Research

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Earliest record of transfer cells in Lower Devonian plants

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Summary

- Key sources of information on the nature of early terrestrial ecosystems are the fossilized remains of plants and associated organic encrustations, which are interpreted as either biofilms, biological soil crusts or lichens. The hypothesis that some of these encrustations might be the remains of the thalloid gametophytes of embryophytes provided the stimulus for this investigation.
- Fossils preserved in charcoal were extracted from Devonian Period (Lochkovian Stage, *c.* 410–419 Myr old) sediments at a geological site in Shropshire (UK). Scanning electron micrographs (SEMs) of the fossils were compared with new and published SEMs of extant bryophytes and tracheophytes, respectively. One specimen was further prepared and imaged by transmission electron microscopy.
- Fossils of thalloid morphology were composed almost entirely of cells with labyrinthine ingrowths; these also were present in fossils of axial morphology where they were associated with putative food-conducting cells. Comparison with modern embryophytes demonstrates that these distinctive cells are transfer cells (TCs).
- Our fossils provide by far the earliest geological evidence of TCs. They also show that some organic encrustations are the remains of thalloid land plants and that these are possibly part of the life cycle of a newly recognized group of plants called the eophytes.

Introduction

Recent palaeobotanical research on early terrestrial ecosystems has demonstrated the existence of a new group of plants called the eophytes (Edwards *et al.*, 2014, 2021). Their discovery and characterization are based on one exceptional geological site in the Welsh Borderland that dates to the early part of the Devonian Period (Lochkovian Stage, *c.* 410–419 Myr ago). Here, fossils are formed of charcoal, which was a product of one of the earliest incidences of wildfire in the geological record (Edwards & Axe, 2004; Glasspool *et al.*, 2006). Although quite fragmentary, the charcoal preserves details of cellular structure with exceptionally high fidelity. The eophytes were very small plants that possessed a distinctive and unique combination of characteristics. The sporophyte phase of the life cycle was simple, comprising leafless, bifurcating axes measuring < 1 mm in diameter. The axes bore terminal sporangia in which permanent polyad spores (known as permanent cryptospores in the dispersed record) developed. Eophytes had an internal vascular system composed of food-conducting cells and a plicate epidermis with occasional stomata (Edwards *et al.*, 2021). The gametophyte phase of the life cycle remains unknown. Alongside the eophytes and other plant remains (e.g. Edwards, 2000; Morris *et al.*, 2011, 2012; Edwards *et al.*, 2014) there are stratified thalli, some of which are thought to be among the earliest lichenized fungi (Honegger *et al.*, 2013a, b). Carbonaceous encrustations of broadly similar general form, but lacking the detail preserved in charcoal, have been recorded more widely in rocks ranging in age from Middle Ordovician to Lower Devonian. These were attributed variously to microbial mats comprising bacteria and cyanobacteria (Tomescu & Rothwell, 2006; Tomescu *et al.*, 2006, 2008, 2009), to embryophyte gametophytes (Strother, 2010), and to appendages of the large fungal sporophore *Prototaxites* (Jonker, 1979; Hueber, 2001). Such widespread carbonaceous encrustations probably represent the remains of important components of early terrestrial ecosystems, yet their composition and affinities are still poorly understood.

Here, we describe very small fragments of two forms of plant preserved in charcoal. One is flattened and thallus-like, whereas the other is axial. We show that both contain cells with distinctive, internal, labyrinthine ingrowths. Comparison with broadly similar inclusions in a wide range of extant embryophytes leads us to reject the possibility that these are endomycorrhizal fungi or actinomycetes and to conclude that they are transfer cells (TCs). In living land plants, TCs are widespread, developing in a variety of tissue systems where they play important roles in absorption or

secretion of nutrients (Offler *et al.*, 2002). We draw comparisons to published scanning electron micrographs (SEMs) of TCs in tracheophytes (Talbot *et al.*, 2001, 2002; Offler *et al.*, 2002) and newly acquired SEMs of bryophytes. Although TCs in extant bryophytes have been described in detail (e.g. Ligrone *et al.*, 1993; Frey *et al.*, 1996; Frey & Hilger, 2001; Carafa *et al.*, 2003), most observations were made using transmission electron microscopy (TEM), which does not allow for a full appreciation of the 3D morphology of the wall ingrowths (Talbot *et al.*, 2002), nor is it easily comparable to the SEM of our fossils. Therefore, we conducted a parallel SEM study of TCs in three species of living mosses and one liverwort to obtain comparative data. The significance of these findings is discussed in relation to the life cycle of the eophytes and to the enigmatic carbonaceous encrustations commonly observed at other geological sites.

Material and Methods

Charcoalified fossils

Two distinct but fragmentary morphotypes of fossil are documented here. One is flattened and thallus-like and the other is axial. These were extracted from a grey siltstone collected from a stream section on the north side of Brown Clee Hill, Shropshire, UK (Edwards *et al.*, 1994). The siltstone is part of the Freshwater West Formation of the Lower Old Red Sandstone, Anglo-Welsh Basin. Palynological assemblages from this bed were studied by John Richardson and assigned to the middle sub-zone of the *micrornatus – newportensis* Sporomorph Assemblage Biozone, which indicates an early to middle Lochkovian age (Richardson, 1996). The locality is a Lagerstätte (i.e. a deposit in which the fossils are exceptionally well preserved). The charred remains of the vegetation preserve the cellular detail of tissues, anatomy and *in situ* spores (Edwards, 1996; Edwards *et al.*, 2014; Morris *et al.*, 2018).

The fossils were extracted by disaggregation of the siltstone in water, followed by standard HF/ HCl maceration, and then air-dried and picked under a stereo light microscope. The specimens then were transferred to a scanning electron microscopy (SEM) stub, coated in gold-palladium, and examined under a Field Emission Gun Environmental SEM (FEI, Hillsboro, OR, USA). For further details on material preparation and accounts of the reconstructed vegetation from this locality, see earlier studies (e.g. Edwards, 1996; Morris *et al.*, 2011, 2018, and references therein). Semi-quantitative Energy-dispersive X-ray spectroscopy (EDS) analysis was performed using INCA software (Oxford Instruments, Abingdon, UK) with a beam current of 1 nA.

One specimen (HD527/06) was prepared for transmission electron microscopy (TEM). It was removed from the SEM stub and immersed in a 1.5% aqueous solution of melted agar. Following solidification, the blob of agar containing the specimen was dehydrated in ethanol, and embedded in modified Spurr resin substitute (Ellis, 2006). After roughly trimming to the axis base, 100-nm-thick longitudinal sections from near the axis centre were cut and collected on formvar coated 2 × 3 mm copper slot grids. Grids with sections were examined with a Jeol-2010 TEM (Jeol Ltd, Tokyo, Japan) operating at 80 kV and images captured with an AMT (Woburn, MA, USA) digital camera.

Specimens with numbers prefixed with the letter 'V' are housed in the Natural History Museum, London (NHMUK).

Extant bryophytes

Gametophytes bearing young sporophytes of the mosses *Polytrichum juniperinum* Hedw., *Mnium hornum* Hedw. and *Funaria hygrometrica* Hedw. and the liverwort *Lophocolea heterophylla* (Schrad.) Dumort. were collected in the field and processed for SEM as described in Duckett *et al.* (2006). Briefly, tissues were placed in 10% ethanol to extract the cytoplasm. The placental region, including the sporophyte foot and the surrounding gametophyte tissue, was cut longitudinally, fixed in 3% glutaraldehyde, dehydrated through an ethanol series, critical-point dried using CO_2 as transfusion fluid, sputter-coated with 20 nm palladium-gold and viewed using an FEI Quanta SEM.

Results

Description of fossils

Thalloid specimens (V 68863, Fig. 1a–c,e,n, 0.8 × 0.9 mm; V 68864, Figs 1d,f–m,o,p, 2a–l; 1.7 × 0.4 mm) The thallus-like fragments are united in being almost entirely composed of cells with labyrinthine contents. Except for a small mound (*c.* 0.4 mm diameter) on the larger fragment, both have flat surfaces. Because they possess almost identical internal anatomy, they are both interpreted as parts of larger plants, although probably from different individuals. Orientation vis-à-vis upper and lower surfaces of the fragments is conjectural. In V 68863, one surface (Fig. 1a) bears occasional adpressed hyphae of possible actinomycete affinity (Fig. 1e). On the reverse side, the surface of the small mound is superficially different. It has a very irregular fibrous texture (Fig. 1c) that we interpret as possibly fused hyphae forming a hyphal mantle as recorded in other fossils elsewhere (work in progress). The fractured margin of this fossil reveals internal features. Both surfaces are bound by a continuous structureless layer, but this is thicker on the surface with the 'fibrous' appearance (Fig. 1n). In the second specimen, one surface is smooth except for occasional protuberances, or pustules, that are variously punctured at their apices (Fig. 1f). This surface also has clusters of fragmentary hyphae (Fig. 1g), contact features left by hyphae (Fig. 1h) and occasional imprints of pyrite crystals. The other is more chaotic, with pustules of varying size (Fig. 1i) and occasional hyphae. In fractured section, the surface layers appear to be quite different. One is homogenized and noncellular, but the other comprises a layer of cells that are irregularly shaped in vertical section (Fig. 1k,l,m,q). A fortuitous tangential split revealed that these cells are irregular, almost sinuous, and associated with small circular 'pits' (Fig. 1o,p). However, there are no perforations in the outer surface. Where this superficial layer is missing, the outermost layer of a tissue, which forms the bulk of the thallus in thicker regions, is visible, and it reveals closely packed cells of varying shapes and sizes with uniformly thickened walls

separated by narrow intercellular spaces (Fig. 1j). Towards the margins, cell lumens are smaller and lack well-preserved contents. The cell walls are relatively thick.

The commonest type of cell within the thallus possesses a lumen that is almost fully occluded by a much-branched structure. The branching elements are mostly smooth surfaced and of more or less constant width of 180–(330)–470 nm, with numerous connections to the wall (Fig. 2a,b). As far as can be ascertained, they do not appear to develop preferentially on particular faces of the cell wall, nor do they show any preferred orientation with respect to the surface of the thallus. The fractured bases of the branching elements reveal their solid nature (Fig. 2c). At numerous points, one can observe that the ingrowths are completely continuous with the wall. In section, the texture of the wall and the ingrowths is homogeneous. This is a consequence of the charcoalification process, which makes it impossible to determine if the branching structures penetrated the wall or were extensions of it. We have very few examples of growths continuing between cells (Fig. 2g). Rare examples bear irregular granules, and these are clustered at branch tips (Fig. 2f) or in very rare cases, granular material extends over the entire surface (Fig. 2d). Occasionally, cells are empty (Fig. 2h) or very rarely partially filled (Fig. 2g). Also rare are cells in which the internal surfaces are simply lined by close-set granules. Fig. 2(l) shows an exceptional type where the lumen is filled with the granules. Scattered, sporadic examples of the latter are superficially similar in size and shape to microcrystalline pyrite. However, elemental analysis (by EDS) shows them to be predominantly carbon-based and chemically indistinguishable from the cell walls or the much-branched ingrowths. There are no other cell contents.

Sterile axial fragment A short, sterile, unbranched, axial fragment (2.1 mm long; Fig. 3a) with smooth surface and typical

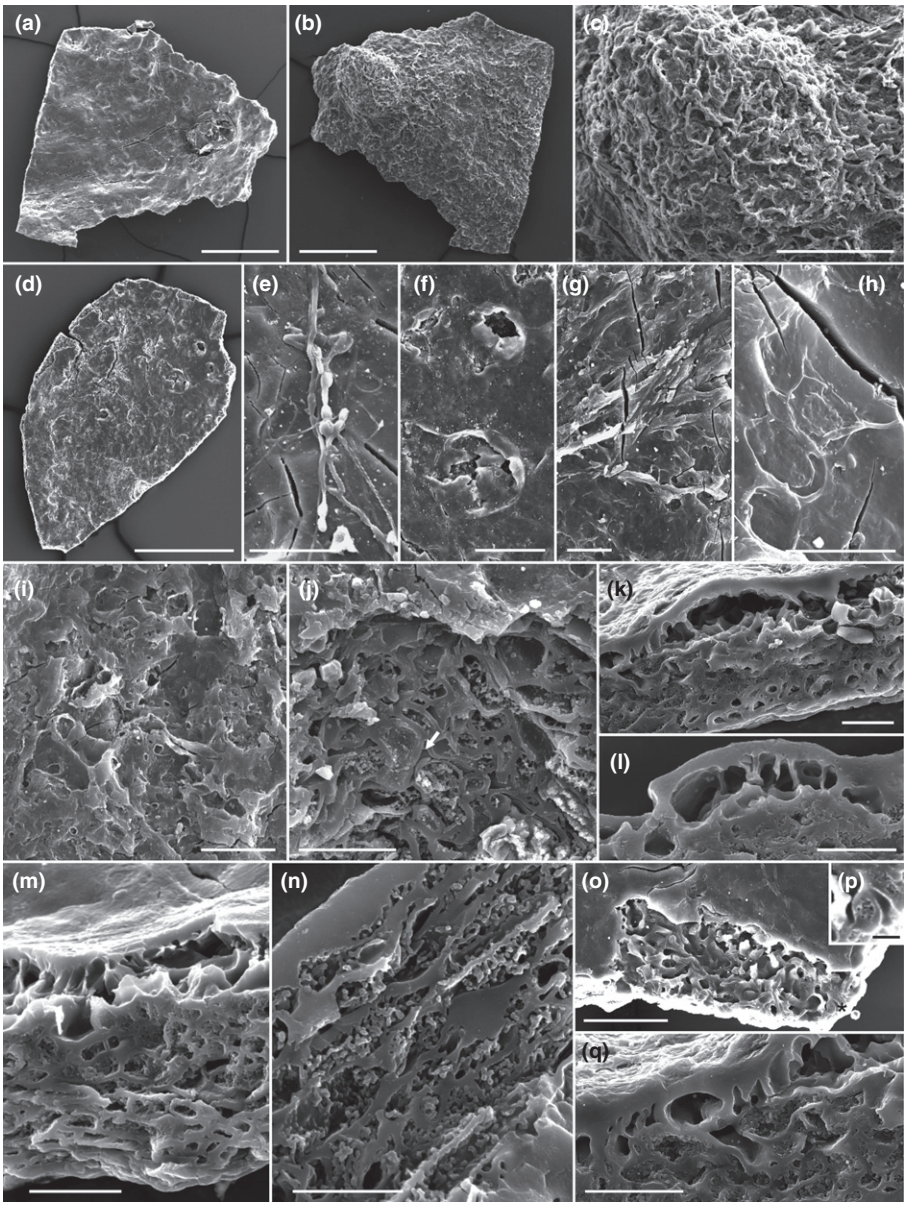

Fig. 1 Thalloid specimens; (a–c, e, n) V 68863 and (d, f–m, o, p, q) V 68864, Lochkovian Welsh Borderland. (a, b) Interpreted upper and lower surfaces of thallus. (c) Mound with irregular surface (? hyphal mantle) on putative lower surface. (d) Putative upper surface of thallus. (e) Hyphal fragments (?actinomycete) on (a). (f) Superficial pustules (?consequent on charring) on (d). (g) Remains of hyphal-contaminants. (h) Hyphal contact features. (i) General view of lower surface. (j) Periclinal fracture of tissue immediately below superficial layer of cells, showing small intercellular spaces (arrow). (k) Longitudinal fracture near margin. (l) LS, cells in superficial layer. (m) Oblique and longitudinal fracture, near margin of thallus. (n) Longitudinal fracture with compressed and aligned cells. (o) Periclinal fracture of superficial layer, star indicate possible pores. (p) Magnification of starred area in (o), illustrating possible pores. (q) Longitudinal fracture through presumed upper surface. Bars: (a, b, d) 500 μm; (c) 200 μm; (e, o) 50 μm; (f) 100 μm; (g, i, k, l, m, q) 20 μm; (h, j, n) 10 μm; p) 5 μm.

eophyte anatomy (Edwards *et al.*, 2021) was split longitudinally and revealed toward its centre a fragment of a single cell, whose lumen is filled with a labyrinthian ingrowth (Fig. 3b). The numerous branches forming this ingrowth are smooth with occasional granules that are aggregated at branch tips (Fig. 3d–f). An abundance of adhering material, possibly the remnants of cell contents, obscures contacts with the cell wall (Fig. 3c). Individual elements vary between 60 and 200 nm in a cell that is ≥ 7.3 µm wide.

Fertile fragment. The specimen is *c.* 0.73 mm long and comprises an unbranched, typically ridged, eophytic stem terminated by a tight cluster of presumed sporangia with pronounced reticulate walls (Fig. 3g). SEM imaging shows that the tissues are almost completely homogenized. However, longitudinal sections prepared for TEM revealed two types of more or less centrally placed cells. In Fig. 3(h), a lumen surrounded by homogenized walls contains discrete structures with irregular, roughly circular outlines and of varying size, that are comparable to the presumed food-conducting cells (FCCs) of eophytes (Edwards *et al.*, 2021). In the second type, walls are more distinct, and the lumen filled with a network of branching structures similar to the labyrinths noted in the sterile axis (Fig. 3i), although even more dense than those seen in the thalloid examples, with individual elements about 40 nm in diameter in a cell ≥ 1.2 µm wide.

Comparison with transfer cells (TCs) in extant bryophytes

Cytological analyses of TCs in mosses and liverworts In all the species analyzed there are several layers of TCs at the sporophyte–gametophyte junction, as illustrated here in the moss *Funaria hygrometrica* (Fig. 4a,b). As shown before (Ligrone *et al.*,

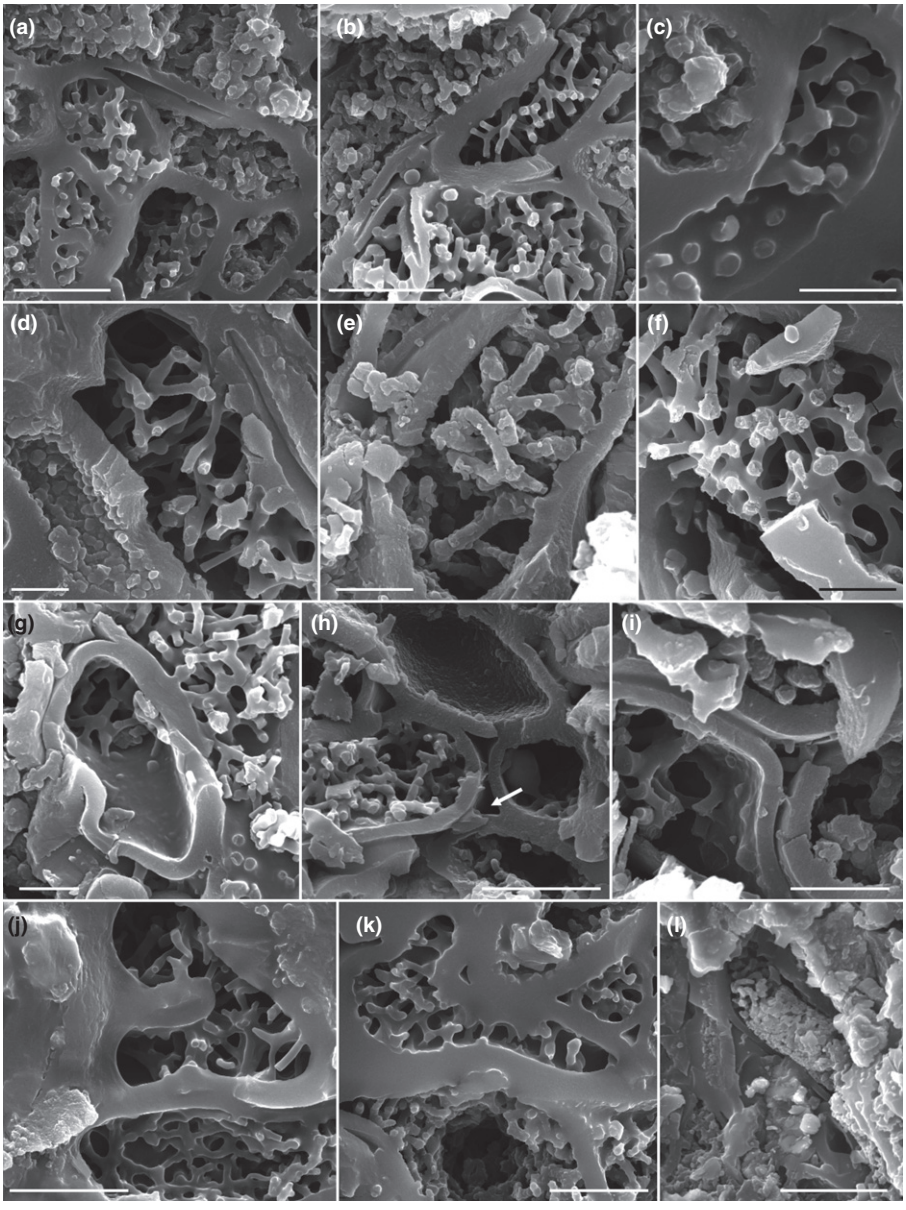

Fig. 2 Thalloid specimen, V 68864, Lochkovian Welsh Borderland. (a, b) Typical longitudinal fracture of labyrinths. (c) Transversely fractured bases of branches, and granular overgrowths (top left). (d) Lining of granular structures, bottom left, slender branches, right. (e) Branches with surface granules. (f) Fretwork of branches, granular terminations. (g) Partially empty single cell. (h) Pronounced intercellular space; note empty cells (arrow). (i) Narrow intercellular spaces between three cells; note terminal clusters of granules, bottom right. Alternatively, the intercellular space may be a collapsed thin-walled cell with a single ingrowth peg. (j, k) Diversely shaped cells, no intercellular spaces. (l) Cell filled with granules. Bars: (a, b, h, j, k) 5 µm; (c, d, e, f, g, i) 2 µm; (l) 10 µm.

1993) bryophyte placental TCs are characterized by extensive wall ingrowths consisting of tubular projections which form complex, labyrinthine networks (Figs 4, 5). These are first deposited as small papillae at discrete loci on the cytoplasmic face of the underlying wall (Figs 4c, 5b), and then they elongate, branch and eventually fuse together with neighbouring ingrowths to form an interconnected labyrinthine network (Figs 4d,f,g, 5c,d). New projections then are deposited, appearing first as small papillate protrusions (Figs 4g, 5d), which either branch again and fuse with neighbouring ones, or may elongate considerably reaching ≤ 5 μm in length (Fig. 4h), forming a looser network (Fig. 4e). The diameter of the wall ingrowths varies between species and within species depending on the developmental stage of the TCs. The smaller diameters, 100–(140)–180 nm, were observed in the liverwort *Lophocolea heterophylla*; those in *Funaria* were 190–(230)–270 nm, with the greatest variation found in *Mnium hornum*, 200–(420)–600 nm, where the elongate protrusions illustrated in Fig. 4(h) reach up to 600 nm in diameter.

Our SEM observations of bryophyte placental TCs confirm that the cells with a complex 3D network of branched ingrowths filling their lumina in the fossil fragments illustrated in Figs 1–3 are most likely TCs. Similarities include the initial deposition of small papillae (cf. Fig. 2f with Figs 4c, 5b), which then elongate, branch and fuse to form a complex labyrinthine network or reticulum, with new projections extending from it (cf. Figs 2f, 3d with 4g, 5d). Similar morphological development of reticulate wall ingrowths also has been observed under the SEM in a range of tissues in various vascular plant species (Talbot *et al.*, 2002) as exemplified by the epidermal transfer cells in cotyledons of *Vicia faba* (Talbot *et al.*, 2001). Dimensions of the ingrowths in the thalloid fossils 180–(330)–470 nm (five cells measured) fall within the range of those found in bryophyte TCs and indeed

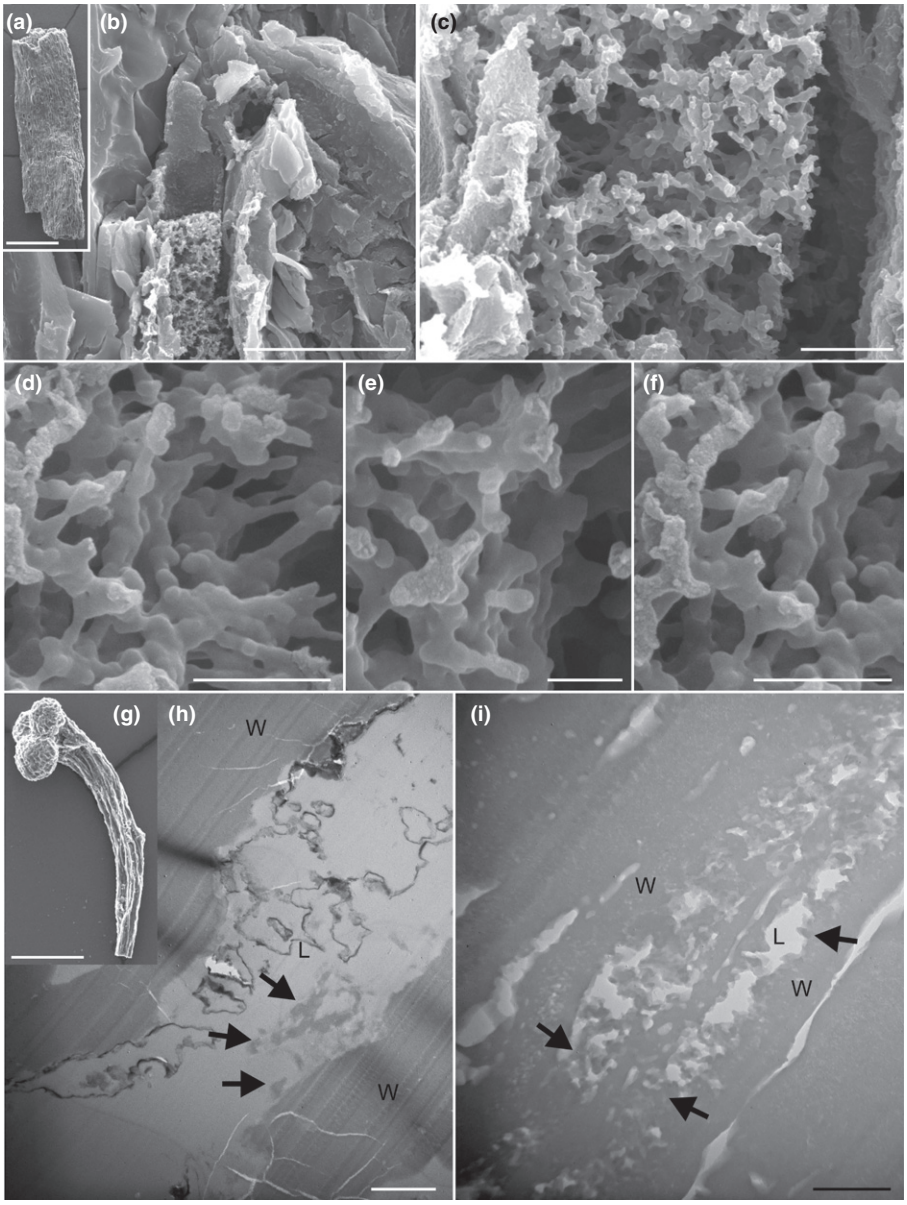

Fig. 3 (a-f) Sterile axial fragment, V 68865, Lochkovian Welsh Borderland. (a) Scanning electron microscopy (SEM) of sterile axis before investigation. (b) Central area of axis after longitudinal fracture. (c) Incomplete longitudinally fractured cell with lumen filled with much branched labyrinth. (d–f, parts of c) magnified showing 'granules' on free ends of branches. (g–i) Fertile fragment, HD527/06, Lochkovian Welsh Borderland. (g) Ridged axis with terminal cluster of three sporangia. (h, i) Transmission electron microscopy images (TEMs) of sections through central area of axis. (h) LS part of cell, with homogenous walls (W) with irregular fragments of globular structures (arrows) in lumen (L). Such cells have recently been interpreted (Edwards *et al*., 2021) as involved in food conduction. (i) LS part of cell filled with branching structures (arrows), here identified as a transfer cell. W, cell wall; L, lumen. Bars: (a) 500 μm; (b) 20 μm; (c) 2 μm; (d, f, h) 1 μm; (e) 500 nm; (g) 200 μm; (i) 400 nm.

across other modern embryophyte lineages (e.g. Talbot *et al.*, 2002). The dimensions of the cell wall ingrowths in the axial fossils are mostly smaller, in the 55–(92)–110 nm range, but overlap at their maximum size with the bryophyte TCs. The overall morphology of both fossil and bryophyte TCs corresponds to the reticulate-type morphology described before in several angiosperms (Offler *et al.*, 2002; Talbot *et al.*, 2002 and literature within).

Discussion

Transfer cells developed in early land plants

The most striking feature of our thalloid fossils is that they appear to be almost entirely composed of cells with labyrinthine ingrowths. Although these ingrowths do bear some resemblance to arbuscules of endomycorrhizal fungi, we exclude this possibility because in the latter a single hypha penetrates the cell with subsequent prolific branching and coiling, and the hyphae are invariably much larger. Members of the arbuscular mycorrhiza forming (AMF) Glomeromycotina are characterized by hyphae with diameters $>2\,\mu m$, whereas hyphae produced by Mucoromycotina fine root endophytes (MFRE) are smaller, usually $>1.5\,\mu m$ in diameter (Orchard *et al.*, 2017; Hoysted *et al.*, 2018, 2019). Both types of fungi form arbuscules or arbuscule-like structures in the plant host cells, the finest ramifications of which have diameters in the range of 700–900 nm, considerably larger than those described here. Further, in both AMF and MFRE colonization, intracellular hyphae form vesicles either terminally (AMF) or both terminally and intercalary (MFRE); we have never observed any vesicles in the fossils.

The labyrinthine ingrowths are closer in dimensions to the hyphae involved in actinorhizal symbioses. Moreover, in actinomycetes there can be multiple entries of the hyphae into an individual cell followed by hyphal proliferation within the cell lumen. However, the hyphae of *Frankia* also develop vesicles; they do not form interconnected 3D networks and infected cells often contain irregular agglomerations of breaking down actinomycete cells (Benson & Silvester, 1993) which we never saw in the fossils. Furthermore, although actinomycetes are ancient, in extant plants this nitrogen-fixing root endosymbiosis occurs only in one clade within rosid angiosperms (Li *et al.*, 2015). We observed probable actinomycete hyphae on the surface of the thalloid fossil (Fig. 1e) but these were very rare internally. We therefore rule out an actinomycete affinity for the labyrinthine ingrowths.

Based on their size, disposition and structural characteristics, we conclude that the labyrinthine cell wall ingrowths are neither endomycorrhizal fungi nor actinomycetes, but instead constitute structures typical of embryophyte transfer cells (TCs) (Gunning & Pate, 1974). One major difference is that the fossil wall ingrowths show no evidence of polarization mirroring the putative direction of nutrient flow, whereas this is almost the rule in extant transfer cells except where multiple layers occur as in the mature nucellar projections cells of *Triticum aestivum* (Talbot *et al.*, 2002) and in the placentas of many bryophytes (Ligrone *et al.*, 1993; Fig. 4).

Polarization aside, similarities to TCs of modern embryophytes are most striking (Ligrone *et al.*, 1993; Offler *et al.*, 2002; Talbot *et al.*, 2002), as confirmed here by comparative SEM analyses of placental TCs in extant bryophytes. The same also is true for the dense reticulum observed in the fractured short axial fragment. TCs are highly specialized plant cells that facilitate transport of nutrients across the symplast/apoplast interface. They are characterized by extensive secondary cell wall invaginations (ingrowths) greatly increasing the surface area of the plasma membrane; they occur throughout embryophytes in a wide range of tissues, including xylem and phloem, at the maternal–filial boundary in developing seeds, in the leaf epidermis of aquatic angiosperms and in parasite haustoria (Pate & Gunning, 1972; Gunning & Pate, 1974; Gunning, 1977; Offler *et al.*, 2002). In bryophytes (liverworts, mosses, hornworts) TCs typically are present at the gametophyte-sporophyte junction (placenta) where they facilitate translocation of sugars from free-living, parental gametophytes to more transient, nutritionally dependent sporophytes (Ligrone & Gambardella, 1988; Ligrone *et al.*, 1993; Regmi *et al.*, 2017). Elsewhere, wall ingrowths are associated with the cyanobacterial colonies in Blasiales, with a clear role in transport between host and endophyte (Duckett *et al.*, 1977), and in the antheridial jacket cells of hornworts where their function is unknown (Duckett, 1973). In our thalloid fossils, except for the epidermal layers, most cells observed appear to express a TC morphology, raising the question of what role they played in the function of the plant. In this respect, the abundance and distribution of TCs in the fossils clearly differs from that in modern thalloid bryophytes.

The ubiquity of TCs in extant land plant lineages, including the early bryophytes, and even extending to the tissues enveloping zygotes in the charalean alga *Coleochaete* (Graham & Wilcox, 1983), suggests that they might have evolved ancestrally in embryophytes, possibly with the same putative role as that attributed to today's bryophyte placental TCs (Ligrone *et al.*, 1993). Therefore, the hitherto lack of fossil evidence of TCs for any plant group, bar one report in the fossil seed coat of the angiosperm *Ehretia clausentia* from the Lower Eocene (Gottschling & Hilger, 2003), is baffling. TCs exhibit considerable variation in the shape and arrangement of their wall ingrowths, from relatively small projections to long branched ingrowths that may interconnect to form a labyrinth or reticulum or, in some cases, elaborate flanges (Talbot *et al.*, 2002).

Different morphologies have been observed in different taxonomic groups and depending on the anatomical location of TCs (Offler *et al.*, 2002). The overall distribution of reticulate-type wall ingrowths across the land plant phylogeny indicates that this morphology is ancestral (Offler *et al.*, 2002). It should be noted, however, that in *Coleochaete* the putative TCs contain just simple wall protuberances (Graham & Wilcox, 1983) and complex labyrinths are unknown in the algae.

In bryophytes, differences also include whether TCs are present or absent in each generation (gametophyte and sporophyte) together with morphological changes associated with sporophyte

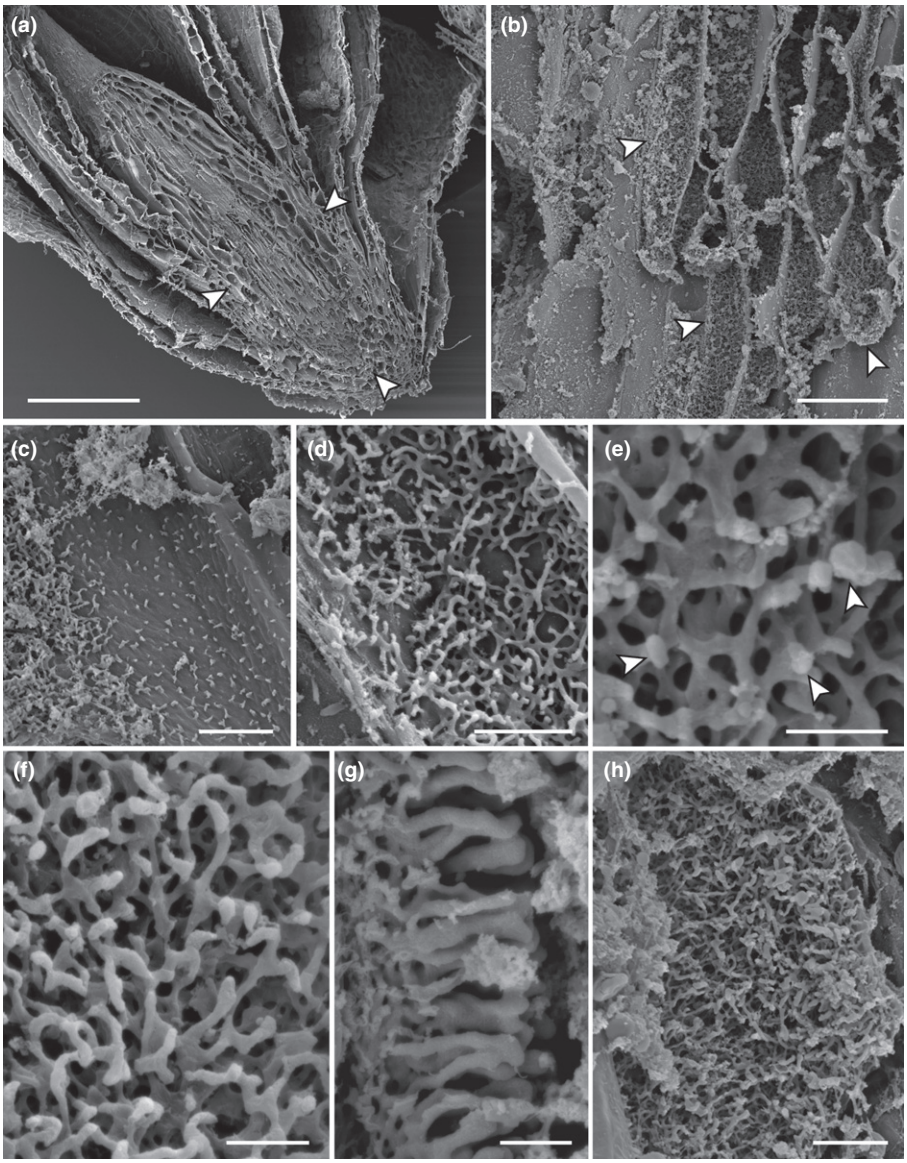

Fig. 4 Scanning electron micrographs of transfer cells in the mosses *Funaria hygrometrica* (a, b, d, f, g) and *Mnium hornum* (c, e, h). (a) Longitudinal section of sporophyte foot penetrating the parental gametophyte, the placenta (arrowed) is at the interface (junction) between the sporophyte foot and the gametophyte. (b) Several layers of transfer cells (arrowed). (c) Wall ingrowths first are deposited as small papillae at discrete loci on the cytoplasmic face of the underlying wall. (d) Elongating and branching wall ingrowths fusing with neighbouring ones to form an interconnected labyrinthine network. (e) New projections (arrowed) arising from the most recently formed layer of wall ingrowths. The new projections either branch and fuse again (f) or become much elongate (g, h). Bars: (a) 300 μm; (b) 20 μm; (c, d, h) 5 μm; (e–g) 2 μm.

development (Ligrone *et al.*, 1993). Nevertheless, their overall morphology, characterized by extensive secondary wall ingrowths (Offler *et al.*, 2002), makes TCs highly distinct and readily recognizable. We conclude that our small charcoalified fossils comprise by far the earliest geological evidence of TCs in plants. This finding is consistent with the hypothesis that the genetic competence to form TCs is deep-rooted and ancestral in embryophytes (Gunning & Pate, 1974).

The affinities of the fossils and the roles of transfer cells

The minute thallus-like fossils documented here clearly are fragments of a land plant. Their coherent, integrated structure of well-defined cells and dermal layers allows us to exclude the possibility that they represent loose consortia of algae, bacteria and fungi, as one might find in fossilized microbial mats (Tomescu & Rothwell, 2006; Tomescu *et al.*, 2008). The absence of internal hyphae and the lack of stratification allow us to rule out early fossil lichens (Honegger *et al.*, 2013a,b), as well as the large, contemporaneous, extinct fungus *Prototaxites* (Hueber, 2001; Honegger *et al.*, 2018) and the more enigmatic thalloid fossils of *Nematothallus* (Edwards *et al.*, 2013). The robust cell walls and the presence of intercellular spaces within the fossils taken together with the sedimentological context indicate that they are unlikely to be fragments of macroalgae. This raises the possibility that these pre-Devonian thalloid encrustations were the gametophytes of embryophytes, as suggested by Tomescu & Rothwell (2006), who subsequently provided evidence from carbon isotopic values of specimens from Ordovician and Silurian rocks from the Appalachians, USA, that were comparable with those from extant liverworts (i.e. bryophyte-grade affinities) (Tomescu *et al.*, 2009). Further support was provided when structures comparable to those seen in some fossils from the same localities were produced on compression of certain liverworts in attempts to mimic the

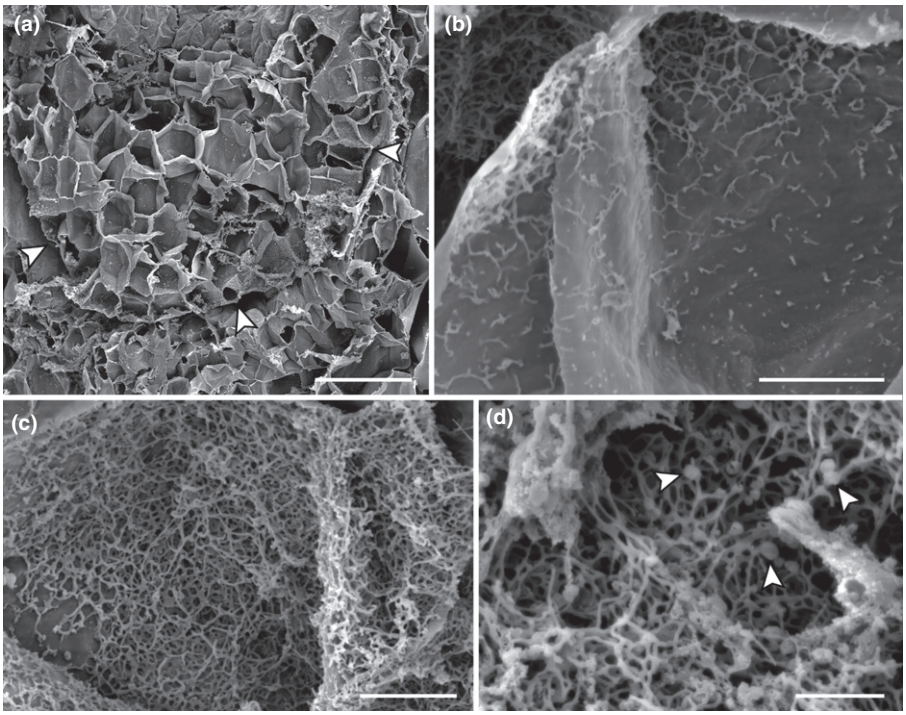

Fig. 5 Scanning electron micrographs of transfer cells in the liverwort *Lophocolea heterophylla*. (a) Longitudinal section of sporophyte foot penetrating the parental gametophyte, the placenta (arrowed) is at the interface (junction) between the sporophyte foot and the gametophyte. (b) Wall ingrowths first are deposited as small papillae at discrete loci on the cytoplasmic face of the underlying wall. (c) Elongate, branched ingrowths fusing with neighbouring ones to form an interconnected, labyrinthine network. (d) New projections (arrowed) arising from the most recently formed layer of wall ingrowths. Bars: (a) 50 μm; (b, c) 5 μm; (d) 2 μm.

taphonomic process (Tomescu *et al.*, 2010). Thus a land plant affinity seems likely, and although the fragmentary nature of the specimens and the lack of diagnostic characters limits identification, the most compelling parallels lie with the thalloid liverworts while leaving open the possibility that they were hornworts. The thalloid fossils share a similar overall form and a low level of internal tissue differentiation with liverworts. Throughout this account, we have avoided using the terms dorsal and ventral, and the situation is further complicated by differences between the two specimens. Our preferred inference of thallus orientation is based on interpreting the epidermal layer with pustules in specimen V 68864 as equivalent to the dorsal photosynthetic region of complex thalloid liverworts (Marchantiales) with subepidermal air chambers bearing photosynthetic filaments subtending air pores. The opposite surface with the thick-walled epidermal cells would therefore be ventral. However, evidence of rhizoids is lacking. Further, based on the relative thicknesses of the epidermal layers, we infer that the smoother surface in specimen V 68863 is dorsal and the surface with a 'fibrous' covering ventral. The fragments of fossil thalli therefore bear some similarities to complex thalloid liverworts, but here we are drawing functional parallels and not implying phylogenetic affinities.

Further insights into the affinities of the fossils and their relations to other plants might be drawn from the nature and distribution of the TCs. Again, our interpretation is tempered by the fragmentary nature of the material. TCs are known to play roles in the trans membrane flux of solutes, which can include secretion and absorption to and from the external environment (Offler *et al.*, 2002). This might have been their role here, with pores or pustules in the thallus surface forming channels between interior and exterior. In the axial fossils, TCs are less prevalent. They are located towards the centre of the axis. In the eophytes, the centrally located vascular system is composed of food-conducting cells (FCCs) (Edwards *et al.*, 2021). Here, the TCs might be playing a role in loading the FCCs as they can do in the phloem of living plants (Gunning, 1977). Another plausible interpretation is that the thalloid and the axial fossils are different parts of a plant life cycle, and that the role of the TCs is to transfer nutrients from a maternal gametophyte (thalloid fossil) to a physiologically dependent sporophyte (axial fossil with sporangia). At first sight it might seem that the chance of finding placental tissue in randomly collected minute specimens would be extremely small. However, in liverworts with their otherwise very simple anatomy, placentas are one of their most prominent and persistent features. The minute size of the spore-producing phase of the eophyte life cycle (Edwards *et al.*, 2021) could mean that it lacked photosynthetic competence (Boyce, 2008) and that it was nutritionally dependent on a larger autotrophic gametophyte in a manner similar to that observed in modern peristomate mosses and most liverworts where TCs are present in both generations (Ligrone *et al.*, 1993). The close matching of the fine details of the fossil TCs with those in bryophyte placentas, and the proximity in mosses of FCCs to the placental TCs, as observed in the eophyte aerial axes, support the idea of a matrotrophic function.

The fossil thalli, together with the sporophytes with similar anatomy described here and eophyte mesofossils where axes are attached to very small fragments of a thalloid nature (Edwards *et al.*, in press), allow reconstruction of small plants with limited branching aerial axes terminating in sporangia arising from a thalloid gametophyte. This fits the concept of the plant body of early polysporangiophytes (embryophytes with branched sporophytes) probably, from their small size, dependent on thalloid gametophytes (Strother, 2010; Gerrienne & Gonez, 2011; Tomescu *et al.*, 2014). The eophytes (Edwards *et al.*, in press)

differ, as in the sporophytes, whereas most bifurcation is isotomous, branching can be complex and, at least in these Lochkovian examples, there was internal tissue differentiation.

Conclusion

The abundant carbonaceous encrustations observed in terrestrially sedimentary sequences deposited during the latter part of the Silurian and early part of the Devonian periods are puzzling and challenging to interpret because cellular structures are seldom preserved (Tomescu & Rothwell, 2006; Strother, 2010). Convincing cases have been made that some of these are microbial consortia, possibly the remnants of biological soil crusts or cyanobacterial mats living on soil surfaces or in shallow pools (Tomescu *et al.*, 2006, 2008, 2009). The high-fidelity preservation of cellular and subcellular features in fossil charcoal at the Brown Clee Hill geological site affords an opportunity to probe and test these ideas. One class of thalloid structure previously documented from this site is a lichen-like consortium (Honegger *et al.*, 2013a,b). Here, we have shown that this early flora also contains a thalloid element with clear affinity to land plants. The carbonaceous encrustations so widely observed at other geological sites might therefore represent the remains of diverse early terrestrial communities, and they merit further scrutiny.

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Author contributions

DE conceived the project initially based on SEMs of JLM and LA, and TEMs of WAT on fossils and latterly, SEMs on extant bryophytes from SP and JGD; PK coordinated and developed the writing of the paper with input from SP, JGD, JLM and DE.

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Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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See also the Commentary on this article by Tomescu, **233**: 1018–1021.