Developmental Changes in Peanut Root Structure during Root Growth and Root-structure Modification by Nodulation

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INTRODUCTION

The capability of legumes to fix atmospheric dinitrogen (N2) using symbioses with specific bacteria has been a research subject of agricultural and plant biology laboratories for a very long time (e.g. Fred et al., 1939; Caetano-Anollés, 1997). As described in a review by Sinclair (2004), this unique feature of legumes underscores their high value as a component of many cropping systems, and it might become increasingly important for alternative cropping systems in both industrial and developing countries. The capability is one reason for the recently increasing number of studies, using molecular biology techniques, of the symbiotic association between legume roots and their bacterial symbionts (e.g. Gleason et al., 2006; Tirichine et al., 2006). However, in many cases basic information related to the root structure of a given species is incomplete; numerous aspects of the relationship between the host and its symbionts thereby remain unresolved.

One legume species that is studied intensively for its agronomic and food value is the peanut (Arachis hypogaea). Yarbrough (1949) provided fundamental anatomical observations of peanut vegetative organs, including data related to root primary and secondary structure, whilst Allen and Allen (1940) described morphological development of peanut nodules. Structural and functional aspects of legume root-nodule initiation and development were documented in detail by Dart (1975). Important aspects of the transport system in leguminous root nodules were characterized by Pate et al. (1969) using transmission electron microscope, and Sen et al. (1986) and Bal et al. (1989) later made detailed investigations of the ultrastructural characteristics of the host–symbiont interaction of peanut. More recently, structural features of peanut nodule cells have been studied from the aspect of lipid storage and metabolism by Bal (1990), Jayaram and Bal (1991) and Khetmalas and Bal (1997).

Two routes of Rhizobium infection have been described for root-nodule formation in legume roots: entry via root-hairs and via cracks. Root-hair entry occurs in most legumes, e.g. soybean and common bean (Phaseolus vulgaris). Crack entry occurs in few legumes: peanut and Sesbania. In peanut, root nodules develop only at the sites of lateral-root emergence (Uheda et al., 2001), where the epidermis and cortex of the parent root are broken by emergence of the lateral root (reviewed in Boogerd and van Rossum, 1997). Using gus-A-marked Bradyrhizobium,
Uheada et al. (2001) visually demonstrated *Rhizobium* infection into the root through the intercellular gap created by lateral-root emergence.

The nitrogen-fixing activity of individual nodules is affected by oxygen diffusion into the inner part of the nodule and by the partition of photosynthesis from the plant to the nodule through the root. These physiological traits are probably affected by the anatomy of the nodule and by the junction between the root and nodule (Brown and Walsh, 1994). The diffusion barrier, which restricts O₂ flux into the nodule, is of vital importance because it protects nitrogenase from inactivation by oxygen (Sinclair and Goudriaan, 1981). The mechanism of changes in the gas permeability of this barrier remain largely unexplained, but some models present the possible role of structural features such as opened or closed pores in the nodule cortex (Denison, 1992). The function and structure of the nodule surface are important subjects that need to be examined.

In this study, structural differences of various peanut root types and their changes during growth and ontogenesis were characterized. Furthermore, the question was addressed of, in structural terms, the incompletely defined root-nodule development, with special reference to the presence and changes of the root apoplasmic barrier, the endodermis, and to the formation and development of peridermal tissues of the root and of the nodule.

**MATERIALS AND METHODS**

**Plant materials**

*Field cultivation of peanut.* For this study, plants of ‘Chibahananchi’, a leading cultivar of peanut (*Arachis hypogaea* L.) in Japan, were grown using conventional Japanese methods in a Kanto loam (humic Andosol) field at the Field Production Science Center of the University of Tokyo, Tokyo, Japan (35°43′N, 139°32′E) in 2004 and 2006. Peanut seeds were inoculated with *Bradyrhizobium* spp. (J2P21; Tokachi Nokyoren, Hokkaido, Japan) and planted in the field by hand at 5 cm depth in May. The row direction was east–west, with row spacing of 0.7 m and plant spacing of 0.3 m. Chemical fertilizer including N, P, and K was applied as a basal dressing before planting at rates of 15, 50 and 50 kg ha⁻¹, respectively. The plants were harvested at 71 d after seeding (DAS, pod-filling stage) in 2004 and 49 DAS (flowering stage) in 2006. After removing the above-ground parts, the root systems were extracted using a shovel and the roots in the topsoil layer (approx. 30 cm depth) were washed out carefully.

*Vermiculite culture of peanut.* Peanuts were grown in vermiculite (Bumpiece S; Technon) using 1-L plastic pots in a growth chamber at 30/25 °C (day/night) with 12 h daily light of 228 μmol m⁻² s⁻¹. Rolled filter papers (10 cm high) holding a seed near the top were placed upright in a beaker and wetted every other day. Roots were sampled at 5 DAS.

**RESULTS**

**Peanut root structure**

Peanut plants have a typical dicotyledonous root system with a single taproot branched by the formation of first-, second- and third-order lateral roots (Fig. 1).

**Primary root (taproot) structure.** After germination, the seminal root is thick and covered on its surface by a thin peripheral zone (Fig. 2A, B). Yarbrough (1949) has characterized the anatomical structure previously; thus, only a brief summary with some added amendments will be given here. This paper does not specifically address the root apical meristem organization as it is outside the scope of our study.

The basic primary structure is typical of leguminous crop plants, with an epidermis formed by small cells, a broad cortical zone of parenchyma cells arranged radially in its inner part and alternately in its peripheral region, and a broad central cylinder with tetrach organization of the vascular system (Fig. 2A–C). The exodermis, as defined by Peterson et al. (1981), is not developed: no Casparian
FIG. 1. Peanut taproot. (A) Taproot root system of a field-grown peanut at the pod-filling stage. (B) Taproot of a field-grown peanut plant at the flowering stage. (C) Peanut seedling (5 DAS). PR, primary root; 1stb, first-order lateral root formed on the basal part of the primary root; 1std, first-order lateral root formed on the distal part of the primary root; 2nd, second-order lateral root; arrowhead, root nodule. Scale bars: (A, B) ¼ 50 mm; (C) ¼ 10 mm.

FIG. 2. Primary root structures. (A–F) Peanut grown in filter paper, (G, H) field-grown plants. (A–H) Hand sections. (A, B, D, E, G) Observed under white light, (C, F, H) observed under UV light. (A) Cross-section 2 cm from the root apex of a seedling root. (B) Magnified image of (A). (C) Primary structure of a central cylinder of the seedling root with fluorol yellow and toluidine blue O staining. (D) Pericycle adjacent to the phloem pole. (E, F) Pericycle adjacent to the xylem pole with several cell layers. (G) Taproot secondary structure of a field-grown plant in the basal part after toluidine blue staining. (H) Taproot secondary structure of a field-grown plant in the distal part. CB, cork cambium; CO, cortex; EN, endodermis; EP, epidermis; LP, lateral root primordium; PC, pericycle; PHF, phloem fibers; PH, phloem; PP, parenchyma pith; SPH, secondary phloem; SXY, secondary xylem; VCB, vascular cambium; XY, xylem; arrowhead, proto-xylem. Scale bars: (A, B, G, H) ¼ 100 mm; (C–F) ¼ 50 mm.
bands are formed in the tissue internally adjacent to the epidermis. In the young, fast-growing primary root of the seedling, the innermost cortical layer, the endodermis, starts its differentiation approximately 10–15 mm from the root-cap junction. Caspian bands (the first state of endodermal development) are identifiable at this distance. The second state, suberin lamellae formation, starts in these roots at a distance of approx. 50 mm from the root tip, by the deposition of suberin lamellae in the cells adjacent to the phloem poles (Fig. 2C). At 100 mm from the root tip, almost all endodermal cells are in the second stage of their development. This is the final stage of endodermal development in roots of peanut plants.

The stele exterior tissue, the pericycle, is not uniform (Fig. 2D–F). The pericycle adjacent to the xylem poles is 3–5 layered (Fig. 2E, F), whilst the zones adjacent to the phloem poles are 1 (–2) layered (Fig. 2D). The xylem elements of the fast-growing seminal root of the seedling mature close to the apex, at about the same distance where the formation of endodermis starts (approx. 10 mm from the root cap boundary). The first narrow protoxylem elements are followed quickly by lignification of metaxylem elements as the process of xylem development proceeds centripetally (Fig. 2E, F). Phloem poles are extensive, and are accompanied peripherally by groups of sclerified primary phloem fibres that give a positive reaction for lignin (Fig. 2F).

Secondary growth of the taproot starts very early. The first tangential divisions of vascular parenchyma cells that start the formation of cambium cells adjacent to the phloem poles were observed approx. 20 mm from the root tip in young seedlings (Fig. 2D–F). The activity of the vascular cambium later fosters formation of extensive secondary vascular tissues. Close to the root base in particular, the taproot becomes considerably thickened – it is apparent at the flowering stage of the plant (Fig. 1B, G). However, the distal part of the same root remains much thinner with a limited secondary growth (Fig. 2H). The secondary xylem is formed by broad vessel elements: libriform and parenchyma cells (Fig. 2G, H). The original tetrarch organization of the taproot primary structure can be identified clearly from the persistence of four groups of prominent primary phloem fibres (Fig. 2C, H). Cork cambium is initiated soon after formation of the vascular cambium, by the activity of pericycle cells (Fig. 2G, H). Peripheral root layers, epidermis and cortical tissues are subject to dilatation growth for some time. This involves cell extension and limited additional cell divisions of some cortical cells, including those of the endodermis. Epidermal and hypodermal cell walls become slightly lignified and suberized, as indicated by phloroglucinol staining (see Fig. 3G). However, in later stages of secondary growth, all peripheral tissues are destroyed and are gradually shed. Lignified and suberized peridermal tissues formed by the cork cambium protect the root surface. The broad parenchyma pith, which is present during root primary growth, might be preserved even during the flowering stage of the plant (Fig. 2G).

First-order lateral roots. The formation of the first lateral roots starts soon after germination. Well-developed lateral root primordia emerging from the primary root are already present 20 mm from the root tip in young seedlings (Figs 1C and 2A, B). The structure of these roots differs from that of the taproot (Fig. 3A–D); they are thinner, and consequently the cortex and stele are also less extensive than in the primary root (Figs 2B and 3A). These lateral roots are diarch or triarch, with two-to-three xylem and phloem poles; however, their structure differs along the root axis. At the base of the taproot, the first-emerging laterals are thick, but later-emerging ones become thinner and their structure is simpler, with a less extensive cortex (Fig. 1A, ‘1std’). First-order lateral roots formed from the distal part of the taproot exhibit a similar structure and probably serve a similar function to that of higher-order lateral roots (see below).

In the central area of the cortex, some very specific cells are developed with wall ingrowths in the form of regularly shaped, semi-globular structures (Fig. 3C, arrowhead). These structures are also abundant in the second-order and third-order lateral roots (Fig. 3E–G; third-order lateral roots are not shown) and exhibit strong autofluorescence and a positive reaction to lignin (Fig. 3F, G). They are present more-or-less regularly in the central cortex, but never in the layer immediately adjacent to the endodermis (Fig. 3C, F). The wall ingrowths develop in the cell ‘corners’ opposite to the intercellular spaces of internally adjacent cells. X-ray analyses coupled with environmental scanning electron microscopy revealed no specific elemental composition of these ingrowths (data not shown). Therefore, we might conclude that these structures are formed purely by organic materials of the cell walls. The endodermis of the first-order laterals, similar to the primary root, matures close to the root apex at a distance of several millimeters by the formation of Caspian bands. Later, the second stage of endodermal development occurs; at 50 mm from the root tip, almost all endodermal cells are covered by a prominent layer of lamellar suberin (Fig. 3B). The pericycle is two- or three-layered (Fig. 3A).

First-order lateral roots exhibit secondary growth that resembles that of the primary roots. Activity of the vascular cambium is followed by formation of cork cambium of pericycle origin (Fig. 3C). At the initial stage of this process, two layers of lignified and suberized tissues are located centripetally to the primary cortical layers: the peripheral one is the endodermis, the internal one is the first layer derived from the cork cambium (Fig. 3C). The number of layers increases gradually; however, the peripheral zones, including the former endodermal layer, are gradually destroyed and detached. Outer peridermal cells become lignified and suberized, as shown by histochemical reactions. Other events occurring in the cortical layers as a reaction to the secondary thickening closely resemble those of the primary roots.

The epidermis is occasionally double-layered and the size of epidermal cells varies (Fig. 3A, G, H, black arrow). Abundant root hairs, some forked or branched (Fig. 3H, I), were formed when the peanut seedlings were grown in wet filter paper. No root hairs were observed when peanut plants were grown in field cultivation or in vermiculite culture.
Second- and third-order lateral roots. These roots differ from both the previously described types. Differences in their structure, and probably their function, are greater when compared to the difference between the primary and first-order laterals. These roots are, and remain, thin with a diameter of 200 μm. They exhibit no secondary growth at the flowering stage. However, in older plants of 90 DAS, some second-order lateral roots were detected with limited secondary thickening. The second- and third-order lateral roots have simple primary structures. Under an endodermis in second state of development, shown by fluorol yellow staining, (C) Secondary structure. (D) Secondary structure of a central cylinder. (E) Simple structure of a second-order lateral root. (F) Second state of the endodermis against phloem poles, detected with fluorol yellow staining and autofluorescence of the cell-wall ingrowths in cortex. (G) Cell-wall ingrowths in cortex detected with phloroglucinol staining. (H, I) Epidermis with root hairs. CCB, cork cambium; CO, cortex; EN, endodermis; EP, epidermis; PC, pericycle; PH, phloem; RH, root hair; VCB, vascular cambium; XY, xylem; white arrow, Casparian band; black arrow, double-layered epidermis; arrowhead, cell-wall ingrowths. Scale bars: (A–F, H, I) = 100 mm; (G) = 50 mm.
the second-order laterals (Fig. 3E). The endodermis also matures via two states in these roots; however, the second state (suberin lamellae deposition) is restricted to the cells that are adjacent to the phloem poles (Fig. 3F). No endodermis was found with all cells in the second state in the second-order lateral roots of plants at the flowering stage. The stelae of these roots is narrow, with a diarch xylem. The pericycle is uniseriate; xylem and phloem poles are formed by a few conductive elements (Fig. 3E, F). For the third-order laterals, only a single protoxylem and a single metaxylem element exist in each xylem pole (data not shown). A group of phloem fibres is formed even in these roots; the pith is absent in both second- and third-order lateral roots.

Nodule structure and development

Peanut root nodules are of a determinate type. In the root systems both of flowering and pod-filling plants, their development is restricted mostly to first-order lateral roots (Tajima et al., 2006). Occasionally, they occur also on the taproot, or on the second-order laterals. Nodules change their size and structure as they mature. Young nodules are white in cross-section, whereas mature nodules are intensively pink-reddish in colour in their centre due to the presence of leghemoglobin (Fig. 4A, B). Even macroscopically, it is possible to distinguish central, coloured, homogeneous infected areas and a white, semi-transparent peripheral zone (Fig. 4B). Light-brown peridermal tissue is formed on the nodule surface. In later stages, senescing nodules turn greenish in their interior (not shown here).

The development of nodules in this species is connected with the development of lateral roots. The nodule body is formed at the junction of the main and lateral roots by the activity of proliferating cells derived originally from the pericycle, which is most clearly apparent in Fig. 4(C, D). In the cross-section of the first-order lateral root, it is possible to distinguish a dark-stained layer of cork cells derived peripherally from the cork cambium. The remainder of the primary cortical and epidermal tissues, including collapsed endodermal cells, is present on the root surface. These layers are shed later, and the nodule surface is then protected by a thin peridermal layer that is impregnated with lignin and suberin, as demonstrated by histochemical reactions (Fig. 4B).

Detailed observations made at higher magnification support the connection of a nodule vascular bundle with vascular bundles of both parent and lateral roots at their junction (Fig. 4E). The connecting vascular bundle is branched to form nodule vascular bundles (Fig. 4E). Individual nodule bundles are covered and surrounded by one endodermal layer with prominent Casparian bands (Fig. 4F–H, arrow).

DISCUSSION

Structures of various peanut root types

The root structures of crops have been studied and documented intensively by earlier plant anatomists. However, most of our knowledge is based on structural analyses of primary (seminal, pole or prop) roots in dicotyledons, and of both primary seminal and adventitious roots in monocotyledons. Very little is known about the structure of lateral roots, their different types and categories, or their different structure-related functions. Lateral root structures have been analysed in detail, to the best of our knowledge, only for a few cereals. Cereal plants form L-type and S-type lateral roots: the former being thick and long whilst the latter are thin and short (Yamauchi et al., 1987a, b). In rice, the L-type are approx. 150 μm in diameter at the base and the S-type are 50–80 μm. Only L-type lateral roots form higher-order lateral roots. Kawata et al. (1977) observed first-order through to fifth-order lateral roots of rice and reported that thick lateral roots (i.e. L-type) have an anatomical structure resembling that of the primary root, whereas thin lateral roots (i.e. S-type) lack the mid-cortex (i.e. aerenchyma in the case of rice) and late meta-xylems in the central cylinder. Consequently, the epi- dermis, exodermis, aerenchyma, endodermis and central cylinder, with phloem and protoxylem, are commonly present in all primary lateral roots, thick and thin. In other monocotyledons, the disparities between the lateral roots might be different. For maize, they have been classified into four different types based on their thickness and anatomy (Varney et al., 1991).

For dicotyledons, available information on this topic is scarce. In dicotyledonous plants, large lateral roots develop secondary thickening and form a permanent root system structure, although small feeder lateral roots do not develop secondary thickening (Zobel, 1986). Differences between large lateral roots and small feeder lateral roots are insufficient to elucidate their anatomical structures and functions, as inferred already by Zobel (1986). For instance, in a recently published monograph related to soybeans, only five lines of text were given to present knowledge about lateral (branch) roots (Lersten and Carlson, 2004). Similarly, in a structural study of peanut seedlings published by Yarbrough (1949), the characteristics attributed to the lateral roots referred to the fact that their vascular pattern does not correspond to that of the primary root: laterals are diarch and without pith. Fundamentally identical information is included in a monograph about peanuts (Ramanatha Rao and Murty, 1994).

Our study revealed a principal difference between first-order and higher-order lateral roots of peanut plants. The first-order lateral roots, at least most of them, resemble the primary seminal root but have simpler organization because of their smaller diameter and lack of pith. They become secondarily thickened and form the skeleton of the entire root system. In contrast, higher-order laterals are very thin and have a simple structure; we might classify them as ‘feeder roots’ (Zobel, 1986).

An interesting phenomenon is the incomplete development of the second state of the endodermis, suberin lamellae deposition, in these roots. Sections of the endodermis opposite the xylem poles remain with Casparian bands only, even during the flowering stage of the plant. Similar incomplete development of the second state of the endodermis was also observed in thin lateral roots of the shrub...
Karwinskia (Lux et al., 2004). This might be an important feature for uptake and transport processes in these ‘feeder’ roots.

An unusual development of the epidermis in peanut was addressed long ago by Yarbrough (1949), who noted a meristem-like hypodermal region producing a multilayered ‘sloughing zone’ and commonly missing root hairs, except at the root base where ‘tuft hairs’ occur. Exceptional shedding of the epidermal and even outer cortical cells from the root surface in this species was examined recently by Uheda et al. (1997). Apparently, root hairs are formed in this species only under specific conditions in first-order lateral roots, in our case in wet filter paper but not in the soil; they might become branched.

Fig. 4. Root nodule and peanut root modified by nodule formation for plants grown in vermiculite. (A) Cross-section of a nodule and first-order lateral root at low magnification without staining. (B) Cross-section of nodules and first-order lateral root at low magnification with red phloroglucinol staining, showing lignification of the root xylem and peridermal layers of the nodules. (C, D) Spurr-embedded semi-thin sections stained with toluidine blue and basic fuchsin. (C) Cross-section of a first-order lateral root modified by a second-order lateral root and nodule formation. (D) Longitudinal section of a first-order lateral root and nodule formation. (E–G) Technovit-embedded sections stained with toluidine blue. (E) Magnified view of a vascular bundle connection. (F) Peripheral structure of a root nodule. (G) Vascular bundle in peripheral tissue of the root nodule. (H) UV-fluorescent view of a hand-section of a nodule vascular bundle stained by berberin, showing Casparian bands. 1st, first-order lateral root; CCB, cork cambium; CO, cortex; EN, endodermis; IA, Rhizobia-infected area; ND, root nodule; PC, pericycle; PD, peridermal layers of a nodule impregnated with lignin and suberin; PH, phloem; ST, stele; VB1, vascular bundle of the first-order lateral root; VB2, vascular bundle of the second-order lateral root; VBN, vascular bundle of the root nodule; VE, vessel; XY, xylem; arrow, Casparian band. Scale bars: (A, B) = 1 mm; (C–F) = 100 mm; (G, H) = 10 mm.
The presence of cell-wall ingrowths, which we detected in the cortical cells, is another uncommon structural feature of the peanut root. Several types of wall ingrowths that are present in the root cortex were well documented by von Guttenberg (1968). The best known are so-called phi-thickenings, which are found in hypodermal or inner-cortical layers in some species. These cells are widely believed to have a mechanical function in the root, but other opinions have also been advanced (Peterson et al., 1981; Weerdenburg and Peterson, 1983; Soukup et al., 2004; Gerrath et al., 2005). Other types of cell-wall thickening were identified recently in the metal hyperaccumulator species Thlaspi caerulescens, but not in a closely related non-accumulator species, T. arvense (Broadley et al., 2007; Zelko et al., in press). The layer of these cells, localized adjacent to the endodermis, was designated as the peri-endodermal layer. Although the precise function of these cells remains unclear, we speculate that they are somehow related to transport processes in the roots of these exceptional plants. Similarly, in the case of the peanut, the regular development of massive, lignified wall ingrowths opposite the intercellular spaces in some peanut cortical cells might have some other function for the root, aside from its purely mechanical function.

Connection of the vascular bundle between the nodule and roots

Both the vascular bundles of the nodule and the lateral root were connected at the same site of the parent root. Grant and Trese (1996) reported that the peanut lateral roots that formed nodules at the base were shorter than lateral roots without nodules, which was inferred to result from competition for photosynthetic products between nodules and the lateral roots through the junction of the vascular bundles. On the other hand, the vascular bundles of nodules were found to be connected with vascular bundles of parent roots, separately from the vascular bundle of the lateral root in soybean (Ikeda, 1955). Moreover, the branching site of the nodule vascular bundle was closer to the junction with the parent root in peanut than in soybean. This interspecific difference might arise from the different positions of the primary infection of Rhizobium in their respective roots: the outer cortex of the root in soybean, and the pericycle in peanut (Kamata, 1958). Our results confirm that this type of nodule formation originates from the pericycle in peanut. The frequently discussed problem of a ‘common endodermis’ of the nodule and the root (Dart, 1975) might be resolved in this species as follows. The early formed peridermal tissue, resulting from periclinal division of pericycle cells (forming the cork cambium) covers both the root and the nodule surface. The first derivative of the cork cambium forms a layer of suberized cork cells. This layer separates the outer root layers, consisting of the epidermal and primary cortical layers. Their cells die and are gradually shed. A network of thin vascular bundles is formed within the peripheral part of the nodule, each one surrounded by endodermis. The root and nodule surface is covered and protected by a thin peridermal layer.

The peanut nodule is formed nearer to the centre of the parent root than the soybean nodule, with larger overlapping of nodule and root tissues. Consequently, the vascular bundle of the peanut nodule branches near the junction with the root vascular bundle. The short distance between the root and nodule in peanut might engender close ecophysiological interactions between the plant and Rhizobia.

Anatomical structures of non-infected peripheral zones in nodules

Legume nodules are of two types – determinate and indeterminate – viewed in terms of the growing periods of the individual nodules. The determinate nodule is oval, whilst the indeterminate nodule has an axis and is elongated by the meristem at the apical part of the nodule (reviewed in Puppo et al., 2005). The peanut nodules are of determinate type (Stalker, 1997). The non-infected layers regulate oxygen diffusion into the inner part of the nodule, which is a very important feature for nitrogen fixation (Serraj et al., 1999). The thin and simple structure of the non-infected peripheral zones in peanut nodules raises the possibility that oxygen diffusion might easily be affected by environmental stresses, thereby decreasing the nitrogen-fixing capability of peanut plants. However, Sinclair and Serraj (1995) compared several legume crops and determined that the suppression of nitrogen fixation by drought stress is less in peanut than in soybean. Although soybean has three peripheral zones, the peanut nodule, with a thin and simple peripheral zone, might have a specific mechanism for the maintenance of nitrogen fixation, even under external stresses.

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LITERATURE CITED


