides* embryo (Fig. 3, D–H), the two longitudinal divisions of the hypophyseal cell occurred in 8-cell stage, and its daughter cells divided longitudinally again to generate a one-cell layer at the basal part of the embryo by the globular stage (Fig. 4, F–H). Subsequently, these derivatives of the hypophysis divided repeatedly as in *Z. lichenoides* (Fig. 4, I–K).

The apical and central embryo domains showed a different developmental pattern from that of *Z. lichenoides*. The apical cells of the 8-cell embryo underwent anticlinal divisions, but no tangential division (Fig. 4G). Thus, there were no inner subdermal cells in the apical domain at the 16-cell stage (Fig. 4G). These outermost cells of the apical embryo domain developed into a protoderm though further anticlinal divisions, failing to initiate inner cells of shoot meristem (Fig. 4H). From the heart stage on, the cotyledonary primordia emerged and were tightly appressed to each other at their base (Fig. 4, I–K). The central vascular initials, throughout the globular to heart stages, did not arise because the subdermal cells of the central domain of the 8-cell

embryo underwent oblique divisions to generate ground tissue underlying the epidermal cell layer (Fig. 4, G–I).

DISCUSSION

Loss of Embryonic Shoot Meristem in Podostemaceae

This study revealed, for the first time, the cell-by-cell based embryogenesis of Podostemaceae that is involved in establishing embryonic shoot and root meristems. We identified three developmental patterns in the apical domain of Podostemaceae embryos, which correspond to the developmental patterns of the primary shoots of seedlings (Fig. 5). The first is the pattern of *Terniopsis brevis*, Tristichoideae, which form a primary shoot with a distinct shoot meristem between the cotyledons (Fig. 5A). This type of embryonic development is comparable to that of most dicots, as represented by *Arabidopsis*. In the 16-cell stage, the inner subdermal cells in the apical embryo domain originate from the apical cells of the 8-cell embryo through tangential divisions. These four subdermal cells and small epidermal cells above the subdermal cells form a distinct shoot meristem initial. After germination, the initial meristem develops into a dome-shaped meristem with tunica-corpus organization, which formed leaves on its flank (Kita and Kato 2005). Thus, four subdermal cells of the apical domain of 16-cell embryo could be compared to OC precursor cells, and the four small epidermal cells above them would form the L1 layer, i.e., the epidermal cell layer of the shoot meristem. *Terniopsis brevis* retains the conventional development of embryonic shoot meristem.

The second pattern observed in *Zeylanidium lichenoides*, Podostemoideae, is less typical in angiosperms. Similar to the seedlings of most Podostemoideae, the *Z.*

lichenoides seedling forms several plumular leaves between the cotyledons, but does not have a distinct shoot meristem with tunica-corpus organization (Mohan Ram and Sehgal 1997; Suzuki et al. 2002). However, our result showed that a morphologically unrecognizable shoot meristem is formed (Fig. 5B). As seen in *T. brevis* and other dicots, the four subdermal apical cells, which might be a putative OC precursor, arise in the 16-cell stage. These putative OC precursor cells divide several times to produce what appears to be a group of shoot meristem cells, although it is uncertain whether all of these cells have OC identity. On the other hand, a distinct L1 layer of a shoot meristem is not formed, because small epidermal cells above putative OC precursor cells do not arise. Therefore, a shoot meristem without a distinct L1 layer is established during the *Z. lichonides* embryogenesis.

The third developmental pattern in the apical domain occurred in *Hydrobryum japonicum*, a species of the derived genus *Hydrobryum*, Podostemoideae. In the seedlings of the several *Hydrobryum* species, e.g., *H. griffithii*, *H.* (syn. *Synstylis*) *micrantherum* and *H. japonicum*, neither a distinct shoot meristem nor a plumular leaf are formed between the cotyledons (Suzuki et al 2002; Koi and Kato 2010; N. Katayama, unpubl. data). During the *H. japonicum* embryogenesis, the first sign of developmental deviation in the apical domain appears from 8-cell to 16-cell embryo stages (Fig. 5C). Anticlinal divisions occur in the apical cells of the 8-cell embryo but not to form inner subdermal cells in the apical domain, and no tangential divisions occur, as in *T. brevis*, *Z. lichenoides*, and other dicots. Consequently, all daughter cells of the apical domain of the 16-cell-embryo proper differentiate into epidermal cells. Thus, the possible OC founder cells do not initiate, resulting in the complete loss of an embryonic shoot meristem (also see discussion below).

Based on the phylogenetic relationship and the seedling morphology in Podostemaceae, the reduced embryonic shoot meristem with only a few leaves of most Podostemoideae is a derived character state, compared with the typical shoot meristem of Tristichoideae and Weddellinoideae. In Podostemoideae, a reduced primary shoot is a plesiomorphic character state and the embryonic shoot meristem was lost completely later in the clade including *Hydrobryum* (Fig.1E; Kita and Kato 2001, 2005; Suzuki et al. 2002; Koi and Kato 2010). Considering the anatomical data obtained in this study and evolutionary relationships in Podostemaceae (Kita and Kato 2001, 2005), we infer that the conventional embryonic shoot meristem was lost via two embryogenetic changes in Podostemaceae (Fig. 1E and 5): (1) Lack of asymmetric divisions in protodermal cells above the OC precursor cells, i.e., the loss of a distinct L1 layer, which causes the reduced embryonic shoot meristem, occurred in common ancestor of Podostemoideae, and (2) the change of divisional plane in the apical cells of the 8-cell embryo, i.e., the loss of the OC, which causes the complete loss of the embryonic shoot meristem, occurred in the *Hydrobryum* clade within Podostemoideae.

In adventitious root-borne shoots of Podostemoideae, a new leaf arises in the basal part of an existing leaf in the absence of a distinct shoot meristem and the repetitive leaf formation occur on the adaxial side of the existing leaf, forming a chain of leaves (Imaichi et al. 2005, Koi et al. 2005). A gene expression analysis revealed that each leaf arises as a shoot meristem at the base of an existing leaf and then differentiates into an apical leaf, resulting in a leaf-like shoot system (Katayama et al. 2010). In our preliminary observations on the seedlings of some Podostemoideae including *Z. lichenoides*, a plumular leaf arises from the cells of a cryptic shoot meristem at the base of cotyledon (N. Katayama, unpubl. data). Such leaf development of primary shoot at

the base of cotyledon is very similar to that of the adventitious root-borne shoot, suggesting that the common developmental program would be used for both shoots.

The central embryo domain shows harmonious development with the apical embryo domain. In *T. brevis* and *Z. lichoides*, putative OC precursor cells arise at the 16-cell stage, and the subdermal cells of the central domain subsequently divide longitudinally to generate provascular initials at the globular embryo stage (Fig. 5, A and B). In *H. japonicum*, which does not have OC precursor cells initiated at the 16-cell stage, the subdermal cells of the central domain undergo oblique divisions and repeat random divisions during later development (Fig. 5C). Thus, in mature embryos, the central region of the *T. brevis* and *Z. lichenoides* embryos is more or less organized with provascular cells, whereas the cells in the central region of the *H. japonicum* embryo are randomly arranged. In the *Arabidopsis* embryo, the provascular cells mediate apical-to-basal auxin transport (Friml et al. 2003). A random arrangement of the inner cells of the *Hydrobryum* embryo suggests that basipetal auxin flow might be disturbed.

A hypothesis on epidermal cell fate during embryogenesis has been proposed that cells exposed to the outside environment develop into a epidermis, i.e., the epidermal traits could be conferred to all cells of the 8-cell embryo proper, and outermost protodermal (and epidermal) cells after the 16-cell stage (Bruck and Walker 1985; Berger et al. 1994). This hypothesis is supported by the expression patterns of two epidermal markers, *ARABIDOPSIS THALIANA MERISTEM LAYER1* and *PROTODERMAL FACTOR2*, in the *Arabidopsis* embryo, as they are expressed in cells where the hypothesis predicts epidermal cell fate (Lu et al. 1996, Abe et al. 2003). Because the apical cells of the 8-cell embryo in *Hydrobryum* undergo anticlinal divisions, all of their descendants face the outside environment. This suggests that all

cells of the apical embryo domain acquire epidermal identity instead of becoming embryonic shoot meristem in *H. japonicum* (Fig. 5C).

Loss of the Embryonic Root Meristem

We found two developmental patterns in the basal region of the embryo corresponding to the presence of a primary root and an embryonic root meristem in seedlings of *Terniopsis brevis* and the absence in *Zeylanidium lichenoides* and *Hydrobryum japonicum* (Fig. 5). In the three species, the hypophyseal cell arises from the uppermost suspensor via a series of cell divisions common to *Indotristicha ramosissima* and *Dalzellia zeylanica* of the subfamily Tristichoideae (Mukkada 1969; Mukkada and Chopra1973). In *T. brevis*, the first two divisions of the hypophyseal cell are longitudinal in the globular to heart stages, and then the daughter cells divide transversely to generate four inner cells within the basal tip of the embryo (Fig. 5A). These cells no longer divide during subsequent embryogenesis, and after germination, the root meristem and the primary root develop further (Kita and Kato 2005). Thus, these four cells of the basal region of the embryo could be considered as QC cells of the root meristem. In most dicots, e.g., *Arabidopsis*, the hypophyseal cell gives rise to four QC precursor cells by the first transverse and subsequent two longitudinal divisions during transition from the globular to heart stage (Johri et al. 1992; Mansfield and Briarty 1991; J•ü•rgens and Mayer 1994). Therefore, the putative QC initials in *T. brevis* are formed by a different divisional sequence from other dicots, although the source, arrangement, and initiation stage of QC precursor cells are consistent with those in other dicots.

In *Z. lichenoides* and *H. japonicum*, the hypophyseal cell divides longitudinally twice by the 16-cell stage, i.e., much earlier stage than that in *T. brevis* and other dicots. Furthermore, the subsequent division is again longitudinal, and the four inner cells of the putative QC founder cells are not formed. It is likely that the developmental change in the hypophyseal cell causes the loss of the root meristem, and the resulting loss of the primary root is reported in various species of Podostemoideae (Fig. 1E; Warming 1882; Willis 1902; Philbrick 1984; Mohan Ram and Sehgal 1997; Jager-Zurn 2000; Uniyal and Mohan Ram 2001; Sehgal et al. 2002; Suzuki et al. 2002). In our preliminary observations, similar to the hypophysis development of *Z. lichenoides* and *H. japonicum*, longitudinal divisions of the hypophyseal cells were repeated during the early embryogenesis of *Weddellina squamulosa*, Weddellinoideae, whose seedling is rootless (N. Katayama, unpubl. data). Taken together, our results suggest that the developmental change in the hypophysis, which fails to initiate the putative QC, occurred in the Weddellinoideae–Podostemoideae lineage.

Molecular genetic studies with *Arabidopsis* demonstrate that the hypophysis specification is required for development of the embryonic root meristem. An auxin-dependent transcription factor *MP* drives the hypophysis specification by promoting auxin transport from the embryo proper to the hypophysis precursor during early root development (Hardtke and Berleth 1998; Weijers et al. 2006). The *mp* seedlings are rootless, because the hypophyseal cell divides aberrantly and fails to initiate QC precursor cells in the globular stage by the misspecification of hypophysis during early embryogenesis (Berleth and Jürgens 1993; Cole et al. 2009; Schlereth et al. 2010). The hypophyseal cell of the 8-cell embryo before cell division in *Z. lichenoides* and *H. japonicum* appears to be larger and more cytoplasm-rich than that of *T. brevis*

and *A.thaliana* (Fig. 2E, 3D, 4E; Mansfield and Briarty 1991; J•ü•rgens and Mayer 1994), suggesting that specification of the hypophysis does not occur during early embryogenesis. Thus, in *Z. lichenoides* and *H. japonicum*, the hypophysis does not maintain its own cell fate and fails to initiate QC founder cells. It may be that this unusual development of the hypophyseal cell, leading to the loss of a primary root and a root meristem, is caused by a change in the root specification network, e.g., auxin-dependent *MP* network.

Conclusions

Differences in cellular embryogenesis, which are involved in the loss of embryonic shoot and root meristems, are present in Podostemaceae and eventually lead to the loss of a vertical body plan. The conventional embryonic shoot meristem, as present in *Terniopsis*, Tristichoideae, was lost in Podostemoideae via two steps: (1) the loss of a distinct L1 layer at the base of the subfamily and (2) the loss of the OC in the derived *Hydrobryum* clade. The loss of the embryonic root meristem occurred with the loss of the QC that might be caused by misspecification of the hypophysis in the Weddellinoideae–Podostemoideae lineage. However, the timing when those cells in the embryonic shoot and root meristem precursors change their own cell fates and the genetic mechanisms underlying these embryonic changes remain to be solved.

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Table 1. Materials examined in this study

Species	Locality	Voucher
Terniopsis brevis	Kaeng Lamduan stream, Yoddome Wildlife Sanctuary, Ubon Ratchathani, Thailand	TNS-8000205
Zeylanidium lichenoides	Huay Kaew stream, Maethakhrai National Park, Chiang Mai, Thailand.	TNS-8000188
Hydrobryum japonicum	Stream near None Phatana village, Phu Luang Wildlife Reservation, Loei, Thailand	TNS-8000075

Figure Legends

Figure 1. Seedling morphology and phylogeny of Podostemaceae. (A) St. John's wort (*Hypericum perforatum*; outgroup) showing a typical body plan with primary shoot and root. (B–D) Seedlings of Podostemaceae before adventitious root protrusion. (B) *Terniopsis brevis* (Tristichoideae) with a plumular leaf between cotyledons and a primary root at the tip of the hypocotyl. (C) *Zeylanidium lichenoides* (Podostemoideae) with a reduced primary shoot and no primary root. Leaf primordium (arrowhead) arising between cotyledons in the absence of a distinct shoot meristem. Rhizoids occupying the tip of the hypocotyl. (D) *Hydrobryum japonicum* (Podsotemoideae) without a primary shoot and root. No shoot meristem is found between cotyledons appressed at their base. Rhizoids occupying the tip of the hypocotyl. (E) Hypothesis on evolutionary changes in seedling body plan of Podostemaceae (modified from Kita and Kato 2005). Arrows and T-shaped lines indicate indeterminate shoots with a conventional shoot meristem and determinate shoots in the absence of a distinct shoot meristem, respectively. ar, adventitious root; as, adventitious shoot; c, cotyledon; h, hypocotyl; pr, primary root; ps, primary shoot; rh, rhizoid. Scale bars = 1 mm

Figure 2. Light micrographs of longitudinal sections of *Terniopsis brevis* embryos. (A) Zygote surrounded by the outer and inner integuments and the pseudo-embryo sac. *Inset* shows magnification of zygote highly polarized with an apically-sited nucleus and a basally-sited large vacuole. (B) One-cell embryo with a small apical cell and a larger basal cell. (C) One-cell embryo with an apical cell and suspensor cells. (D) Four-cell embryo with 4-cell embryo proper, a hypophysis and suspensor with haustrial cell. (E) Eight-cell embryo comprising 8-cell embryo proper and suspensor. (F) Sixteen-cell

embryo with embryo proper comprising protoderm and inner cells. (G) Globular embryo. Arrowheads indicate the initiation of small epidermal cells (= L1 layer initials). Note that the hypophyseal cell is more conspicuously vacuolated than are the embryo proper cells. (H) Transition stage from globular to heart-shaped embryo. Cotyledonary primordia have emerged, and the hypophyseal cell divided longitudinally. (I) Heart-shaped embryo with cotyledonary primordia elongating. Daughter cells of the hypophysis divided transversely, forming four inner cells (= putative quiescent center precursor cells). (J, K) Sagittal (J) and frontal (K) sections of the torpedo embryo. ac, apical cell; bc, basal cell; ha, haustrial cell; hy, hypophysis; ii, inner integument; oi, outer integument; pse, pseudo-embryo sac; su, suspensor. asterisks ; putative organizing center precursor cells. cells marked X ; hypophysis and putative quiescent center precursor cells. Scale bars = 20 μm

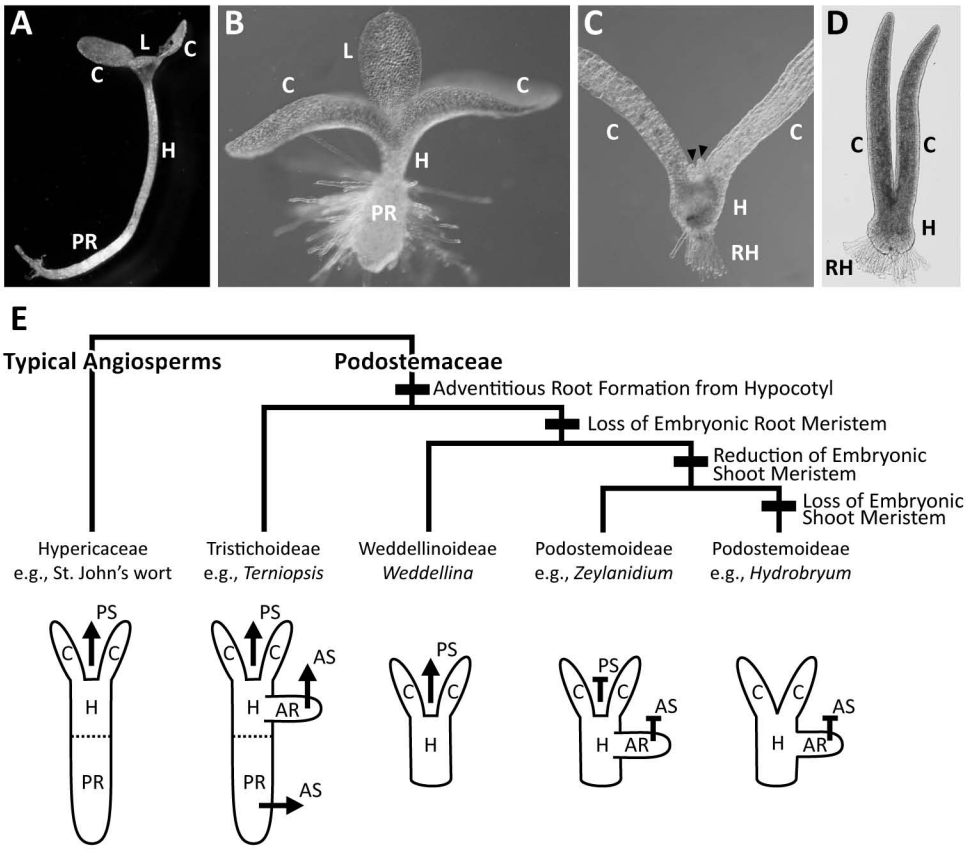
Figure 3. Light micrographs of longitudinal sections of *Zeylanidium lichenoides* embryos. (A) Zygote showing highly polarized cells with an apically-sited nucleus and a basally-sited large vacuole. (B) Elongating 1-cell embryo with an apical cell and a basal cell. (C) Four-cell embryo with 4-cell embryo proper and suspensor cells. (D) Eight-cell embryo with embryo proper, hypophysis, and haustrial cell. Note that hypophyseal cell is as cytoplasm-rich as the embryo proper cells. (E) Eight-cell embryo with the hypophyseal cell divided longitudinally. (F) Sixteen-cell embryo with embryo-proper comprising a protoderm and inner cells. Asterisks indicate the putative organizing center (OC) precursor cells. (G) Globular embryo. Arrowheads indicate that initial cells of L1 layer did not form above putative OC precursor cells. Provascular

initials arising in the central domain. Daughter cells of hypophyseal cell divided longitudinally. (H) Transition stage from globular to heart-shaped embryo. Cotyledonary primordia have emerged and are tightly appressed to each other at their base. Four daughter cells of the hypophysis are arranged in a row in the section. (I, J) Heart-shaped embryo with elongating cotyledonary primordia. Oblique divisions occur in putative OC precursor cells, and the daughter cells of the hypophysis divided in random planes. (K) Torpedo embryo. Cotyledons are elongate by this stage and tightly appressed. Cryptic shoot meristem comprising a cell group derived from putative OC precursor cells (asterisks) is seen. Organized provascular tissue and root meristem did not form. ac, apical cell; bc, basal cell; ha, haustrial cell; hy, hypophysis; ii, inner integument; oi, outer integument; pse, pseudo-embryo sac; su, suspensor. asterisks ; putative organizing center precursor cells. cells marked X ; hypophysis and its daughter cells. Scale bars = 20 μm

Figure 4. Light micrographs of longitudinal sections of *Hydrobryum japonicum* embryos. (A) Polar-organized zygote with a nucleus at the apical end and a large vacuole at the basal end. (B) One-cell embryo with an apical cell and a basal cell. (C) One-cell embryo with an apical cell and suspensor cells. (D) Four-cell stage embryo with 4-cell embryo proper and hypophyseal cell in the uppermost suspensor. (E) Eight-cell embryo. Note that hypophyseal cell is as cytoplasm rich as embryo-proper cells. (F) Eight-cell embryo with the hypophyseal cell divided longitudinally. (G) Sixteen-cell embryo. Eight apical cells of the embryo proper formed by vertical divisions. (H) Globular embryo. Outermost cells divided anticlinally to form protoderm. Inner cells of central domain underwent oblique divisions. Daughter cells of

hypophyseal cell divided longitudinally and are arranged in a row in the section. (I) Transition stage from globular to heart-shaped embryo. Cotyledonary primordial are elongating and inner cells of the embryo divided in random planes. (J) Heart-shaped embryo with cotyledonary primordia elongated and tightly appressed at their base. (K) Torpedo embryo. Inner cells of embryo proliferated and are randomly arranged. Organized provascular tissue, and shoot and root meristems do not form. ac, apical cell; bc, basal cell; ha, haustrial cell; hy, hypophysis; ii, inner integument; oi, outer integument; pse, pseudo-embryo sac; su, suspensor. cells marked X ; hypophysis and its daughter cells Scale bars = 20 μm

Figure 5. Comparison of embryogenesis in three species of Podostemaceae; *Terniopsis brevis* (A), *Zeylanidium lichenoides* (B), and *Hydrobryum japonicum* (C). Red-colored cells indicate putative organizing center (OC) precursor cells and their derivatives, and red-lined cells indicate cell divisions that cause a failure of OC initiation. Blue-colored cells indicate the hypophyseal cell and putative quiescent center (QC) precursor cells, and blue-lined cells indicate the hypophyseal cell divisions that cause a failure of QC initiation. Solid arrowheads indicate the initiation of L1 layer initials, and open arrowheads indicate that initial cells of L1 layer do not form.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Typical Angiosperms
Podostemaceae
Adventitious Root Formation from Hypocotyl
Loss of Embryonic Root Meristem
Reduction of Embryonic Shoot Meristem
Loss of Embryonic Shoot Meristem
Hypericaceae
e.g., St. John's wort
Tristichoideae
e.g., Terniopsis
Weddellinoideae
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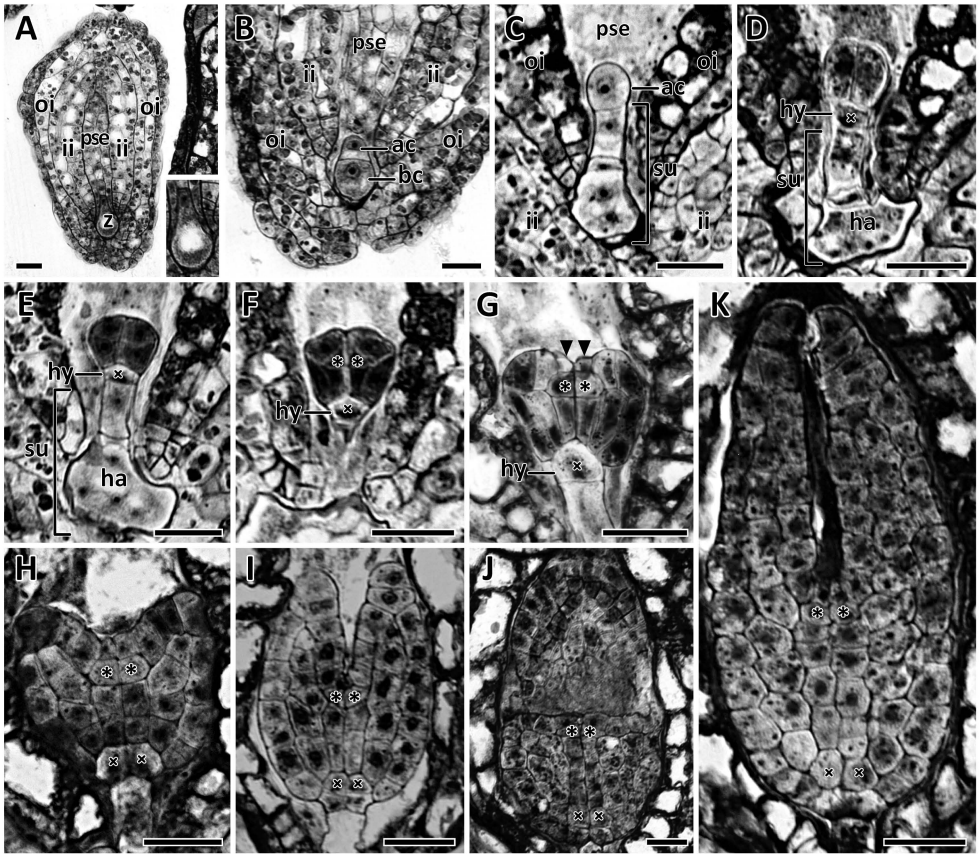
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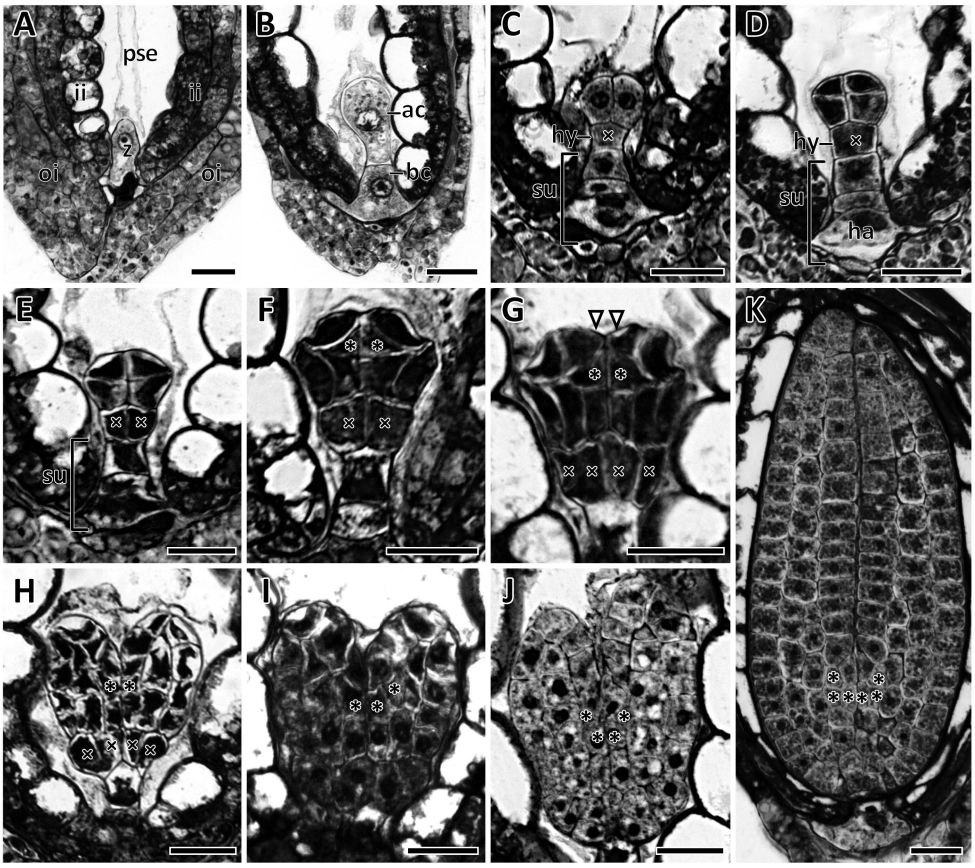
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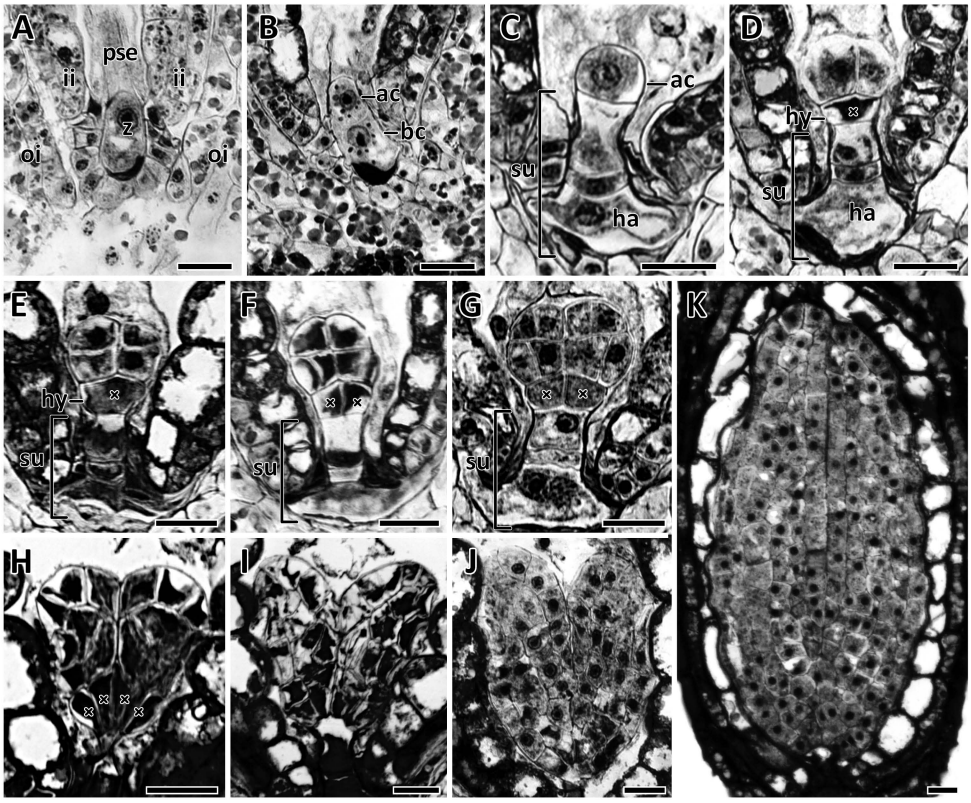
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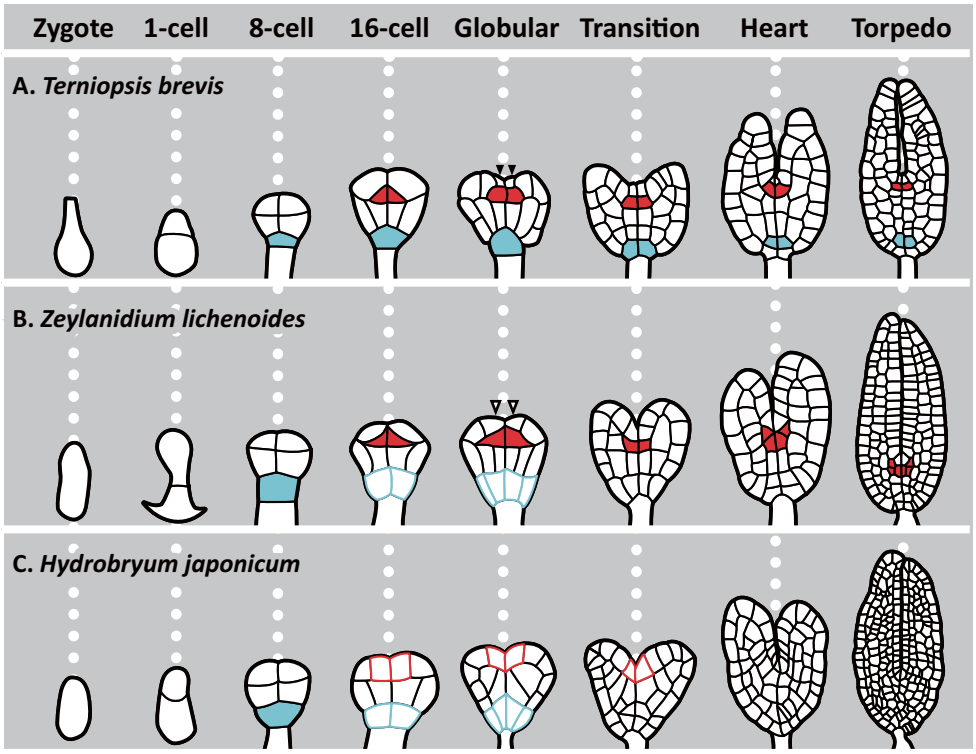
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B. Zeylanidium lichenoides
C. Hydrobryum japonicum