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Development of the Root System in *Spirodela polyrhiza* (L.) Schleiden (Lemnaceae)

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The root structure in members of the Lemnaceae is important to plant researchers, because changes during cell differentiation can more easily be monitored in short roots with determinate growth. Here, the structural organization and cellular differentiation of the root system was assessed in the highly reduced *Spirodela polyrhiza*. While protected by a prophyllous sheath, rapid cell division occurred in the apical and vascular regions of the immature roots. Concentric rings of endodermis with Casparian strips, cortex, and epidermis enclosed a single vascular strand. The cytoplasmic density of the cortex was high at the apex, but decreased progressively along the root. The root root cap junction, closely attached at initiation, later became a distinct boundary layer filled with fibrillar materials. Chloroplasts were well distributed. Numerous plasmodesmata indicated the likely symplastic movement of ions and metabolites in the root system as well as further into the reduced plant body. A high cytoplasmic density at the apex and extreme vacuolization along the cortex provided possible explanations for the considerable distribution of weight along the roots of the plant body. These conditions probably enable the root tip to serve as a pendulum against water motion.

Keywords: cellular differentiation, electron microscopy, root development, *Spirodela polyrhiza*, structural organization

The root structure of unusually reduced free-floating hydrophytes is of great interest when studying plant structure-function relationships, because changes that occur during cell differentiation in such species can be followed from initiation to maturity within short roots with determinate growth. The roots of the water fern *Azolla*, have previously been examined with regard to various aspects of structural differentiation, e.g., characteristics of cell division and differentiation, changes in the meristem (Gunning et al., 1978), chloroplast development (Whatley and Gunning, 1981), and nuclear and cytoplasmic alterations associated with early differentiation (Barlow et al., 1982). Furthermore, vascular maturation has been clearly demonstrated to occur in a precisely defined pattern arising from the zone of cell differentiation near the root apical meristem (Gunning and Steer, 1996). The roots of *Lemna*, have proven to be the most appropriate region for investigating sieve-element development (Melaragno and Walsh, 1976; Walsh and Melaragno, 1976). Likewise, those tissues are frequently used as a botanical tissue samples for low-temperature scanning electron microscopy to illustrate the advantages and disadvantages of that technique (see Echlin, 1992).

The root system in *Spirodela* and *Lemna* of the Lemnaceae are adventitious, arising from the extremely reduced shoots at the node beneath the abaxial frond (Hillman, 1961). A multiple root system develops on each frond in *Spirodela* but only on a single root in *Lemna*, despite their morphological similarities. Roots are thin (300-400 μm diam. at maturity) and elongated, and have prominent root caps (RC). Within the same strain, root lengths may vary on different fronds, depending on internal and external factors.

With regard to their structural aspects, the general features have been described for *Lemna minor* root tips (Melaragno and Walsh, 1976; Echlin et al., 1979, 1980, 1981) and overall morphology of its fully differentiated roots (Echlin et al., 1982). Melaragno and Walsh (1976) also have revealed the occurrence and precise arrangement of phloem tissue while studying the development of sieve elements in this species. Moreover, Echlin et al. (1982) have employed low-temperature X-ray microanalysis to examine the diffusible elements and have adopted a frozen-hydrated bulk material approach to demonstrate the gross cellular morphology and various developmental stages in the root vascular tissue. A schematic representation of cross-sections and several scanning electron micrographs have led to a depiction of a highly organized root structure. Of particular interests have been the disposition, number, and relative sizes of different tissue types, and an approximation of vascular development from the root tip. More recently, Echlin (1992) has obtained several scanning electron micrographs of frozen-hydrated root tips again when he discussed the subject of low-temperature microscopy and analysis. However, that research group has not been able to gain any detailed cellular information from those schematic and photographic representations (Echlin et al., 1982, 1992). Thus, the objective of this current study was to assess the structural organization and cellular differentiation during the development of the root system in *Spirodela polyrhiza*. An examination of the ultrastructural features of the fronds and connective stalks in this species will be treated separately.

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Abbreviations: B, boundary layer; C, chloroplast; Cc, companion cell; Ci, inner cortical layer; Cm, middle cortical layer; Co, outer cortical layer; Cw, cell wall; E, epidermis; En, endodermis; F, frond; G, Golgi body; I, intercellular space; M, mitochondria; m, microorganism; mt, microtubule; N, nucleus; P, P-plastid; Pd, plasmodesmata; Ps, prophyllous sheath; R, root; Rc, root cap cell; er, endoplasmic reticulum; S, starch grain; Sc, sieve cell; T, tracheary element; V, vacuole; Vt, vascular tissue. All figures are TEM, unless specified as SEM.

MATERIALS AND METHODS

Plant Material

Plants of *Spirodela polyrhiza* (L.) Schleiden were collected from the Upo Wetland in Korea during 2003 and 2004. At least 10 plants with 2 to 3 generations of offspring fronds were sampled for transmission and scanning electron microscopy.

Electron Microscopy

For transmission electron microscopy (TEM), tissue samples were fixed in 3% glutaraldehyde in 0.02 M phosphate buffer for 3 h at room temperature, then post-fixed in 2% osmium tetroxide at 4°C for 2 to 16 h (Kim, 2006). After three rinses in the same buffer, the materials were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. Approximately 60- to 90-nm ultra-thin sections were made with a diamond knife on an Ultracut-S ultramicrotome. These sections were mounted on 0.25% dichloroethane-coated copper grids and stained with 2% aqueous uranyl acetate, followed by 1% lead citrate. The sections were examined and photographed with a Hitachi H-7100 TEM operated at 75 kV.

For scanning electron microscopy (SEM), materials were either processed by modifying the methods of Lemon and Posluszny (2000) or fixed and dehydrated as they were for TEM. When the latter procedure was used, the tissues after

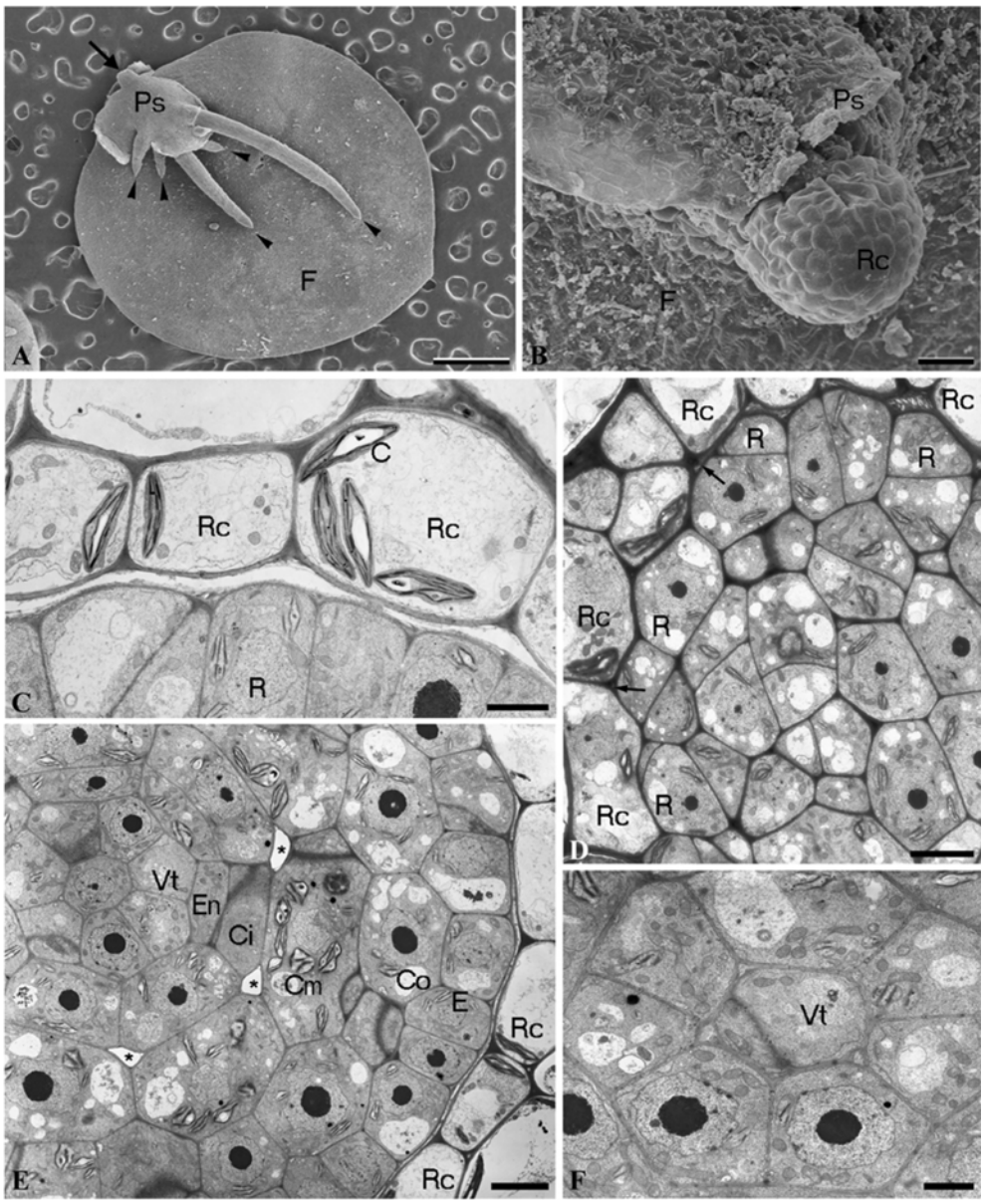

Figure 1. Early root development. **A,** Initiation of root system. Arrow indicates detached connective stalk; arrowheads show developing roots. SEM. Scale bar = 500 μm. **B,** Immature root system elongating through prophyllous sheath. SEM. Scale bar = 20 μm. **C,** Innermost RC cells with chloroplasts. Scale bar = 2.5 μm. **D,** Active cell divisions in root apex. Arrows indicate boundary layer. Scale bar = 5 μm. **E,** Transverse section of immature root tip showing epidermis, concentric layers of cortex and vascular region. Note the limited intercellular spaces (asterisk) in cortex. Scale bar = 5 μm. **F,** Vascular region in polygonal shape. Scale bar = 1 μm.

dehydration were treated with isoamyl acetate three times and stored at 4°C. Following that substitution, the samples were dried to the critical point, coated with 20- to 30-nm platinum-palladium and examined with a Hitachi S-4200 SEM operated at 15 kV.

Chlorophyll Determinations

Chlorophylls from immature and mature root samples were spectrophotometrically estimated in 80% acetone, after the tissues were extracted with N,N-dimethylformamide according to the methods of Moran and Porath (1980). Pigment concentrations were calculated using the extinction coefficients proposed by Inskeep and Bloom (1985): Chlorophyll (mg g^{-1}) = 17.90 A_{647} + 8.08 $A_{664.5}$. Immature roots (approx. 1 to 3 mm long) and mature roots (ca. 10 to 12 mm long) were collected for the assays. Five replicates were made for each extract.

RESULTS

Immature Roots

At least five *Spirodela polyrhiza* roots arose from the meristem at a greatly reduced node located beneath the abax-

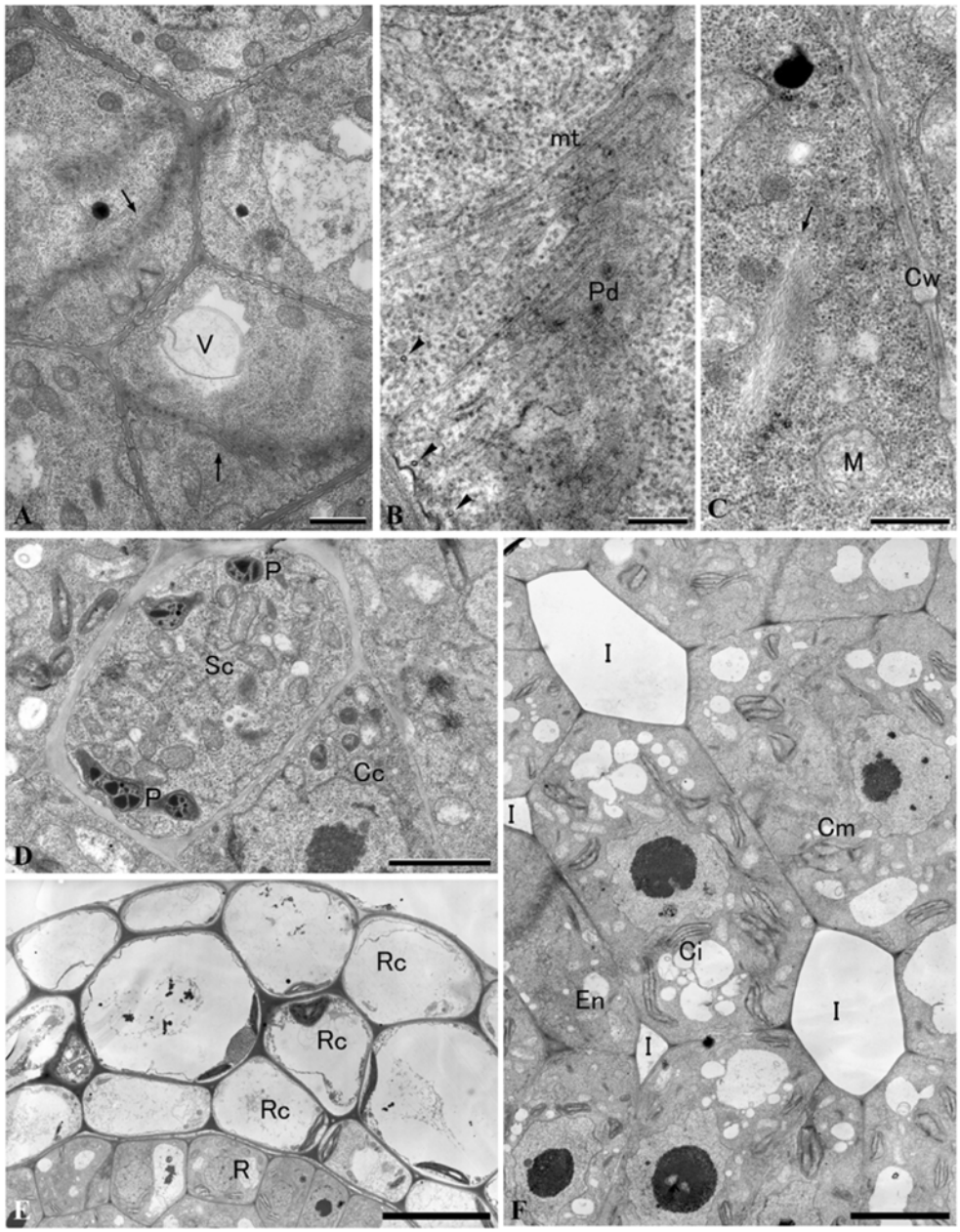

Figure 2. Details of immature root system. **A,** Irregular cell division (arrows) in root apex. Scale bar = 1 μm. **B,** Glancing section showing arrays of microtubules (cross-sectioned, arrowheads) and plasmodesmata. Scale bar = 250 nm. **C,** Bundles of filaments (arrow) found in dense cytoplasm. Scale bar = 500 nm. **D,** Differentiating sieve cell with companion cell. Scale bar = 2 μm. **E,** Three prominent RC layers over root epidermis. Scale bar = 10 μm. **F,** Intercellular spaces formed between inner and middle cortical layers, and between endodermis and inner cortical layer. Scale bar = 5 μm.

ial frond (Fig. 1A). Although a prophyllous sheath, formed at the onset of plant development, initially covered the inconspicuous root primordia, the root system soon elongated through that sheath. Immature roots differentiated rapidly while being protected by the sheath and root cap (Fig. 1B). Young RC cells were somewhat large having chloroplasts with mostly small starch grains (Fig. 1C), and organelles scattered throughout the cytoplasm.

In the root proper, the growing root tip, 40 to 200 μm in diameter, was a very active zone (Fig. 1D). Despite the close attachment of the root-RC junction at initiation, no plasmadesmata were established. That junction loosened over time, and the distance between those two structures eventually reached 0.7 to 0.8 μm. Cross sections revealed several concentric layers of cells surrounding a single central cell in a polygonal pattern (Fig. 1E, F). Intercellular spaces were very limited, showing only six or seven small lacunae at the corners between cortical layers. Irregular cell divisions (Fig. 2A) and a dense cytoplasm with numerous plasmodesmata and microtubules were clearly visible in all sections during this early stage (Fig. 2A and B). Further, bundles of filaments were often scattered in the cytoplasm (Fig. 2C). At this stage, more rapid divisions and cellular differentiation took place in the vascular region, especially in the phloem (Fig. 2D). The RC consisted of three prominent cell layers, where the innermost cells were small with slightly more cytoplasm. In the outer two layers, vacuolization exceeded other features of this cellular construction (Fig. 2E). Chloroplasts were distributed throughout the root from the central cell to the epidermis. Additional intercellular spaces were formed between the inner cortex and the endodermis, while intercellular spaces that were initially formed between the inner and middle cortical layer expanded with time (Fig. 2F). Young roots were thin and, even at maturity, they were usually less than 0.5 mm in diameter. No root hairs developed at the root surface.

Mature Roots

The roots produced by fronds usually numbered 5 to 8 roots at maturity (Fig. 3A). In both transverse and longitudinal sections, the RC, root apical meristem, and mature regions were easily distinguished by their respective cellular characteristics. Approximately 38 to 45 epidermal cells enclosed the 3 distinct cortical layers. In the outer cortex, the parenchyma consisted of about 22 to 25 small cells without intercellular spaces, with 7 to 8 much larger cells being found in the middle layer (Fig. 3B). The inner cortex was also made up of 7 to 8 intermediate-sized cells. Each of the 6 or 7 intercellular spaces was clearly visible between the endodermis and inner cortex, and between the inner and middle cortex. In the latter case, the spaces that initially formed expanded up to 10 to 12 μm during maturation and, occasionally, a small cell with a nucleus and several

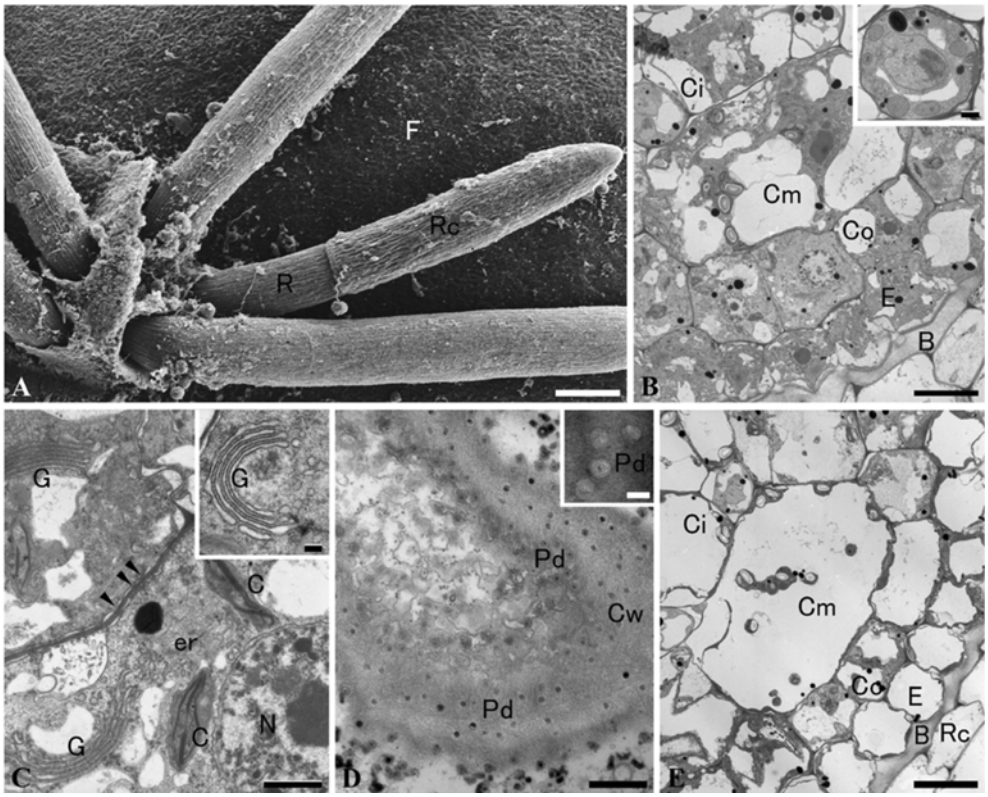

Figure 3. Structural features of mature root system (I). **A**, Five roots arising from abaxial frond surface. SEM. Scale bar = 100 μm. **B,** Transverse section showing epidermis and outer, middle, and inner cortex. Scale bar = 5 μm. Inset: Small cell found in intercellular space. Scale bar = 500 nm. **C,** Portion of cortical cells with large nucleus, Golgi body, chloroplasts, endoplasmic reticulum, and polysomes. Arrowheads indicate plasmodesmata. Scale bar = 1 μm. Inset: Concentrically arranged Gogi bodies studded with ribosomes. Scale bar = 200 nm. **D,** Plasmodesmata found between cortical cells. Scale bar = 0.5 μm. Inset: Close-up of plasmodesmata. Scale bar = 100 nm. **E,** Highly vacuolated root epidermis and cortical cells. Scale bar = 5 μm.

mitochondria filled up the space where it widened (Fig. 3B inset). In the absence of small cells, the air lacuna were narrow, the lacunose cortex represented by a series of longitudinal spaces separated by groups of cells. In general, the cytoplasm of the cortex had a large nucleus and was rich in rough ER, polysomes, mitochondria, and concentric cisternae of Golgi bodies studded with ribosomes (Fig. 3C). Chloroplasts were present throughout all the root cells: those in the cortical parenchyma usually had 3 to 6 thylakoids per granum. In addition to particularly numerous plasmodes-

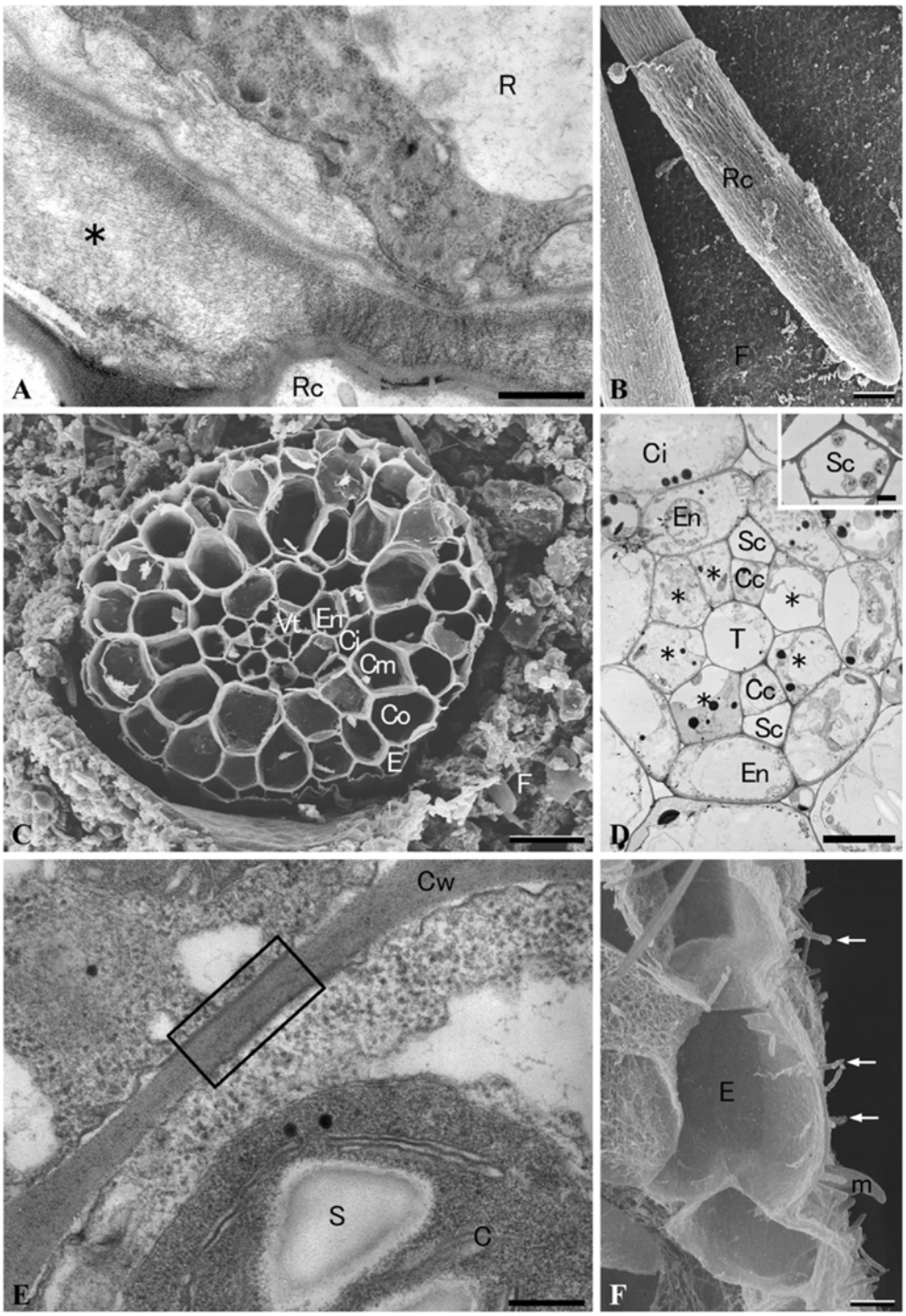

Figure 4. Structural features of mature root system (II). **A,** Boundary layer between root and RC filled with fibrillar materials (asterisks). Scale bar = 500 nm. **B,** Prominent RC with obtuse tip. Scale bar = 50 μm. **C,** Dissected root showing epidermis, cortical layers, endodermis, and central vascular cylinder. SEM. Scale bar = 15 μm. **D,** Vascular tissue of one tracheary element, two sieve elements, and six phloem parenchyma cells (*) enclosed by endodermis. Scale bar = 5 μm. Inset: Fully differentiated sieve cell. Scale bar = 400 nm. **E,** Casparian strip (box) in radial wall of two adjacent endodermal cells. Note the plasmalemma firmly attached to Casparian strip. Scale bar = 500 nm. **F,** External colonization by microorganisms (arrows). SEM. Scale bar = 3 μm.

mata that traversed the cortex to interconnect cells (Fig. 3D), the plasmodesmatal connections were obvious among various root tissues. The cytoplasmic area occupied by vacuoles increased as the root elongated and the apical region matured. Furthermore, there was a progressive decrease in cytoplasmic density along the cortex from the undifferentiated area near the root tip to a differentiated area further away. In the upper regions of the elongated root, enormous vacuoles developed in most of the epidermal and cortical cells (Fig. 3E).

When development was initiated, the root and RC differentiated quite close to one another, leaving very little space without plasmodesmatal connections (Figs. 1D, E, 2E). At maturity, however, the root-RC junction became a prominent boundary layer, ca. 100 to 200 μm, filled with fibrillar materials (Fig. 4A). It was consistent with the upper regions of the RC, most likely allowing the nearly empty root cap to adhere. The tip was obtuse and RC lengths within the same strain but in different fronds varied from 1.2 to 2.1 mm (Fig. 4B). The vascular cylinder that had initiated from a group of cells at the root tip at initiation expanded only slightly in number and size compared with other root areas. At maturity, this cylinder was composed of one xylem cell, a few sieve elements and vascular parenchyma cells (Fig. 4C). The central xylem cell, most likely a tracheid, had unthickened or poorly lignified walls. Two sieve cells, each having a companion cell, contained plastids with electron-opaque, proteinaceous inclusions and smooth ER. Five to six phloem parenchyma cells were situated between the two sieve elements (Fig. 4C and D). The endodermis surrounding the vascular cylinder had rather thin walls, but possessed a Casparian strip in the radial wall (Fig. 4E). No pericycle was detected in the vascular area.

Colonization by numerous bacteria, diatoms, and other small microorganisms was common on submerged regions of the plant body. However, heavy colonization occurred most frequently on the abaxial surface of the mature fronds, where the roots were attached. These colonies appeared to be only external (Fig. 4F), i.e., none of the above microorganisms were detected in any epidermal cells, deeper mesophyll cells, or cortical cells. Almost no colonization was seen at the point of root-RC insertion, and no heavy bacterial colonies were visible in the rhizosphere of immature roots.

Chlorophyll Concentrations

Chlorophyll concentrations were measured to determine whether the immature or the mature roots had more chlorophyll. Although both types exhibited numerous chloroplasts in their ultrastructures, the immature roots appeared to be greener. With chlorophyll contents being variable, the chloroplasts presumably were distributed more widely in the immature roots, based on appearance. However, contrary to common assumption, higher concentrations were measured in the mature roots (content of 0.154 mg g^{-1} ± 0.018 vs. 0.113 mg g^{-1} ± 0.032 for the immature tissues).

DISCUSSION

The structure of the root system in plants of Lemnaceae has been largely speculated to be of relatively simple and undifferentiated organization, because it comprises only single or multiple roots and RC without any branching or root hairs. However, the anatomy and ultrastructure of the root system examined here in *Spirodela polyrhiza* revealed a precise cellular organization. With an unusually reduced morphology, the root followed different pathways to maturation along a carefully defined pattern, producing epidermis, cortex, endodermis, and a stele with xylem, sieve elements, and phloem parenchyma. Little variation in overall root structure has previously been reported for *Lemna minor*, with evidence presented in such features as 1) having cortical layers and an endodermis enclosing the vascular cylinder, 2) a range of cell sizes and numbers within the cortical layers, 3) the presence of intercellular spaces in certain areas of the cortex, and 4) a single vascular strand with a central xylem cell and few sieve elements (Echlin et al., 1979, 1980, 1981, 1982). In contrast, the root structure from *S. polyrhiza* differs in the following characteristics: three layers for the RC, fewer epidermal cells, Casparian strips in the endodermis, and materials filling the root-RC junction. That closely attached root-RC junction at root initiation becomes a distinct boundary layer filled with moderately dense fibrillar materials, unlike that reported by Echlin et al. (1982) for *Lemna minor*, in which a prominent water-filled gap is found between the RC and the root proper.

Initiation of the adventitious roots occurs early at the abaxial frond node (Landolt, 1998; Lemon and Posluszney, 2000), while a prophyllous sheath covers several photosynthetic root primordia. Many root cells contain photosynthetically active chloroplasts. It is expected that photosynthetic rates are much lower in translucent mature roots than in the greener immature roots. Although data on carbon fixation rates in these tissues are not available, their chlorophyll contents do differ. The root, with well-organized chloroplasts and the ability to photosynthesize, seems to utilize a different strategy for fulfilling its organic carbon demand rather than by having fronds with numerous air chambers (Landolt, 1998). Because the photosynthetic ability of a submerged organ strictly depends on the availability of CO_2 entrapped in its intercellular spaces (Rascio et al., 1991), whether this hypothesis can be applied in the same way to the root system of *S. polyrhiza*, where intercellular spaces are quite limited, remains to be elucidated.

As stated above, many root cells contain photosynthetically active chloroplasts, but the functional importance of the root is chiefly as an anchor to keep the fronds right-side-up, to form the tangled masses that possibly aid in dispersals, and as protection against water motion (Hillman, 1961; Noboru, 1990). Here, meristematic activity and differentiation in *S. polyrhiza* occurred synchronously in the vascular regions, while the root system developed more rapidly. Expansion of the cytoplasmic area occupied by vacuoles was distinguished during root development: extreme cases were noted in the RC and root cortical cells. The consequence of a number of underlying control processes has been speculated for these different rates of vacuole development, e.g., in cell types of *Azolla* roots (Barlow et al., 1982). According to Barlow et al. (1982), the fraction of the cytoplasmic area that is occupied by vacuolar profiles

increases as the root elongates and the apical cell age in that species. Here, progressively upward vacuolization in the root cortex of *S. polyrhiza* began in regions enclosed by the RC, then moved to areas up along the elongated root. This process placed more weight toward the apical region, probably playing an important role in retaining a more or less stable center of mass to serve as a pendulum against water motion.

In the vascular tissues of submerged angiosperms, acropetal water transport generally translocates inorganic nutrients from the roots to the fronds (Pedersen and Sand-Jensen, 1993). The concentration of diffusible ions is slightly higher in more actively dividing root tip cells than in the less differentiated tissue of *L. minor* (Echlin et al., 1982). However, the extent of root involvement in nutrient uptake in *S. polyrhiza* is still controversial – both effective and ineffective examples are known. Barnabas and Arnott (1987) have shown that water and ions moves effectively through the xylem in roots. However, little contribution by the roots to nutrient uptake has been demonstrated when inverted fronds continue to multiply as roots develop upward into the air (Meijer and Sutton, 1987). In many *Lemna* and *Spirodela* species, water and nutrient absorption supposedly occur on the abaxial frond surface (Muhonen et al., 1983; Ice and Couch, 1987; Meijer and Sutton, 1987). The numerous plasmodesmata observed here throughout the root most likely demonstrate the symplastic movement of ions and metabolites in the root system and further within the entire reduced body. Compared with the roots of *Lemna minor*, which lack a Casparian strip (Barnabas, 1996), *S. polyrhiza* showed further development of that feature into the endodermal radial walls, blocking the passage of substances through the apoplast. The presence of wall ingrowth reported within a certain time period for the developing fronds of *S. polyrhiza* (IS Kim, unpublished data) can reasonably be correlated with the function of absorption, because wall proliferation increases contact of the surface area with absorbed materials. However, a definite involvement of the fronds in nutrient absorption remains to be clarified because wall ingrowth and plasmalemma proliferation are present only briefly, during early development. Further evidence comes from the nature of the vascular tissue. In *Spirodela* species, poorly developed vascular bundles, usually having a single xylem element isolated from the phloem tissue, are common. Because reduced vascular bundles and poorly developed xylem are characteristics largely associated with submerged aquatics (Sculthorpe, 1967), the xylem is generally regarded to be non-essential because all plant surfaces are in contact with water. The unthickened or poorly lignified walls of a single tracheary element found in *S. polyrhiza* also support this notion.

Aquatic plants take up xenobiotic compounds from the water and bio-transform them in conjunction with the associated microbiota (Federle and Schwab, 1989). As also observed in this study, numerous roots of *Lemna* and *Spirodela* species showed routine colonization by a variety of microorganisms including bacteria, cyanobacteria, and diatoms (Zuberer, 1984). Such colonization by large populations of epiphytic bacteria can be either deleterious or beneficial to the plant. For example, Duong and Tiedje (1985) have reported that cyanobacteria appear to benefit more than the duckweed by using the plant for physical support, protection against direct sunlight, and as a source of carbohydrates and growth factors, although commensalism has been suspected. Nevertheless, senescence is significantly higher in *Lemna* when plants are inoculated with a natural population of bacteria (Underwood and Baker, 1991). One might then conclude that the occurrence of colonization in *S. polyrhiza* is not harmful, because, in the current study no frond senescence or mechanical penetration of the host cell wall by cyanobacteria or bacteria was observed.

Based on the results presented here, the root system of *Spirodela polyrhiza* appears to be rather simple in its morphology, having only elongated roots and an RC without any branches or root hairs. However, its relatively short and thin root system, with a well-organized root and well-suited RC, is a clear indication of its adaptation for life in aquatic environments. The distribution of dense cytoplasm at the root tip, protected by extremely vacuolated RC cells, and a drastically reduced cytoplasm upward along the elongated root might account for the considerable distribution of weight along the roots of the plant body. One can also speculate that these traits enable the root tip to serve as a pendulum against water motion. This likely plays an important role in balancing and maintaining the plant body in a stable, upright position.

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