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Holoparasitic Rafflesiaceae possess the most reduced endophytes and yet give rise to the world's largest flowers

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• **Background and Aims** Species in the holoparasitic plant family Rafflesiaceae exhibit one of the most highly modified vegetative bodies in flowering plants. Apart from the flower shoot and associated bracts, the parasite is a mycelium-like endophyte living inside their grapevine hosts. This study provides a comprehensive treatment of the endophytic vegetative body for all three genera of Rafflesiaceae (*Rafflesia*, *Rhizanthes* and *Sapria*), and reports on the cytology and development of the endophyte, including its structural connection to the host, shedding light on the poorly understood nature of this symbiosis.
• **Methods** Serial sectioning and staining with non-specific dyes, periodic–Schiff's reagent and aniline blue were employed in order to characterize the structure of the endophyte across a phylogenetically diverse sampling.
• **Key Results** A previously identified difference in the nuclear size between Rafflesiaceae endophytes and their hosts was used to investigate the morphology and development of the endophytic body. The endophytes generally comprise uniseriate filaments oriented radially within the host root. The emergence of the parasite from the host during floral development is arrested in some cases by an apparent host response, but otherwise vegetative growth does not appear to elicit suppression by the host.
• **Conclusions** Rafflesiaceae produce greatly reduced and modified vegetative bodies even when compared with the other holoparasitic angiosperms once grouped with Rafflesiaceae, which possess some vegetative differentiation. Based on previous studies of seeds together with these findings, it is concluded that the endophyte probably develops directly from a proembryo, and not from an embryo proper. Similarly, the flowering shoot arises directly from the undifferentiated endophyte. These filaments produce a protocorm in which a shoot apex originates endogenously by formation of a secondary morphological surface. This degree of modification to the vegetative body is exceptional within angiosperms and warrants additional investigation. Furthermore, the study highlights a mechanical isolation mechanism by which the host may defend itself from the parasite.

Key words: Comparative morphology, endophyte, gigantism, holoparasitism, host–parasite relationship, heterochrony, proembryo, Rafflesiaceae, *Rafflesia*, *Rhizanthes*, *Sapria*, *Tetrastigma*.

INTRODUCTION

Plant parasites have evolved at least 11 times from free-living ancestors (Barkman *et al*., 2007). Eight of these parasitic plant clades consist entirely of holoparasitic species, which are incapable of photosynthesis and thus depend exclusively on their host plants for nutrition (Kuijt, 1969; Heide-Jørgensen, 2008). Among these holoparasites, the endophytic lifestyle is the most extreme. An important distinction between the endophytic holoparasites and the other holoparasites [e.g. dodders (*Cuscuta*, Convolvulaceae), beechdrops (*Epifagus*, Orobanchaceae)] is that although endophytic strands are present in both groups, the non-endophytes retain a shoot external to the host that persists throughout the plant's life cycle. In contrast, all endophytic parasites have vegetative bodies resembling a mycelium that exists in the host stem or root and otherwise emerges from within the host only when they flower (Kuijt, 1969; Heide-Jørgensen, 2008). Like other holoparasites, the endophytic holoparasites are completely dependent on host-derived resources.

Angiosperm holoparasites typically develop a haustorium in lieu of a root system, which attaches the parasite directly to its host for water and nutrient uptake (Kuijt, 1977). Although a primary haustorium facilitates the initial attachment to and invasion of the host during seed germination, this structure does not persist in endophytic parasites (Kuijt, 1969; Heide-Jørgensen, 2008). Instead, in these species the seedling epicotyl dies soon after germination, while the endophyte continues to spread invasively and intrusively through the host, eventually losing contact with the initial site of penetration (Kuijt, 1969). Although the endophyte absorbs nutrients from the host, the endophyte is not a haustorium because it does not connect an external shoot to the host (Heide-Jørgensen, 2008). The endophyte is very cryptic and typically cannot be discerned macroscopically. The most conspicuous part of the parasite is the floral shoot. It

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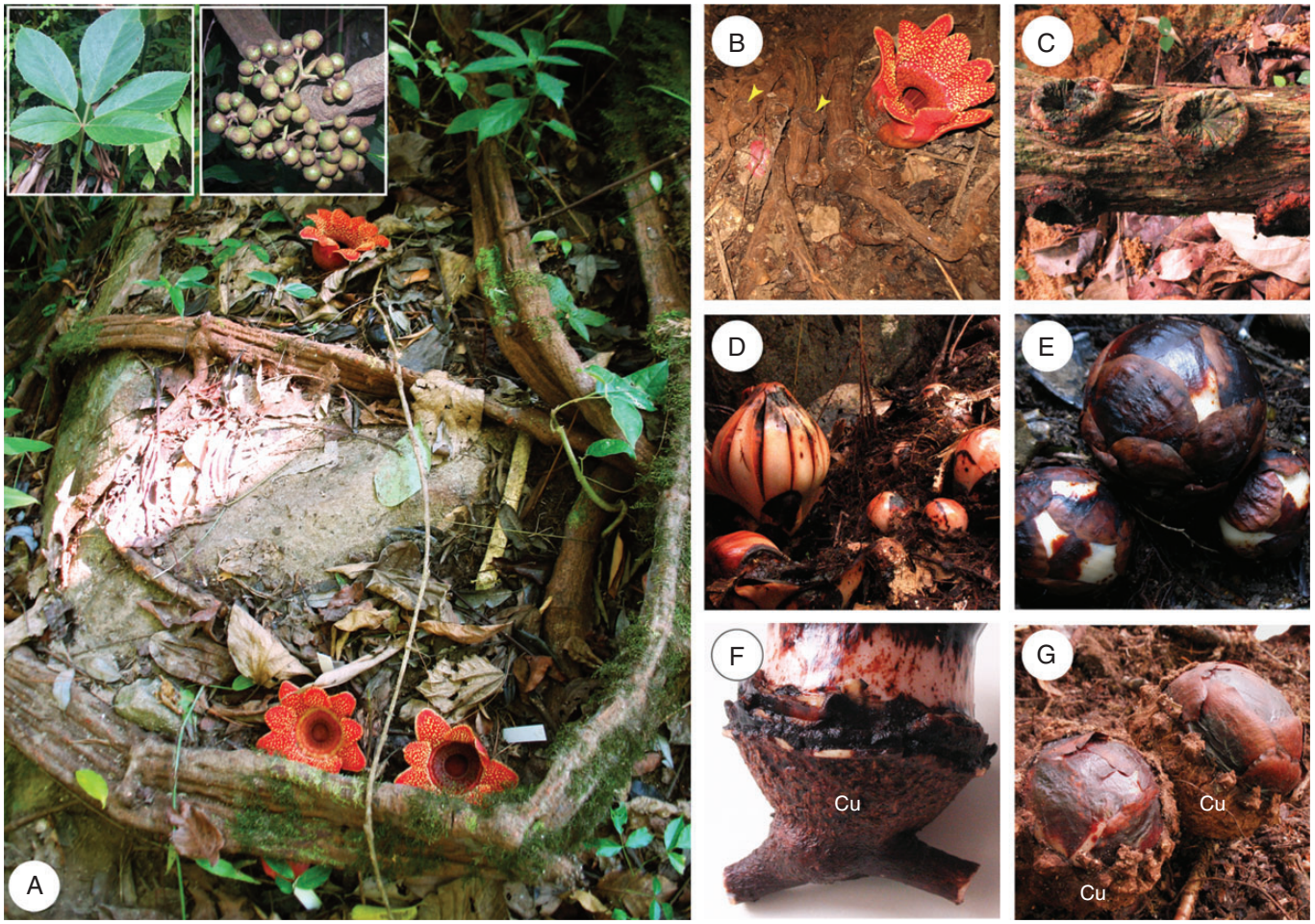

FIG. 1. Macroscopic observations of Rafflesiaceae. (A) *Tetrastigma* vine infected with *Sapria himalayana*. Three open flowers are visible. The insets in the upper left corner depict a leaf and fruits from the host. (B) *Sapria himalayana* cluster with one open flower, emerging buds, and scars from previous flowerings (yellow arrowheads). (C) Deep scars on the host from previous flowerings of *Rafflesia cantleyi*. (D) A cluster of *Rhizanthes lowii* floral buds in different stages of development. (E) A cluster of *Rafflesia cantleyi* buds. (F) Conical cupule (Cu) of *Rhizanthes lowii* (bracts removed). (G) Globose cupules (Cu) of *Rafflesia tuan-mudae*.

emerges from the host and is connected to the host vasculature via a chimeric structure, which is partly produced by the host as a wound response caused by the burst of the parasite buds, and partly by the parasite itself (Fig. 1). This chimeric structure is sometimes referred to as the cupule (Kuijt, 1969, 1977), and it serves to support the robust floral shoot at its base.

The majority of the endophytic angiosperms have traditionally been included within the Rafflesiales (Cronquist, 1988; Takhtajan, 1997). This order has generally been divided into four families, with some differences of opinion on the taxonomic ranks of these divisions. Those species with large solitary flowers (including the species with the world's largest flowers, *Rafflesia arnoldii* Brown), unisulcate or uniporate pollen grains and anatropous ovules have been placed into the family Rafflesiaceae *sensu stricto* (*s.s.*; *Rafflesia*, *Rhizanthes* and *Sapria*) (Takhtajan *et al*, 1985; Cronquist, 1988; Takhtajan, 1997). In contrast, Cytinaceae [*Cytinus*, *Bdallophyton* and a recently described genus, *Sanguisuga* (Fernández-Alonso and Cuadros-Villalobos, 2012)] are characterized by intermediate-sized flowers organized in inflorescences, diporate to multiaperturate pollen and orthotropous ovules (Takhtajan *et al*., 1985; Cronquist, 1988; Takhtajan, 1997). The third family, Apodanthaceae (*Apodanthes*, *Pilostyles* and a third genus sometimes recognized, *Berlinianche*), have small solitary flowers, triporate and atectate pollen, and anatropous ovules (Takhtajan *et al*., 1985; Cronquist, 1988; Takhtajan, 1997). Finally, the monogeneric Mitrastemonaceae have small flowers with superior ovaries, dicolpate pollen and anatropous ovules (Takhtajan *et al*., 1985; Cronquist, 1988; Takhtajan, 1997). Endophytism is also found in some Santalalean species that have long been recognized as separate from Rafflesiales, such as *Tristerix aphyllus* Tiegh. ex Barlow & Wiens, *Viscum minimum* Harv. and *Arceuthobium douglasii* Engelm. (Kuijt, 1969; Heide-Jørgensen, 2008).

Recent phylogenetic analyses have shown that the four families in the former Rafflesiales are distantly related (Nickrent *et al*., 2004; Barkman *et al*., 2007). Rafflesiaceae *s.s.*, which are the focus of our study, are placed in the rosid order Malpighiales (Barkman *et al*., 2004; Davis and Wurdack, 2004); Cytinaceae, in Malvales (Nickrent *et al*., 2004); Mitrastemonaceae, in Ericales (Barkman *et al*., 2004; Nickrent *et al*., 2004); and Apodanthaceae, in Cucurbitales (Filipowicz and Renner, 2010). An alternative placement of Apodanthaceae in Malvales has also been hypothesized based on morphology (Blarer *et al*., 2004). Thus, the endophytic habit has evolved independently

several times in angiosperms (Barkman *et al*., 2004; Nickrent *et al*., 2004).

Much effort has been invested in the study of angiosperm parasites because of the economic detriment caused by some crop pests (e.g., *Striga* spp.), particularly in developing countries (Parker and Riches, 1993; Heide-Jørgensen, 2008; Westwood *et al*., 2010; Parker, 2012). However, the endophytic plant parasites have received far less attention because of their cryptic vegetative nature. A better understanding of these morphological extremes may provide greater insight into the way in which parasites establish and maintain connection with their hosts. For Rafflesiaceae especially, this connection is very intimate and the endophyte is well integrated into the tissues of their hosts, lianas in the genus *Tetrastigma* (Vitaceae). The structure of the endophyte in Rafflesiaceae remains poorly studied even though their exceptionally large flowers have attracted a great deal of attention (Barkman *et al*., 2004; Davis *et al*., 2007; Nikolov *et al*, 2013, 2014). Isolated observations of the endophytic stage of Rafflesiaceae are reported from several species (e.g. Schaar, 1898, for *Rafflesia rochussenii*; Brown, 1912, for *Rafflesia manillana*; Cartellieri, 1926, for *Rhizanthes zippelii*; Stirling, 1939, for *Sapria himalayana*), but a parallel comparative structural study of all three genera in the family has never been performed. Here, we build on these initial observations to provide a more comprehensive treatment of the vegetative body and cytology of the endophyte in all three genera of Rafflesiaceae. We also describe the anatomy of the *Tetrastigma* host roots and the structural changes that occur as the parasite floral shoot emerges.

MATERIALS AND METHODS

Plant material

Roots of infected host *Tetrastigma* vines were collected in the field from Gunung Puey, Sarawak (*Rafflesia tuan-mudae* Becc.); Kampung Giam, Sarawak (*Rhizanthes lowii* (Becc.) Harms); Ulu Geroh, Peninsular Malaysia (*Rafflesia cantleyi* Solms-Laubach); and Queen Sirikit Botanic Garden, Thailand (*Sapria himalayana* Griffith). The taxonomy of *Tetrastigma* is notoriously difficult, and host species could not be reliably identified. Based on the literature (Nais, 2001; Veldkamp, 2008), however, the likely identity of the *Rafflesia* host is *Tetrastigma rafflesiae* Miq., the *Rhizanthes* host is *T. tuberculatum* (Blume) Latiff and the *Sapria* host is *T. cruciatum* Craib & Gagnep. Plant material was preserved in formalin–acetic acid (FAA), and transferred to 70 % ethanol for long-term storage. Vouchers are deposited at the Harvard University Herbaria (A).

Histology

Specimens were embedded in Kulzer's Technovit (2-hydroethyl methacrylate) for serial microtome sections (Igersheim and Cichocki, 1996). A stepwise infiltration was conducted with 50:50, 25:75 and 0:100 ratios of 100 % ethanol to Technovit solution. Embedded material was sectioned using a Microm HM 355 Rotary microtome with a conventional knife D. The mostly 7 μm thick consecutive sections were stained with ruthenium red and toluidine blue, and mounted in Histomount (Invirogen). In addition, histochemical staining was performed with aniline blue for callose, and periodic–Shiff's reagent (PAS) for carbohydrates. Permanent slides of the microtome sections are deposited at the Harvard University Herbaria (A).

RESULTS

Macroscopic observation of the infection

The architecture of the *Tetrastigma* host is complex and can be best described as a liana that alternates between the ground and the high canopy. In this respect, field identification of a single host individual is difficult. The liana produces profuse stem-derived adventitious roots (herein referred to as roots) that grow laterally as runners on the forest floor, which is the most common site of Rafflesiaceae infection (Fig. 1A). An infected liana can be recognized by evidence of flowering, including scars of past flowering events on the roots or, rarely (*Rafflesia cantleyi* Solms-Laubach), on the aerial shoots of their vine hosts (Fig. 1B, C). A single infected liana produces parasite flowers for many consecutive years, often forming clusters of buds at different stages of development along with open flowers (Fig. 1D, E). This consistent blooming from a single liana suggests a chronic infection in which the parasite resides vegetatively inside the host, presumably for an extended period of time. Based on the host's external appearance, the presence of the parasite does not have an obvious effect on the health of the host, especially in its vegetative stage.

The Rafflesiaceae floral shoots originate within the host and eventually rupture the host bark. This process results in the formation of a collar-like cupule, which encircles the base of the flower (Fig. 1F, G). The cupule is covered by host-derived periderm, forming a rhytidome in the *Rafflesia* host, with secondary xylem incorporated at its base. The cupule is morphologically variable across the family. In *Rafflesia*, it is massive, cup-shaped to globose, with pronounced polygonal cracks of the exfoliating host bark (Fig. 1G). In *Sapria* and *Rhizanthes*, it is more slender, obconical to cylindrical, and non-exfoliating and lenticellate on the surface (Fig. 1F). The base of the cupule forms a deep scar in the host root or stem after floral senescence (Fig. 1B, C). Buds of the parasite (*Rhizanthes*) have been observed on roots as thin as 2 mm, although it is unlikely that these buds advance to flowering. The roots that give rise to the majority of the buds we investigated are usually much larger (up to 30 cm in diameter), especially in *Rafflesia* and *Sapria*. The host of *Sapria* in particular has ribbon-like roots, which are up to 40 cm wide. Floral buds in *Sapria* are occasionally formed under the soil surface before they emerge above-ground, and excavation of the host root was necessary to examine early-stage buds. This is in contrast to the aerial or shallower buds in *Rafflesia* and *Rhizanthes* (Fig. 1B–E, G).

Anatomy of the Tetrastigma *host roots*

Tetrastigma host roots have the typical anatomy of an eudicot liana root with secondary growth (Fig. 2A; Supplementary Data Fig. S1). The *Tetrastigma* host root of *Rhizanthes lowii* has mostly tetrarch primary xylem without pith. The secondary xylem is paratracheal and composed of mostly solitary, wide vessels, closely associated with much narrower vessels and xylem parenchyma (Fig. 2B). Perforations of the wide vessels are usually scalariform. Thin-walled tyloses are present

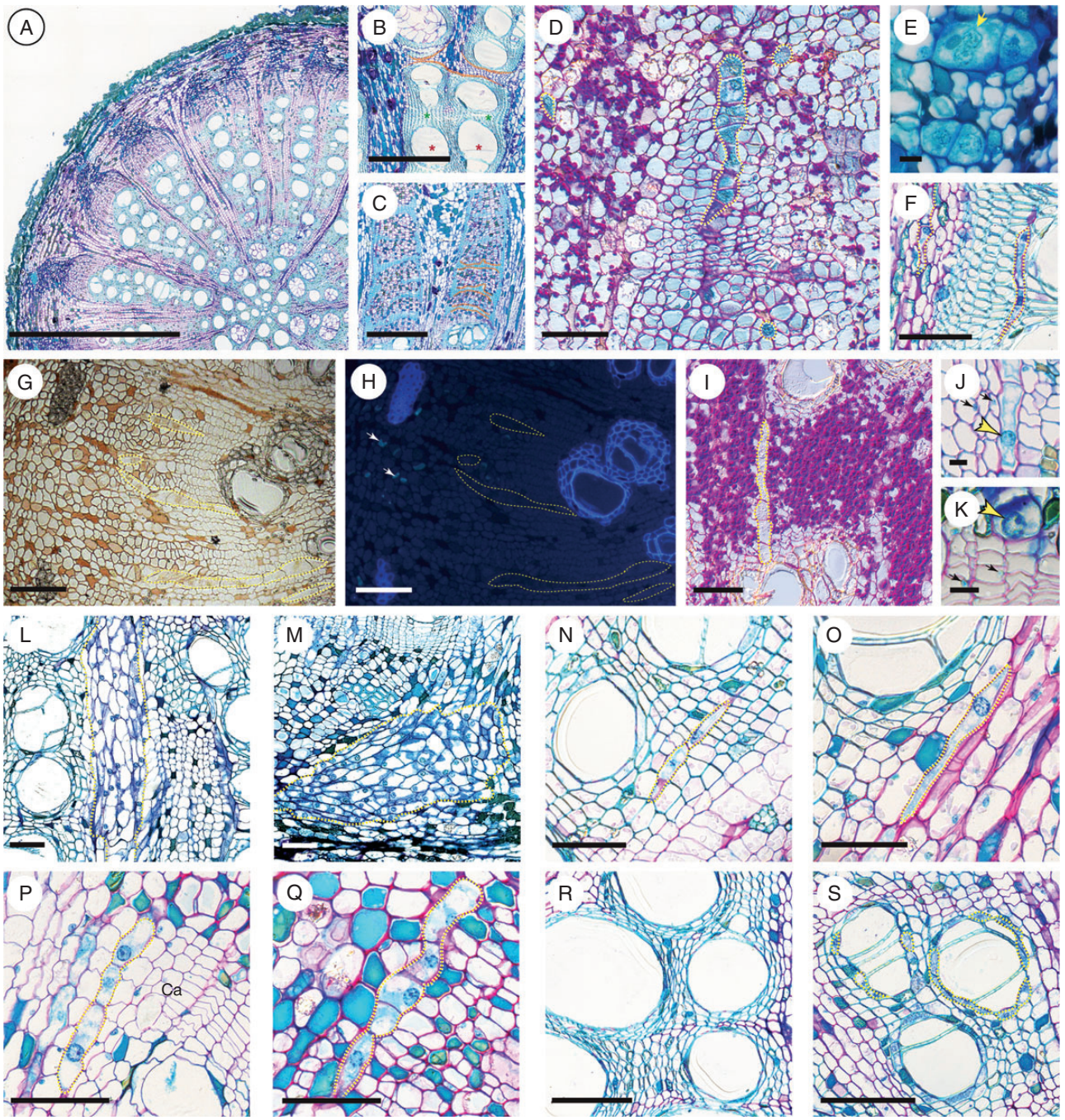

FIG. 2. Micrographs of the Rafflesiaceae host infection, stained with ruthenium red and toluidine blue (similarly in all histological sections unless otherwise noted). Endophyte cells are always marked with dotted yellow lines. (A) Transverse section of the root of the *Tetrastigma* species host of *Rhizanthes lowii*. (B) Xylem of the *Tetrastigma* host of *Rafflesia cantleyi*, depicting vessel dimorphism with large vessels (red asterisks) and small vessels (green asterisks), and parenchyma septa (dotted orange lines) connecting adjacent rays. (C) Secondary phloem of the *Tetrastigma* host of *Rafflesia cantleyi* with dilatating parenchyma rays and sclerenchyma septa (dotted orange lines). (D) *Rhizanthes lowii* endophytes in the host phloem; abundant starch grains are present in the host rays (PAS/toluidine blue contrast staining). (E) *Rhizanthes* endophyte cells with large nuclei, single nucleoli and proteinaceous inclusions; one of the cells is binucleate (yellow arrow). (F) *Rafflesia cantleyi* endophyte inside the host xylem; the cell walls of the parasite stain distinctly red. (G) Bright field image of unstained *Tetrastigma* root with *Rhizanthes* endophytic strands. (H) Fluorescent micrograph of the same area as (G), treated with aniline blue to stain the callose deposits in the sieve elements of the phloem (arrows); callose deposition is not detected from around the endophyte. (I) *Rhizanthes* endophyte strand in the tightly packed with starch xylem parenchyma rays of the host (PAS staining). (J) Difference in the nuclear size of an endophyte cell of *Rhizanthes lowii* (yellow arrow) and the phloem parenchyma cells of its *Tetrastigma* host (black arrows). (K) Difference in the nuclear size of an endophyte cell of *Sapria himalayana* (yellow arrow) and cambium cells of its *Tetrastigma* host (black arrows). (L) Multiseriate endophytic strand of *Rhizanthes* in the xylem ray of the host. (M) A cluster of endophyte cells of *Rhizanthes* preceding protocorm formation in the phloem of the host. (N) *Rhizanthes* endophyte among the small vessels of the host. (O) *Rhizanthes* endophyte strand at the border of a xylem parenchyma ray and the axial vessel elements. (P) *Rhizanthes* endophyte traversing the cambium (Ca) of the host. (Q) *Rhizanthes* endophyte in the phloem of the host. (R) Uninfected vessel elements from the *Tetrastigma* host of *Rhizanthes*; compare relative to (N), (O) and (S). (S). *Rhizanthes* ring-like structures in transverse section, surrounding large vessels of the *Tetrastigma* host. Scale bars: A (2 mm); B, C, R, S (500 μm); D, F, G, H, I, L, M, N, O, P, Q (100 μm); E, J, K (20 μm).

especially towards the centre of the root (Fig. 2A). The radial xylem rays are multiseriate, 4–10 cell layers wide, and are heavily loaded with starch. The xylem rays of the *Tetrastigma* hosts of *Rafflesia* and *Sapria* possess mucilage cells (Fig. 2B). The rays do not always reach the centre of the root but are always dilatated at the phloem. Tangential bands of parenchyma cells traverse the axial vascular system, sometimes connecting neighbouring rays, especially in the *Rafflesia* host (Fig. 2B). The cambial zone is a well-defined area several cell layers wide. The position of the obliterated primary phloem is identifiable by a band of primary phloem fibres (Supplementary Data Fig. S1A, B). Tangential groups of thick-walled, lignified secondary phloem fibres alternate with unlignified sieve tubes and parenchyma cells in the secondary phloem of the *Rafflesia* and *Sapria* hosts (Fig. 2C; Supplementary Data Fig. S1B, C). Occasional islands of sclerenchyma cells are observed in the periderm (Supplementary Data Fig. S1A). The phelloderm and the dilatated phloem rays harbour numerous raphide-containing mucilage idioblasts, tanniniferous cells and druse-containing cells (Supplementary Data Fig. S1D). Rhytidome is present in the host of *Rafflesia* but it is not apparent in the roots of the *Rhizanthes* and *Sapria* hosts, where the phellogen appears to originate from cells in the outer cortex.

Cytology of the endophyte

Based on our previous studies of early parasite bud development (Nikolov *et al.*, 2014), we were able to confirm and expand on earlier observations of cell characteristics, which describe the endophyte of Rafflesiaceae as rows of cells with nuclei larger in size in comparison with the nuclei of the host (Schaar, 1898; Brown, 1912). The endophyte cells exhibit thin cell walls and dense cytoplasm, and are quite inconspicuous and well integrated in the root structure of *Tetrastigma* (Fig. 2D). The endophyte cells are most easily recognized by the large size of their ovoid nuclei, which are comparable among genera and of average diameter 18 μm (s.d. ± 3 μm) for *Rhizanthes*, 18·5 μm (s.d. ± 2 μm) for *Rafflesia* and 16·5 μm (s.d. ± 3·5 μm) for *Sapria* (Fig. 2J, K). These dimensions greatly exceed the size of the host's ellipsoidal nuclei (cell nuclei sizes of all measured Rafflesiaceae hosts are comparable, having an average length of 6·25 ± 0·7 μm, $n = 20$). Such a difference in size translates into an order of magnitude difference of the volumes of the host and the parasite nuclei. The endophytes grow vegetatively as uniseriate strands interspersed among the host tissue (Fig. 2D, E) and, during transition to flowering – as small clusters or multiseriate strands (Fig. 2P, Q). The endophyte cells towards the periphery of the host xylem tend to be anisotropically elongated but are more isodiametric in the host phloem. The cell walls stain distinctly red with ruthenium red (Fig. 2F). However, we did not detect significant callose depositions by the host around the endophyte cells after aniline blue staining (Fig. 2G, H). The cell walls are uniformly thin and do not exhibit the invaginations or sculptural elaborations increasing the surface area of transfer cells found in other parasites (e.g. *Cytinus*, De Vega *et al.*, 2007). Endophyte cells have dense cytoplasm, and often one to several small vacuoles, the largest reaching the size of the nucleus. Some cells accumulate large proteinaceous inclusions, but starch accumulation has not been observed (Fig. 2E, I). The nucleus of each endophyte cell is located more or less centrally, in contrast to the parietal nuclei of the host. In addition, binucleate endophyte cells are occasionally present (Fig. 2E). We could not identify morphological characters that distinguish the three genera, which is not surprising given the extreme reduction of the vegetative body.

Distribution of the endophyte within the host

Based on the parasite distribution in consecutive transverse sections, Rafflesiaceae endophytes appear to form uniseriate strands that are oriented in an approximately radial direction within the root (Fig. 2G, H). No differentiation of cells is observed in different regions of the host – the cytology of endophyte cells in a strand is quite uniform throughout. Mitotic figures were not observed in the endophyte. However, the orientations of the cell walls of neighbouring endophyte cells in a single transverse section, and the presence of the same radially oriented strand in only a few adjacent consecutive transverse sections, suggest that they grow primarily by anticlinal divisions (i.e. division plates form perpendicular to the axis of the strand; Fig. 2P, Q). However, periclinal divisions (i.e. division plates forming parallel to the axis of the strand) may also occur to generate the small clumps of parasite cells occasionally observed toward the host bark that may give rise to protocorms and subsequently flowering shoots (Figs 2L, M and 4C). Branched strands in transverse sections and independent strands that appear to coalesce into a single strand over a series of adjacent transverse sections were rarely observed. All of these patterns seem to suggest that the body of the parasite is largely fragmented, due either to multiple infections or to subsequent cell separation after infection by a single seed. The endophyte appears equally distributed throughout the xylem (both vessel elements and rays) and phloem, often traversing the cambium (Fig. 2N–Q). The parasite tends to occur at the border of rays and tracheary elements (Fig. 2O). The endophyte appears capable of growing intrusively, as shown by ring-like formations surrounding developed vessel elements in transverse sections, where the endophyte has inserted itself between mature host tissues (Fig. 2R, S). The intercalation of endophyte strands between host sclerenchyma fibres supports this conclusion (Supplementary Data Fig. S1E).

Shoot formation and host response

The strands that succeed in forming an incipient floral shoot, known as a protocorm, most often appear to originate from endophyte strands located in the parenchyma rays of the xylem (Figs 3A and 4). Dilatation of the uniseriate endophyte strands by periclinal divisions (much like dilatation of the host rays themselves) and subsequent distortion of the host anatomy usually begins in the xylem, before traversing the cambium, and becomes more pronounced towards the bark. The cells of the incipient shoot are undifferentiated early on. Some differentiation is observed at later stages [i.e. parenchymatization, vascular elements with spiral cell wall thickenings in the periphery of the shoot (Nikolov *et al.*, 2014)]. Mitotic figures are commonly observed in the expanding protocorm and cormus, suggesting an increased rate of cell division as well as a greater variety in cell division orientation during this stage.

Observations in the field have suggested that Rafflesiaceae buds exhibit a high rate of mortality due to resource limitation,

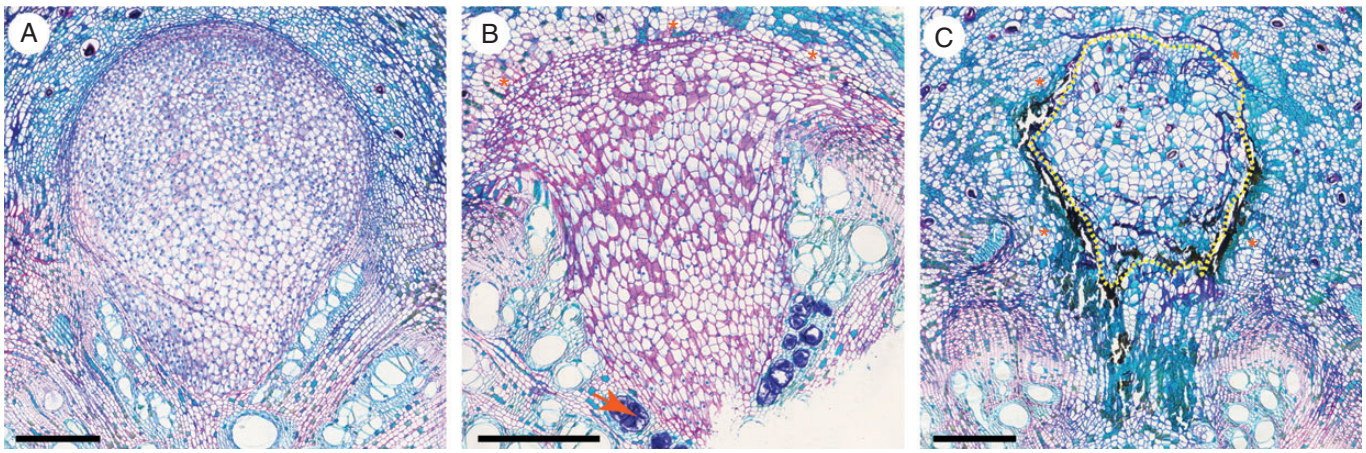

FIG. 3. Reaction of the host during the emergence of the protocorm of *Rhizanthes lowii*. (A) A healthy, tear-shaped protocorm (adapted from Nikolov *et al*., 2014). (B) The broad front of the crescent-shaped protocorm is blocked by a phellogen-like layer from the host (orange asterisks). Mucilage plugs block the vessels near the base of the protocorm (yellow arrow). (C) Dead protocorm (dotted yellow outline) completely isolated by the host phellogen-like layer (orange asterisks). All scale bars are 500 μm.

herbivory and pathogen attack (Nais, 2001; L. A. Nikolov, pers. obs.). Floral mortality before the bud has emerged from the host, however, has not been explored, except for observations by Brown (1912), who reported the isolation of floral buds while still inside the host by host-derived cork tissue. Healthy protocorms are compact, tear-shaped and have smooth, semi-circular front and round flanks (Figs 3A and 4) It appears that in such cases the parasite does not elicit any obvious defence response from the host (e.g. inducing tylose formation, cell wall thickening or cork formation). The only structural reaction by the host appears to be mechanical distortion due to expansion of the endophyte. In other cases, however, the host reacts by clogging its own vessel elements with mucilage near the base of the protocorm (Fig. 3B). Concomitantly, host parenchyma near the front of the protocorm de-differentiates to form phellogen-like layers, with cells that divide periclinally into strict files (Fig. 3B, C). The parasite seems to respond by increasing lateral growth at the flanks (Fig. 3B), with the protocorm transforming from semi-circular to crescent-shaped at the front, and from tear-shaped to bell-shaped overall. Cytologically, the nuclei of the protocorm cells shrink, and become pycnotic. At later stages, host phellogen surrounds the whole protocorm and the host cell layers adjacent to the parasite differentiate into phellem (cork tissue), which isolates and blocks the parasite from the host resources (Fig. 3C). This appears to result in parasite cell death (Fig. 3C). The parasite collapses and its space is subsequently occupied by cells of the host.

DISCUSSION

Reconstruction of Rafflesiaceae endophyte development

The highly reduced endophyte of Rafflesiaceae consists of uniseriate filaments. There is no differentiation at either the cell or the tissue level – all cells have dense cytoplasm and no apparent structural elaborations of the cell wall. The growth of the filaments is presumably slow, once the endophyte is established, because no mitotic figures were observed. In contrast, mitotic divisions are common in the precursor of the flower shoot, the protocorm, whose cells then expand rapidly from a noticeable bump on the surface of the host to an open flower. This takes place in 9–16 months in *Rafflesia arnoldii* Brown, which possesses the largest floral diameter in the family (Meijer, 1997). The endophyte cells are cytologically quite uniform and resemble the undifferentiated proembryo cells in the seed of Rafflesiaceae (i.e. high nuclear–cytoplasmic ratio and small vacuoles), where a true embryo does not develop (Fig. 4; Solms-Laubach, 1874*a*, 1898; Ernst and Schmid, 1913). The similarity between endophyte and proembryonic cells was also recognized by Brown (1912). The angiosperm proembryo is the immediate post-zygotic stage of the sporophyte that precedes embryo differentiation and, therefore, lacks discernible features such as plumule, radicle or hypocotyl (Kawashima and Goldberg, 2009). Thus, since the proembryo does not appear to develop into an embryo in Rafflesiaceae, and although germination and host invasion have proven difficult to study, it is likely that the undifferentiated proembryo gives rise directly to the endophytic vegetative stage. Interestingly, there is some precedent for prolonged proembryonic growth in taxa such as *Tropaeolum* spp. and *Sedum acre* L. where the proembryo or undifferentiated suspensor produces elaborate haustorial outgrowths, which invade neighbouring tissue (Yeung and Meinke, 1993). The Rafflesiaceae endophyte subsequently gives rise directly to reproductive protocorms, in which a shoot differentiates after exposure of a secondary morphological surface (Fig. 4; Nikolov *et al*., 2014).

Based on these observations, we hypothesize that the only analogue of the filamentous and undifferentiated Rafflesiaceae endophyte in the developmental trajectory of other angiosperms is the proembryonic stage. This hypothesis suggests that the vegetative stage of Rafflesiaceae exhibits a prolonged period of proembryonic growth, after which it advances directly to protocorm and flower shoot formation. If this hypothesis were supported, the condition in Rafflesiaceae would reflect two novel heterochronic shifts. The first is the arrest in the proembryonic stage (i.e. protracted juvenilism) of the putative vegetative stage, which can be considered an example of neoteny (Box and Glover, 2010). The second is the accelerated advancement of the undifferentiated endophyte to sexual maturity (flowering) by a loss of the typical

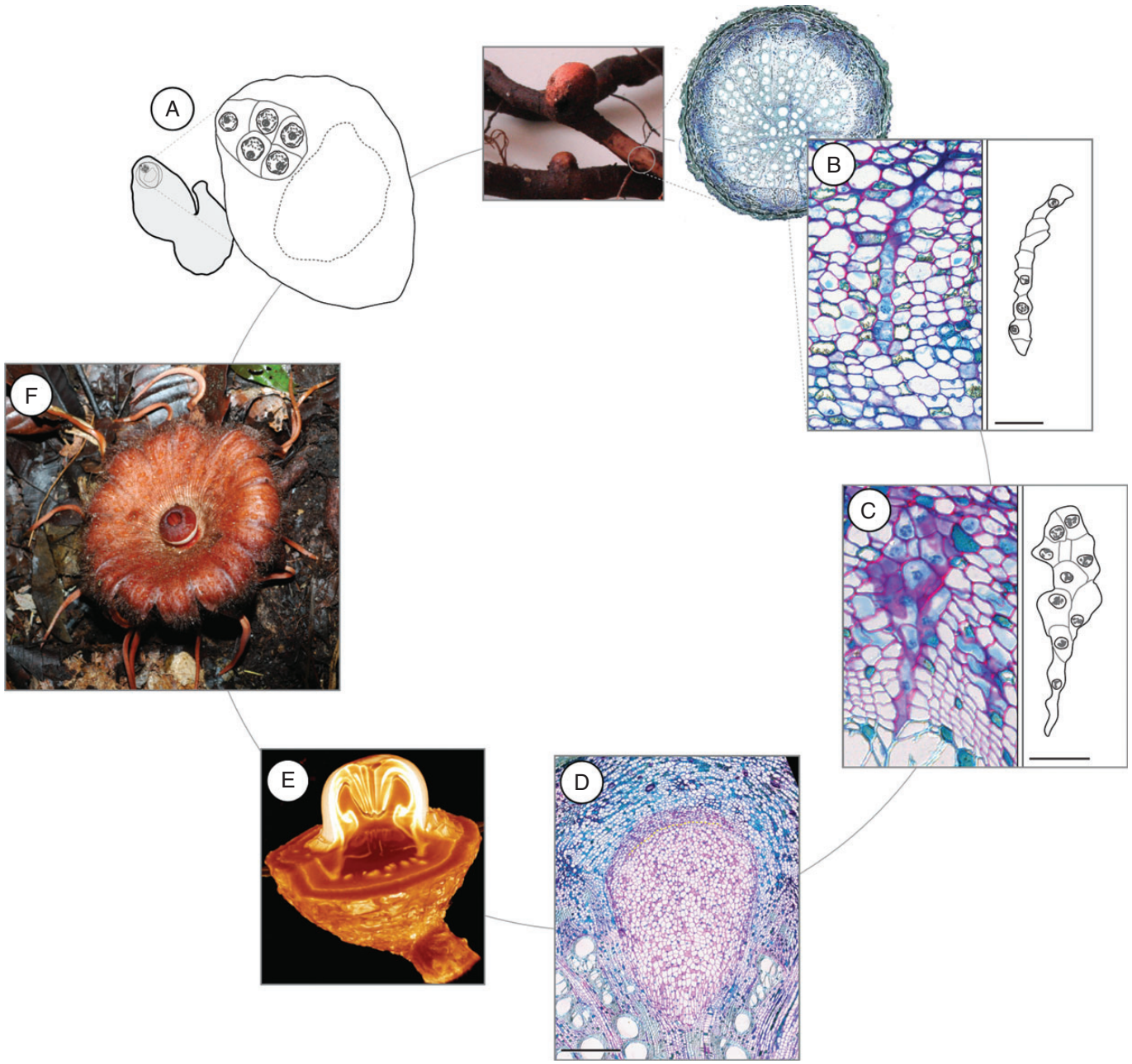

FIG. 4. Development of Rafflesiaceae. All, except (A), depict *Rhizanthes lowii*. (A) Section of a seed of *Rafflesia patma*, showing a young sporophyte (proembryo) composed of two tiers of cells above a single cell (modified from Ernst and Schmid, 1913). (B) *Tetrastigma* host root external appearance and transverse section with a uniseriate filament, the predominant vegetative form. (C) Initiation of protocorm formation by periclinal divisions to give rise to a multiseriate filament. (D) The protocorm is transformed into a cormus by the separation of a sheet of tissue (dotted yellow line) at the front of the growing tear-shaped body. (E) Young floral shoot emerging from the host with completely differentiated floral organs. (F) Anthetic plant. Scale bars: B, C (100 μm); D (500 μm).

vegetative shoot maturation, which is technically vestigial in nature. This sort of heterochronic shift might be a consequence of the transition to parasitism in the early ancestors of Rafflesiaceae, perhaps protecting the endophyte from detection by the host and allowing for the expenditure of resources only at the latest stage of reproductive development (see also below). Obviously, angiosperm development normally does not include a stage of prolonged filamentous growth, and better understanding of the homology of such a form would require analysis of various early-stage proembryonic markers. We appreciate, however, that the endophyte is a completely unique growth form exhibiting extreme morphological reduction, which makes assessing its correspondence to normally observed seed plant structures challenging.

Comparison of Rafflesiaceae s.s. *with other endophytic angiosperms*

In contrast to the largely uniseriate and undifferentiated body of Rafflesiaceae, the endophytic system of the Mediterranean

Cytinus (Cytinaceae), which parasitize Cistaceae, forms clumps of cells in the host phloem and towards the periphery of the host xylem. These clumps expand laterally to produce continuous sheaths of parasitic tissue at later stages (De Vega *et al*., 2007). Secondary growth of the host buries these sheaths inside the host wood and new sheaths are later formed toward the periphery. These successive layers collectively communicate through radially oriented strands that traverse the xylem, called sinkers. The mature endophytes have well-developed vascular systems of both phloem and xylem. *Pilostyles* (Apodanthaceae), which parasitizes Fabaceae, is similar in developing both cortical strands and radial sinkers, but does not seem to produce uninterrupted sheaths of parasitic tissue. Instead, the cortical strands form an anastomozing cortical complex (Solms-Laubach, 1874*b*). Three different cell types have been observed in the cortical complex, including putative sieve elements (Kuijt and Olson, 1985; Solms-Laubach, 1874*b*). *Mitrastemon* (Mitrastemonaceae), which parasitizes Fagaceae, forms multiseriate, spindle-shaped, vascularized strands in the host cambium and phloem, which anastomose outside of the host cambial layer and occasionally form sinkers into the xylem (Watanabe, 1936, 1937). Finally, the Santalalean endophytes *Arceuthobium douglasii* Engelm., *Tristerix aphyllus* Tiegh. ex Barlow & Wiens and *Viscum minimum* Harv. also form multiseriate strands (and radial sinkers and longitudinal strands in *Arceuthobium*), which have differentiated phloem and xylem elements (Mauseth, 1990; Lye, 2006; Mauseth and Rezaei, 2013). This is equally true across other parasitic angiosperms that produce endophytic tissue as part of a haustorium, such as parasitic Orobanchaceae (Joel, 2013).

Despite the extreme modifications of these endophytic parasites, all of them possess some histological complexity and are composed of several cell types in their more advanced vegetative stages. This is not the case in Rafflesiaceae *s.s.*, which have only one cell type in the endophyte and are not vascularized but persist as uniseriate filaments throughout their vegetative stage. The endophyte of at least some other parasitic angiosperms is not comparable with that in Rafflesiaceae because it is not proembryonic, but instead originates from a shoot. Therefore, Rafflesiaceae, which produce the world's largest flowers, also produce the most reduced endophyte. The endophytic system of all three genera in the family is very uniform and shows little variation that could be attributed to specific growth conditions. Thus, while it does not appear to be phylogenetically informative in the family, this cellular homogeneity does serve to distinguish Rafflesiaceae further from the other former members of Rafflesiales.

Nuclear size

The nuclear diameter of endophyte cells is comparable with the nuclear diameter of floral meristem cells of Rafflesiaceae (Nikolov *et al*., 2014). It exceeds the diameter of the nuclei of their hosts by at least 2-fold, making it a convenient landmark to differentiate host from parasite. Presumably, this large nuclear volume translates into a large genome size, but this remains unknown for Rafflesiaceae. The large size of the nuclei of Rafflesiaceae is comparable with that of *Lilium longiflorum* (Liliaceae), which possesses among the largest angiosperm genomes (approx. 90 Gb) (Price *et al*., 1973). Interestingly, other endophytic parasites, such as *Cytinus hypocistis* and *V. minimum*, also possess nuclei much larger than those of their respective hosts (De Vega *et al*., 2007; Mauseth and Rezaei, 2013). It is unclear whether this evolutionary convergence toward larger nuclear sizes in endophytes has an adaptive advantage, or if it represents a passive process as a consequence of the relatively unlimited host resources [e.g. the accumulation of repetitive elements or host-to-parasite gene transfer (Davis and Wurdack, 2004; Xi *et al*., 2013*a*, *b*)]. The large genome size may determine slow growth due to the increased time needed for genome replication. Slow growth of the endophyte is also suggested by the apparent absence of mitotic figures in our material.

Topology of the parasite endophyte strands within the host

There is little endophyte tissue relative to the host, i.e. the endophyte cells in one strand are more likely to be in direct contact with host cells than with other endophyte cells. As such, individual endophyte strands could be described as individuals, and their number per unit volume of host is high, especially in *Rhizanthes* (L. A. Nikolov, pers. obs). One way to explain this pattern is to invoke multiple infections of the host. Field observations note ants and rodents, which consume the fleshy pulp of the mature fruits, as potential agents of seed dispersal (Bouman and Meijer, 1994; Meijer, 1997; Bänziger, 2004; Pelser *et al*, 2013). It has been documented that the passage through the rodent's digestive tract does little damage to the seeds and may actually be required for germination because their exotegmen is lost in the process (Bänziger, 2004). Given that numerous seeds are ingested together, it is possible that many seeds may infect a single vine in one inoculation event. In this case, neighbouring endophytes might be closely related but not necessarily genetically identical to each other if a single fruit was consumed. Of course, endophytes residing in a single host could be distantly related in the case of multiple independent inoculations from different genotypes. This scenario is unlikely, however, due in part to the general scarcity of ripe fruits because successful fertilization events are apparently rare (Nais, 2001). Alternatively, neighbouring endophytes may be genetically identical clones that result from fragmentation of an initially single strand due to localized disruptions by host cells or localized endophyte cell death. This might represent a kind of asexual reproduction in Rafflesiaceae, but only within a single host because dispersal of the 'progeny' is not likely to occur. Population genetic studies will greatly help to clarify these scenarios.

Parasite–host interactions

The parasite cells do not accumulate starch; however, the host rays and phloem parenchyma in closest proximity to most endophytic strands are heavily loaded with starch and probably represent an abundant and important resource for the parasite. We did not detect significant depletion or excess of starch around individual endophytic strands. It appears that the host does not recognize the endophyte as a pathogen when it is present as a uniseriate filament. For instance, the host does not isolate the parasite with callose, which is a common response following pathogen invasion (Nürnberger and Lipka, 2005). This raises a question about the nature of the host–endophyte interaction. Although unable to photosynthesize and obtaining its

resources from the host, it is interesting to consider the possibility that the Rafflesiaceae endophyte could be a commensal or mutually beneficial partner to *Tetrastigma*, for example by providing biochemical versatility or by increasing host immunity (e.g. Bonfante and Genre, 2010). Along these lines, the host appears to react only when the parasite grows significantly to form an incipient shoot. This suggests that there could be a mechanical stimulus to this reaction (and possibly chemical, if the surface properties of the endophyte change during protocorm expansion). The parasite also appears to be capable of modulating its development, as observed in the transformation of the leading apical boundary of the protocorm from semi-circular to crescent-shaped in response to the host phellogen blocking its growth. The functional significance of these processes may be 2-fold. On the one hand, isolating and effectively killing the parasite probably allows for control and reduction of the host investment in the parasite's growth. On the other hand, it may select for parasites that develop quickly before their growth is arrested by cork isolation. Under these circumstances, the proposed heterochronic shifts associated with a prolonged proembryonic stage and the direct advancement to flowering are not surprising. This developmental trajectory might be selected for as a result of the intimate dynamics between the host and the parasite that progressively reduced the vegetative stage of the parasite until it was primarily present as an endophytic strand eliciting no obvious defence response from the host. Thus, the loss of photosynthetic function as a result of parasitism might be only one explanation for the reduced vegetative morphology of Rafflesiaceae. Another intriguing possibility is the adaptive significance of the lack of vegetative elaboration as a way to decrease the duration of host control on the parasite.

Conclusions

Rafflesiaceae have the most reduced endophytic system of all parasitic angiosperms, and lack discernible cell differentiation along their endophytic filaments. These are well integrated into the host tissue and are identifiable by the large nuclear size and the dense cytoplasm of their cells. The cytological similarity between endophyte cells and previously observed proembryonic cells suggests that the vegetative body of Rafflesiaceae exhibits an extended period of proembryonic growth (protracted juvenilism). The transition between their vegetative and reproductive phase is morphologically abrupt. The host inoculation and early development of the endophyte starting from the proembryo remain elusive. Along with a better understanding of the connection and the physiological interaction between Rafflesiaceae and their hosts, this stage of parasite development should be considered a promising avenue for future research.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: images of the anatomy of the *Tetrastigma* host roots.

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