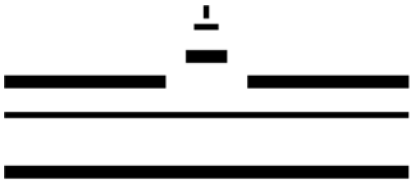

Sporophyte development in *Rhynia gwynne-vaughanii*, an Early Devonian vascular land plant from the Rhynie chert, Aberdeenshire, UK

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2015

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Summary

While the developmental patterns and strategies of extant land plants are widely studied, the same cannot be said for the earliest land plants. The studies in this volume describe the development of the Early Devonian Rhynie chert plant, *Rhynia gwynne-vaughanii*, a morphologically simple vascular land plant, devoid of the many specialised organs and structures that characterise extant vascular land plants, i.e. roots and leaves. Such studies are possible because the Rhynie chert preserves some of the earliest land plants in exceptional detail, down to the cell level. Asexual vegetative propagules of *Rhynia gwynne-vaughanii* as well as mature shoot axes were studied using a combination of petrographic thin sectioning, light microscopy (LM) and synchrotron X-ray tomographic microscopy (SXRTM).

Asexual vegetative propagules played an essential role in understanding the basic developmental pattern of the *Rhynia gwynne-vaughanii* shoot. Using LM, propagules are shown to have developed from two different locations on the shoot axis, from multiple epidermal cells adjacent to mature stomata, and from single cells on the hemispherical projections that dot the surface of the shoot. Propagule development in *Rhynia gwynne-vaughanii* resembles somatic embryogenesis, a form of asexual reproduction that occurs commonly in extant land plants, and has been studied extensively *in vitro* in order to understand the mechanisms controlling shoot development. The preservation of propagules at various points of shoot development allowed for the documentation of specialised tissue formation as well as overall shoot developmental morphology. Comparison of developmental morphology in *Rhynia gwynne-vaughanii* with that of extant land plants revealed some basic similarities with the ancestral bryophytes but in general shoot development was most similar to that of the extant early divergent lycophyte and monilophytes groups.

Both LM and SRXTM show that development of the *Rhynia gwynne-vaughanii* shoot was directed from the shoot apical meristem (SAM). The structure of the SAM, with its tetrahedral and probably transient apical cell, core zone of isodiametric cells and superficial layer of larger square-rectangular cells, resembles that of the lycophytes and the monilophytes. Stomata, rhizoids and hemispherical projections developed from cells derived from the apical cell. For stomata and the water-conducting tracheid cells, the SAM played a primary role in their formation and ontogenetic patterns for both of these specialised tissue complexes fit within modern accepted classification schemes for the same in extant land plants. The SAM of the more primitive non-vascular Rhynie chert plant *Aglaophyton major* was also examined and is shown to be structurally different to that of *Rhynia gwynne-vaughanii*, with the cells of the core of the SAM, the superficial layer and the cells of the peripheral zone around the SAM to be of similar dimensions; the limit of the SAM within the plant axis was relatively more difficult to pinpoint than in *Rhynia gwynne-vaughanii*. In addition, the

SAM of *Aglaophyton major* comprises several divisional zones of vertical files of cells, deep into the core of the SAM. The SAM of *Rhynia gwynne-vaughanii* represents a change in SAM complexity and structure between two evolutionary important groups of land plants, a trend also seen throughout the evolution of the extant land plant groups. This increase in SAM complexity and structure within the vascular land plants accounts for the slightly more complex morphology and physiology of *Rhynia gwynne-vaughanii*, compared to that of *Aglaophyton major*, and was fundamental to the evolution of the increasingly more complex vascular plant sporophyte, as well as to terrestrial adaptation and radiation.

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Chapter 1

Introduction and Review: Land plant biology, reproduction, phylogeny and evolution

1.0 Introduction

The evolution of land plants had a profound effect on the terrestrial environment. The radiation of land plants in the late Silurian and Early Devonian affected weathering and fluvial regimes, nutrient delivery to marine habitats and set the scene for widespread colonisation of the land by many other organisms (Algeo *et al.* 2001; Małkowski and Racki 2009; Gibling and Davies 2012). While the developmental strategies and mechanisms that control growth in extant land plants have been studied extensively (see Chapter 4), albeit mainly within the seed plant groups, developmental strategies within the earliest evolved land plants are poorly known. The studies in this volume set out to obtain developmental data on one such early land plant, the morphologically simple Early Devonian Rhynie chert vascular plant *Rhynia gwynne-vaughanii*. Both asexual vegetative propagules and individual axes of *Rhynia gwynne-vaughanii* were studied using a combination of petrographic thin sectioning, light microscopy (LM) and synchrotron X-ray tomographic microscopy (SRXTM). These investigations sought to establish which developmental strategies have been conserved throughout the evolution of land plants, by comparing the development of *Rhynia gwynne-vaughanii* with that of the early divergent extant plant groups (i.e. lycophytes and monilophytes), as well as with development in the ancestral bryophyte group. In addition to understanding basic morphological development, it was also necessary determine the structure of the shoot apical meristem in *Rhynia gwynne-vaughanii*. The shoot apical meristem is a specialised zone at the apex of all vascular land plant shoots that drives plant development. Land plants became morphologically and developmentally more complex over geological time, with the most recently evolved groups

showing the most complex morphologies, reproductive strategies and developmental pathways.

As a one of earliest known anatomically preserved vascular plants in the fossil record, *Rhynia gwynne-vaughanii* provides a unique opportunity to establish the evolutionary timing of the basic developmental strategies of this group. Further to basic shoot development these studies sought to obtain information on the formation of specialised tissues such as stomata and vascular tissues, and whether their development corresponds to currently accepted ontogenetic classification schemes for extant land plants.

Before presenting results it is necessary to provide a background to the current understanding of the evolutionary relationships between land plants and their algal ancestors, as well as the physiological and morphological characteristics that define land plants. This will give an evolutionary context for the plants of the Rhynie chert, described in Chapter 2 as well as the results and discussion in this volume (Chapters 3 and 4).

Despite extensive and intense research across the various plant-based sciences the evolutionary relationships between plant groups are still very much open to discussion and exploration, as are the timing of the origins of the earliest land plants and the evolution of major morphological traits. The relatively poor fossil record of the earliest land plants is highlighted as an additional major stumbling block in creating a consensus across disciplines.

1.1 Land plant groups

Extant land plant groups (the embryophytes) are united by specialised reproductive organs, and the retention of the embryo on the gametophyte for an extended period of time in order to obtain nutrients during the earliest stages of sporophyte development. They are divided into two groups: a basal, non-vascular group comprising the bryophytes of which the liverworts (Marchantiophyta), mosses (Bryophyta) and hornworts (Antherocerophyta) are the constituent phyla (Crum 2001), and a vascular group which contains all other extant plant groups, i.e. lycophytes, monilophytes, gymnosperms and angiosperms (Figure 1.2), as well as extinct members of this group. Vascular plants are also referred to as the tracheophytes, (Tracheophyta). Vascular and non-vascular plants are distinguished from each other based on morphology, physiology, and generation dominance/reproductive strategy. Vascular plants are also further divided into the spore producing and seed producing groups. A major morphological feature distinguishing non-vascular plants and vascular plants from each other is specialised water conducting tissues. In vascular plants water is conveyed from the base of the plant to the uppermost regions of the shoot via a system of highly specialised and hollow tube-like cells (xylem/tracheids), reinforced with lignin-enriched secondary cell walls, which can be arranged around the cells in distinctive patterns. In contrast the relatively

tiny bryophytes have comparatively weaker water conducting tissues without cell wall reinforcement, and such specialised cells are absent altogether in the hornworts.

In addition to these two groups a third extinct group, the protracheophytes, evolved which had vascular conducting tissue that resembling the mosses but a morphology, reproductive strategy and life cycle resembling the vascular land plants. Protracheophytes are known only from the Rhynie chert and form an intermediate group between the vascular and non-vascular land plants. The protracheophytes and vascular land plants, especially fossilised early representatives and the more morphologically complex lycophytes and monilophytes (Grewe *et al.* 2013; Karol *et al.* 2010), are also referred to as the polysporangiates, i.e., branching plants with a conspicuous sporophyte, where sporangia are laterally or terminally placed on the branches. In addition, vascular land plants that reproduce via spores are also called pteridophytes, this includes the lycophytes and the monilophytes as well as some earlier evolved vascular land plants such as *Rhynia gwynne-vaughanii*. Generalised terminology such as pteridophyte, or polysporangiate used to describe groups of plants with similar characteristics, but which can be systematically distinguished from each other will be avoided as much as possible in this volume, e.g. monilophytes (Kenrick and Crane, 1997) will be used instead of ferns, and the rhyniophytes, lycophytes and monilophytes will not be referred to as 'pteridophytes' as each group is evolutionarily significant.

1.1.1 Phylogeny of land plants – algae, bryophytes and vascular land plants

Figure 1.1 and 1.2

Discerning the evolutionary relationships among land plants is a continuous process involving research across many fields from plant genetics to palaeobotany. Current evidence strongly supports the land plants as the descendants of the charophycean algae (Charophytes/Charophyta) (Qui 2008). The Charophyte lineages most closely related to land plants are the Coleochaetales, Zygnamatales and Charales (Figure 1.1) and each order possesses several characteristics in common with land plants (Graham *et al.* 2000; Bowman 2013 for overview), for example, branching morphology, apical cells, sporopollenin, and plasmodesmata. Furthermore, many gene families encoding for various enzyme syntheses and controlling regulatory processes such as auxin transcription response and transport pathways are found in land plants and in members of the charophycean lineage (De Smet *et al.* 2011; Popper *et al.* 2011).

Karol *et al.* (2001) used plastid, mitochondrial and nuclear DNA sequences of 48 taxa to determine the relationships between the green algal groups and their land plant descendants, with the charophycean algae resolved as the most likely common ancestor of the land plants, and the Charales holding favour over the Coleochaetales as the sister taxon to the land plants. Other more

recent studies analysing nuclear DNA sequences found that the Zygnamatales or a Zygnamatales plus Coleochaete group are sister to the land plants (Wodniok *et al.* 2011; Finet *et al.* 2010, 2012; Laurin-Lemay *et al.* 2012).

The phylogenetic relationships between the bryophytes and the vascular land plants still remain somewhat unclear despite more than a decade of rigorous genetic analysis. However, the bryophytes are consistently resolved as a basal lineage. Lewis *et al.* (1997) recognised a monophyletic relationship among the liverworts, but found a paraphyletic relationship among the bryophyte groups. Nishiyama *et al.* (2003) using chloroplast genome sequences supported a monophyletic relationship among the bryophytes although they found no strong evidence of a similar relationship among the vascular plants. Qiu *et al.* (2006) using a multigenomic approach, resolved hornworts as the sister group to the vascular land plants, however, the chloroplast genome sequencing data in that study also supported a liverwort plus moss clade and a hornwort plus lycophyte clade. Results from nucleotide data and amino acid translations of the same gene set by Karol *et al.* (2010) also found that the hornworts are sister to all vascular plants. Each of these two methods also returned different results regarding the relationships among the bryophyte groups. A monophyletic relationship among the bryophytes was supported by the amino acid data and a paraphyletic relationship was supported by the nucleotide sequence data. These results have recently been challenged by Cox *et al.* (2014), where the paraphyletic results of both Karol *et al.* (2010) and Qiu *et al.* (2006) are postulated to be affected by phenotype changing synonymous substitutions, causing phylogenetic artefacts. The authors' reanalysis of the Karol *et al.* (2010) amino acid data supported a monophyletic relationship among the bryophytes. A monophyletic relationship among the bryophytes is further supported by transcriptome data of Wickett *et al.* (2014).

As well as evidence from molecular genomic analyses, several synapomorphies unite the vascular land plants with the bryophytes, with strong support for hornworts as the sister group to the vascular plants. Synapomorphic features shared by the hornworts and vascular land plants include, embedded gametangia, largely nutritionally independent sporophyte, the interdigitation of the cells of the sporophyte and gametophyte at their junction (Sporne 1962), presence of xylans in the secondary cell walls of vascular plants and the pseudoelaters and spores of hornworts (Carafa *et al.* 2005). In addition the Rhynie chert protracheophyte plant *Horneophyton lignieri* possesses features that ally it with the hornwort group, e.g. a bulbous rhizome and a distinct columella within the sporangium (Edwards 2003a, Taylor *et al.* 2008).

1.1.2 Alternation of generations and reproductive biology in land plants

The life cycles of the algae are complex and varied in form and can be greatly affected by environmental conditions (Bold and Wynne 1978; Sharma 1986; Van den Hoek *et al.* 1995). Asexual and sexual reproduction both occur extensively, and in some cases sexual reproduction remains unconfirmed in some groups, e.g. Euglenophyta, (Bold and Wynne 1978). In the charophycean green algae the alternation of two separate life phases, defined by ploidy number, in order to complete the life cycle, is one key feature that has been passed into the land plant lineages, although in a relatively more complex form. In land plants the alternation of generations involves the production of two separate vegetative plant bodies within the life cycle of the plant: a haploid gamete producing generation (gametophyte) and a diploid generation that produces haploid spores (sporophyte). In general, 'alternation of generations' refers to the life cycle of land plants. In land plants both of these generations are multicellular, and in the case of extant land plants, are morphologically dissimilar (heteromorphic) and this type of life cycle is referred to as diplobiontic (Renzaglia *et al.* 2000; Qiu *et al.* 2012). Most algae are not diplobiontic but for algae that do display a diplobiontic life cycle there is evidence this type of life cycle evolved twice, once in the charophycean algae and once in the ulvophycean algae, a lineage within the Chlorophyte green algae with whom the Charophytes share a common ancestor (Graham and Wilcox, 2000; Lewis and McCourt, 2004; Niklas and Kutschera, 2009). Other reproductive features that define land plants and separate them from their algal ancestors include the development of sperm producing organs (antheridia) and egg producing organs (archegonia) on the gametophyte, and the retention of the zygote within the archegonium, from where it derives nutrients, before the development of the shoot.

Within the land plants the non-vascular bryophytes are unique in having a gametophyte dominant life cycle, where the gametophyte is the more conspicuous, and long-lived, vegetative generation (figure 1.3 and figure 1.4A–C). This type of generational organisation places emphasis on the successful dispersal of sperm cells and fertilisation of the egg cell rather than spore production and dispersal, further evidenced by the presence of just a single unbranched spore-producing axis in the sporophyte generation. Bryophytes are heavily dependent on the presence of water to disperse their gametes and are commonly found in damp or wet environments, or in environments with only periodic desiccation. The sporophyte remains in contact with the gametophyte throughout its existence, receiving nutrients from the gametophyte via a foot organ (matrophagy). Bryophyte gametophytes have two distinct morphologies, thalloid or axial. The gametophytes of the hornworts and of the Metzgeriidae and Marchantiopsida groups of liverworts have a thalloid morphology. Jungermanniales liverworts and the mosses possess axial leafy gametophytes that, although

considered aerial, are still relatively close to the substrate (Crum 2001) and additionally the leafy axes of liverworts are dorso-ventrally flattened. All bryophyte sporophytes are monosporangiate, with the sporangium terminally placed in the liverworts and mosses, but in the hornworts much of the axial sporophyte is composed of the sporangium. The sporophyte generation of the bryophytes can be short, stubby vegetative growths projecting from the archegonia in some liverworts (e.g. *Marchantia polymorpha*) but also elongate aerial, axial vegetative structures as seen in all bryophyte groups. All other seedless extant land plants are sporophyte dominant with a physically and nutritionally independent sporophyte. Emphasis is placed on the production and dispersal of haploid spores from branched axial sporophytes. However, for these plants there is still some initial dependency on the gametophyte for nutrition, e.g. in the monilophyte *Ceratopteris richardii*; this dependency diminishes as the embryo develops (Johnson and Renzaglia 2009). Identifying the gametophyte stage of lycophytes and monilophytes, which possess several morphological types, has been historically difficult due to their relatively small size compared to the sporophyte, and generally inconspicuous nature (Farrar *et al.* 2008).

For the heterosporous seed plants the reproductive cycle is more complex than in seedless plants. For a comprehensive account of angiosperm and gymnosperm reproduction see Niklas (1997). Over all, the reduction of the gametophyte generation and elaboration of the sporophyte is a trend seen across vascular land plant phylogeny and played a key role in the successful domination of the terrestrial landscape by vascular plants.

1.2 Physiological adaptation to land

The transition from a primarily aquatic environment, to a primarily terrestrial one, required major physiological adaptations for plants (Graham *et al.* 2000). Terrestrial plants would need to be less reliant on the aquatic environment for the dispersal of their gametes, control water loss, prevent desiccation or become desiccation tolerant. Characteristics particular to land plants and favourable to successful terrestrial colonisation include, an increasingly dominant sporophyte generation, stomata or pores within the epidermal layer for gas exchange and control of water loss, vascular conducting systems for water transport, a waxy external cuticle secreted by epidermal cells to control internal water and gas properties, dominance of the sporophyte generation and development of the terrestrial spore (Brown and Lemmon 2011). Vascularised water conducting tissues promote the upright growth of the shoot and the dispersal of spores over a wider area. The cuticle provides a protective layer against UV-B radiation, which, during the late Silurian-Early Devonian was at much higher levels than today. In addition, oxygen levels were lower than today, and the ozone layer, which filters UV-B radiation, was much less developed. Aquatic plants are

protected from UV-B rays by the water, which filters out harmful UV-B rays (e.g. see Caldwell 1979 and Krauss et al. 1997). However, not all of these characteristics are necessary for survival in a terrestrial environment, e.g. bryophytes have relatively weak vascular systems and are gametophyte dominant.

1.2.1 Evolution of the terrestrial sporophyte shoot

The evolution of a multicellular sporophyte generation was a leading factor in the success of vascular land plants, and of land plants in general. However, the origins of the sporophyte, and ultimately the diplobiontic alternation of generations within the land plant clade, are still unresolved. Traditionally two competing hypotheses dominate the debate despite, being proposed in the late 19th and early 20th centuries. Pringsheim (1878) introduced the idea of the Homologous Theory whereby the sporophyte is hypothesised to represent a modification of the gametophyte. Support for the Homologous Theory is inferred by the presence of two separate but morphologically similar generations in some algae, e.g. *Ulva*. This does not account for heteromorphic generations in nearly all extant land plants, but is supported by the near-isomorphic gametophytes of the Rhynie chert plants. The 'Antithetic Theory' proposed by Bower (1908), and later modified and renamed the 'Interpolation Theory' (Bower 1935), proposed that the sporophyte developed through repeated mitosis of a zygote retained on the haploid thallus of an ancestral green alga – the "amplification of the zygote" led to a sporic form of reproduction in contrast to a zygotic one. The Antithetic Theory does not presuppose that an ancestral alga with isomorphic generations was necessary for the development of the sporophyte. It requires just the haploid stage in an ancestral algal species, and the development of the sporophyte as a new feature specific to growth in a terrestrial environment. The evolution of the polysporangiate sporophyte itself is also of considerable interest and its origins ultimately lie with discussions regarding the development of the sporophyte in general. Kato and Akiyama (2005) provided support for the intercalation of the polysporangiate sporophyte as a new organ between the foot and sporangium of an ancestral bryophyte. Ligrone *et al.* (2012b) interpret the polysporangiate sporophyte as the sterilisation of the shoot, i.e. retention of juvenile characteristics and a delay in the development of the reproductive organ (sporangium). Comprehensive reviews of the arguments regarding the evolution of the sporophyte and the polysporangiate sporophyte are given by Blackwell (2003), Bennici (2008), and Ligrone *et al.* (2012b).

Tomescu *et al.* (2014) separated the debate regarding the evolution of the sporophyte and the polysporangiate sporophyte in to three arguments: 1. The sporophyte originated as a branching of a bryophyte-like ancestor; 2. The sporophyte represents a new organ inserted into the life cycle (in

concordance with the Antithetic Theory, and Kato and Akiyama (2005); 3. The polysporangiate sporophyte evolved by a sterilisation of a sporangial axis, as per Ligrone *et al.* (2012b), and vegetative growth continues until reproductive developmental mechanisms are expressed in the apical meristem. In addition the authors suggest a further hypothesis, the Apical Growth Hypothesis, whereby the change from vegetative to reproductive development in the sporophyte marks the critical point that separates the polysporangiates from their bryophyte-like ancestors, much in the same way as the sterilisation hypothesis. Although the origins of the sporophyte may still be unclear, the development of the sporophyte shoot is somewhat easier to comprehend, as there are multiple examples to work from across the land plant groups of which growth and development is well known. In all, the origins and development of the sporophyte shoot may be disputed for many years to come. The development and evolution of the sporophyte shoot is further discussed in Chapter 4 in reference to the results of the studies described in this volume.

1.2.2 Sporopollenin and a delay in spore production

Haploid spores are produced via meiosis by both land plants and algae. In land plants the production of spores is delayed and a multicellular sporophyte develops indeterminately by mitosis. This may have provided additional adaptive fitness to early land plants which probably occupied periodically desiccating conditions, with meiosis commencing once favourable environmental conditions for spore dispersal returned. In land plants the outer layer of the spore is covered with a sporopollenin coat. Sporopollenin is a strong and stable biopolymer of uncertain composition imparting a high level of protection to the spore against damage in the environment, allowing for relatively long distance transport, as well as protection against biotic and other abiotic factors. In addition, sporopollenin is one of the most degradation-resistant natural substances, hence the preservation of spores in the geological record prior to the preservation of the larger vegetative structures of land plants. Sporopollenin is considered a synapomorphy of land plants (Graham 1993). In green algae sporopollenin is found in the cell walls of zygotes and may represent an adaptation in algae that may be exposed to periodic aerial desiccation, protecting the cells. In addition sporopollenin would provide a mechanical barrier to pathogens (Delwiche *et al.* 1989; Graham and Gray 2001).

1.2.3 Stomata

Stomata were one of the major physiological adaptations to the terrestrial environment. Stomata consist of two specialised epidermal cells controlling access to a pore and a sub-epidermal chamber. These stomatal guard cells open and close in response to changes in guard cell turgor and the

exchange of ions with surrounding cells (Jarvis and Mansfield 1981). They control gas exchange and water loss and are found in all land plant groups except the liverworts. On the thalloid liverwort gametophyte, small air pores open into an air chamber connected to the parenchymatous tissues. Plants bearing stomata form the Stomatophyte group. Stomata are found in the earliest known fossil land plants such as *Cooksonia* (Edwards *et al.* 1986), and are even found on both generations of the Early Devonian Rhynie chert plants *Rhynia gwynne-vaughanii* and *Aglaophyton major* (Kerp *et al.* 2003; Taylor *et al.* 2005) This is in stark contrast to the bryophytes where true stomata are restricted to the sporophytes alone. The presence of stomata on the gametophytes of the sporophyte dominant Rhynie chert plants suggest that they were relatively better adapted to the terrestrial environment regarding gas exchange and water loss maintenance than their extant bryophytic relatives. In mosses stomata are implicated in capsule dehiscence, forcing some researchers to question the evolutionary function of stomata in early land plants (Duckett *et al.* 2009; Cox *et al.* 2010; Volkmar and Knoop, 2010; Chang and Graham, 2011). In hornworts the gametophyte bears pores with slime-filled cavities (Renzaglia *et al.* 2007). Some lineages of hornworts also have lost their stomata, and the air pores on the hornwort gametophyte are stoma-like have a similar developmental pattern to true stomata but do not open and close (Ziegler 1987).

The evolution of stomata has been interpreted as a single evolutionary event (Ligrone *et al.* 2012b) and common mechanisms controlling stomatal function in mosses and vascular land plants support this assertion (Bowman 2011; Chater *et al.* 2011), e.g. mutants of the flowering plant *Arabidopsis thaliana* lacking in the necessary abscisic-acid regulatory protein kinase to control stomatal opening became functional by the substitution with the homologous regulatory protein found in the moss *Physcomitrella patens*. However, nucleotide sequences and functional morphology support a separate evolution of stomata within the mosses and a hornwort-like ancestor of tracheophytes (Cox *et al.* 2004; Duckett *et al.* 2009).

1.2.4 Water acquisition and physical support

A specialised internal water conducting system of elongate xylem cells is one of the defining features of vascular land plants and internalises the mechanisms controlling the hydration of the plant (homoiohydry) (Raven 1999a-b). This is in contrast to plants such as the bryophytes where hydration is generally controlled by the external environment, (poikilohydry) (Raven 1999a-b), and their small size negates the requirement for such highly specialised internal cells; although mosses and some liverworts do have internal water conducting cells. Poikilohydry imparts a certain amount of desiccation tolerance, controlled by the cuticle and stomata, and allowing bryophytes to dry-out and recover. Poikilohydry is considered an ancestral trait among land plants, retained only in the

spores, seeds and pollen of vascular land plants and a trait particular to the bryophytes, optimal for their small size, ecology and biology (Proctor 2007).

Xylem is comprised of elongate tracheid elements and/or vessel elements. All xylem cells bear secondary cell wall deposits of lignin, a chemically complex hydrophobic monolignol (Wertz and Bédué 2013), which can be arranged in various patterns around the cell. Lignin has also been reported in the red algae *Calliarthron cheilosporioides* (Martone *et al.* 2009), pointing to either a convergent or conserved ability to produce lignin among the Archaeplastida (i.e. red algae, green algae and land plants).

Tracheids are elongate but relatively short cells with tapered ends. Pits in common cell walls facilitate the passage of water and solutes between tracheids (Esau 1965). Tracheids are the main water conducting cells of the lycophytes, monilophytes and gymnosperms. They are also the only xylem cells that conduct water in the vascular Rhynie chert plants. Transport rates in Rhynie chert plants were lower than what is found in extant mesophytic leaves, however water use efficiency is considered to have been higher, attributed to the much higher concentration of atmospheric carbon dioxide during the Early Devonian (Roth-Nebelsick and Konrad 2003). Tracheids gave some structural support to the Rhynie chert plants but further support was provided by turgor pressure. The development of the tracheid cell likely played an essential role in the permanent colonisation of the terrestrial landscape and set the stage for an irreversible radiation of the vascular land plant. Vessels are generally found in angiosperms but vessel-like cells have been reported in *Equisetum* and *Selaginella* (Duerden 1934; Schneider and Carlquist 2000a-b) as well as in the permineralised gigantopterid *Vasovinea tianii* (Li and Taylor 1999) from the Upper Permian Xuanwei Formation of the Guizhou Province, China. Vessels are more efficient than tracheids and are connected end to end with perforations allowing the free passage of fluids between vessels elements, essentially forming long continuous tubes.

Xylem imparts biomechanical strength (via lignin) to the plant axis but xylem cells are primarily concerned with transporting water up through the plant axis in contact with the surface interface. Xylem is further characterised by fact that at maturity the cells are dead, following the expulsion of all cell contents after the deposition of the cell walls, essentially providing an empty tube through which fluids can pass. Phloem cells accompany the xylem cells to complete the vascular tissue system. Phloem cells carry nutrients such as sucrose formed via photosynthesis through the plant. In contrast to xylem, phloem cells are living cells. The phloem is composed of sieve elements, the sieve cells and the sieve tube members. Sieve cells are found in seedless vascular plants and gymnosperms but sieve tube members are found only in angiosperms.

Although lacking lignified vascular tissues the water-conducting cells (WCCs) in the bryophytes,

especially the mosses show a relatively large degree of morphological diversity. Many groups of mosses bear hydrolysed, generally uniformly thin-walled WCCs called hydroids. The basal-most group to contain hydroids are the Polytricopsid group. In this group hydroid morphology is characterised by thin areas with scattered thicker areas but immunocytochemical evidence shows that these thicker areas are not secondary walls (Ligrone *et al.* 2008). Hydroids are absent in Sphagnopsida, a group of mosses found in peaty, boggy environments and instead hyalocytes serve this role, a specific adaptation to bog habitats (Bragina *et al.* 2012). WCCs have been described for some liverworts (Hébant 1978; Ligrone and Duckett 1996), but only in the gametophyte. Unusually, hornworts do not have specialised WWCs. This may be attributed to the basal position of its meristem. The majority of the hornwort sporophyte shoot is composed of the sporangium and does not require a system of WCCs.

1.3 Fossil evidence for early land plants

Over the past two decades several studies have attempted to pinpoint the origins of land plants. Molecular phylogenetic analyses have provided surprising results and in most cases results clashing with the fossil record regarding the timing of the emergence of land plants. Studies involving molecular clock analyses (e.g. Bromham and Penny 2003 and references therein) place the origins and divergences of land plant groups within the Ordovician (485 – 443 Ma), Cambrian (541 – 585 Ma) and even in the Neoproterozoic (1 Ga – 541 Ma). Clarke *et al.* (2011) place the origin of crown group land plants (embryophytes) at between 568 and 815 Ma, the divergence of the liverwort-stomatophyte lineages at 670 Ma, the hornwort-tracheophyte groups at 524 Ma, and the moss-tracheophyte divergence at 632 Ma. Analyses by Hedges *et al.* (2004) placed the divergence of the chlorophyte algae and vascular land plants at 968 Ma and mosses and tracheophytes at 707 Ma; Heckman *et al.* (2001) put plants on land by 700 Ma. Sanderson (2003) provide the only analysis, which places the origins of land plants within the timeframe they appear in the fossil record, 425 - 490 Ma. Tentative evidence of streptophyte (green) algae on land by at least the Cambrian was provided by Graham *et al.* (2012). They subjected terrestrially grown *Coleochaete* to artificial degradation and found that it resembled Cambrian microfossils.

The earliest unequivocal evidence in the fossil record for the existence of plants with features characteristic of a terrestrial existence come from spores found in the early Middle Ordovician (475 Ma) strata of the Sierra Subandinas in north-western Argentina (Rubenstein *et al.* 2010). The authors described five genera of relatively well-preserved monad and tetrad cryptospores and proposed that the initial colonisation of land by plants occurred in the eastern part of Gondwana with later expansion into more western parts of the continent. Further certainty of plants at the liverwort-

grade of organisation by the Middle Ordovician is provided by spore tetrads and dyads with spore wall ultrastructure similar to that seen in modern liverworts (Taylor 1996). Late Ordovician (425 Ma) fossil spores (Wellman *et al.* 2003) from the Safiq Formation of Oman also show affinities to the bryophytes, specifically to the liverwort group. Enigmatic fossils such as the early middle Cambrian *Parafunaria sinensis* (Yang *et al.* 2004), the Silurian-Devonian *Cosmochlaina* (Edwards 1986), banded tubes (Taylor and Wellman 2009), and the nematophytes (e.g. Lang 1937; Strother 1988; but see Smith and Butterfield 2013) have been tentatively proposed as affiliates of marchantoid liverworts (Graham *et al.* 2004) or mosses (Yang *et al.* 2004), further indicating that bryophyte-grade plants were present at least in the Ordovician and Silurian. The discovery of trilete spores from the Upper Ordovician of Saudi Arabia strongly points to a Late Ordovician origin for vascular plants (Steemans *et al.* 2009).

The macrofossil record of the earliest land plants comprises almost exclusively of the polysporangiates with simple branching, protracheophyte or vascular sporophytes with stomata, rhizoids and terminally or laterally placed sporangia (Taylor *et al.* 2008 for a comprehensive review). Late Silurian to Early Devonian plant fossils, assigned to the genus *Cooksonia* are considered representatives of the earliest vascular land plants (Edwards and Feehan 1980). Cooksonioid plants had simple, naked isotomously branching axes with broad, rounded, terminal sporangia that may also have a cup-like appearance (Edwards *et al.* 1992; Gerrienne *et al.* 2006). The rhyniophytes (named after plants found in the Rhynie chert) are an extinct group of early polysporangiate land plants most commonly found in upper Silurian and Lower Devonian sedimentary sequences (Taylor *et al.* 2008). They are characterised by leafless bifurcating axes with terminal sporangia, and are rootless. The rhyniophytes do not constitute a true phylogenetic group and generally contain both vascular and protracheophyte early land plants along with other plants of uncertain vascular affinities.

1.3.1 Lack of macrofossils for earliest land plants

The paucity of very early land plant macrofossils prior to the late Silurian is most likely due to a combination of environmental and plant morphological factors. Despite some evidence of bryophyte-like fossils from the Cambrian to Devonian, the general lack of recognisably bryophyte-like macrofossils is one of the major stumbling blocks in deciphering the early evolution of land plants and it is uncertain how these plants would have appeared morphologically. Despite strong support as the ancestral clade to vascular land plants, the most confidently accepted earliest macrofossil record of bryophytes is from middle Late Devonian siltstones and shales of the Catskill Delta of New York (Van-Aller Hernick *et al.* 2008). Over 150 thalli were recovered showing distinct

morphological features similar to those of liverwort gametophytes. In addition, one sporophyte also characteristic of liverworts was also identified. The oldest accepted evidence for mosses comes from Mississippian (Late Viséan) strata of eastern Germany (Hübers *et al.* 2012), where three types of mosses were described, inferring a more widespread distribution of mosses in the Carboniferous than previously considered. Hornworts make their first appearance in the fossil record in the Cretaceous (Archangelsky and Villar de Seoane 1996) despite clear evidence, should molecular phylogenetics be correct, that a hornwort-like plant must have existed during at least the Silurian.

The earliest land plants most likely occupied habitats close to aquatic environments, probably with an almost permanent water supply. The presence of a regular, dynamic water supply would be somewhat destructive on very small bryophyte-like ancestral plants that could be easily destroyed and transported away in these types of environments, impacting negatively on any preservation potential. However, experimental data show that certain constituents in extant bryophyte are as resistant to decay over short time intervals as those of vascular plants (Hemsley 2001) and in addition bryophytes have relatively slow decomposition rates (Glime 2007). Many bryophytes decay from the bottom upwards (Glime 2007), with aerial apical areas continuing to grow, indicating that if applicable to the earliest bryophyte-like land plants, vegetative parts would have decayed long before any possibility of fossilisation could occur. In this case only strong degradation resistant materials such as sporopollenin, found in spore walls could be preserved, hence the appearance of spores before vegetative body fossils. However, applying experimental results regarding extant bryophytes to possible bryophyte-like ancestral plant forms should be done with caution considering the hundreds of millions of years of evolution between the extant bryophytes and the ancestral bryophyte-like land plants. The poor representation of bryophytes in the fossil record remains a significant problem for palaeotany, although several have been described preserved in amber, e.g. Hedenäs *et al.* 2014; Heinrichs *et al.* 2012; Bell and York 2007, suggesting that bryophytes were not particularly rare. Compression fossils of bryophytes are rare however with a few examples from the Permian of Angara (Neuberg 1960) and the Jurassic Jiulongshan Formation of Inner Mongolia, China (Heinrichs *et al.* 2014)

The presence of vegetation in terrestrial environments with more ephemeral water availability, and plants with more resistant constituent materials, such as lignified vascular tissues, cuticles, as wells as sporopollenin, likely contributed to the preservation of land plants much later in the fossil record than would have been possible in the early stages of terrestrial colonisation. In addition, the effects of larger groups of terrestrial plants binding soils and stabilising the substrate may also have contributed to a later preservation of bryophytes in the fossil record, e.g. in Van-Aller Hernick *et al.* (2008) liverwort-like fossils occur with other vegetative remains.

Figure 1.1

Phylogenetic relationships among the charophycean algae. Modified from Bowman (2013).

Figure 1.2

Phylogenetic relationships among the algae and land plants. Based on Kenrick and Crane (1997).

Figure 1.3

Schematic representations of the morphologies of the three bryophyte groups.

Figure 1.4

Examples of bryophytes (A, B and C), lycophyte (D) and monilophytes (E and F)

A: Thalloid gametophyte of liverwort (overlying axial gametophyte of bryophyte).
Photo courtesy of Zuzanna Wawrzyniak, University of Silesia, Poland.

B: Moss gametophytes with axial sporophytes (*Mnium hornum*). Photo P. Kearney.

C: Hornwort thalloid gametophytes (unidentified genus/species) with branches of an axial leafy liverwort (Jungermanniales species- *Scapania*?) gametophytes pushing through. Photo courtesy of Zuzanna Wawrzyniak, University of Silesia, Poland.

D: Lycophyte. Probably *Lycopodium clavatum*. Photo courtesy of Zuzanna Wawrzyniak, University of Silesia, Poland.

E: *Equisetum telmateia*. Wesfälische Wilhelms-Universität, Botanical Gardens Münster. Photo P. Kearney.

F: *Polystichum aculeatum*. Wesfälische Wilhelms-Universität, Botanical Gardens Münster. Photo P. Kearney.

Figure 1.1

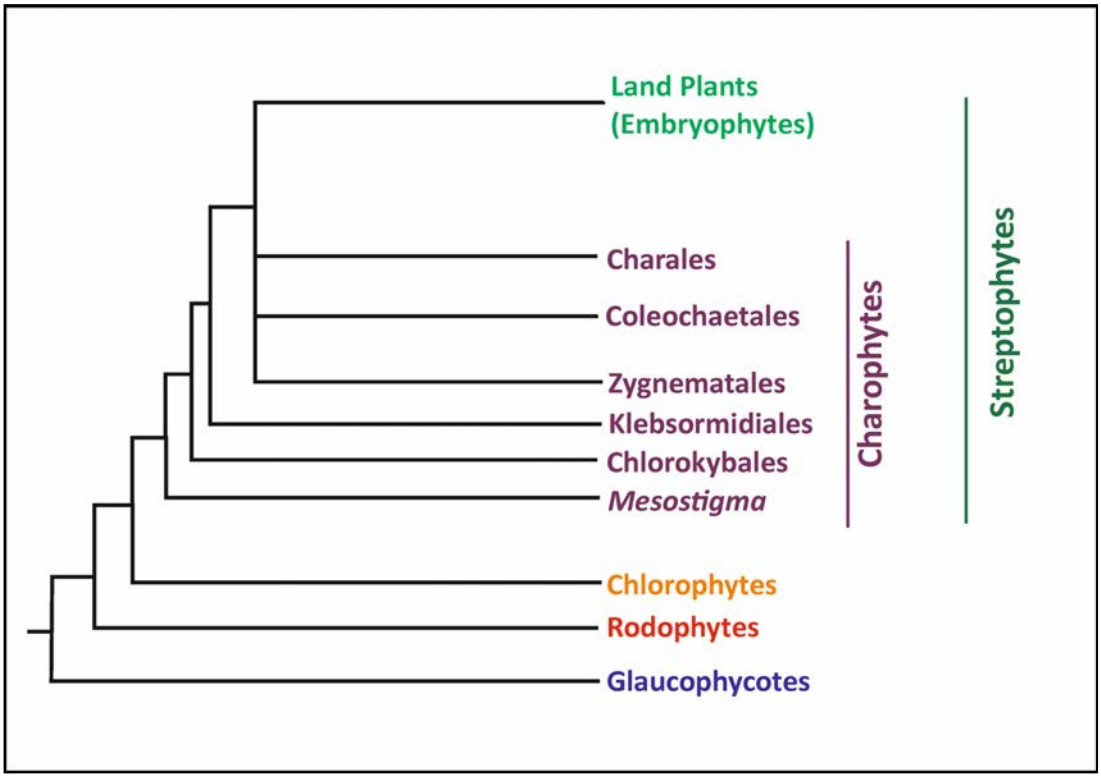


Figure 1.2

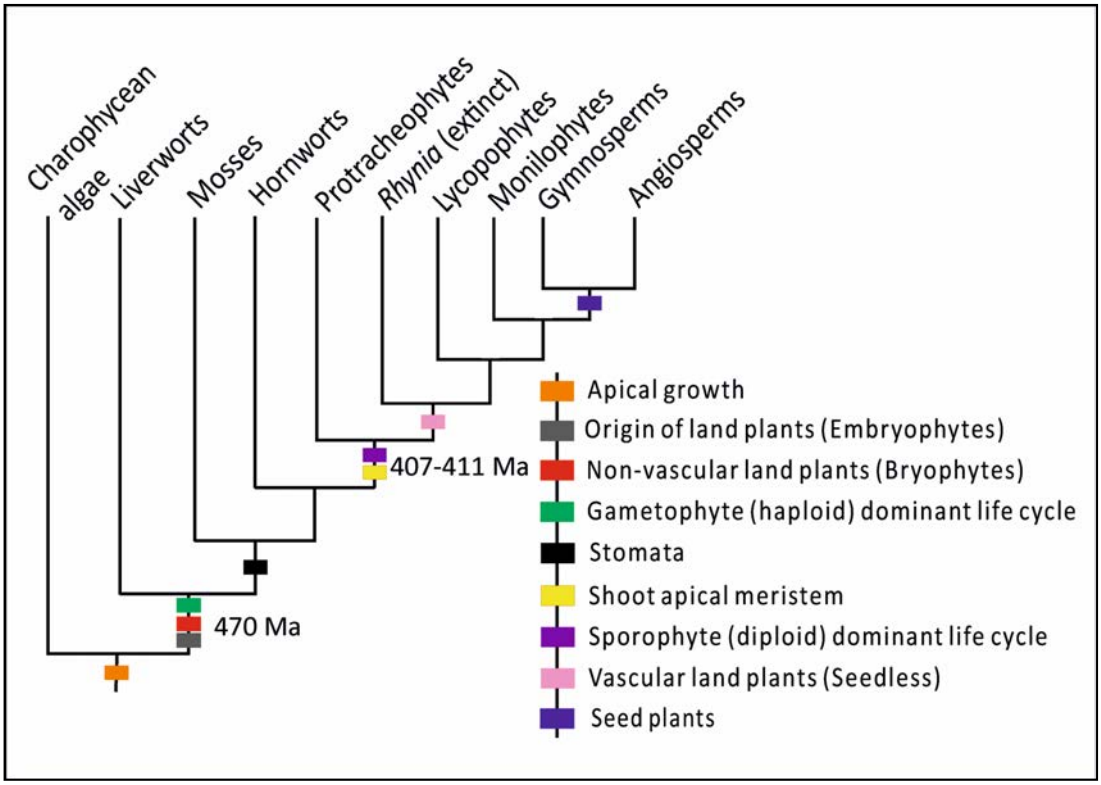


Figure 1.3

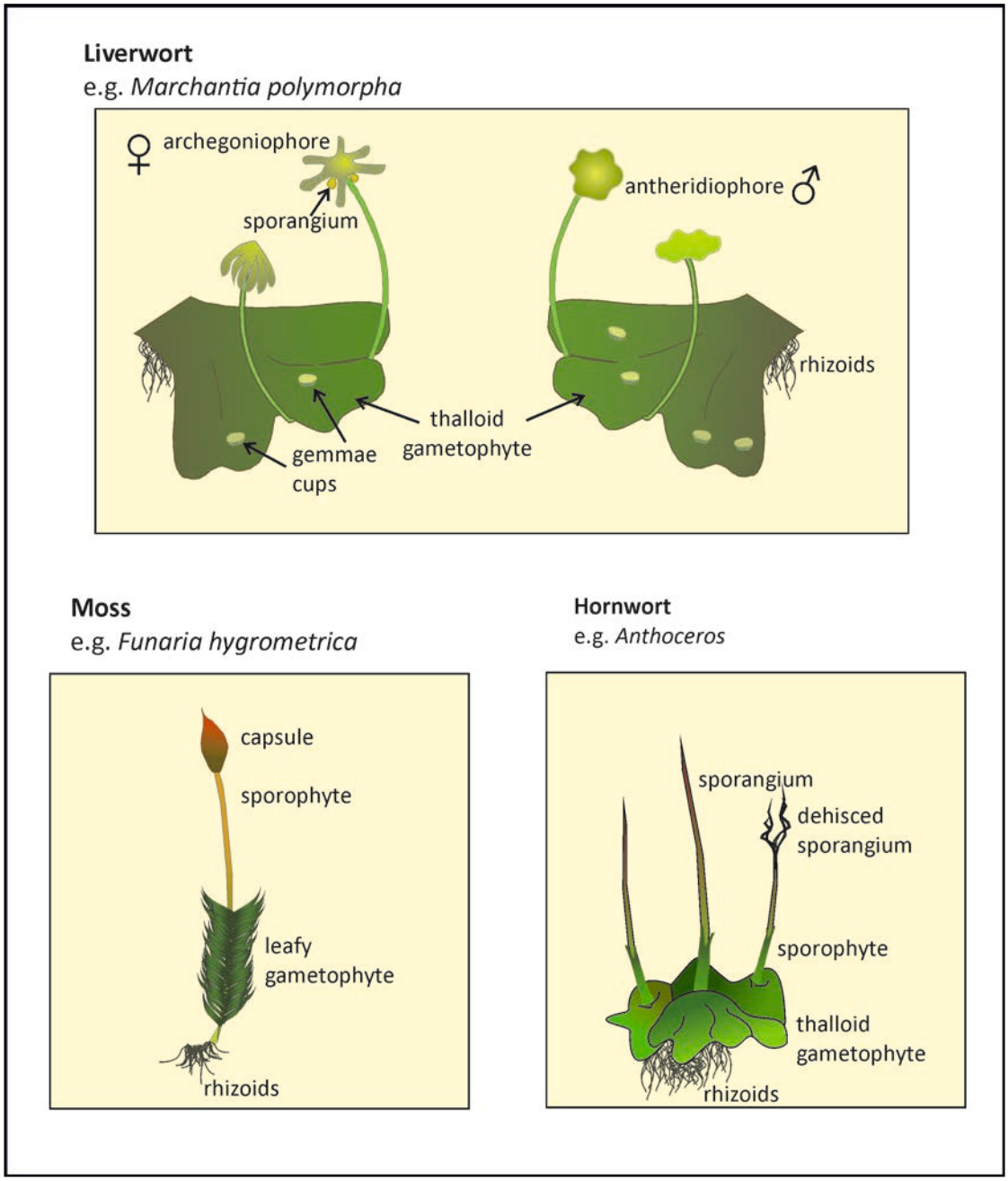

Liverwort
e.g. Marchantia polymorpha
archegoniophore
sporangium
antheridiophore
rhizoids
thalloid gametophyte
gemmae cups
Moss
e.g. Funaria hygrometrica
capsule
sporophyte
leafy gametophyte
rhizoids
Hornwort
e.g. Anthoceros
sporangium
dehisced sporangium
sporophyte
thalloid gametophyte
rhizoids

Figure 1.4

Chapter 2

Rhynie chert: History of research, geology, flora and analytical techniques

2.0 The Rhynie chert: discovery and main scientific contributors

The Rhynie chert has been studied and analysed for over a century, following its discovery by William Mackie while carrying out a geological survey in the Rhynie area of Aberdeenshire (Mackie 1913; Trewin 2003). As *in situ* chert is not exposed at the surface the first trenches were dug between 1913 and 1916 (Horne *et al.* 1916) showing interbedded sandstones, shales and chert. Robert Kidston and William Lang (Kidston and Lang 1917, 1920a, 1921a-b) carried out the first systematic descriptions of the Rhynie chert plants. *Rhynia gwynne-vaughanii* was one of the first plants to be described, with later work morphologically distinguishing *Rhynia gwynne-vaughanii* from *Aglaophyton major* (then, *Rhynia major*, but later recognised as a separate genus by Edwards D.S. 1986). Later volumes described *Asteroxylon mackiei*, *Nothia aphylla*, *Horneophyton lignieri*, and the charophyte alga *Palaeonitella*. Further work on the Rhynie chert carried out by Geoffrey Lyon in the 1950's and 1960's, described germinating spores (Lyon 1957), *Nematoplexus* (Lyon 1962) and the fertile structures of *Asteroxylon mackiei* (Lyon 1964). Further trenching during the 1960's and 1970's produced large quantities of material, which were enthusiastically examined and studied by Winfried and Renate Remy from the late 1970's up to the early 1990's at Westfälische Wilhelm-Universität, Münster. These studies produced the first descriptions of gametophytes in the Rhynie chert (Remy 1980; Remy and Remy 1980a-b). Throughout the 1990's and the early twenty-first century several studies brought descriptions of fungi, (Taylor *et al.* 1992; Hass *et al.* 1994; Remy *et al.* 1994; Taylor *et al.* 2003) new animals (Dunlop *et al.* 2003) the description of more gametophytes (Kerp *et al.* 2003) and new, detailed descriptions of the Rhynie chert flora (Edwards 2003a; Daviero-Gomez, *et al.*

2005; Kerp *et al.* 2013).

2.1 Stratigraphy and structural geology

Figures 2.1, 2.2 and 2.3

The Early Devonian Rhynie chert lagerstätte from Aberdeenshire, UK consists of a suite of permineralised plant, fungal and arthropod remains preserved in silica, recording an exceptional example of an early terrestrial ecosystem. The chert was deposited in a hot-spring environment with a geyser supplying a regular stream of warm silica-rich water to the surrounding landscape (Rice *et al.* 2002; 2003; Baron *et al.* 2003). The Rhynie chert occurs within the Dryden Flags Formation, a unit of laminated shales and sandstones. A unit of mixed sandstone, shale and conglomerate underlies the Dryden Flags Formation. The underlying basement consists of Ordovician basic igneous rocks and Precambrian metasediments. The boundary between the basement and overlying sediments is marked by an unconformity. The basin itself is considered a half-graben system with the western margin on the northern side a low angle listric fault zone (Rice and Ashcroft 2003). The basin can also be interpreted as a pull-apart basin due to evidence for strike slip faulting in northern Britain during the early Devonian (Strachan *et al.* 2002), which could further account for the localised, as opposed to regional (Tosdal and Richards 2001), volcanic activity seen in the Rhynie lithologies.

Based on high-precision $^{40}Ar/^{39}Ar$ dating of K-feldspar from two vent feeding conduit veins the chert has been dated to an age of 403.9±2.1Ma (Mark *et al.* 2011). In addition, an age of 411.5±1.3 Ma (Parry *et al.* 2011) was determined by U-Pb dating of an andesitic lava flow occurring within the Lower Devonian Rhynie Outlier. Dispersed spore assemblages within the chert are dated to the Pragian-?earliest Emsian (Wellman 2006).

2.2 Plant biota

The Rhynie chert is renowned for the extraordinarily detailed preservation of early land plants. Six species of land plant with varying degrees of morphological complexity have so far been identified in the chert, each exhibiting the various cellular structures common in land plants, such as stomata, epidermal and cortical cells and conducting tissues (Edwards 2003b). The Windyfield chert (figure 2.2), situated at a location of approximately 700m northeast of the Rhynie chert locality, preserves a similar depositional environment and hosts the same plant species, plus one other species, *Ventarura* (Powell *et al.* 2000). The Windyfield chert may be slightly younger in age than the Rhynie chert, based on its geological setting, although it occupies the same Biozone as the Rhynie chert (Fayers and Trewin 2003), and also occurs within the Dryden Flags Formation.

The plants preserved in the Rhynie chert are not only more complex than the extant bryophytes

but also show varying degrees of morphological complexity. The simplest of the plants in the chert include the protracheophytes *Aglaophyton major* and *Horneophyton lignieri* and the vascular plant *Rhynia gwynne-vaughanii*. *Asteroxylon mackiei*, a stem lycophyte, is the most complex plant found in the chert possessing short, but unvascularised, leaf-like enations, root-like subsurface axes and a distinct actinostele (Kerp *et al.* 2013). For four of the six species of Rhynie chert plant, gametophytes have been described, however the occurrence of gametophytes in the chert is very rare and in several cases only one example for a species is known. For *Aglaophyton major* and *Rhynia gwynne-vaughanii* full life cycles have been successfully illustrated (Remy and Remy 1980; Remy and Hass 1996; Kerp *et al.* 2003). Both the female and male gametophores are carried on separate gametophytic axes. Remarkably the gametophyte generation of these two species are very similar to their sporophyte counterparts, but much smaller, and bear stomata and rhizoids, although they are not branched. This isomorphic, or near-isomorphic, morphology between both generations of a plant species is virtually unknown in any extant land plant group. The closest exception is *Psilotum nudum* (a monilophyte, Karol *et al.* 2010), (Bierhorst 1977), where the gametophyte is axial, but branched, and similar to the sporophyte.

The chert plants shared their environment with a host of other organisms such as arthropods and fungi. Fungi are ubiquitous in the chert and most likely played a key role in the cycling of nutrients through the ecosystem. Fungi occur as saprophytes, parasites and mutualists underlining the important role they played in the development of terrestrial ecosystems (Taylor *et al.* 2003). Despite the wealth of information provided by the Rhynie chert there are still many outstanding questions regarding the detailed developmental morphology of the plants found therein. Unravelling this information can broaden our understanding of early land plant development and especially allow for meaningful comparisons with the lower non-vascular extant land plants such as the bryophytes, and the extant early divergent higher vascular land plants like the lycophytes and monilophytes. Crucially, the development of the diploid sporophyte on the haploid gametophyte has never been convincingly seen for any of the Rhynie chert plants (but see Kerp *et al.* 2003 and figure 4.4). Although early ontogeny in plant embryos can vary across plant groups, the discovery of this type of information could provide an important link between the lower land plant groups and the Rhynie chert plants, leading to a better understanding of sporophyte evolution and development.

Preservation varies in quality throughout the Rhynie chert succession, with the best preservation restricted to those plants that primarily occupied the regions closest to the geyser vent, such as *Aglaophyton major* and *Rhynia gwynne-vaughanii*, with comparatively poorer preservation in better-drained soils further out, where *Asteroxylon mackiei* was more common. Examples of exceptionally

preserved *in situ* plant material, including the preservation of sperm being ejected from the antheridia as well as internal organs of arthropods (Dunlop *et al.* 2003, Anderson *et al.* 2003, Kerp *et al.* 2003), point to what was in many cases extremely rapid preservation in an aquatic medium highly enriched in silica. Modern studies of hot spring environments and the preservation of the biota therein show a preservation time of several months for some plant material (Channing and Edwards 2003, 2009a-b, 2013), but the Rhynie chert in many cases shows such exquisite preservation in parts that can only point to immediate fixation in a viscous silica-rich medium, immediate death of cellular functions and preservation over the scale of days or weeks. An amorphous silica gel is implicated in the partial silicification of a branch of a pine tree, (*Pinus contorta*), found at a hot spring in Yellowstone National Park, USA (Hellawell *et al.* (2015). The branch has been dated to approximately 140 years (^{14}C) and supports the assertion that the silicification process can occur reasonably rapidly and within the lifetime of a plant.

2.2.1 *Rhynia gwynne-vaughanii*

Figure 2.4 and 2.5

Rhynia gwynne-vaughanii is one of the most intensely studied vascular plants of Rhynie chert. A relatively small plant that grew only to about 18cm in height, *Rhynia gwynne-vaughanii* was first described by Kidston and Lang (1917, 1920a), and subsequently reconstructed and examined at a deeper level to include more complex morphological features (Edwards D.S. 1980; Edwards 1993, 2003a; Edwards *et al.* 1998). The aerial axes were branched with terminal sporangia and had a distinct external epidermal pattern with elongate lozenge-shaped cells marked with a dark central line. Hemispherical projections on both prostrate and aerial axes could bear rhizoids and/or a stoma. *Rhynia gwynne-vaughanii* lacked a rooting system; instead rhizoids on aerial and rhizomatous prostate axes fulfilled the function of moisture absorption, probably aided by glomeromycotan mychorrhizae; rhizoids on prostrate axes also provided anchorage to the ground surface. The terminal fusiform sporangia of *Rhynia gwynne-vaughanii* have been rarely encountered in the chert, with Edwards D.S. (1980) giving the most comprehensive review of their morphology. Internally, *Rhynia gwynne-vaughanii* possessed vascular tissues of tracheids with distinct helical secondary wall thickenings, a parenchymatous zone, and an epidermal cell layer. *Rhynia gwynne-vaughanii* is considered a polysporangiate and true vascular plant, i.e. a tracheophyte, and sits phylogenetically between non-vascular plants such as bryophytes and the vascular lycophytes (Kenrick and Crane 1997).

Although the sporophyte *Rhynia gwynne-vaughanii* occurs abundantly throughout the chert, its gametophyte counterpart was described and named only in 2003. *Remyophyton delicatum*, identified by

Kerp *et al.* (2003), is represented by a single monospecific stand of unbranched unisexual axes. Examination of the vascular tissue, as well as other histological aspects revealed similarities between *Remyophyton delicatum* and the immature axes of *Rhynia gwynne-vaughanii* helping to confirm that *Remyophyton delicatum* represents the gametophyte stage.

2.2.2 *Aglaophyton major*

Although these studies focused primarily on *Rhynia gwynne-vaughanii*, some new information is given on *Aglaophyton major*. *Aglaophyton major* on first glace bears a strong resemblance to *Rhynia gwynne-vaughanii* and both plants were long considered to be representatives of the same genus (*Rhynia*). However, *Aglaophyton major* was a slightly larger plant, with a relatively larger shoot diameter and did not have hemispherical projections. *Aglaophyton major* possesses further characteristics identifying it as a member of the extinct protracheophytes (Edwards D.S. 1986). This classification is based on the lack of true xylem tissues and a vascular system that resembles the hydroids of mosses. *Horneophyton lignieri*, another Rhynie chert plant, is also considered a protracheophyte further supporting evidence for a transitional group between the non-vascular bryophyte-like ancestors and the earliest vascular land plants. However, it is clear that by the Early Devonian, as is evidenced by the Rhynie chert plant *Asteroxylon mackiei*, and even in the latest Silurian by *Baragwanathia longifolia*, land plants had evolved complex morphologies and the sporophyte-dominant life cycle was well established (Kidston and Lang 1920a-b; Lang and Cookson 1935; Rickards 2000).

2.3 Analytical techniques

2.3.1 Light microscopy

The chert has traditionally been studied as petrographic thin sections on standard slides and viewed under a light microscope (Hass and Rowe 1999). The studies described here followed the same procedure.

Prior to sectioning, blocks of chert of varying sizes were observed under low magnification to identify interesting targets within. The blocks were then cut to fit on a glass slide (28 mm x 48 mm), lightly ground down using silica carbide powder on the targeted side, washed, and heated (120°C) on a hot plate. The block was then coated on the ground side with a thermoplastic resin and left to cool completely. A glass slide was ground in a similar fashion to the block of chert, washed and left to dry. The slide was also heated on the hot plate and a thin layer of thermoplastic resin is applied. The block was adhered to the slide carefully applying some gentle pressure to remove any air bubbles that may form between the slide and the block of chert. The specimen was then removed

from the hot plate and left to cool completely.

The cold specimen was cut on an automatic saw (here, Buehler Isomet 4000) to the desired thickness (usually approximately 700–800 μm). The result was a thin sliver of chert that could be further ground down with silicon carbide powder on a glass plate in order to view interesting targets more easily. The slice of chert can also be released from the slide by heating it to soften the resin, carefully turned around, reattached and further ground down if required. Rather than a cover slip the specimen was covered with a light layer of cedar wood oil allowing for better observation under the microscope. For this study the chert was cut in serial sections through a block, to see as much of the morphology through the targeted axes as possible.

The thin section images for these studies were photographed using Leica DM 750 and MZ 16 microscopes and images were processed using Leica imaging software LAS 4.5.

Thin section specimens used for this study are housed at WWU labelled 'RA' and 'RB'.

Traditional methods of chert examination, using petrographic slide preparation and light microscopy, have clearly proven fruitful over the past century, however there are a number of shortcomings with this type of technique. Cutting the chert using a petrographic saw can be destructive with approximately a 1–2 mm thickness of chert and thus potential information lost each time the chert is mounted and a new slide made. In addition, in order to properly observe and photograph specimens, the chert must be ground, mostly by hand, to a thin sliver using silica carbide powder, producing slides where plant specimens become just fragmented slices of the original object of interest. Furthermore, especially in thick specimens, interesting three-dimensional targets are difficult to observe through oblique slides and may also be orientated obliquely in the specimen making any significant studies of important developmental areas such as the shoot apical meristem impossible. In petrographic thin sections proper assessment of the apical area of the shoot can regularly be difficult as commonly these thin-walled and undifferentiated cells do not preserve as readily in the silica medium or in such fine detail as more mature or differentiated cells, for example the shoot apical meristem is often preserved as a zone of thin walled cells low in detail and lighter in colour to the rest of the cells in the specimen.

2.3.2 Synchrotron X-ray tomographic microscopy

Opportunities to subject the Rhynie chert to a different method of analysis arose which provided the potential to visualise the chert plants in unprecedented detail. *Rhynia gwynne-vaughanii* specimens were subjected to scanning using phase contrast synchrotron X-ray tomographic microscopy (SRXTM). SRXTM is now a commonly used analytical technique for examining the fine detail of small (millimetre-scale and smaller) three-dimensional fossil materials (Donoghue *et al.* 2006; Betz *et*

al. 2007; Friis *et al.* 2007). For this study, chert specimens from relatively thick thin sections (~ 700μm–1 mm thickness) were removed from their slides, by heating the thermoplastic resin. Suitable plant fragments could be isolated from the chert matrix by cracking, using a flat head tweezers along natural weak points such as where the plant fossil meets the matrix. In most cases however the targeted specimen was not separable from the surrounding chert and such specimens were trimmed down as much as possible. All preparation of the chert for SRXTM was done under a Leica EZ4 stereomicroscope. Specimen sizes were kept approximately ≤1 mm in width and length. In total 19 specimens of *Rhynia gwynne-vaughanii* were scanned. Specimens were typically mounted on brass SEM stubs with clear nail polish, but very small (≤1 mm) specimens were adhered to a metal pin, stabilised on the stub using wax. All specimens were scanned using propagation based phase contrast synchrotron X-ray tomographic microscopy at the TOMCAT beamline (Stampanoni *et al.* 2006) at the Swiss Light Source, Paul Scherrer Institute in Villigen, Switzerland. The beam energy was set between 15 and 21 keV depending on the specimen thickness. For each scan, 1501 equiangularly distributed projections over 180° were acquired. The transmitted and refracted radiation was converted to visible light by a LAG:Ce 20 μm thick scintillator screen, magnified by a 10x or 20x microscope objective and digitised by a sCMOS detector (PCO.edge). The resulting voxel sizes were 0.65 μm and 0.325 μm, respectively. The sample-detector distance was 30 mm for most specimens. Prior to tomographic reconstruction using a direct Fourier method based on a regridding scheme (Marone and Stampanoni 2012), each dark- and flat-field corrected projection was phase retrieved according to the single distance algorithm by Paganin *et al.* (2002). In this way, it was possible to boost the contrast between the plant material and the chert matrix, while preserving high spatial resolution in particular for completely embedded fossilised plant fragments. For fragments exposed to the air, a bright white border was obscuring any cellular detail within the upper cell layers. The parameter adjustments in the phase retrieval algorithm necessary to remove this border would have severely compromised the spatial resolution. For selected specimens, the high frequency component of the original (prior to phase retrieval) dataset was included to minimize this loss in spatial resolution (Irvine *et al.* 2014). Tomographic volumes for each scanned specimen are stored as .tif files, essentially virtual thin sections, and were analysed using VSG-3D Avizo Standard® rendering software. In each specimen, cells of interest were individually labelled, and reconstructed, by tracing the cell walls through the virtual slices. All .tif files stored electronically at WWU are under file names RTK 3–21. In total 19 specimens of *Rhynia gwynne-vaughanii* were scanned.

Thin sections of the shoot apical meristem of *Aglaophyton major* form part of the permanent collection at WWU, were prepared by Hagen Hass.

Figure 2.1

Generalised geological map of the Rhynie area. Modified from Baron *et al.* (2003).

Figure 2.2

Detailed geological map of the Rhynie area (plus Windyfield). Modified from Baron *et al.* (2003).

Figure 2.3

Stratigraphic section of the geology of the Rhynie area and position of the Rhynie chert. Modified from Rice and Ashcroft (2003).

Figure 2.4

Schematic representation of the morphology of *Rhynia gwynne-vaughanii* and its gametophyte *Remyophyton delicatum*.

Figure 2.4

Branched axis of *Rhynia gwynne-vaughanii*. p. = propagules, vt. = vascular tissue, hp. = hemispherical projection. See also figure 3.12 E – H. Slide RA 4.0. Scale 2 mm.

Figure 2.5

Composite image of a branched *Rhynia gwynne-vaughanii* axis. hp. = hemispherical projection; p. = propagule; vt. = vascular tissue. Slide RA 4.0. Scale 2 mm.

Figure 2.1

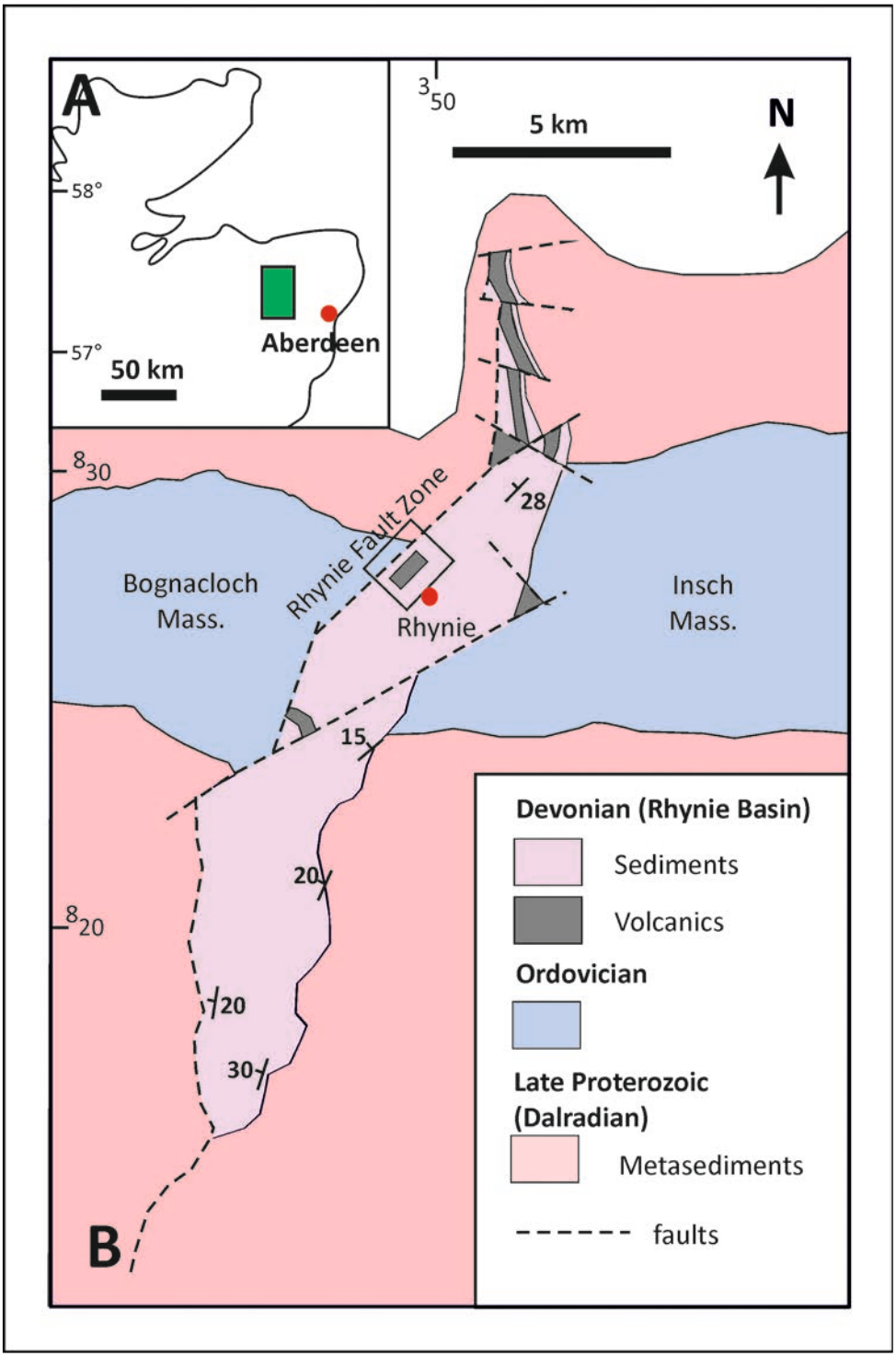

A
58°
57°
50 km
Aberdeen
3 50
5 km
N
8 30
8 20
Rhynie Fault Zone
Rhynie
Bognacloch
Mass.
Insch
Mass.
28
15
20
20
30
B
Devonian (Rhynie Basin)
Sediments
Volcanics
Ordovician
Late Proterozoic
(Dalradian)
Metasediments
faults

Figure 2.2

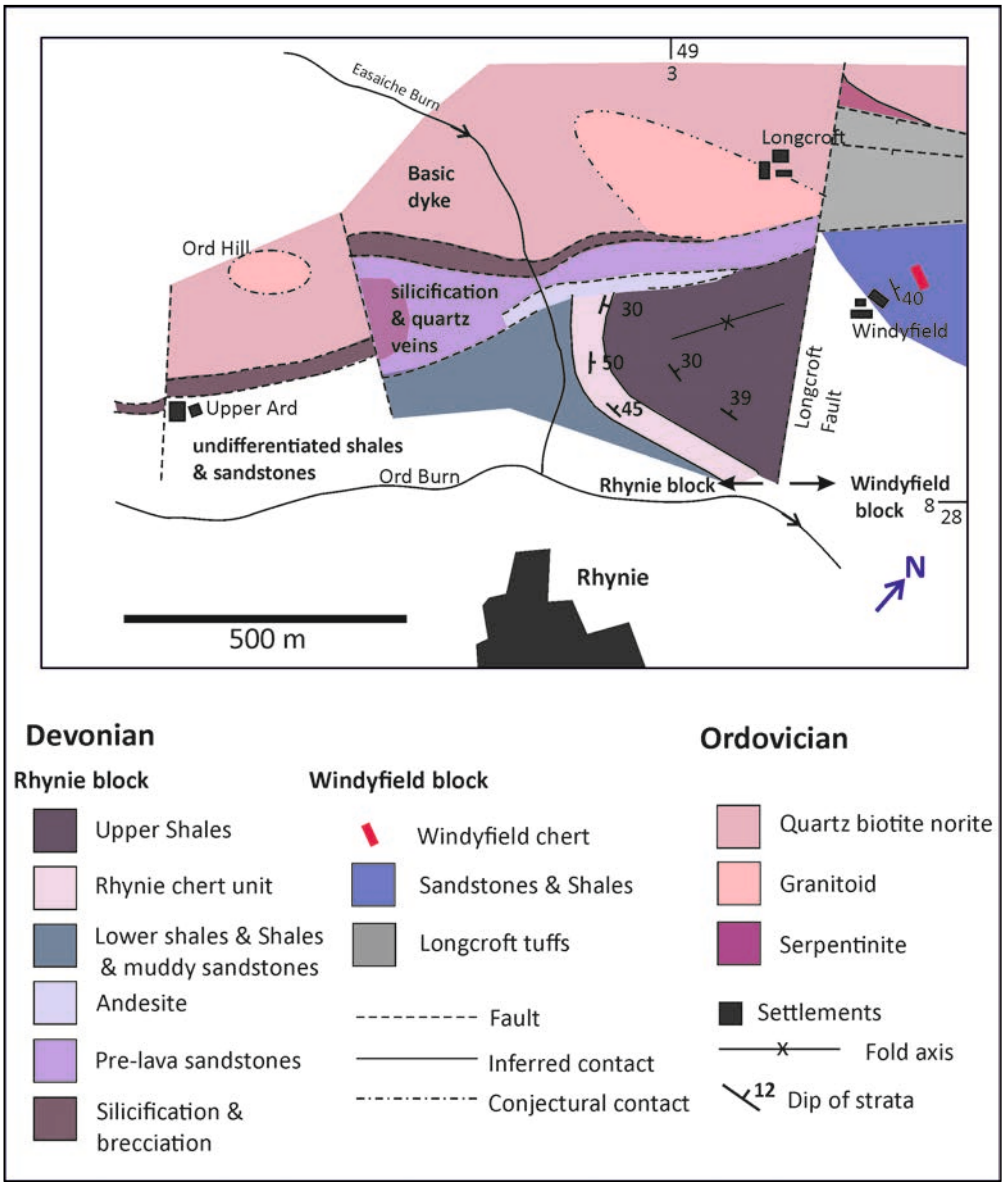

Easaiche Burn
49
3
Longcroft
Basic dyke
Ord Hill
silicification & quartz veins
30
50
30
45
39
40
Windyfield
Longcroft Fault
Upper Ard
undifferentiated shales & sandstones
Ord Burn
Rhynie block
Windyfield block
8
28
Rhynie
N
500 m
Devonian
Rhynie block
Upper Shales
Rhynie chert unit
Lower shales & Shales & muddy sandstones
Andesite
Pre-lava sandstones
Silicification & brecciation
Windyfield block
Windyfield chert
Sandstones & Shales
Longcroft tuffs
Fault
Inferred contact
Conjectural contact
Ordovician
Quartz biotite norite
Granitoid
Serpentinite
Settlements
Fold axis
12
Dip of strata

Figure 2.3

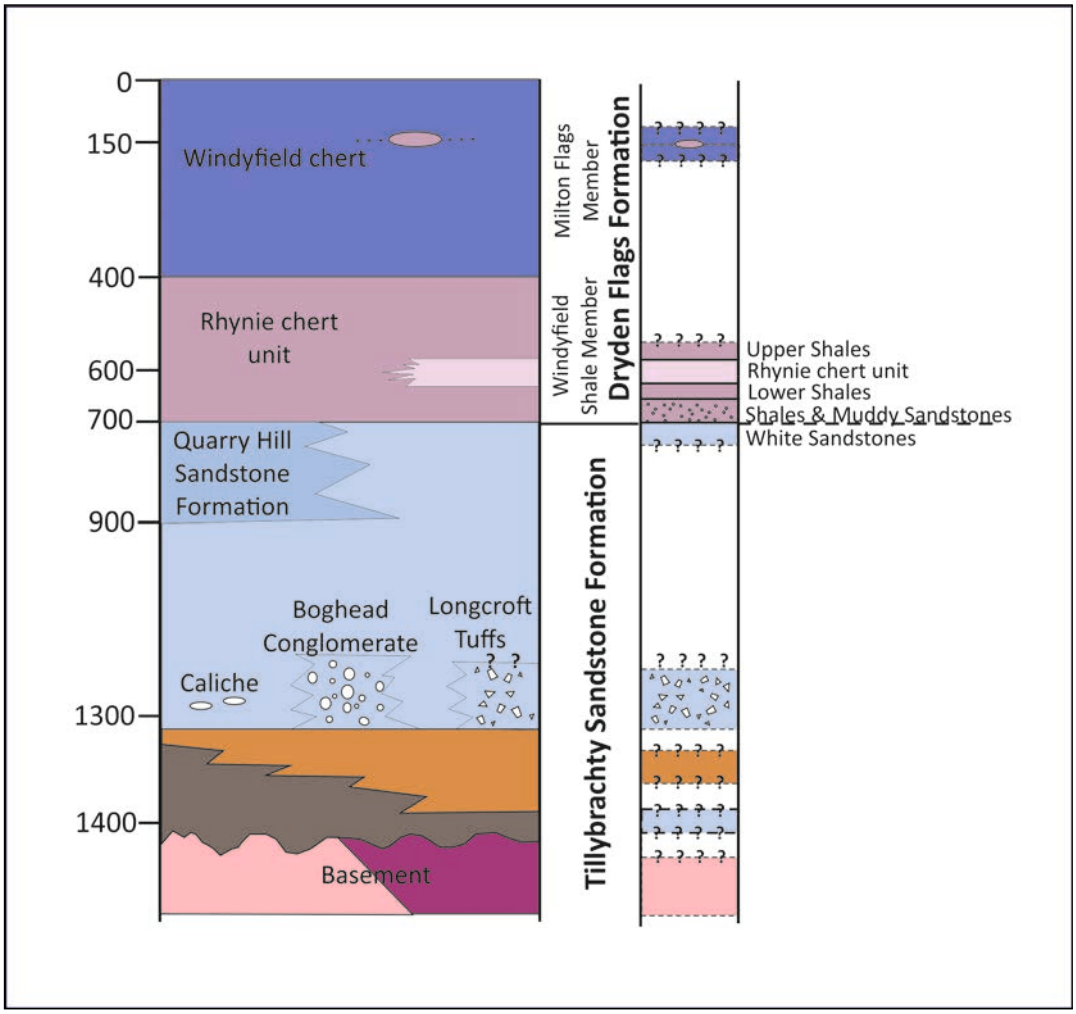

0
150
400
600
700
900
1300
1400
Windyfield chert
Rhynie chert unit
Quarry Hill Sandstone Formation
Boghead Conglomerate
Longcroft Tuffs
Caliche
Basement
Milton Flags Member
Windyfield Shale Member
Dryden Flags Formation
Tillybrachty Sandstone Formation
Upper Shales
Rhynie chert unit
Lower Shales
Shales & Muddy Sandstones
White Sandstones

Figure 2.4

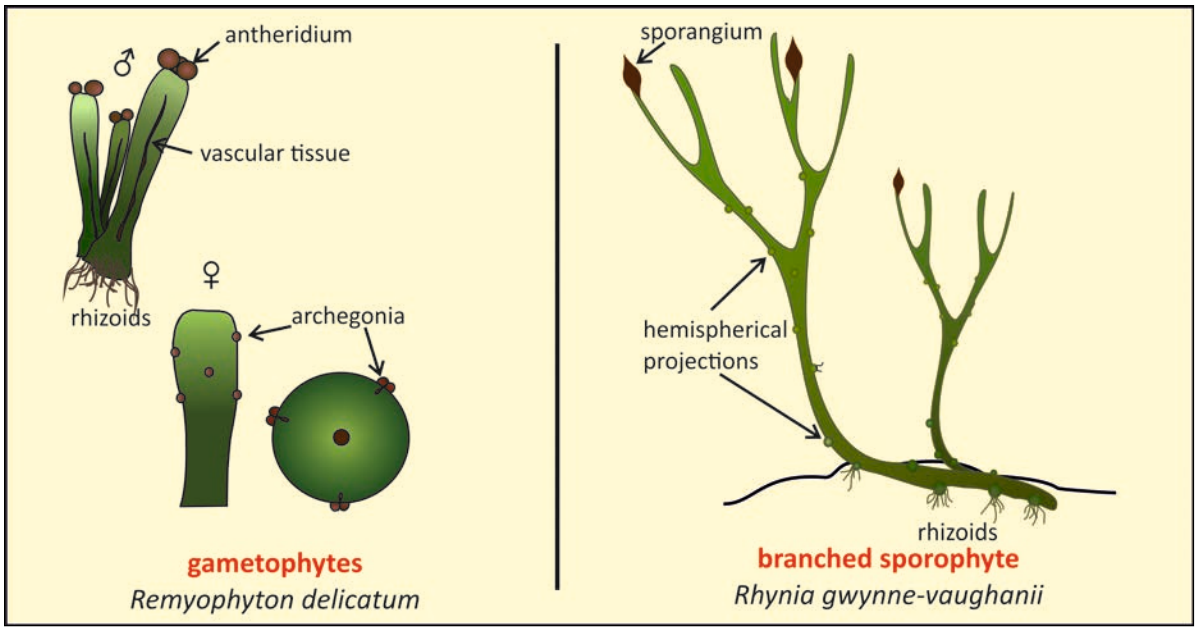
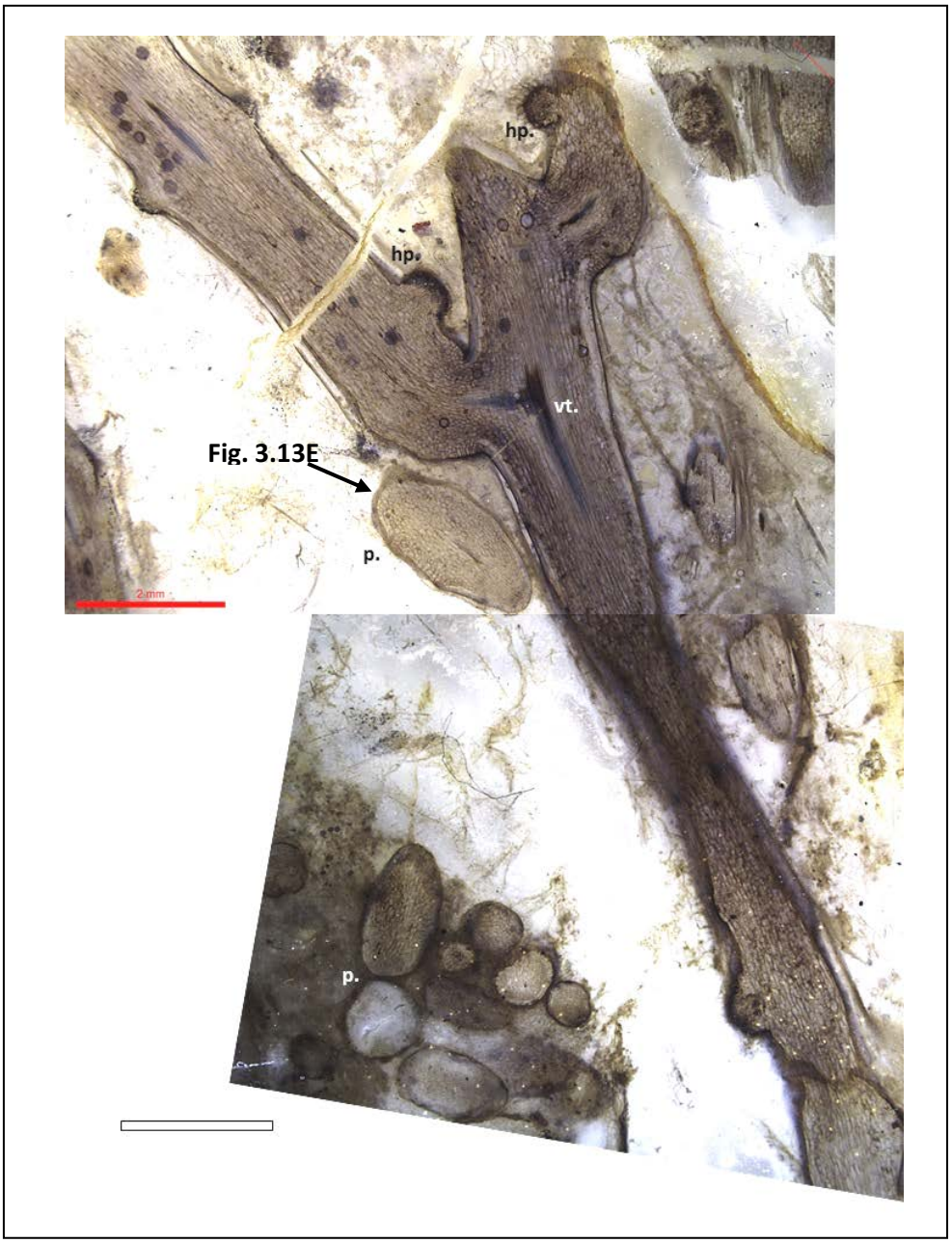

antheridium
♂
vascular tissue
♀
rhizoids
archegonia
gametophytes
Remyophyton delicatum
sporangium
hemispherical projections
rhizoids
branched sporophyte
Rhynia gwynne-vaughanii

Figure 2.5



Chapter 3

Results: Light microscopy and synchrotron X-ray tomographic microscopy of asexual propagules of *Rhynia gwynne-vaughanii*, and light microscopy of the apical meristem of *Aglaophaton major*.

3.0 Introduction

Asexual propagules of *Rhynia gwynne-vaughanii* were examined to obtain information regarding the detailed morphology and general developmental pattern of the shoot as well as detailed information regarding the formation of major organs and tissues within the shoot system. The results are separated into three major case studies: sections 3.1 and 3.2 describe the propagules and their development using light microscopy (LM); sections 3.3 and 3.4 describe the structure of the shoot and shoot apical meristem of *Rhynia gwynne-vaughanii* using both LM and synchrotron X-ray tomographic microscopy (SRXTM). The results of LM examinations of the shoot apical meristem of the protracheophyte *Aglaophyton major* are also given; section 3.5 describes the development of stomata, rhizoids and tracheids in *Rhynia gwynne-vaughanii* using data from both LM and SRXTM. The implications of these results are discussed in Chapter 4.

3.1 Development of asexual propagules in *Rhynia gwynne-vaughanii*

Figures 3.1, 3.2, 3.3, 3.4

Asexual propagules of *Rhynia gwynne-vaughanii* occur commonly within *Rhynia*-rich chert specimens and can be observed in clusters or as single dispersed globular or golf-club-shaped propagules (figure 3.1A and B). Alone, these propagules are ideal study objects, however knowledge of the origin of these propagules is also an important factor. This section describes these asexual

propagules, the axes that produced and bore them, and the developmental stages seen. Propagules developed from two separate areas on the *Rhynia gwynne-vaughanii* axis, from epidermal cells surrounding stomatal guard cells and from the cells of the hemispherical projections.

Three distinct stages of development are seen in the propagules attached to *Rhynia gwynne-vaughanii*.

- A Pre-propagule Stage I, before the formation of the propagule, consisting of cell divisions within the epidermal cells around the stoma, and a small dome of cells ranging from 82 μm to 162.5 μm from the base to the highest point
- An Early Globular Stage II, the development of a globular propagule and distinction of the suspensor organ attaching the propagule to the plant axis
- A Late Globular Stage III, with extensive histogenesis and apical growth. Stages I and III are not observed in propagules attached to hemispherical projections.

Propagule producing axes were encountered frequently in the chert throughout this study, although quality of preservation, the number of attached propagules, and number of developmental stages seen on each axis varied between specimens. Some of these axes are seen in figure 3.1C and figure 3.2G. Attached propagules are conspicuous in that they project out from the epidermis of the plant via a multi-celled suspensor-like organ. The propagules are also in many cases shrivelled in appearance, especially at Stage II, impacting on obtaining accurate measurements. Propagules that have broken loose from their axes are characterised by a dark necrotic patch of cells at the basal attachment point. Similar necrotic patches are seen on shoot axes (e.g. figure 3.1E). Propagule-bearing axes are in most cases characterised by large areas of decay, and/or the pervasive presence of fungi in all of the cells. Preservation quality of both axes and propagules is variable. In many cases the cell walls are only faintly visible in some axes and propagules. In other specimens cells and cell walls are clearly visible.

In total 22 slides with propagules were used for this study. For one single *Rhynia gwynne-vaughanii* axis 16 slides were prepared, showing attached propagules at various stages of development (RA 10.0–10.12; RA 9.1–9.2). Four other slides bearing attached and detached propagules were prepared from separate chert specimens representing separate *Rhynia gwynne-vaughanii* axes showing various stages of development (RA 12.0, RA 18.1, RA 32.3, RB 10.0).

3.1.1 Propagules developing from epidermal cells

Figure 3.1E–H; Figure 3.2C, D, E, G, H; Figure 3.3

From one single chert sample, serial thin sectioning revealed a single *Rhynia gwynne-vaughanii* axis

bearing 11 attached propagules, showing all three stages of development (figure 3.1D–H, figure 3.2D and E, figure 3.3A–H). This axis forms the basis of most of the descriptions and figures here. The axis was prepared as both longitudinal and cross sectional thin sections. Cross sections of the axis show a roughly elliptical shape. It is unclear if this was the true shape of the axis or if this was a result of the axis lying at a slight angle to the cutting plane of the thin sections, thus producing the more elliptical shape. Some shrinkage of the axis has occurred which also impacts the shape of the axis, e.g. figure 3.1D and figure 3.3C. The maximum distance between the axis and the axial shrinkage trace left in the chert matrix is approximately 90 μm. Two axial measurements of the propagule-bearing axis were taken, once across the major axis, and once across the minor axis, with each measurement bisecting the central vascular trace as accurately as possible. The axis has a maximum major axis of 2.13 mm and a minimum of 1.92 mm. The minor axis maximum is 1.80 mm, and the minimum, 406 μm. Longitudinal sections were measured at the widest and narrowest points and generally comprised just partial longitudinal sections of the axis. Maximum length was 4.46 mm. It is estimated that the full propagule-bearing axis in this specimen originally measured approximately one centimetre in length prior to cutting. Propagule length was measured from the point of attachment of the suspensor to the axis to the furthest point of the vegetative structure or apex if present. Propagule width measures the two estimated widest points on the propagule, perpendicular to the length. Stage II propagules found on this axis range in length from 337 μm to 668 μm. Width ranges from 317 μm to 499 μm. One Stage II propagule (figure 3.2C), on a separate axis and at a very early point in development, has a length of 167 μm. The globular propagule measures 86 μm in length, and 122 μm at the widest points. Stage II propagules varied in length depending on the development point, and range from 799 μm to 2.33 mm. Propagule widths range from 424 μm to 894 μm.

3.1.2 Propagules developing from hemispherical projections

Figure 3.2A, B and F

Asexual propagules attached to hemispherical projections on *Rhynia gwynne-vaughanii*, do not show a full sequence of sporophyte development such as seen for the propagules developing from the epidermis. Propagules that developed from hemispherical projections were also encountered much less often in the chert than those developing from the epidermal cells and in total just four separate occurrences were found during this study. The propagules attached to hemispherical projections are relatively immature and represent only Stage II. The propagules developed from a single cell in the projection and the earliest point seen is a multi-celled globular body attached via a stalk-like unicellular suspensor to the projection (figure 3.2A and B, very early Stage II). This propagule is

attached to a fragmented *Rhynia gwynne-vaughanii* axis with a broad patch of decay. The propagule measures 138 μm from the point of attachment to the hemispherical projection to the furthest point of the globular propagule. The suspensor is approximately 49 μm wide. Cell walls of some of the cells within the propagule can be seen. The next stage of development seen is a later phase Stage II, (figure 3.2F). These propagules do not have an apparent suspensor and consist mainly of the globular propagule.

3.2 Stages of development

3.2.1 Pre-propagule Stage I

Figure 3.1C–H

Pre-propagule Stage I is identifiable by divisions of the cells of the epidermis (figure 3.1E) surrounding the stomatal guard cells. This stage marks the first cell divisions prior to the development of the actual propagule. Anticlinal divisions (at right angles to the surface of the length of the axis) in the epidermal cells, produce four smaller cells. Periclinal divisions (parallel to the surface axis length) of these cells produce a domed structure (Proembryogenic mass – PEM – see section 4.1.3) protruding from the axis surface figure 3.2D, F, G and H).

3.2.2 Early Globular Stage II

Figure 3.2

The Early Globular Stage II is characterised by a globular cell mass that projects out from the axis (figure 3.2A–C). In figure 3.2A–C preservation of individual cells is poor (although best in figure 3.2B), but the morphology is quite clear and the structure forms two distinct but linked sections. The lower section, the suspensor which attaches the propagule to the plant axis, is multicellular in the case of propagules developing from the epidermis (figure 3C), and single-celled in propagules attached to hemispherical projections (figure 3.2A and B). The upper globular section is the propagule. One cell can be seen the upper left of the propagule in figure 3.2C and based on its size and the size of the propagule, it is suggested that there are at least eight cells. The propagule is flanked to the left by stomatal guard cells (s.), to the right of which is a dome of cells of the same structure described in the Pre-propagule Stage I above. This propagule has also broken through and displaced the surrounding cuticle (cu.) layer of the 'parent' plant. Continued cell division of the cells in the propagule increases the size of the globular mass (figures 3.2D, E and F). It is at this stage that the propagules show shrinkage and shrivelling. The propagules are a dark brown colour and a dark brown-black internal globular patch can be seen in one propagule (figure 3.2E). No differentiation of cells is observed, although in many cases the propagule is too poorly preserved

and shrivelled to confirm this. Consequently it is possible that these later Stage II propagules may belong to Stage III and the poor preservation obscures any identification of specialised cell types, e.g. stomata. Suspensor cells are a dark brown-black colour and necrotic in some cases.

3.2.3 Late Globular Stage III

Figure 3.3

The Late Globular Stage III is characterised by extensive histogenesis. Stomata, rhizoid-bearing bulges (i.e. hemispherical projections), rhizoid development, vascular tissue and apical growth are all visible. Stomata form early and as do the rhizoidal bulges from where rhizoids develop (figure 3.3A). Rhizoids (figure 3.3B arrows) begin developing from the basal region from bulges of cells similar to hemispherical projections. These early rhizoids have an ovoid shape and project slightly from the bulge. Definition of the apical meristem and apical growth follows and in some cases the apical meristem has divided to form a branched propagule (figure 3.3F). Apical growth in one propagule is well developed although it is not possible to confirm the presence of an apical cell at the apex due to the preservation in silica not providing enough contrast to the delicate cell walls (figure 3.3E). In figure 3.3F a branched propagule has two separate strands of vascular tissue in each branch. One strand appears more developed than the other, (but this may be a preparatory artefact) and shows elongation of the vascular cells and formation of secondary wall thickenings. Tracheid precursor cells are initially ovoid in shape, but begin to elongate and take on a darker, more orange colour with a pitted appearance during elongation of the shoot (figure 3.3G). More elongate tracheids with secondary wall patterning develop synchronously with shoot elongation and are seen only in propagules with relatively well-developed sporophyte axes (figure 3.3F, G, H). As histogenesis proceeds the suspensor becomes increasingly necrotic, allowing the propagule to detach from the main axis with a dark necrotic patch of cells remaining on the axis and the propagule (figure 3.3H).

3.3 Synchrotron X-ray tomographic microscopy of *Rhynia gwynne-vaughanii*

This study set out to assess the suitability of the Rhynie chert material for examination using synchrotron X-ray tomographic microscopy (SRXTM) with the intention of obtaining high-resolution virtual thin sections and constructing three-dimensional models of the specialised cells and cell complexes in *Rhynia gwynne-vaughanii*. Asexual propagules, as previously described, were chosen as appropriate models for study because of their small size and because they preserve various stages of sporophyte development. Specimens included Late Globular Stage III propagules without elongate sporophyte shoots or early very early shoot development, and globular propagules

with short sporophyte axes but no apex. Individual apices of *Rhynia gwynne-vaughanii* not associated with any propagules were also examined.

Overall, *Rhynia gwynne-vaughanii* specimens without a covering of chert material returned the best results for surface views of vegetative propagules. However, in such specimens tomographic slices showed a strong white outline around the specimen obscuring the cell walls in epidermal cells. Most specimens were either entirely encased in chert or only partially exposed. RTK8 is the only specimen to be completely free of any surrounding chert matrix, where the plant fossil surface and chert formed a natural weak point and became disconnected. RTK 8 was the first specimen to positively indicate the involvement of at least one apical cell in the development of the *Rhynia gwynne-vaughanii* shoot and served as a guide for other specimens. In total 19 specimens were scanned. Preservation quality varied between the specimens with some specimens showing large areas of decay or damage to the surface of the plant fossil, and in many specimens near the apex. The quality of the results of SRXTM varied also between specimens despite the same settings used across all specimens. In well preserved specimens, mature or fully differentiated cells such as stomatal guard cells, epidermal cells and rhizoids returned the best resolution with their relatively thick cell walls displaying a strong contrast to the surrounding chert. *Rhynia gwynne-vaughanii* specimens that returned the best results illustrating all of the developmental and morphological features of interest are described here in detail. Specimens that did not return good results or did not give any useful morphological information are described in the Appendix. Potential information in such specimens was mainly affected by poor preservation quality and in many cases a banded pattern to mineralogy of the silica which obscured cell detail. Some specimens were scanned more than once to correct for errors such as off-centre positioning of the specimen in front of the detector and the bright white outline to specimen RTK 8, which obscured the cells in the uppermost layers.

3.3.1 Specimen RTK 4a/RTK 4b

Figures 3.5 and 3.6

This specimen was scanned in two separate sessions, (RTK 4 and 4b), as it was too large for the field of view for the tomographic microscope. The specimen consists of a single Late Globular Stage III propagule with an elongate sporophyte axis (figure 3.5A). The apex of the shoot was not preserved.

RTK 4 represents the apical end of the specimen and RTK 4b illustrates the basal end. This specimen returned some of the best results regarding the reconstruction of the specialised tissues of *Rhynia gwynne-vaughanii*. The propagule bears two hemispherical projections (hp.), with rhizoids (rh.)

at its basal end and an elongate sporophyte axis (figure 3.5B and C). Internally a vascular trace runs through the centre of the plant specimen comprising of several elongate tracheid cells. Tracheid cells are longest, with a smaller diameter towards the apical end, and shorter and more numerous near the base of the propagule. The individual tracheid cells are coloured differently to illustrate clearly their shape (figure 3.5C and D). Water conducting cells near the basal end of the shoot (figure 3.6C) are shorter and wider, lacking secondary wall deposition, but bearing simple round pit-like structures. Secondary walls form a helical pattern around the tracheid cells further from the basal end, but this pattern is not continuously traceable throughout each tracheid, producing only partial reconstructions in tracheids with secondary walls (figure 3.5H). The lumen of the tracheids varies in shape, from roughly spherical to oval. In some sections the lumen has straighter edges, forming a pentagonal or lozenge shape. This shape is due to the inwardly pointing helically arranged secondary wall structure. Stomatal complexes could be readily illustrated and rhizoids were easily reconstructed. Stomatal complexes in *Rhynia gwynne-vaughanii* consist of two guard cells overlying a ring of eight oblong cells that form the pore of the stoma (figure 3.5E, F and G). The stomatal guard cells overly this pore.

3.3.2 Specimen RTK 8

Figure 3.7 and Figure 3.8

RTK 8 consists of one Late Globular Stage III propagule with one sheared edge from initial preparation using a rock saw. The propagule bears four stomata (S1–4, figures 3.6G and 3.7A–D) one of which, S1, is found on a small hemispherical projection. S4 has been mostly sheared off by the saw blade and consists of two fragmentary guard cells. However the stomatal chamber below the guard cells is clearly visible in tomographic sections (figures 3.7D and 3.8G and H), including two of the oblong cells that form the pore. A triangular cell (figures 3.7B and 3.8B–H) stands out among the cells near S4 and is designated as an apical initial cell. In surface view the apical cell has two longer sides and one shorter side. The apical cell could be traced only partially through the tomographic sections, however this was enough to show that the apical cell has an irregular tetrahedral shape (figure 3.8E). Apical derivatives divide from the apical cell in a spiral manner (figure 3.8F).

3.3.3 RTK 10

Figure 3.9A and B

RTK 10 consists of a single Globular Stage III propagule with initial stages of apical growth. The assumed former attachment point shows a large zone of decay. Preservation quality is poor to

moderate. Cell walls can be faintly seen, however the cells closer to the surface that show the strongest contrast. An apical cell could not be confidently identified even though the general position of the apex could be pinpointed. The tomographic sections do show the presence of some stomata. An isosurface rendering of the propagule shows strong resolution of the epidermal cells, with the central line characteristic of *Rhynia gwynne-vaughanii* epidermal cell visible in some cases. The area near the apex returned very poor three-dimensional rendering indicating poor preservation quality.

3.3.4 Specimen RTK 15

Figure 3.9C–H

RTK 15 is an isolated, apical fragment and was tested to establish the presence of an apical cell, as seen in RTK 8, during later (non-globular) stages of shoot ontogeny. Rendering of these propagules as isosurface models show that although cell walls are clearly defined, the apex appears to have been destroyed at some point either during the preservation process or during the life of the plant, something that could not be clearly seen in the specimen alone using a light microscope (figure3.9H). However, cells are clearly identifiable within the virtual tomographic sections (figure 3.9D and E) and the rounded, isodiametric cells forming the core of the Shoot Apical Meristem (SAM) (blue) are distinguishable from the more ovoid cells below (grey). The core SAM cells (blue) are overlain by a layer of slightly larger and rectangular shaped cells (green). Cells lying just outside of the SAM zone (yellow) show periclinal cell divisions with the development of a vertical file of cells. The presence of an apical cell could not be confidently established. Just one cell within the SAM area showed a resemblance to an apical cell (figure 3.9F and G); despite the strong triangular out line of this cell could be traced through only a few tomographic sections and in just one spatial direction (through the xz plane).

3.3.5 RTK 17

Figure 3.10A–D

RTK 17 is an isolated *Rhynia gwynne-vaughanii* apex. Internally, cell walls are poorly resolved, and the surface of the apex is damaged extensively (figure 3.10B) impacting any positive identification of an apical cell. Figure 3.9C and D shows isolated strands of tracheid cells with helical secondary thickenings. These tracheids were easily reconstructed in three dimensions (figure 3.10C and D) showing the helical arrangement of the secondary walls around the cell.

3.3.6 RTK 18

Figure 3.10E–H

RTK 18 is also an isolated apex. This specimen returned excellent resolution of internal cells. Cells near the apex and in the area of the SAM were also easily seen. The plant specimen shows a slight irregularity in shape near the apex (figure 3.11E inset). A single apical cell was identified and could be traced through the virtual slices and reconstructed in three dimensions. It was possible to trace the cell walls from the xy, yz and xz planes allowing for a near complete reconstruction in three dimensions of the apical cell (figure 3.11G–I). The apical cell is tetrahedral in shape. The derivative cells surrounding the apical cell are not as easily traced and could not be reconstructed. A single stoma flanks the apical cell, similar to seen in specimen RTK 8 and appears to be separated from the apical cell by only one other cell, although it is difficult to confirm this with a high degree of certainty due to the relatively poorer resolution of the cells surrounding the apical cell. Overall SAM structure was not as readily seen as in specimen RTK 15.

3.4 Light microscopy and the structure of the shoot apical meristem in *Rhynia gwynne-vaughanii* and *Aglaophyton major*

Figure 3.11; also see figure 4.5

In order to further demonstrate that *Rhynia gwynne-vaughanii* possessed a tetrahedral apical cell and a defined shoot apical meristem structure, preparation of a single *Rhynia gwynne-vaughanii* propagule with excellent SAM preservation using traditional hand-grinding techniques was carried out. Figure 3.11A and B shows a longitudinal cross section through the SAM of a Late Globular Stage III *Rhynia gwynne-vaughanii* propagule that had begun to display a more elongate axial morphology. A triangular cell at the apex with thin cell walls is seen, accompanied by two stomata to the left. This supports the assertion that the tetrahedral cells seen in the SRXTM specimens RTK8 and RTK 18 are indeed single apical cells and that *Rhynia gwynne-vaughanii* possessed one tetrahedral cell at its apex. The preparation of this specimen highlighted the shortcomings for obtaining detailed morphological data from petrographic slides, i.e. most of the specimen had to be destroyed in order to obtain basic information on the SAM.

As a direct coeval comparison, 15 petrographic thin sections from the permanent Rhynie chert collection at WWU bearing the apices of *Aglaophyton major* were examined. Examination revealed that the SAM of *Aglaophyton major* differed structurally to that of *Rhynia gwynne-vaughanii*. An apical cell was not identified in any of the specimens examined and the SAM consists of a defined zone of tightly packed files of isodiametric cells, with what appear to be multiple zones of division, figure 3.11E–H. The superficial layer overlying the apex also bears isodiametric cells of a similar size to

those of the core meristematic area. Near-vertical files of cells can be seen in the areas adjacent to the main meristematic area, as was seen in the *Rhynia gwynne-vaughanii* specimen RTK 15. It is important to consider, however, that these slides bear just slices through the SAM of *Aglaophyton major* and for that reason the presence of a single apical initial cannot be completely discounted. A single specimen with a poor surface view of the SAM of *Aglaophyton major* shows no defined apical cell and consists of separate zones of cell division (Appendix figure 26).

3.5 Sporophyte development: shoot apical meristem, stomata, rhizoids and tracheids

In addition to demonstrating asexual reproduction and highlighting the fine detail of the morphological structure of *Rhynia gwynne-vaughanii*, the above specimens provide a unique opportunity to examine the development of an early vascular land plant sporophyte and the specialised cells that allowed the plant to function. General development of the shoot from the apical cell, development of stomatal guard cells and tracheid development can all be demonstrated. In general, shoot development was directed from a single apical cell, from which all specialised cells and tissues were derived. Stomata and rhizoids developed directly from apical cell derivatives and *de novo* development of stomatal guard cells along older sections of the *Rhynia gwynne-vaughanii* shoot was not seen during the course of these investigations, however this cannot be ruled out completely.

3.5.1 Cell division in the shoot apical meristem

Figure 3.12A and B

The SAM of *Rhynia gwynne-vaughanii* was characterised by a single tetrahedral apical cell and specimen RTK 8 shows a sequence of cell division from this apical cell. Cell division from the apical cell proceeded in a spiral manner with apical derivative (1) the youngest in the sequence. Apical derivative (2) had already divided into two cells, the lowermost of which was forming an epidermal cell. Apical derivative (3) is the oldest in the sequence and had differentiated into an epidermal cell, taking on the distinctive lozenge-shape morphology characteristic of mature epidermal cells of *Rhynia gwynne-vaughanii* (figure 3.12B). It is more difficult to determine cell division sequence from the longitudinal profile of the SAM due to the relatively poor resolution of the internal structure of the propagule.

3.5.2 Stomatal guard cell and rhizoid development

Figure 3.12 C and D

The apical cells shown in specimens RTK 8, RTK 18 and figure 3.10A–D, all show at least one stoma in close proximity to the apical cell indicating that stomata developed quickly after the first

cell divisions of the apical intial and were among the first specialised cells to form during shoot development. Specimen RTK 8 shows a distinct zone of cell division to the left of the apical cell (merophyte). It is this area of development that is proposed as the site of stomatal and rhizoidal development. Applying accepted classification schemes (e.g. see Fryns-Claessens and Van Cotthem 1973; Rudall *et al.* 2013) in stomatal ontogeny to RTK 8 (figure 3.12A and figure 4.6), a sequence of cell divisions can be traced back to a single apical derivative:

1: A single apical derivative in figure 3.12C(i) divides into two unequal daughter cells, (figure 3.11C(ii)). Adjacent cells to the left, products of a previous apical cell division also divide, but symmetrically - figure 3.12C(ii) and (iii).

2: The lowermost, and smaller cell in figure 3.12C(ii), (the stomatal lineage ground cells/meristemoid mother cell) further divides asymmetrically to produce one distinct triangular-shaped cell, the guard mother cell (GMC), which should divide symmetrically to form two stomatal guard cells. This specimen was preserved just prior to the division of the GMC.

To the left of this zone of cell division a file of four roughly rectangular cells, derived from a separate apical derivative to those that formed the GMC, most resemble cells that form rhizoids as per Edwards (1998). It is also worth noting the similarity of this file of four cells, and those described by Edwards (1998), to cells seen in the area surrounding the stomatal guard cells during the development of the propagules described in section 3.1.1 and figure 3.1E. It is possible that the entire zone of cell division was in the process of developing a new hemispherical projection, of which two are commonly found at the basal end of more mature propagules (figures 3.1B and 3.4A).

3.5.3 Tracheid development in *Rhynia gwynne-vaughanii*

Figure 3.13

Tracheid morphology in *Rhynia gwynne-vaughanii* has been described in detail in various studies (reviewed in Chapter 4), but tracheid development has largely been neglected. Tracheids in *Rhynia gwynne-vaughanii* are tightly packed and are hexagonal or pentagonal in cross section. Secondary wall thickenings in *Rhynia gwynne-vaughanii* are helical and point in towards the cell lumen (figure 3.13B). Tracheids are connected to each other along a broad zone at the tapered ends of the tracheid. Tracheids in *Rhynia gwynne-vaughanii* run through the centre of the shoot and are surrounded by parenchymatous tissues. Phloem cells and their companion cells are readily identifiable in cross section (figure 3.13A).

The first specialised cells associated with water-conducting tissues seen in *Rhynia gwynne-vaughanii* are oblong in shape and brown-orange in colour (figure 3.13D). Each cell is pitted with vesicles.

These cells are to be found near the base of Globular Stage III propagules without sporophyte elongation as wells as with developing elongate axes. They are also found at the base of new branches (figure 3.13G) developing from mature *Rhynia gwynne-vaughanii* axes. In propagules with elongate sporophyte axes and in 'mature' branches these cells develop into recognisable elongate tracheid cells, develop only once apical growth commences. In the Late Globular Stage III propagule RTK 4 (figure 3.5H), early tracheids are somewhat short and have a wider diameter to tracheids found axes that have axes greater than 1–2 mm in length. However, these cells do bear secondary walls marking them out as tracheids. These shorter tracheids remain at the basal end of propagules throughout the development of the shoot and are visible in figure 3.3F, where a single propagule has branched dichotomously and developed two separate strands of tracheid tissues. Tracheids found primarily in the elongate shoot axis are narrower in diameter and longer in length but retain the characteristic helical secondary wall pattern. In some axes with developing tracheids, the earliest stages of formation after cell elongation are characterised by small pits on the cell walls. This gives the cells an appearance similar to that of the water conducting cells in *Aglaophyton major* (figure 3.12C and figure 3.5C). During the development of secondary walls, circular pits are arranged across the tracheid cell in a straight line, (figure 3.13E and F). The position of future secondary wall-thickenings are marked by an orange-brown band (figure 3.13E). In figure 3.13 tracheids are more developed towards the basal end of this propagule (highlighted in figure 2.5, adjacent to the axis of *Rhynia gwynne-vaughanii*; the apex of the propagule is orientated to the bottom right of the image).

Figure 3.1

Asexual propagules and the Pro-propagule Stage I

A: Cluster of globular propagules (p.) with clusters of *Rhynia gwynne-vaughanii* spores. Slide RA 3.10. Scale 1 mm.

B: Stage III, detached propagule with elongate sporophyte axis. Propagule bears two hemispherical projections (hp.) and a single tracheid trace (tr.). Slide RA 10.0. Scale 1 mm.

C: Thin section through mature *Rhynia gwynne-vaughanii* axis showing multiple necrotic detachment points (arrows). One single propagule with and elongating axis is seen to the right (pr.). Slide RA 2.3. Scale 100 μm.

D: Cross section through propagule bearing axis. Star marks necrotic detachment point. Arrows indicate new areas of very early propagule development (PEM – Proembryogenic mass). Central tracheid trace (tr.) and significant section of decay to the top right. Slide RA 10.1. Scale 100 μm.

E: Surface view of initial stage of cell division in the vicinity of a single stoma (s.). Slide RA 9.1. Scale 100 μm.

F: Cross section of propagule bearing axis with domed mass of cells (PEM). Slide RA 10.7. Scale 100 μm.

G: Detail of stoma (s.) with flanking PEMs. Slide RA 10.4. Scale 100 μm.

H: PEM detail. At least four cells visible. Note the pervasive presence of fungi in the cells of the plant specimens in each image. Slide RA 10.4. Scale 100 μm.

Figure 3.2

Early Globular Stage II

A: A single asexual propagule (pr.) on a hemispherical projection (hp.). rh. = rhizoid; su.= suspensor. Slide RB 10.0. Scale 100 μm.

B: Detail of propagule in A. Scale 100 μm.

C: Propagule (pr.) attached to a mature *Rhynia gwynne-vaughanii* axis. cu. = cuticle; s. = stoma; su. = suspensor. Slide RA 18.1. Scale 100 μm.

D: Two Early Globular Stage II propagules (pr.). Slide RA 10.8. Scale 100 μm.

E: A single Early Globular Stage II or possible earliest late Globular Stage III propagule. Very shrivelled in appearance. Dark shadow within propagule may be indicative of the development of a procambium, thus potentially a Stage II propagule. f. = fungi; tr. = tracheids. Slide RA 9.2. Scale 100 μm.

F: Single propagule (pr.) Stage II attached to hemispherical projection. f. = fungi. Slide RA 12.0. Scale 30 μm.

G: Poorly preserved axis with two Stage II propagules (pr.) and a single PEM. Slide RA 32.3. Scale 1 mm.

H: Well preserved Stage II propagule (pr.) attached to mature *Rhynia gwynne-vaughanii* axis; no histogenesis visible. tr. = tracheids. Slide RA 3a.2. Scale 100 μm.

Figure 3.3

Late Globular Stage III

A: Single propagule, ovoid in shape. Single stoma to the bottom left (s.). Dark central areas indicate initial development of specialized water conducting tissues. Suspensor (su.) has become necrotic and individual cells are difficult to discern. s. = stoma. Slide RA 9.3. Scale 100 μm.

B: Reverse side of A. Rhizoid initial cells near base of propagule (rh.). Central development of water conducting cells clear, dark brown – orange discoloration of cells. One single stoma seen near the top (s.), at an early point in development, guard cells faintly seen. Slide RA 9.3. Scale 100 μm.

C: Cross section of axis with multiple propagules. Two propagules (pr.) with apical growth, but just the lower propagule has an intact apex. tr. = tracheids. Slide RA 10.2. Scale 100 μm

D: Detail of C. A single stoma (s.) is seen between the two propagules. Guard cells are difficult to see, but stoma is well defined. Scale 50 μm.

E: Detail of lower propagule in C. Apex is intact but cell detail of the shoot apical meristem (SAM) is poor. Stomata (s.) are seen at the top right and near the bottom centre of the image. Scale 100 μm.

F: Single branched propagule (pr.) with two separate tracheid traces (tr.). The lower trace appears further developed to that in the upper branch, but has lost its apex. Suspensor is necrotic. Slide RA10.6. Scale 100 μm.

G: Detail of tracheids (tr.) in lower branch of propagule in F. Basal-most cells are pitted and comparatively short. Arrow points in direction of basal end of propagule at the suspensor. Secondary wall thickenings are apparent in cells closer to the apical end and tracheids are longer and narrower. Slide RA 10.6. Scale 100 μm.

H: Detached propagule (pr.) with extensive shrinkage; dashed line shows original dimensions. One single stoma (s.) and a tracheid trace are visible. To the bottom left, a single PEM. Slide RA 10.7. Scale 100 μm.

Figure 3.4

Schematic representation of propagule development from multiple cells in the epidermis of *Rhynia gwynne-vaughanii* and propagule development from a single cell on a hemispherical projection on the shoot of *Rhynia gwynne-vaughanii*.

Figure 3.5

SRXTM Specimen RTK 4a

A: Single propagule similar to Figure 3.1B. RTK 4 shows apical end. hp. = hemispherical projections; tr. = tracheids. Scale 100 μm.

B: Apical end of propagule. Overlay of rendered surface on tomographic slice in the yz plane. tr. = tracheids; s. = stoma; rh. = rhizoids. Scale 100 μm.

C: View of tomographic slice in yz plane. Arrows point to reconstructed individual, elongate tracheid cells which become fewer, longer and narrower towards the apical end. s. = stoma; rh. = rhizoids. Scale 100 μm.

D: View in the xz plane of A, showing individual tracheid cells (arrows). s. = stoma: epi. = epidermal cells. Stoma and epidermal cells are on the dorsal side of the propagule. Scale 100 μm.

E: Detail of stoma in D (xz plane). Two stomatal guard cells (s.) surrounded by lozenge shaped epidermal cells (arrows). Scale 100 μm.

F: Ring of eight cells directly beneath the stomatal guard cells (black arrows) that delineate the stomatal pore. Epidermal cells (white arrows) overly some of these cells. Scale 100 μm

G: Subsurface view of stoma (s.) in E and F. Ring of cells show an elongate form beneath the guard cells (s.). (yz plane). Scale 100 μm.

H: Reconstruction of spiral secondary wall thickenings (red; white arrow) in tracheids (black arrow). Central lumen also reconstructed (yellow arrow). Scale 100 μm.

Figure 3.6

SRXTM Specimen RTK 4b

A: As in Figure 4A. Scale 100 μm.

B: Basal region of propagule. Arrow indicate position of short, central water conducting tissues. hp. = hemispherical projection; rh. = rhizoid. Scale 100 μm.

C: Detail of elongate water conducting cells with pitted ornamentation on the cell walls. Scale 100 μm.

D: reconstruction of water conducting cells. Cells are individually coloured to distinguish each cell. rh. = rhizoid; hp. = hemispherical projection. Scale 100 μm.

E: Reconstruction of rhizoids (rh.), green, blue, and purple, with overlying companion cells. Scale 100 μm.

Figure 3.7

SRXTM Specimen RTK 8

A: Light microscopy of Late Globular Stage III propagule. Scale 100 μm.

B: Detail of area of interest in propagule. Arrow indicates triangular shaped cell. Scale 100 μm.

C: Further detail of zone of interest. Scale 100 μm.

D: Sheared end of propagule. Single substomatal chamber (white arrow) visible to the top centre-left. (S4) = stomatal guard cells. Scale 100 μm.

E: Surface rendering of propagule and tomographic slices. Scale 100 μm.

F: Tomographic slices in all three planes. Scale 100 μm.

G: Surface rendering of propagule without tomographic slices. Black arrow indicate direction of growth of propagule. S1 and S2 = stomata; xz plane of view; broken like outline missing fragment of the propagule. Scale 100 μm.

H: View of propagule in xz plane but from opposite side. S3 = stoma, faintly visible from this angle. Scale 100 μm.

Figure 3.8

SRXTM Specimen RTK 8

A: View from yz plane. S3 more clearly seen (blue arrow). S3 has formed on a small hemispherical projection. Area of primary interest is seen at the top-centre of the image. Scale 100 μm.

B: View from xy plane. S2, 3, 4 = stomata. Black arrow points to triangular cell. Scale 100 μm

C: Further detail B. Scale 100 μm.

D: Cells surrounding the triangular cell, an apical initial cell. Black broken lines delineate apical derivatives. Blue broken lines mark out a preserved merophyte zone of cell division. The partial stomatal guard cells of S4 are highlighted also. Scale 100 μm.

E: Partial reconstruction of triangular cell in D (pink) with overlay of surface rendering. Broken yellow line mark the subsurface limits of the cell wall. View from xz plane. Scale 100 μm

F: Spiral division of apical derivatives from apical cell. Scale 100 μm.

G: Tomographic slices, without surface rendering, showing reconstruction of S4 guard cells and substomatal cells and the apical cell. Black broken lines help delineate the shape of the apical cell (pink) in three dimensions, Scale 100 μm.

H: Further detail of G, in yz plane. Scale 100 μm.

Figure 3.9

SRXTM Specimens RTK 10 and RTK 15

A: RTK 10 surface rendering. Poor internal results. Well-developed epidermal cells with central line (black arrows). Scale 100 μm.

B: RTK 10, poor internal resolution of cells. Scale 100 μm.

C: RTK 15, *Rhynia gwynne-vaughanii* apex in tomographic sections. Orange broken line delineates the apical surface of the shoot, in the xz axial plane. Scale 100 μm.

D: RTK 15, xy plane, detail of apex in a single tomographic section. Cells are well defined. Cell walls are seen in white. Scale 100 μm.

E: RTK 15 with different types of cell in the apex highlighted. Black broken line marks the boundary of the shoot apical meristem (SAM). Green = superficial layer; Blue = isodiametric cells of the SAM; Yellow = cells of a peripheral zone of cell division. Cells below SAM (dark grey) are more elongate. Scale 100 μm.

F: Surface rendering of RTK 15. Surface of the apex (yz plane) shows a path of destroyed cells (white arrows). Scale 100 μm.

G: Potential apical cell in RTK 15. Black arrows indicate subsurface edges of cells; xz plane. Scale 100 μm.

H: Partial reconstruction of cell in G. Cell shape could only be traced for a few slices. Scale 100 μm.

Figure 3.10

SRXTM Specimens RTK 17 and RTK 18

A: Poor internal resolution of cell in the apex of RTK 17. Scale 100 μm.

B: Rendering of the surface of RTK 17 shows poor preservation and rendering in apical area. Scale 100 μm.

C: Isolated tracheid cells (x2) from specimen RTK 17. Longitudinal section of secondary walls (black arrows) in xz plane. Scale 100 μm.

D: Partial reconstruction of a single tracheid cell showing spiral secondary wall thickenings (red, white arrow) and lumen (white, black arrow). Scale 100 μm.

E: RTK 18, tomographic slice of *Rhynia gwynne-vaughanii* apex; xy plane. Black arrow = apical cell; white arrows = stomatal guard cells. Inset shows irregular shape of shoot apex. Strong resolution of cells within the shoot. Scale 100 μm.

F: RTK 18 with marking of apical cell and stomatal guard cells.

G: Reconstruction of apical cell (pink). Triangular shape in yz plane. Scale 100 μm.

H: Reconstruction of apical cell. Triangular shape in xy axis. Scale 100 μm.

I: Three dimensional view of apical cell. Cell also has triangular shape in the xz plane. Yellow broken lines help delineate the tetrahedral shape of the cell. Scale 100 μm.

Figure 3.11

Shoot apical meristem structure in *Rhynia gwynne-vaughanii* and *Aglaophyton major*

Light microscopy

A: Thin section of apex of Late Globular Stage III propagule of *Rhynia gwynne-vaughanii*. Arrow indicates position of apical cell. s. = stoma. Slide RA 34.2b. Scale 50 μm.

B: Line drawing of apical cell and stoma in Figure 3.11A. Scale 50 μm.

C: Detail of apical cell in Figure 3.11A and B (arrow). Scale 25 μm.

D: Line drawing of Figure 3.11C for clarity. Scale 25 μm.

E: Apex of *Aglaophyton major* shoot. Slide *Aglaophyton major* apices No. 2030. Scale 300 μm.

F: Detail of Figure 3.11E. Red broken line delineates the apical meristem area, the superficial layer of cells and the zoned division of core of meristematic cells. Slide *Aglaophyton major* apices No. 2030. Scale 300 μm.

G: Detail of apex of *Aglaophyton major*. Slide *Aglaophyton major* apices No. 2030. Scale 200 μm.

H: Detail of Figure 3.11G. Red broken lines indicate the files of cells within the apical meristem, forming vertical files of dividing cells. Slide *Aglaophyton major* apices No. 2030. Scale 200 μm.

Figure 3.12

Stomatal development in *Rhynia gwynne-vaughanii*

A: SRXTM Specimen RTK 8. Apical cell, apical derivatives; merophyte zone to the left. Scale 100 μm.

B: Line drawing of cells of interest. Star marks apical cell. Broken lines indicate cell divisions. Scale 100 μm.

C: Schematic diagram of sequence of cell divisions leading to cell arrangement and probable stoma formation seen in Figure 3.12A and B. Figure C(iii) represent the phase of development preserved in Figure 3.12A.

D: Classification of stomatal development. Modified from Rudall *et al.* (2013). Stomatal development type seen in *Rhynia gwynne-vaughanii* highlighted in red box.

Figure 3.13

Tracheid morphology and development

A: Detail of vascular tissues of *Rhynia gwynne-vaughanii*. Dark central tracheid strand with surrounding phloem cells (black arrows) with phloem companion cells (blue arrow). Slide RA 20.2a. Scale 100 μm.

B: Longitudinal section of tracheids in *Rhynia gwynne-vaughanii* shoot. Black arrow points to tapered end of tracheid cell. White arrows indicate spongy secondary wall thickenings that point in towards the cell lumen. Slide RA 4.0. Scale 100 μm.

C: Water conducting cells of *Aglaophyton major*. Note the absence of secondary wall thickenings and the pitted nature of the cell walls. Slide *Aglaophyton* teaching slide. Scale 50 μm.

D: Early development of water conducting cells at the basal end of an asexual propagule of *Rhynia gwynne-vaughanii*, from Figure 3.4F and G. Note the pitted appearance of the cells walls and the short cell length with tapered ends. Slide RA10.6. Scale 10 μm.

E: Development of secondary walls in the tracheids of a Stage III asexual propagule (see figure 2.5). Note the linear arrangement of large pits at the top right and bottom left. Slide RA 4.0. Scale 20 μm

F: Line drawing of Figure 3.13E. Scale 20 μm.

G: Water conducting cells from the base of a single branch, growing from a *Rhynia gwynne-vaughanii* shoot axis. Note similarity in appearance to cells Figure 3.13D. Slide RA 4.0. Scale 100 μm,

H: Poorly preserved tracheids and water conducting cells in a single branch of *Rhynia gwynne-vaughanii*. Secondary walls (white arrow9 are visible but not well preserved. Pitted cells to the right are also present (black arrow). Slide RA 4.0. Scale 25 μm,

Figure 3.1

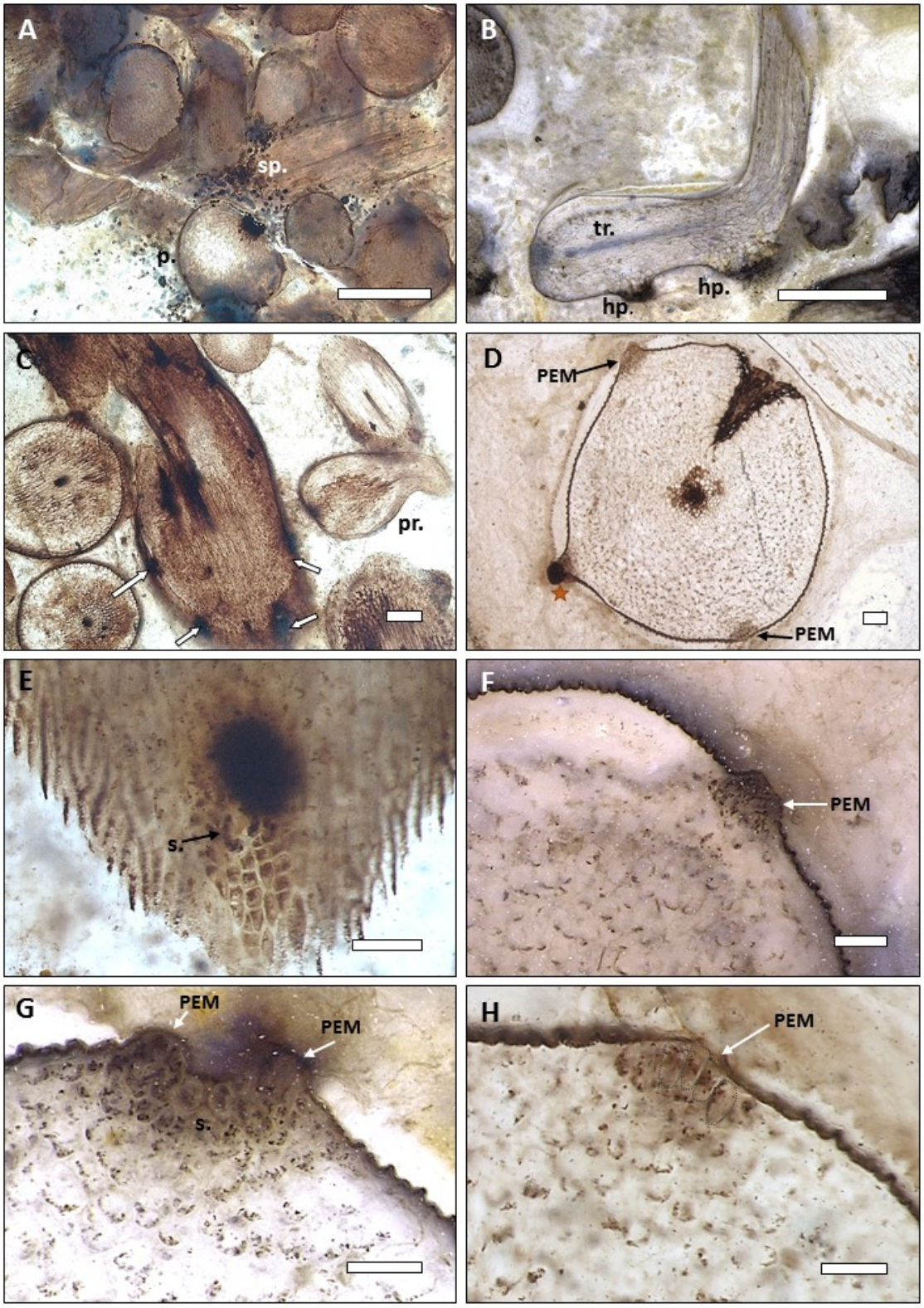
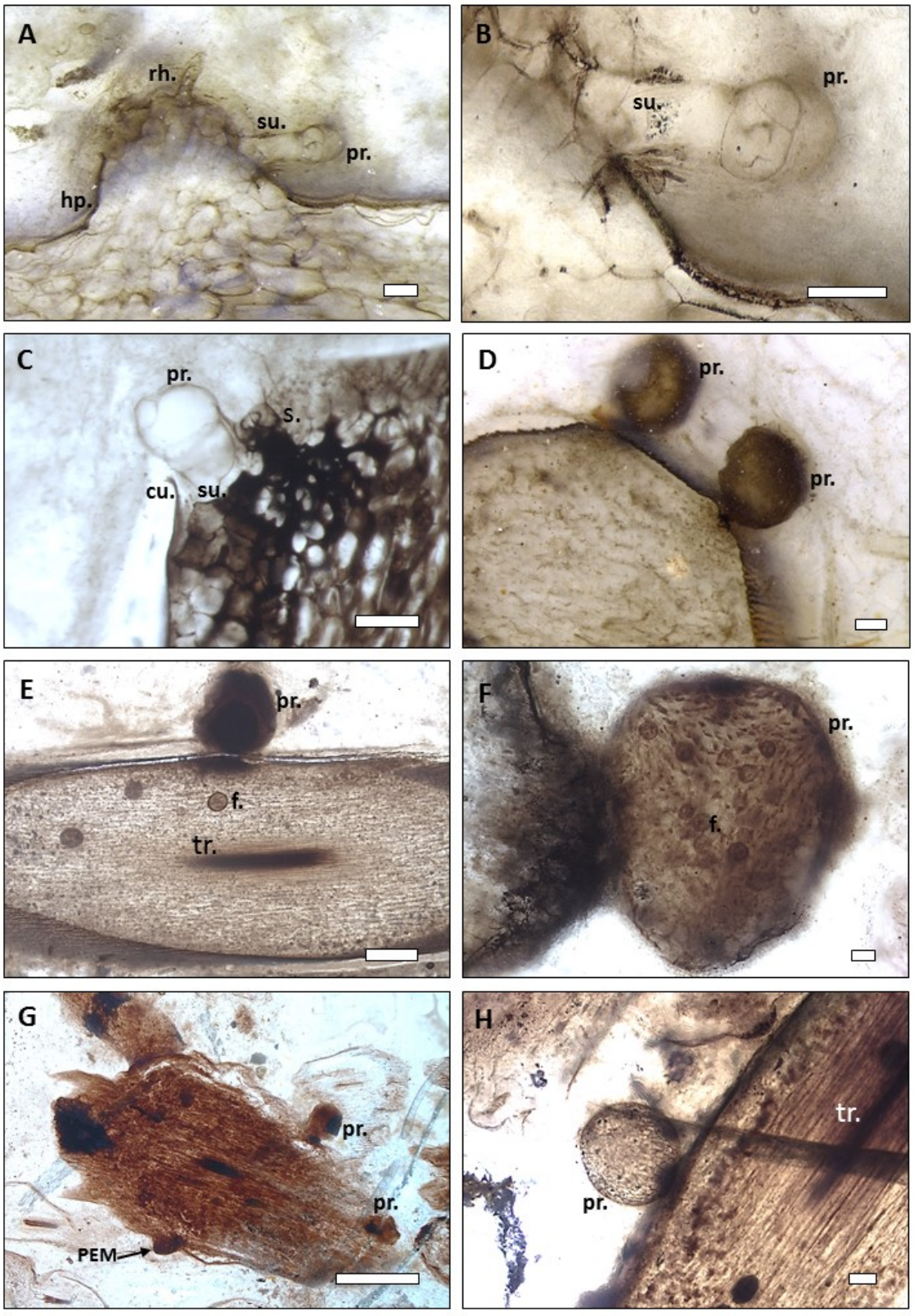


Figure 3.2


A
rh.
su.
pr.
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B
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pr.
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F
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Figure 3.3

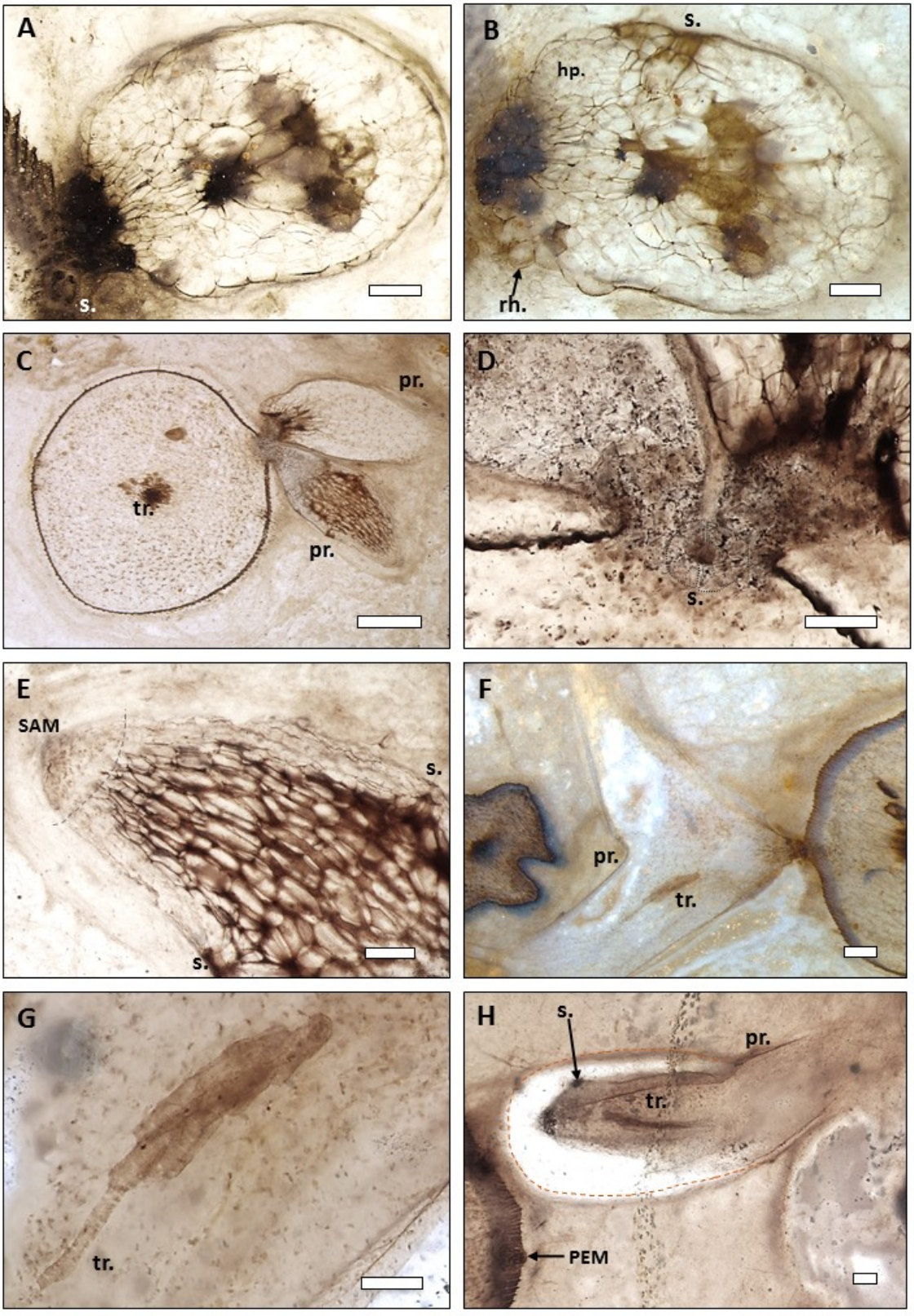


Figure 3.4

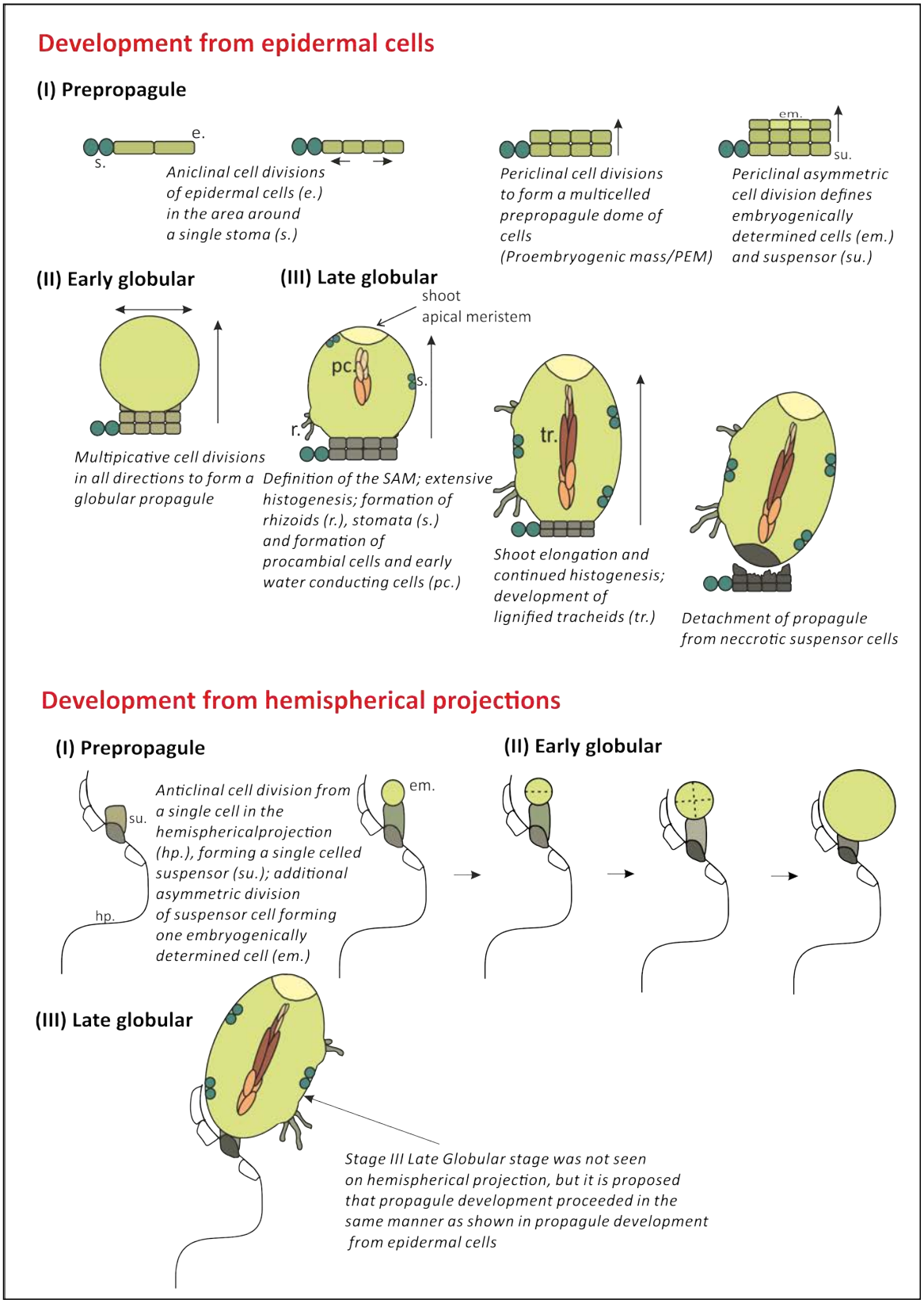
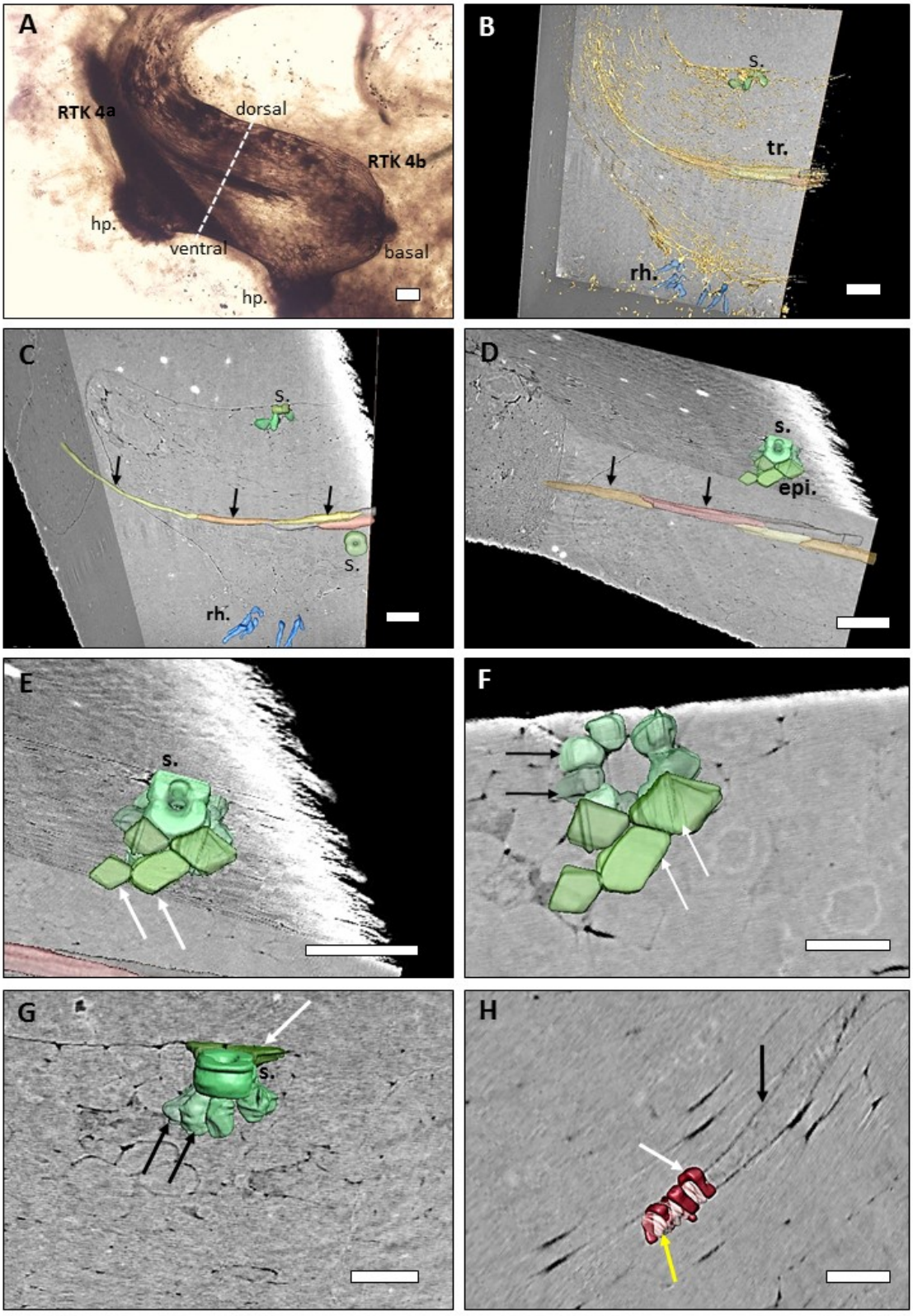

Development from epidermal cells
(I) Prepropagule
e.
s.
Aniclinal cell divisions of epidermal cells (e.) in the area around a single stoma (s.)
Periclinal cell divisions to form a multicelled prepropagule dome of cells (Proembryogenic mass/PEM)
em.
su.
Periclinal asymmetric cell division defines embryogenically determined cells (em.) and suspensor (su.)
(II) Early globular
Multipicative cell divisions in all directions to form a globular propagule
(III) Late globular
shoot apical meristem
pc.
s.
r.
Definition of the SAM; extensive histogenesis; formation of rhizoids (r.), stomata (s.) and formation of procambial cells and early water conducting cells (pc.)
tr.
Shoot elongation and continued histogenesis; development of lignified tracheids (tr.)
Detachment of propagule from neccrotic suspensor cells
Development from hemispherical projections
(I) Prepropagule
su.
hp.
Anticlinal cell division from a single cell in the hemisphericalprojection (hp.), forming a single celled suspensor (su.); additional asymmetric division of suspensor cell forming one embryogenically determined cell (em.)
em.
(II) Early globular
(III) Late globular
Stage III Late Globular stage was not seen on hemispherical projection, but it is proposed that propagule development proceeded in the same manner as shown in propagule development from epidermal cells

Figure 3.5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RTK 4b
hp.
ventral
basal
hp.
B
s.
tr.
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H

Figure 3.6

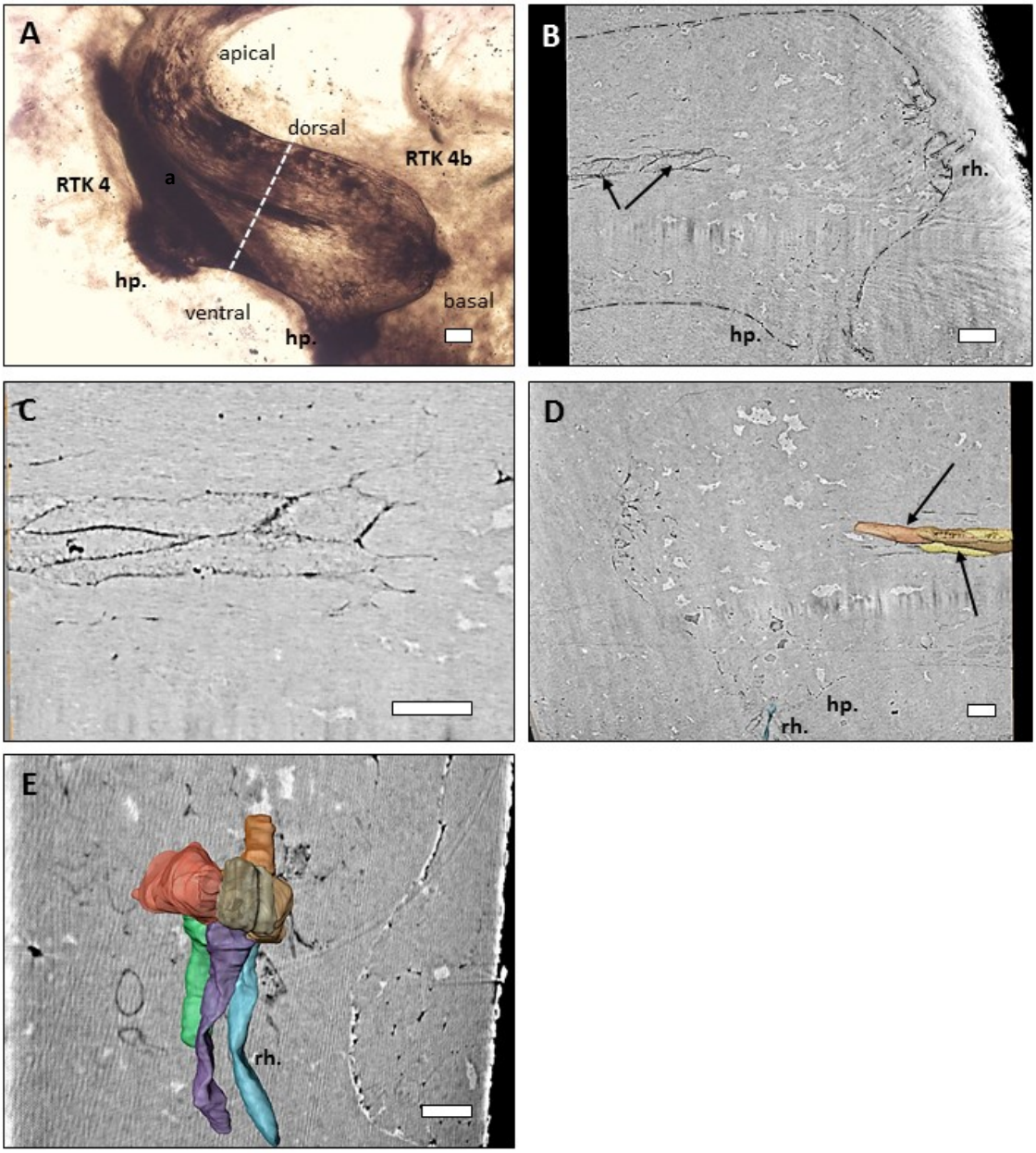
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Figure 3.7

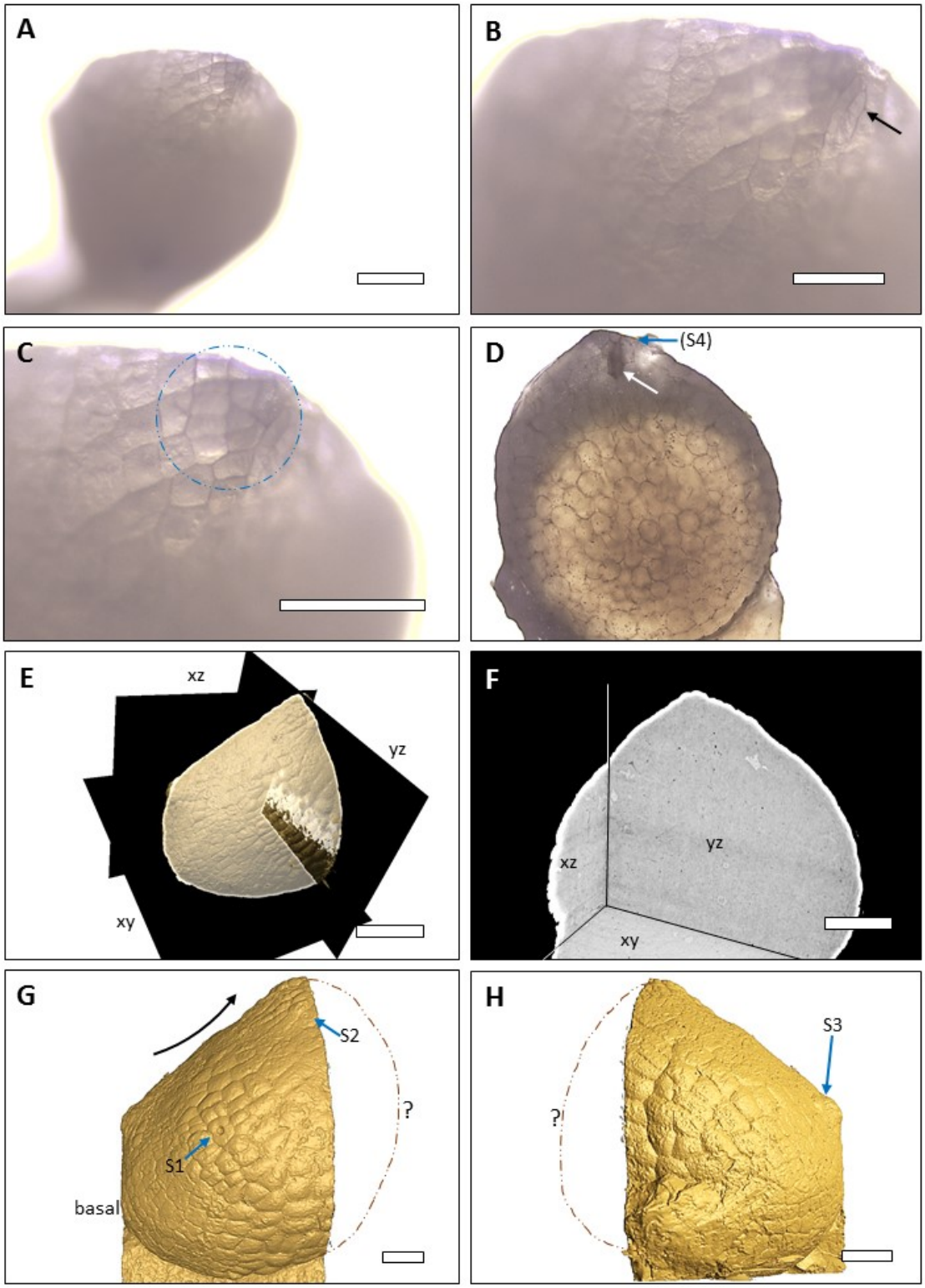


Figure 3.8

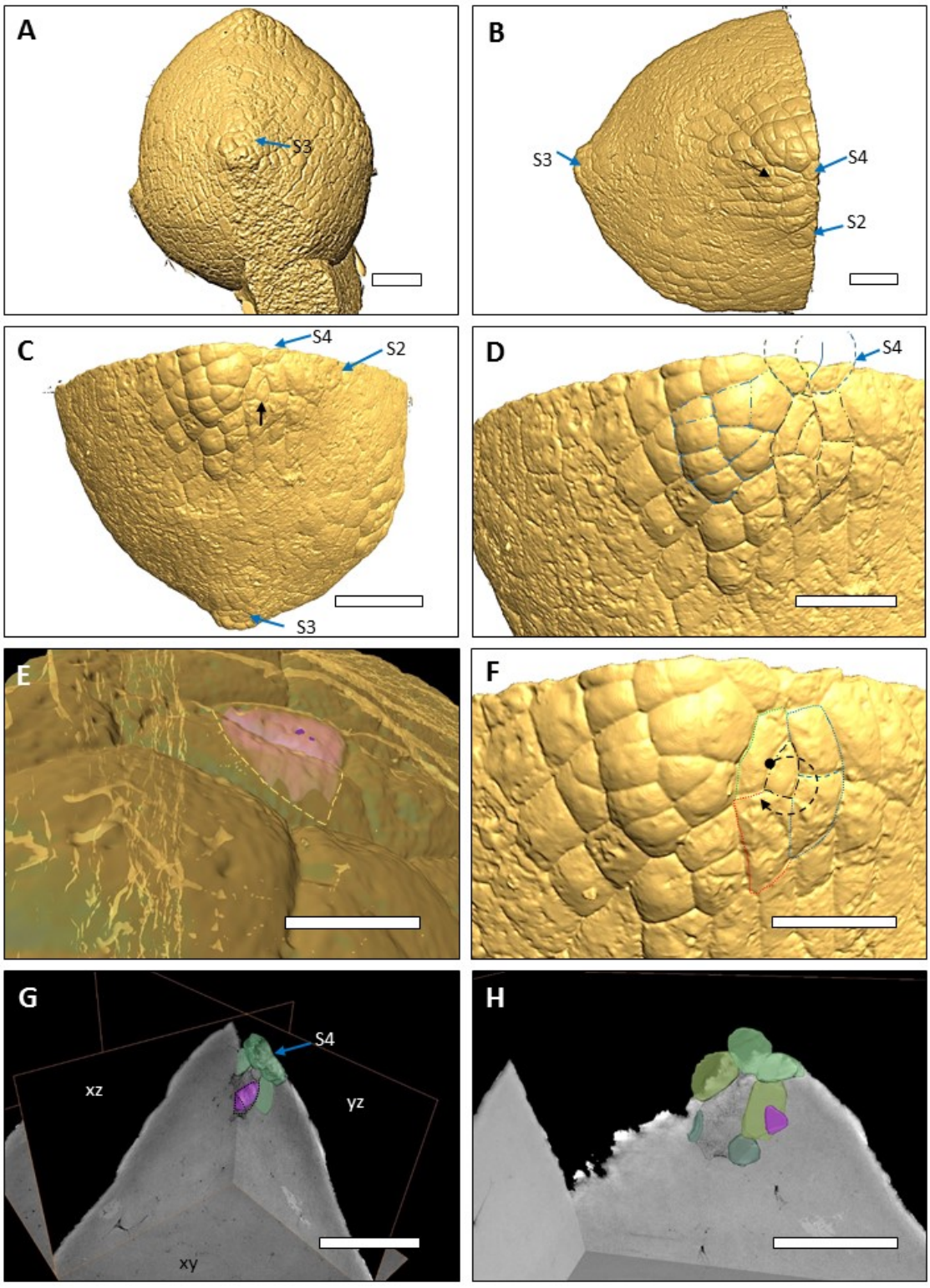
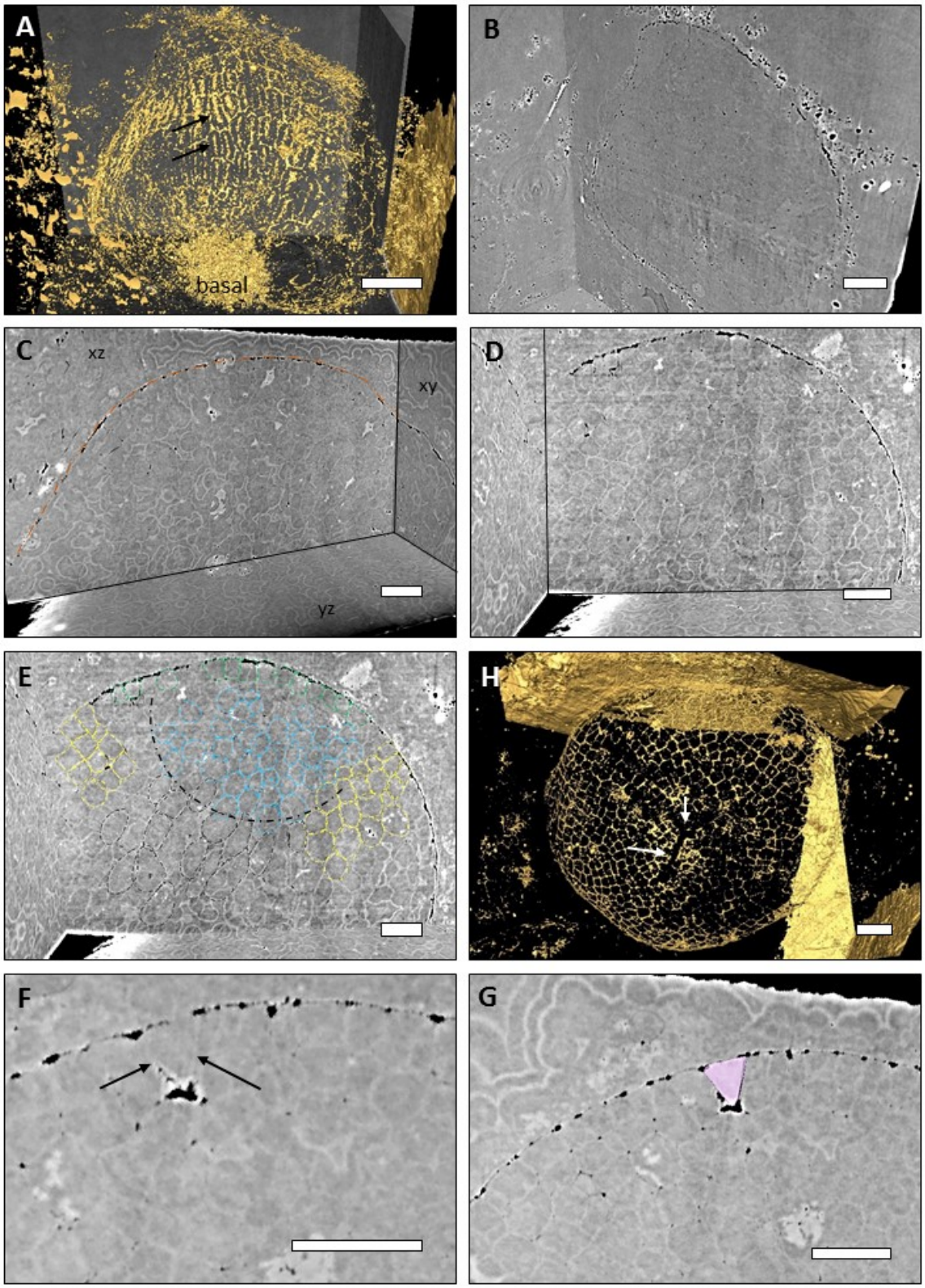


Figure 3.9



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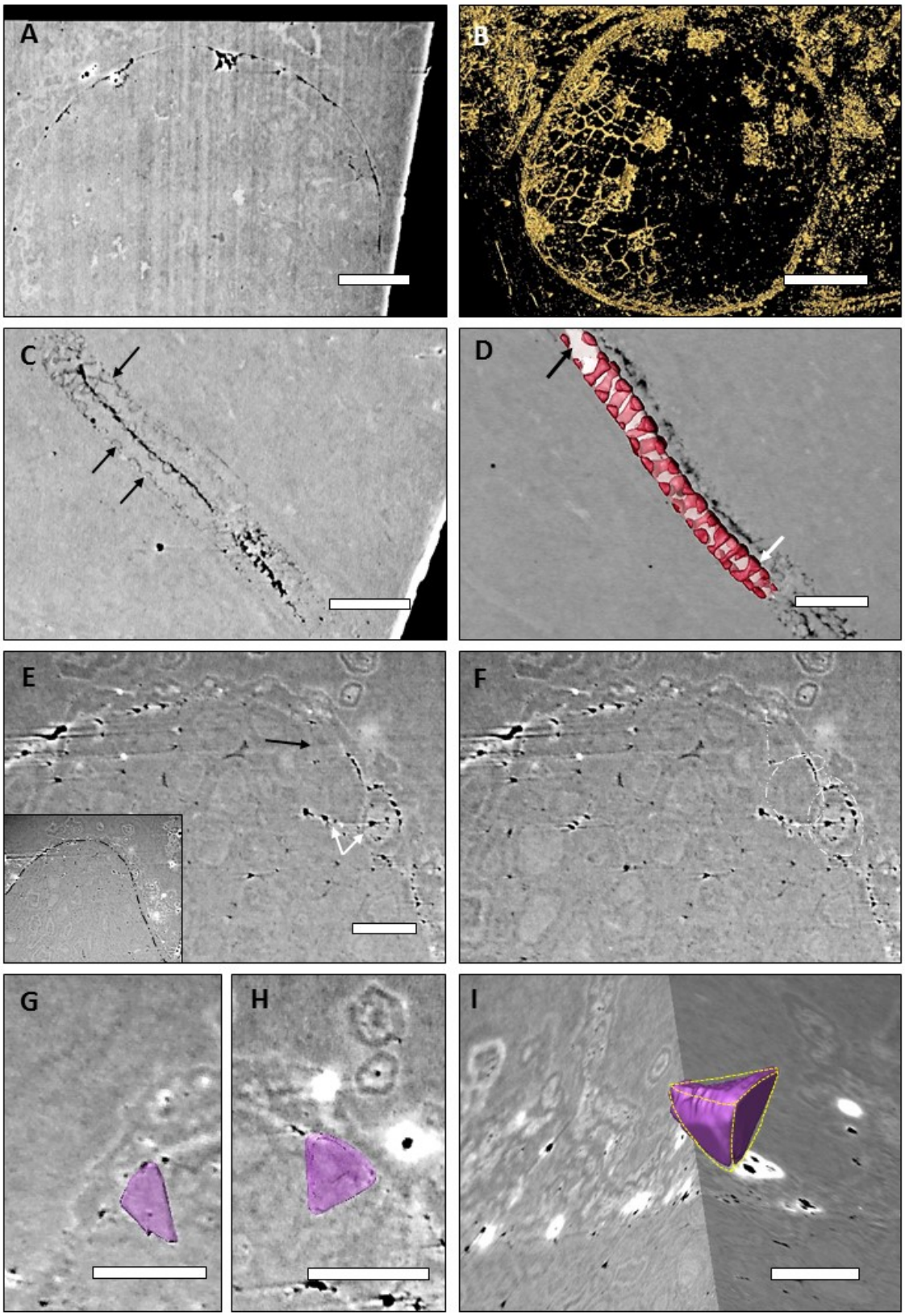


Figure 3.11

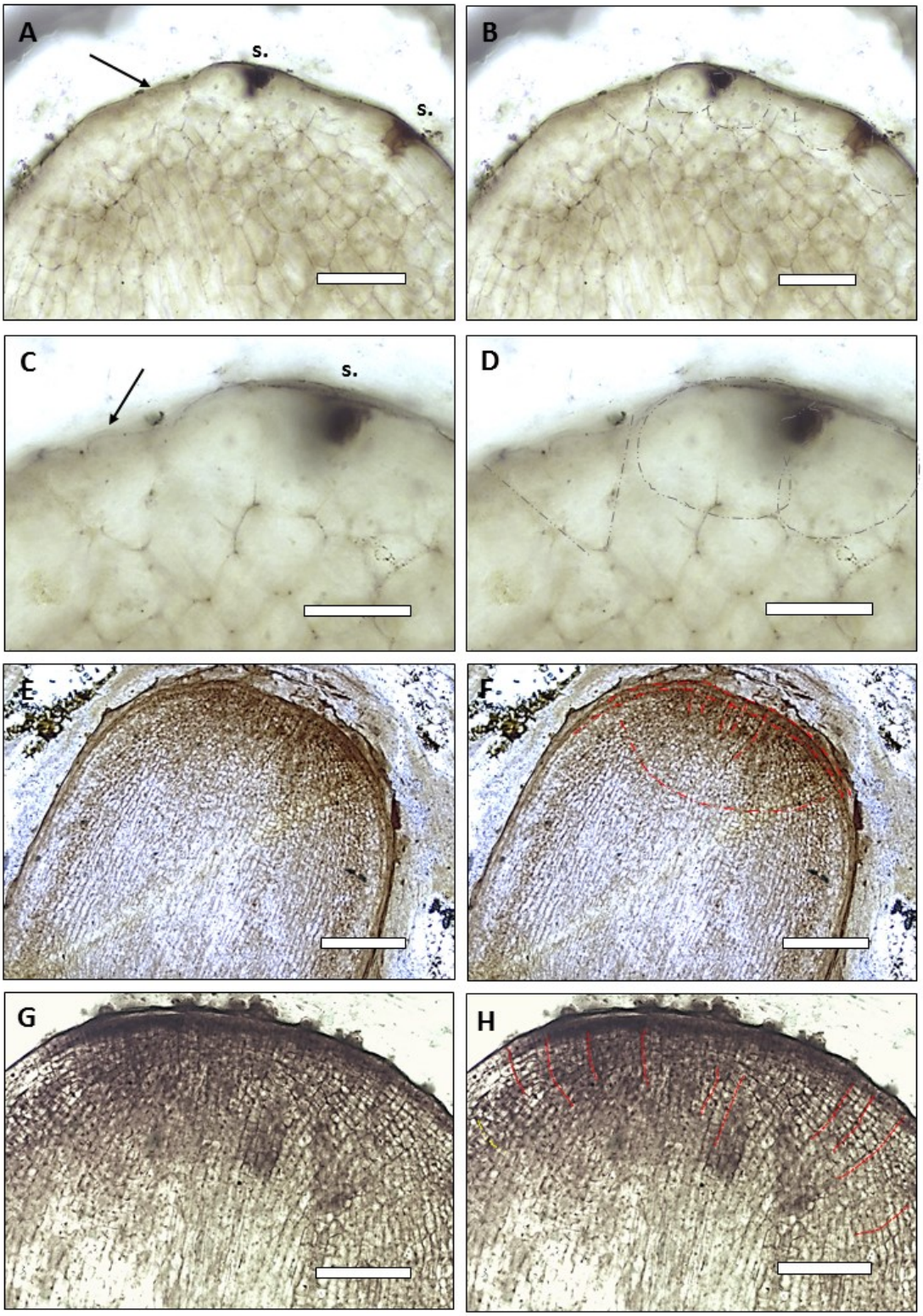


Figure 3.12

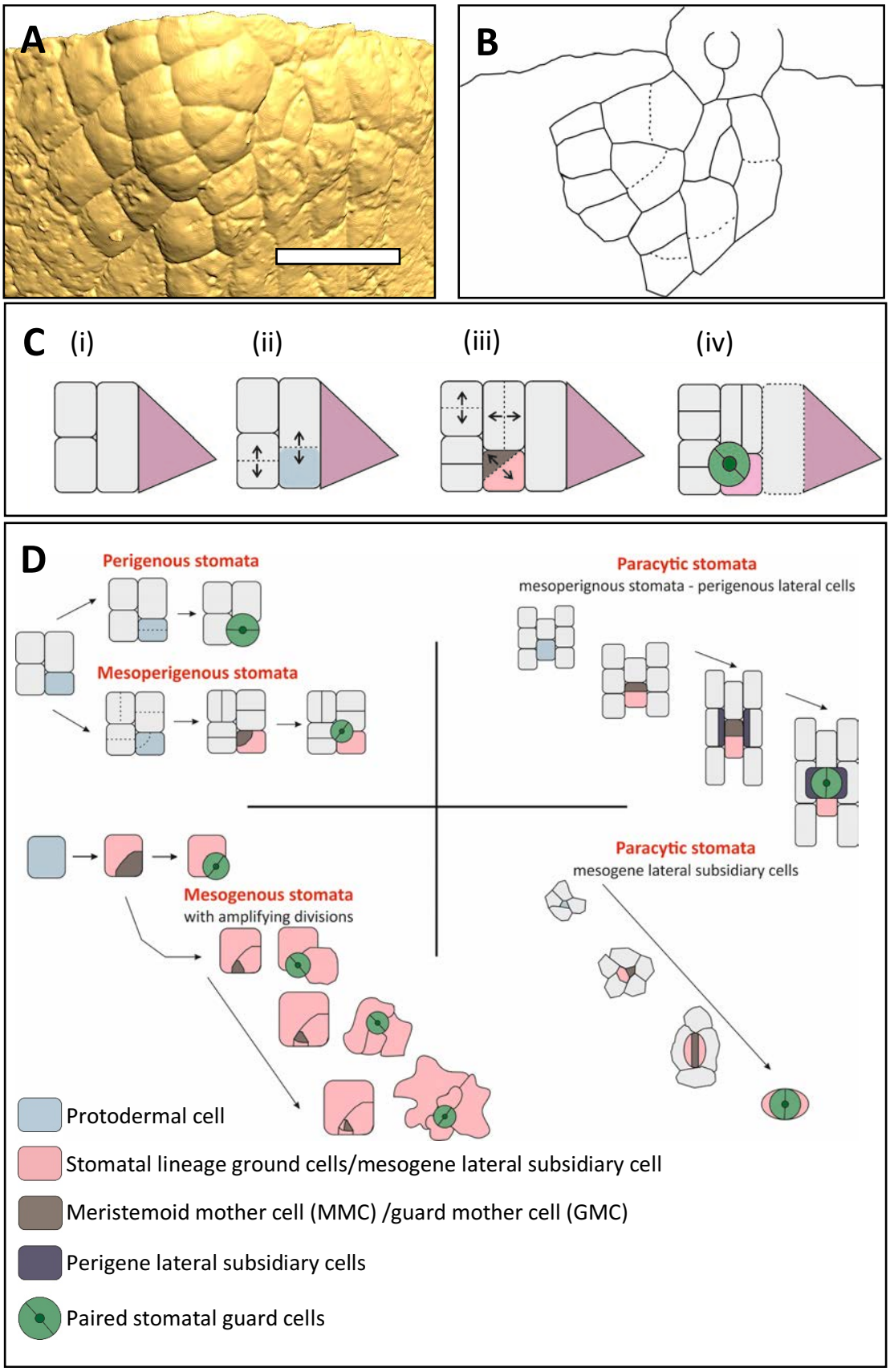
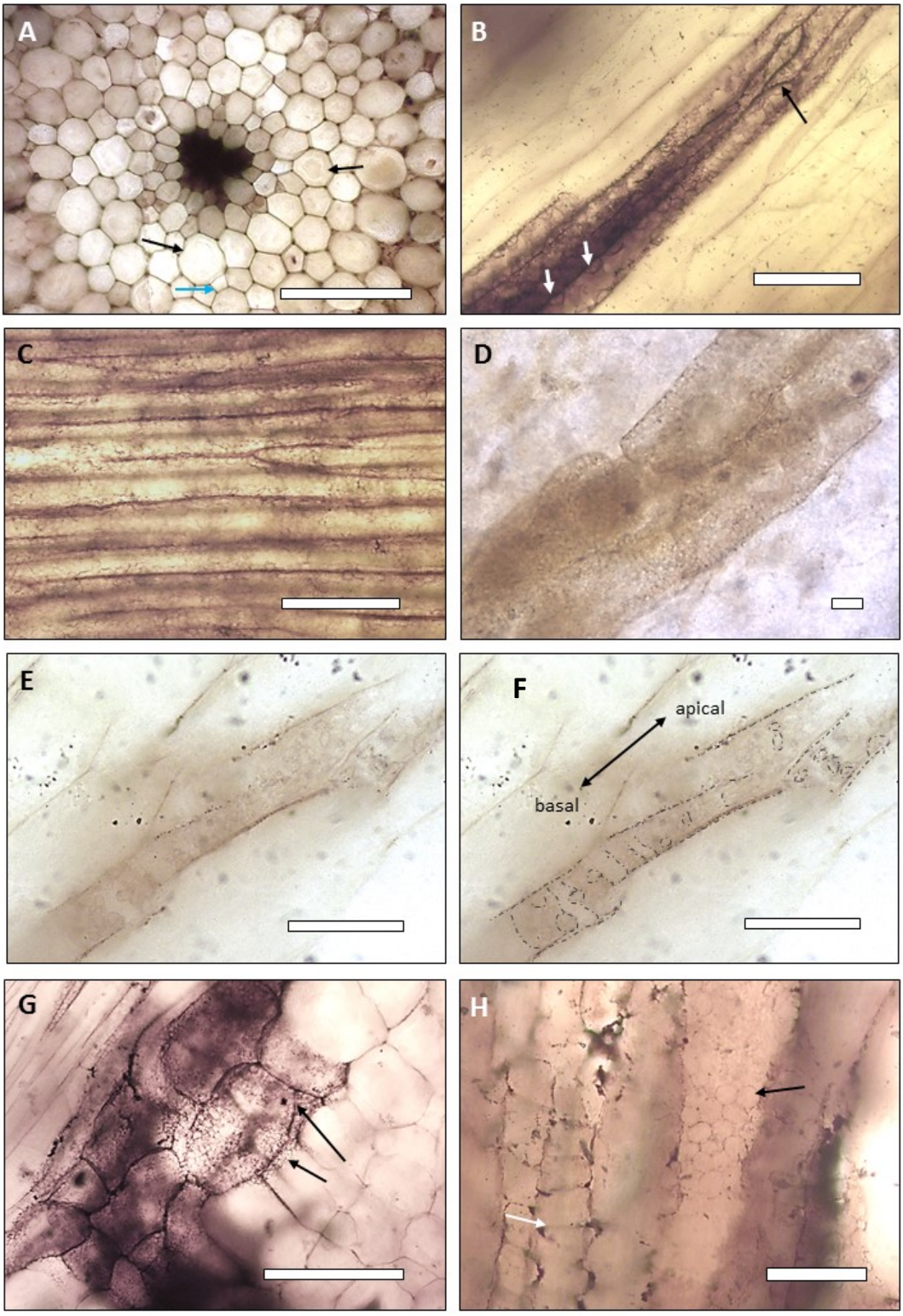

A
B
C
(i)
(ii)
(iii)
(iv)
D
Perigenous stomata
Mesoperigenous stomata
Paracytic stomata
mesoperignous stomata - perigenous lateral cells
Mesogenous stomata
with amplifying divisions
Paracytic stomata
mesogene lateral subsidiary cells
Protodermal cell
Stomatal lineage ground cells/mesogene lateral subsidiary cell
Meristemoid mother cell (MMC) /guard mother cell (GMC)
Perigene lateral subsidiary cells
Paired stomatal guard cells

Figure 3.13



Chapter 4

Sporophyte development in *Rhynia gwynne-vaughanii* and the evolution of sporophyte morphological complexity

4.0 Introduction

As *Rhynia gwynne-vaughanii* represents an early divergent vascular land plant, it is necessary to discuss the results given in Chapter 3 in the context of current knowledge regarding vascular-plant evolution and development. The development of asexual propagules is comparable to a similar form of asexual reproduction in extant vascular land plants (somatic embryogenesis) and shoot development is discussed in reference to developmental patterns in extant land plants. The contrasting structures of the shoot apical meristems of *Rhynia gwynne-vaughanii* and *Aglaophyton major* are compared with those of extant land plants also and discussed in reference to the evolution of complexity in sporophyte morphology in land plants. In addition the development of specialised tissue complexes is discussed in relation to the same in extant land plants.

4.1 Asexual Reproduction

Asexual reproduction is common across the plant kingdom performing an essential role in the colonisation of habitats. It can be the predominant form of reproduction in some plant species, and in many cases the only form of reproduction, especially in some bryophytes (Longton and Miles 1982, Longton and Schuster 1983; Newton and Mishler 1994). The production of asexual propagules takes many forms such as from specialised organs as gemmae cups in liverworts (Frey and Kürshner 2011), on the rhizomes of *Psilotum nudum* (Bierhorst 1971) or on the leaves of the succulent *Kalanchoë daigremontiana* (Garces *et al.* 2007). The large quantity of asexual propagules

encountered in the chert in comparison to the single known example of a mature *Rhynia gwynne-vaughanii* gametophyte, as well as the relative rarity of sporangia, demonstrates the prevalence of asexual reproductive strategies over sexual reproduction in this early land plant. *Rhynia gwynne-vaughanii* occupied what was at times a partially aquatic habitat with warm silica-rich fluids regularly inundating the environment. Such conditions should be and are generally favourable to the dispersal of gametes and spores, but despite this the environment appears not to have been stable enough for extensive gametophyte germination and thus for prevalent sexual reproduction to successfully take place. Asexual reproduction via propagules provided an alternative mechanism for *Rhynia gwynne-vaughanii* to move out farther into the environment using the semi-aquatic nature of its habitat as a conduit.

Although clonal propagation provides a quick means of habitat and resource acquisition, especially in a fragmented and unstable environment where sexual reproduction may have been seasonal, a high level of clonal reproduction within a plant population is not without problems. Increased clonal propagation can permanently inhibit a plant population's ability to reproduce sexually, which impacts directly on sexual recruitment and genotypic diversity (Eriksson and Ehrlén 2001). Clonal populations also limit the space available within the habitat for sexually reproduced plants to germinate and establish, and longer timeframes between the sexual reproduction of clonal plants result in a decrease in successful reproduction with a population (Honnay and Bossuyt 2005; Klekowski 1988, 1997).

4.1.1 Asexual reproduction in *Rhynia gwynne-vaughanii* via somatic cells

The propagules produced from epidermal cells surrounding the stomatal guard cells develop in a manner similar to somatic embryogenesis (SE). This form of asexual reproduction in plants produces genetically identical 'plantlets' from somatic cells in the plant body. Somatic cells are any cells in the plant body not normally associated with reproduction. The development of the propagules from epidermal cells in the stomatal complex reveals something fundamental about this part of the *Rhynia gwynne-vaughanii* plant. Primarily it indicates that in some cases, after the development of the stomatal guard cells, the surrounding cells retained all of their genetic information, could undergo a reactivation of the cell cycle and resume division. These cells were biochemically induced to follow an embryogenic developmental pathway (Tisserat *and* Demason 1980; Sharp *et al.* 1982). This behaviour is in contrast to that of the guard cells themselves, which were likely completely differentiated and probably still functioning during the development of the propagules.

Somatic embryogenesis occurs naturally in plant species such as, *Kalanchoë daigremontiana* (Garces

et al. 2007) , but *in vitro* studies of SE in angiosperms and gymnosperms have been successfully carried out on many species, following the pioneering studies by Steward *et al.* (1958) and Reinert (1958) on *Daucus carota*. These studies reveal the inherent ability of higher land plants to reproduce via SE, despite many not doing so naturally outside of the laboratory. More recently, SE has been confirmed in the monilophyte *Cyathea delgadii* (Mikuła *et al.* 2015) where somatic embryos form from epidermal cells, with a pro-embryogenic stage similar to that of *Rhynia gwynne-vaughanii*, i.e. several multiplane cell divisions of single epidermal cells, and a domed pro-embryo.

In vitro studies have contributed significantly to the understanding of the developmental pathways of SE and its molecular mechanisms (Dudits *et al.* 1991, 1995; Rose and Nolan 2006). As somatic embryos largely follow the same developmental pathways as zygotic embryos, these studies provide vital information on the regulation of embryo development. SE can be either direct from the cells of the plant, or, more commonly, indirect via an intermediary callus stage from which somatic embryos then develop. These two pathways are considered to be not too different in reality, as both require embryogenic cells to form somatic embryos (De Jong *et al.* 1993). Morphologically somatic embryos must be bipolar with root and shoot growth arising at opposite ends (von Arnold *et al.* 2002; Chawla 2002). *Rhynia gwynne-vaughanii* lacks roots, however, it does have both apical and basal organisation with the initial rhizoids developing at the opposite end to apical growth. As well as this simple morphological arrangement, *Rhynia gwynne-vaughanii* propagules also bear the basic organisation of the adult sporophyte from early in Late Globular Stage III. It is also important that in SE the propagule should be nutritionally independent from the parent plant during development, i.e. no vascular connection. There is no evidence for a vascular connection between the *Rhynia gwynne-vaughanii* propagules and the parent plant, and the suspensor organ becomes increasingly necrotic as the propagule grows, essentially cutting the propagule off from its source.

4.1.2 Universal growth regulators controlling plant development

Plant-growth regulators (PGRs) driving the development of somatic embryos have also been widely examined through the management of explant material and the study of the effects of various phytohormones on somatic embryo production. Although studies have focused on higher land plants, the phytohormones involved are active across the plant kingdom from bryophytes to angiosperms and thus it can be inferred that the same/similar PGRs operated in the production of the *Rhynia gwynne-vaughanii* propagules. Auxin, cytokinin and abscisic acid (ABA) play key roles in the initiation and development of somatic embryos with auxin considered one of the most important phytohormones involved in the regulation of somatic embryo development. It may also have played a significant role in the early radiation of land plants (Cooke *et al.* 2003; Poli *et al.* 2003). Auxin is

found naturally (as indole-3-acetic acid – IAA) in the apical and root meristems and young leaves, and also plays an important role in the induction of cell polarity and asymmetrical division. Even though the effects of exogeneous cytokinin in *in vitro* studies can vary, the presence of high levels of auxin and cytokinin at the initial stages of SE have shown to be essential, however the level of auxin decreases as SE proceeds to the later stages (Michalczuk *et al.* 1992; Ribnicky *et al.* 1996, 2002; Schiavone and Cooke 1987).

Cytokinin alone is implicated in the induction of SE in some species (Remotti 1995; Zdravkovic-Korać and Neskovic 1999; Iantcheva *et al.* 1999 Sagare *et al.* 2000), however it regularly works with auxin to produce somatic embryos. Both cytokinin and auxin function in the growth and development of both vascular and non-vascular plants indicating that their roles have been conserved (Poli *et al.* 2003; von Schwartzenberg *et al.* 2007).

Abscisic acid (ABA) also has an effect on the development of somatic embryos (Nishiwaki *et al.* 2000; Su *et al.* 2013). Nishiwaki *et al.* (2000) showed that ABA drove the development of somatic embryos directly from epidermal cells of small (< 30 mm) *Daucus carota* seedlings; the authors found an increase in the number of embryos per seedling with an increase in concentration of ABA. They also established that in seedlings with removed apices, ABA did not induce the formation of SEs, concluding that endogenous substances formed in the apex worked in tandem with the exogenous ABA to induce somatic embryo development. In addition, and in agreement with previous studies (Trolinder 1988), the authors determined that juvenile plant tissue holds the best potential for the development of somatic embryos and in relation to ABA this is acceptable as more juvenile, i.e. undifferentiated somatic tissues, may contain more ABA (Srivastava 2002). This may hold some significance for the *Rhynia gwynne-vaughanii* axes described above and the epidermal cells of surrounding the stomatal guard cells. It is suggested that these cells were not terminally differentiated and therefore 'young' and open to reprogramming to an embryogenic state to produce vegetative propagules. In vascular plants ABA is associated with many functions from desiccation and dehydration tolerance and other stresses (Reynolds and Bewley 1993; Liu *et al.* 2008), growth inhibition and regulation (Swami and Raghavan 1980; Chia and Raghavan 1982) to control of sex determination (Hickock 1983). ABA synthesis in the non-vascular bryophytes (Nishiyama *et al.* 2003; Takezawa *et al.* 2011), indicates that ABA has been conserved across the plant kingdom from a very early point in the evolution of land plants, playing a role in desiccation and dehydration tolerance, an important adaptation to terrestrial environments. In mosses ABA is associated with the inhibition of protonemal growth (Menon and Lal 1974, Chopra and Kapur 1989), causing the development of asexual gemmae structures (Goode *et al.* 1993, Schnepf and Reinhard 1997), as well as the inhibition of gametophyte development (Valadon and Mummery

1971; Chopra and Kapur 1989; Christianson 2000). Considering that ABA is a stress-induced phytohormone capable of reacting to a stressful event and eliciting an appropriate response (Fehér *et al.* 2003) it may have played an important role in the production of the somatic embryos seen on the decaying and fungi-ridden *Rhynia gwynne-vaughanii* axes described here. The role of stress in the production of somatic embryos is reviewed in detail by Zavattieri *et al.* (2010).

4.1.3 Developmental pattern of asexual propagules

Figure 3.4 and Figure 4.1A and B

During the development of the propagules in *Rhynia gwynne-vaughanii*, epidermal cells divide to form several smaller cells, with dark internal colouration, possibly representing an increased density of cytoplasm (figure 3.1D–H). A central nucleus and other physiological aspects diagnostic of embryogenic competent cells (Šamaj *et al.* 2006) have not been preserved. Any stages between the Pre-propagule I dome of cells (figures 3.1D–H), and the Early Globular Stage II propagule (figure 3.2B and C) were not observed. The dome of cells in Stage I is comparable to the pro-embryogenic mass (PEM) formed during SE, where a group of embryogenically determined cells (EDCs) develops prior to the development of the embryo-proper (Sharp *et al.* 1982; Williams and Maheswaran 1986). Further, Williams and Maheswaran (1986) consider the development of somatic embryos with multi-cellular origins to be from pre-embryogenically determined cells (PEDCs), i.e. cells that do not require induction in to an embryogenic state by external stimuli. It is not clear what occurs following the development of EDCs to produce a propagule with a multi-cellular suspensor in *Rhynia gwynne-vaughanii*. Considering figure 3.2C shows a distinct division in Stage II into separate propagule and suspensor zones it is proposed that the EDCs divide asymmetrically to form a small globular body, which although derived from these cells is morphologically and biochemically distinct from the suspensor-forming cells below. On continued cell division and growth of the propagule, designation of the meristematic shoot apical meristem zone occurs, and thus development of stomata and procambium follows. Williams and Maheswaran (1986) considered this type of multi-celled origin the result of a group of cells in the same embryogenic state synchronously acting to produce an asexual propagule. This multi-cellular origin of the propagule also accounts for the relatively broad patch of necrotic cells at the base of some loose propagules. A single-cell origin for the propagules would not leave such a mark. For *Rhynia gwynne-vaughanii* propagules that developed from a single cell in the hemispherical projections a small or absent necrotic patch would be more likely. Some loose propagules in the chert do show this feature. Kidston and Lang (1917) recognised that adventitious branches which developed from hemispherical projections also left a zone of necrotic cells after detachment. They further

documented that some of these branches possessed narrower stalk-like bases of attachment than others and it is possible that these particular 'branches' were in fact well developed asexual propagules formed in the manner described in this study. Further work on *Rhynia gwynne-vaughanii* by Kidston and Lang (1921a) recognised necrotic zones which extended into the epidermal layer, and the authors even postulated a possible relationship between these necrotic areas, stomata and hemispherical projections.

The Late Globular Stage III sees the formation of stomata, cuticle, rhizoids, and vascular tissue as well as extensive apical growth. The development of the various organs in some respects shows some organisation with the development of stomata evident from the earliest point in Stage III ontogeny before any indications of apical growth and vascular differentiation. Tracking the timing of the development of rhizoids is somewhat more problematic with loose Late Globular Stage III propagules showing both rhizoids and stomata, and, attached well-developed propagules with apical growth and vascular tissue displaying no rhizoid growth. It should be considered that the absence of rhizoids on some well-developed propagules might be a preparatory bias with the loss of some chert material unavoidable during the cutting process, as well as preservation bias. Loose well-developed propagules regularly bear rhizoids on projections with an increase in the size and number of projections and rhizoids as the propagule grows, e.g. figure 3.1B. Shoot elongation commences after the development of stomata but just prior to the formation of the vascular tissue, and tracheid elongation and development proceeds along with shoot elongation.

As the propagule proceeds through the three stages of development, the cells in the suspensor become increasingly necrotic and by Stage III the suspensor cells are dark brown and in most cases indistinguishable from each other. The initiation of programmed cell death (PCD) in the cells of the suspensor of the *Rhynia gwynne-vaughanii* propagules likely played a role in driving the development of the somatic embryo itself (Filanova *et al.* 2000). However, as previously mentioned, loose propagules at various developmental stages are seen in the chert, so it is unclear why in some cases PCD in the suspensor seems to have proceeded at a faster rate allowing the propagules to detach for example at Stage II before histogenesis seemingly has occurred. Detachment of the propagules from the axis was probably promoted by wind or by the movement of water through the habitat.

Latest Stage II and Stage III show shrinkage of the propagules in some cases. Stage II propagules can have a wrinkled morphology, and Stage III propagules can also show extensive shrinkage, e.g. figure 3.3H. This shrivelling is attributed to the dehydrating nature of the preserving silica medium on these juvenile propagules which probably had an under developed, or perhaps absent, cuticle during these stages.

4.2 Comparative developmental morphology: *Rhynia gwynne-vaughanii* and extant land plants

Figures 4.2 and 4.3; see also figure 3.4

The large number of propagules preserved in the chert displaying multiple stages of development, not only presents a unique opportunity to study the development of an extinct early land plant but also allows for comparison with the developmental morphology of extant land plants. As the stages seen in the development of zygotic embryos in angiosperms and gymnosperms, are reflected in the development of their somatic embryos, it is proposed that the propagules described here are also proxies. This section compares the ontogeny of the three developmental stages of the *Rhynia gwynne-vaughanii* propagules to shoot development in the bryophytes, lycophytes, monilophytes, as well as in the more distantly related gymnosperms and angiosperms. Early embryology in all plants is characterised (Gifford and Foster 1989) by the plane of division of the zygote in relation to the axis of the archegonium neck and the orientation of the growing plant, (figure 4.2A) (Johnson and Renzaglia 2009); prone, exoscopic, endoscopic without suspensor, endoscopic with suspensor. Although within the lycophytes and monilophytes more than one early embryology type may exist among the different genera, the bryophytes have exclusively exoscopic orientation of the early embryo.

4.2.1 Bryophytes

The sporophytes of the non-vascular bryophytes (liverworts, mosses and hornworts) show a distinctive embryology but each lineage has a different developmental process. At a basic level bryophyte embryos undergo a brief globular phase before entering a phase of shoot elongation (figure 4.2B) (Niklas 2008), which is reflected the development of the sporophyte of *Rhynia gwynne-vaughanii*, a major difference being that the bryophyte sporophyte develops determinately towards the formation of the sporangium/capsule. Sporangium formation is delayed in the polysporangiates. Although displaying growth from meristematic cells, the bryophytes do not show the same level of organisation in the apex seen in the vascular plants and the mode of sporophyte development is different in each of the three separate bryophyte groups. Epibasal and hypobasal cells contribute to growth in the liverworts, later defining a foot, seta and spore-bearing capsule. A basal meristem controls growth in the hornworts and the majority of the mature sporophyte body consists of an elongate spore-filled capsule (Crum 2001; Niklas 2008). The sporophyte of hornworts may grow indefinitely, however because the sporophyte is still somewhat dependent on the gametophyte, any factors affecting the growth and development of the gametophyte can affect the growth of the sporophyte. For mosses sporophyte growth is directed by a sub-apical meristem near the seta but

also from the apex (Ligrone and Duckett 1998), which may or may not have an apical cell (Crum 2001). Although Johnson and Renzaglia (2009) define exoscopic embryology as without a suspensor, Niklas (2008) reports the presence of a unicellular suspensor in the exoscopic embryos of the liverworts *Pellia epiphylla* and *Porella bolanderi*.

4.2.2 Lycophytes and monilophytes

Like the bryophytes, the lycophytes and monilophytes have a defined foot organ, but these two groups have evolved several other organs that define them morphologically: the root, microphylls, possibly a suspensor, the shoot apical meristem and vascular system. In lycophytes following the initial division of the zygote, the organ precursor cell is orientated away from the archegonium neck (figure 4.3A). The early embryo defines a foot organ, root and first leaf precursor early and the foot is embedded into the gametophytic tissues. Nutritional independence is relatively quick in lycophyte, and monilophyte embryos via mycoheterotrophy, i.e. fungi, or through photosynthesis, and impacts the development of rooting organs, delaying them and forming a rhizome instead (Johnson and Renzaglia 2009). Given the rhizomatous morphology of *Rhynia gwynne-vaughanii* as well as the pervasive presence of fungi in both propagule producing axes and the propagules themselves, it is proposed that nutritional independence was relatively quick, mycoheterotrophy was extensive and affected basic morphology. Early independence of the *Rhynia gwynne-vaughanii* sporophyte is further evidenced by the development of stomata and rhizoids early in shoot ontogeny, before any extensive elongation of the shoot axis.

In the majority of the monilophytes, the shoot developmental pattern is set at a very early stage in ontogeny (Wardlaw 1955; DeMaggo 1977), and four initial cells define the first foot, leaf, shoot apex and root (figure 4.4). Studies of somatic embryogenesis in *Cyathea delagii* by Milkuła *et al.* (2015) shows the formation of a four-segmented somatic embryo, indicating that even in non-sexually reproduced embryos this segmentation is detrimental to the development of the organism and determination of cell fates is set very early in ontogeny. The authors further describe three developmental stages of the shoot. Initial segmentation of the *Rhynia gwynne-vaughanii* asexual propagule is not clearly evident although figure 3.1H shows four cells within the pre-propagule dome. The significance, if any, of these four cells with regard to the embryology and development of *Rhynia gwynne-vaughanii* can only be realised if sexual embryos would be discovered in the Rhynie chert.

4.2.3 Gymnosperms and Angiosperms

On comparing development in *Rhynia gwynne-vaughanii* and the gymnosperms, some basic similarities exist, (figure 4.3B,) e.g. the development of the zygote also follows three distinct stages: proembryogeny (before suspensor elongation), early embryogeny (after suspensor elongation but before root meristem development) and late embryogeny (formation of different tissues and the root and shoot meristems) (von Arnold *et al.* 2002). However, the formation of multiple embryos and suspensors is a distinctive feature of gymnosperms during the early globular stage, bearing no resemblance to any stage in *Rhynia gwynne-vaughanii* development.

Within angiosperms, morphological similarities persist up to the late globular stage prior to the formation of the cotyledons, when the protoderm, meristem ground tissues and procambium are all established (figure 4.5B). Later in development the angiosperm globular embryo forms a more triangular shape from which the cotyledons grow. The angiosperm embryo goes through several other stages of development before reaching maturity and forming a seed coat.

These comparisons show that any ontogenetic differences between extant land plants arise very soon after the initial cell divisions of the embryo, with the biochemical signals driving ontogeny to produce the differing morphologies among vascular land plants initiated very early in development, e.g. ontogenetic pattern was shown to be set as early as the PEM stage in some *in vitro* studies of SE in the common grape vine (Jayasankar *et al.* 2003). In addition, the diversity of gametophyte morphology and habit among land plants is hypothesised to have played a significant role in the diversity of embryological form (Bower 1935; Johnson and Renzaglia 2009).

In conclusion, sporophyte morphological development in *Rhynia gwynne-vaughanii* bears the basic two-stage 'globular-to-shoot elongation' path of development of the bryophytes, but with a more complex external morphology and internal physiology, similar to the lycophytes, minus the development of rootingstructures and microphylls.. Examination of gametophytes from the Rhynie chert during these studies returned just one single specimen bearing an archegonium with a potential early stage embryo (figure 4.5). This archegonium of *Lyonophyton rhyniensis*, the gametophyte of the protracheophyte *Aglaophyton major*, is well preserved. The potential embryogenic structure shows at least two defined zones and possibly a third. The developing embryo can be orientated in an endoscopic fashion, similar to that seen in the lycophytes, *Isoëtes* and *Selaginella* (figures 4.1 and 4.2), but also in an exoscopic direction similar to bryophytes and some monilophytes, such as the morphologically simple *Psilotum nudum*. Endoscopic embryo organisation of the protracheophyte embryo would strongly suggest the same within *Rhynia gwynne-vaughanii*, and the switch of embryo orientation could have played a significant role in establishing the vascular land plant lineage; by distinguishing them developmentally from their bryophyte-like ancestors.

How the orientation of the early embryo may have affected the evolution of plant morphology is not clear. It is equally possible that the early vascular plants retained the exoscopic orientation, which may account for its occurrence in *Psilotum nudum*, which has other 'primitive' morphological characteristics compared to other genera in its phylum. For *Tmesipteris*, which belongs to the same group as *Psilotum nudum*, there is a similarity in embryo morphology early in development with the hornwort *Anthoceros*, both bearing a mulitlobed haustorial foot embedded in the archegonial tissue (Sporne 1962). However, whether or not these two features are homologous is unclear. Further evidence of early embryology in the Rhynie chert plants is crucial, as well as a better understanding of how gametophyte morphology affects embryo development.

4.3 Structural evolution of the shoot apical meristem

Although apical growth can be found across all phyla of land plants and even in the charophycean algae, the Shoot Apical Meristem (SAM) is considered a distinct and phylum-defining organ found in vascular land plants, but as the results of these studies show it was also present in the non-vascular ancestral protracheophyte lineage. The SAM of *Rhynia gwynne-vaughanii* had a distinct structure, and was characterised by a single tetrahedral apical initial from which all other cells in the shoot body were either directly or indirectly derived, and divided out from in a spiral manner. The SAM of *Aglaophyton major* differed in structure, lacked any apparent apical initial and consisted of several zones of meristematic activity, consisting of vertical files of cells, which went deep into the core of the SAM.

Three hypotheses currently dominate discussions regarding the evolution of the shoot apical meristem:

1. The SAM evolved from the transient apical meristem of the moss embryo, which became indefinite (Albert, 1999).
2. The SAM evolved through a displacement of the basal meristem of a hornwort-like ancestor (to become apical and indeterminate) Ligrone *et al.* (2012b).
3. The vascular plant SAM arose as a *de novo* specialised organ (Kato and Akiyama, 2005).

The protracheophyte *Aglaophyton major* is the most primitive plant in the fossil record to show a distinct SAM and confirms that the SAM is not exclusively a vascular land plant trait. Therefore the initial development and elaboration of the SAM most likely took place in a non-vascular bryophyte-like plant ancestral to both vascular plants and protracheophytes, and the main organ initiating the evolution of indeterminate, complex sporophyte morphologies. It would not be necessary for this ancestral form to be independent of its gametophyte, branched or sporophyte dominant, and would

only require a shift of meristematic activity to the apex of the sporophyte (as per Ligrone *et al.* 2012b). Even without branching, delaying spore production would permit a longer vegetative growth period, producing a taller aerial sporophyte shoot. Such strategies would be driven by pressure from the external environment with decreasing available space favouring an axial shoot morphology. Further pressure in new habitats with more unstable environmental conditions would also favour plants with the ability to control growth and delay sexual maturity until the most favourable conditions occur. The development of a more complex and branching sporophyte with high nutritional demands, as well as intense symbiotic nutritional relationships with fungi may have contributed to the uncoupling of the two vegetative generations until eventually both generations were completely independent.

A number of genes control SAM initiation and maintenance in land plants. Although the majority of such studies are carried out within the members of the seed plant group, homologs for many of the regulatory gene groups in the seed plants are found in the moss *Physcomitrella patens*. The regulatory gene groups controlling SAM initiation and maintenance as well as shoot and organ development are given a comprehensive review by Floyd and Bowman (2007) and are summarised in Table 4.1.

Examination of the structure of the SAM is of particular interest to all fields of plant research as it drives the development of the plant body, allowing the plant to continue to grow and function. The SAM allows for indeterminate growth of the sporophyte shoot before the development of the sporangium and facilitates branching of the sporophyte to potentially produce multiple sporangia. The SAM differs structurally across the major plant groups, showing an overall increase in morphological complexity over geological time, in line with the evolution of more complex shoot morphologies (figure 4.5). The structure of the shoot apical meristem was first appreciated by Wolff (1759) who recognised it as a zone of meristematic cells, the 'punctum vegetationis', which contributed to the growth and development of the plant shoot. The term 'meristem' is attributed to Nägeli (1858) who developed the Apical Cell theory, recognising the single apical initial controlling development across many plant types. Hanstein (1868) was the first to recognise that the Apical Cell theory did not fully account for the more complex structure of the SAM in angiosperms and identified a zonation of the cells within the SAM, delineating three separate zones of cells with different developmental fates. In the twentieth century Schmidt (1924) further divided the angiosperm SAM into the tunica and corpus. The tunica consists of one or two layers of anticlinally dividing cells. The corpus is a body of cells located at the centre of the meristem and cell divisions occur in all planes. However Foster (1938), on recognising that this tunica-corpus theory was not suitable for the SAM of gymnosperms, recognised cytohistological layering within the tunica,

specifically working on *Ginkgo biloba*. Clowes (1961) and Gifford and Corson (1971) further superimposed this layering theory on the SAM of angiosperms. In addition to these descriptions, Buvat (1952) proposed the much less acknowledged Meristem d'Attente theory. In this case the SAM remained quiescent until the reproductive stage was reached upon which meristematic activity resumed.

Much of the more recent classification and reclassification of SAM structure (Popham 1951; Newman 1965; Philipson 1990) arose from the variability of structure across phyla. Popham (1951) described seven different SAM forms, with the single apical cell, fern-type meristem the simplest and the angiosperm meristem as the most complex. Three broad divisions are recognised as per Newman (1956, 1961, 1963): (1) Monoplex, pyramidal apical cell pteridophyte-type common, *Equisetum*, *Psilotum*, *Tmesipteris* and *Selaginalla*; (2) Simplex seed plant-type found in gymnosperms plus *Lycopodium*, *Phyloglossum*, *Isoëtes* and *Stylites*; (3) Duplex seed plant-like found in angiosperms and some gymnosperms. However, even recently, the SAM of the lycophyte *Selaginella krausiana* was shown to bear more than one apical cell (Harrison *et al.* 2007); - Jones and Drinnan (2009) challenged this interpretation showing that only one apical cell with two cutting faces could be assigned. Only the lycophytes show a cross over regarding SAM structure. Further evaluation of the number of SAM types was made by Philipson (1990) who recognised just two, the pteridophyte SAM and the seed plant SAM. Philipson (1990) ruled out any transitional types, which had been described by Popham (1951).

The organisation of an organ such as the SAM, essential to plant development, requires significant levels of cell-to-cell communication. Land plants (Popper and Tuohy 2010; Raven 1997), and some algae, Cook *et al.* (1997), transport various biomolecules from cell to cell via networks of tubes in the cell walls called plasmodesmata (PD). Studies by Imaichi and Hiratsuka (2007) demonstrate that the SAMs of vascular land plants can be divided into two main types based on the density of PD: a fern-like group with a high PD density SAM, and a seed plant-like group with a low PD density SAM. Low densities were found in the angiosperms, gymnosperms, Lycopodiaceae and Isoëtaceae, all of which bear plurial apical initials. SAMs with single apical initials (*Psilotum nudum*, *Equistem* sp., *Selaginella*) have much higher densities. The ability of seed plant-like SAMs to produce secondary PD, i.e. *de novo* PD development in cell walls, may also be a critical factor that allows for the complex SAM structure found in angiosperms and gymnosperms (Cooke *et al.* 1996). This would permit increased communication between not only cells of the same lineage but between cells of different lineages and may have allowed for better organisation of the SAM. Cell to cell communication in fern-like plants with only primary PD, which forms during cytokenesis, would be restricted to cells of a common lineage. It is also worth noting the existence of both types of SAM

in the lycophytes, a cross over also seen in their SAM structures.

Although access to fossil SAMs is a unique opportunity, interpretation of fossilised SAMs which were once dynamic zones of cell division should be approached with some caution as over interpretation of static fossilised cells can lead to false assumptions and it must be accepted that this type of analysis cannot be as reliable as would be if examined in living and actively developing plant axes; even though it is clear that even in extant plant SAM studies, interpretations are open to scrutiny and perhaps misinterpretation (e.g. Harrison *et al.* 2007 versus Jones and Drinan 2009).

The SAM of *Rhynia gwynne-vaughanii* shows strong similarities to the pteridophyte-like monoplex and high PD density SAMs of the leptosporangiate monilophytes and some of the lycophytes, e.g. the Selaginellaceae, although within this group apical cells vary in cutting-face number (Siegert 1974; Imaichi and Kato 1989, 1991; Imaichi 2008). In *Rhynia gwynne-vaughanii* and these two groups the SAM consists of a superficial layer of anticlinally dividing cells overlying a core zone of smaller, isodiametric cells (figure 4.5). The SAM of *Rhynia gwynne-vaughanii* was previously described as lacking a single apical initial, with Edwards (1994), and Kidston and Lang (1920b, p.606) describing the apex as consisting of small, closely packed, isodiametric cells with no apparent organisation. Cells within the small fraction of the apices described in both cases show roughly square-shaped packets of cells arranged in fours. However these specimens, like the *Aglaophyton major* specimens described in this volume, exist only as thin sections and thus represent a small fraction of the overall structure of the plant. In addition, the apices are assigned to *Rhynia gwynne-vaughanii* based on their occurrence with mature *Rhynia gwynne-vaughanii* shoots. Assuming that these apices are those of *Rhynia gwynne-vaughanii*, it is likely that the apical cell of *Rhynia gwynne-vaughanii* was transient and its absence altered the appearance of the SAM at the surface. For several of the apices analysed using SRXTM an apical cell could not be identified despite careful searching through all tomographic sections in each specimen further suggesting that the apical cell of *Rhynia gwynne-vaughanii* could have been transient. Hypothetically, the presence of a distinct tetrahedral apical cell may have been dependent of the growth stage of the shoot, i.e. present during points of additive growth, but absent during periods when shoot elongation was necessary (the absence of a single apical initial, and the presence of many meristematic cells may have acted as protection against damage of this important initial cell), or when favourable environmental conditions for growth were not present. During these periods the SAM at the surface would consist just of small meristematic cells, without a specialised cell. Development of an apical cell would occur from these cells. Impermanent apical initials are in general considered a characteristic of seed plant meristems but as shown by Harrison *et al.* (2007) the SAM of *Selaginella krausiana* may have two transient initials. If the apical cell of *Rhynia gwynne-vaughanii* was also transient then potentially this trait is fundamentally an ancestral one within at least

the vascular plants. For some mosses the apical cell of the sporophyte has been reported to be transient during the early stages of embryology (Renzaglia *et al.* 2000; Cooke *et al.* 2003; Friedman *et al.* 2004).

In the SAM of *Aglaophyton major* the cells appear to be of a similar size in both the superficial zone and the core meristematic area. This similarity of cell size may be indicative of an overall simpler mode of development as is shown by the relatively simpler shoot morphology. The core meristematic area consists of vertical files of cells, similar to those seen on the periphery of the *Rhynia gwynne-vaughanii* apex, just outside the core cells of the SAM (figure 3.8D and E).

4.4 Developmental pattern in shoot ontogeny

A simple spiral ontogeny from a single apical initial with three cutting faces underlies the ontogeny of the *Rhynia gwynne-vaughanii* shoot, a pattern that is echoed in the development of helical phyllotaxy of the lycophyte microphylls (leaf-like enations that project from the shoot) and in shoot ontogeny in the monilophytes (Imiachi 2008). Lycophyte microphylls develop basipetally and have no lateral apical or marginal meristems (Imaichi 2008). Hemispherical projections are the only cell protuberences that occur on *Rhynia gwynne-vaughanii*, but it is unclear if these are primitive representatives of the vascularised microphylls in lycophytes or of the similar, but non-vascularised, enations found in *Asteroxylon mackiei*. Kidston and Lang (1921a, pp. 833, plate III, figure 10) document the elongation of a hemispherical projection from the axis to form an emergence-like structure that may be comparable to the microphyll emergences of stem lycophytes such as *Asteroxylon mackiei*. Hemispherical projections can appear to be arranged helically around the *Rhynia gwynne-vaughanii* shoot but not exclusively, and projections occur directly opposite each other on the same horizontal or vertical plane (figure 1.5; as seen in RTK 4, figure 3.5A). Kidston and Lang (1921a) describe hemispherical projections as developing from below a stoma (perhaps accounting for projections with a stoma) and from cells within the epidermis and the hypodermis, echoing the basipetal development of the microphylls of the lycophytes. One other Rhynie chert plant, *Nothia aphylla* (Daviero-Gomez *et al.* 2004, 2005) also bears dome-like protuberances on its aerial shoot. These protuberances are arranged helically around the stem. *Nothia aphylla* is classified as a Zosterophyll, an extinct group of plants with (perhaps vague) links to the lycophytes and the rhyniophytes (Kerp *et al.* 2001). The irregular organisation, of the hemispherical projections in *Rhynia gwynne-vaughanii* compared to those of *Nothia aphylla* and the enations of *Asteroxylon mackiei* highlights the increasingly more complex arrangement and evolution of lateral appendages during early vascular plant evolution.

Kidston and Lang (1921a) ruled out the development of hemispherical projections as part of the

primary developmental pattern of the *Rhynia gwynne-vaughanii* shoot, but this appears not to be the case as results here show that hemispherical projections formed during the primary development of the axis and played an important role in increasing the surface area of developing shoots, bearing stomata and rhizoids during early development.

Hemispherical projections that form *de novo* on mature *Rhynia gwynne-vaughanii* axes can be attributed to the development of adventitious branches and asexual propagules from somatic cells on the shoot. Kidston and Lang (1921a) recognised a link between the tissues that form intumescences on modern plants (e.g. see Pinkard *et al.* (2006) and references therein), callus cells (important in asexual propagation) and the hemispherical projections of *Rhynia gwynne-vaughanii*; this may have some significance as the hemispherical projections produce asexual propagules.

Spiral cell division from a single three-faced apical cell is also seen in the gametophytes of some mosses, e.g. *Rhizobium novae-hollandiae*, which also bears a single tetrahedral apical cell from which derivatives divide out in a spiral manner (Crum 2001); in addition the 'leaves' of the gametophyte shoot are arranged helically. It is reasonable to conclude that this spiral developmental pattern in axial aerial shoots was inherited from a bryophyte-like ancestor and the mechanisms and pathways controlling this type of development were conserved in the earliest polysporangiates and carried over into divergent lycophyte and monilophyte groups. Overall, the SAM was the defining organ that organised the development of the specialised tissue complexes of the sporophyte and over time modifications to these pathways contributed to more complex SAMs and more complex shoot morphologies.

4.4.1 Gametophyte development

The developmental pathway of the independent axial, unisex and almost isomorphic gametophytes seen for *Rhynia gwynne-vaughanii* and *Aglaophyton major* for now remains undetermined. Taylor *et al.* (2005) show that gametophyte of *Lyonophyton rhyniensis* had a single apical cell suggesting that the basic developmental patterns between the sporophyte and the gametophyte, of at least this group of land plants, was similar. Further clarification as to the evolution of the generational arrangement among the vascular plants is required as in contrast to *Rhynia gwynne-vaughanii* and *Aglaophyton major* and other chert plants, lycophytes and monilophytes have small thalloid gametophytes; a notable exception is the gametophyte of *Psilotum nudum* (Bierhorst 1977) which, although less than 2 mm in length, is axial and branched. The stem lycophyte *Asteroxylon mackiei* is evidence of a relatively deep split within the vascular land plant group, but critically it is not known whether its gametophyte was thalloid or axial. An isomorphic, axial and aerial gametophyte for *Asteroxylon mackiei* would indicate that this generational organisation is a synapomorphic feature of

early polysporangiates rather than just the rhyniophytes, and later lycophyte groups evolved their thalloid gametophytes independently.

4.5 Development of specialised tissues in *Rhynia gwynne-vaughanii*

Information regarding the development of specialised tissues and organs, e.g. stomata, vascular tissues, from early land plant fossils is virtually non-existent. Inadequate preservation quality and the relative paucity of early land plant fossils can seriously impede any significant observations. By combining light microscopy (LM) and synchrotron X-ray tomography (SRXTM) these studies have shed new light on the development of such specialised tissues.

4.5.1 Stomata

Many studies since the 1930's have demonstrated the complex nature of stomatal development across plant phyla (Florin 1931, 1933, 1950, 1951; Pant 1965; Pant and Mehra 1964a-b; Pant and Kidwai 1968; Fryns-Claessens-Van Cotthem 1973; Rudall *et al.* 2013); figures 3.11 and figure 4.6). The development of stomata is generally concerned with the development of the guard cells and general classification of stomatal development refers to the formation of the guard cells and their neighbouring cells rather than the full stomatal complex, which includes the substomatal chamber. Accepted terminology for the ontogenetic and morphological classification of stomata is given in figure 3.11D, figure 4.6. Studies of ontogeny in fossil stomata can be somewhat problematic with a lack of specimens showing early stage ontogeny, although there are exceptions (Barbacka and Boka 2000).

The stomata of *Rhynia gwynne-vaughanii* are anomocytic, lacking any specialised neighbouring cells. This type of stomatal complex is also found among the mosses, hornworts, the extinct Trimerophytes, the Psilotaceae (*Psilotum* and *Tmesipteris*) and the lycophytes (Rudall *et al.* 2013). Reconstruction of the stomata in specimen RTK4, figure 3.4E, F and G, show the pore of the *Rhynia gwynne-vaughanii* stomata as a ring of eight oblong hypodermal cells, also previously described by Edwards *et al.* (1998), over which the stomatal guard cells lie. The stomatal pore is a defined structure separate to that of the guard cells, but the developmental relationship between the pore and the guard cells in *Rhynia gwynne-vaughanii* is not known. The stomatal complex of *Aglaophyton major* bears a similar ring of hypodermal cells below the guard cells and a similar structure is also seen in the 'glandular' pores found in *Horneophyton lignieri* (Edwards *et al.* 1998). This marks the guard cells out as distinctly specialised epidermal cells. Stomatal guard cells are ultimately evidence of increased terrestrialisation and control over the internal functioning of the plant shoot and its relationship with the external environment. This is supported by the lack of any specialised guard

cells with the pores in the liverwort group, the basal-most group of land plants.

Unusually for any plant, either extinct or extant, results from LM and SRXTM show that in *Rhynia gwynne-vaughanii* stomata formed early in shoot development, and are seen in close proximity to the apical cell. Specimen RTK 8, (figure 3.11, section 3.5.2), shows that stomatal development in *Rhynia gwynne-vaughanii* was mesoperigenous (ranunculous) (see figure 3.11C and figure 4.6) and is somewhat more complex than would be assumed for a plant as 'primitive'. In this case, the cells surrounding a stoma are not only derived from the same direct cell lineage as the GMC but also from cells of a different cell lineage, i.e., the file of four cells immediately to the left of this zone of cell division in figure 3.11A–C which can be also attributed to an earlier apical derivative. It is suggested that at least one, if not all, of these cells would have further differentiated into rhizoids (Edwards *et al.* 1998). Perigenous anomocytic stomata are considered the most primitive, (Rudall *et al.* 2013), and are found in *Psilotum nudum* (Mickle 2012). Stomatal development in bryophytes and lycophytes is also considered to be perigenous but this requires re-examination (Rudall *et al.* 2013). It cannot be ruled out however that perigenous ontogeny did not occur in *Rhynia gwynne-vaughanii* as more than one ontogenetic type could occur in the same plant.

4.5.2 Rhizoids

The unicellular rhizoids of *Rhynia gwynne-vaughanii* are relatively simple tissues compared to stomata or tracheids and their development appears equally as simple, with just one cell division from undifferentiated cells within the epidermis or from similar cells on hemispherical projections. Rhizoids, like stomata, form early in shoot development. Early rhizoids are short and ovoid/peg-like (figure 3.3A and B), but gradually elongate. Rhizoids can be found on Late Globular Stage III propagules, but not always. Propagules at this stage may have several stomata, but no rhizoid development. Given their role in moisture absorption and anchorage of the rhizome to the substrate, rhizoid development may have been prompted by external environmental factors, such as contact with the substrate or a deficiency in water and essential minerals (rhizoids are also found on aerial axes). The lack of rhizoids may also be a preservation bias or additionally a preparation artefact, where any early rhizoid development may have been destroyed in some specimens during preparation of the petrographic thin sections.

In extant land plants rhizoids grow by tip growth, whereby a highly polarised cell produces an elongate, tubular cell (Vidali and Bezanilla 2012; Rounds and Bezanilla 2013). Although the developmental mechanisms involved in rhizoid development in *Rhynia gwynne-vaughanii* can never be known, it has been shown that it is likely a similar gene regulatory network controls the development of rhizoids in the bryophytes and root hairs in vascular plants (Jones and Dolan 2012),

so it is reasonable to assume that mechanisms controlling rhizoid development in *Rhynia gwynne-vaughanii* were essentially similar to that of the bryophytes and rhizoid growth was via tip growth.

4.5.3 Tracheids

Studies of xylem in well-preserved fossilised early land plants show that by the early Devonian a reasonably diverse level of tracheid morphology had evolved. Tracheid morphology in early land plant fossils, and in early divergent extant plants such as the lycophytes, is classified by the longitudinal section of the lignified secondary walls (figure 4.7). The simplest, the S-type tracheids, are named for the Early Devonian *Sennicaulis hippocrepiformis* from which they were first described (Kenrick *et al.* 1991). S-type tracheids can also be found in *Rhynia gwynne-vaughanii*, (figure 3.9C and D and figure 3.12B), (Kenrick and Crane 1991), as well as in other Early to Middle Devonian plant taxa (Edwards 2003b). S-type tracheids have secondary walls with a spongy inner zone and an outer layer with closely spaced holes. G-type tracheids named for the Early Devonian plant *Gosslingia breconensis* (Kenrick and Edwards 1988 and references therein) are characteristic of fossil lycophytes and are also found in *Asteroxylon mackiei* (Kenrick and Crane 1991). G-type tracheids have annular, helical or near-reticulate secondary wall patterns. The cell walls have two layers, a degradation resistant inner layer with simple pitting between wall thickenings, and a non-resistant outer layer. Other tracheid types are the C-type, described in *Cooksonia pertoni* (Edwards *et al.* 1992), I-type found in unidentified early Devonian coalified fossil plant axes from Wales, UK (Edwards and Axe 2000; Edwards 2003b) and P-type named for *Psilophyton* (Edwards 2003b). Classification of the comparatively smooth and uniformly thick-walled water conducting cells of the protracheophyte *Aglaophyton major* and *Nothia aphylla* confirms similarities with members of the mosses rather than the vascular plant groups (D.S. Edwards 1986; Kerp *et al.* 2001; Edwards *et al.* 2003b). A single origin for tracheids in land plants is supported by a consistency in secondary wall structure between relatively primitive extant vascular plants such as the lycophyte *Huperzia*, *Equisetum* of the Equisetales, and late Silurian to Early Devonian fossil land plants (Cook and Friedman 1998; Friedman and Cook 2000).

During vascular plant development cells determined to become xylem and phloem (procambial cells) are specified in the procambium, a defined set of meristematic cells derived from the apical meristem. In seedless land plants the development of the xylem can be divided into two stages, the development of protoxylem, the first xylem cells to mature and the metaxylem, which matures after the protoxylem. Xylem that matures from the outside of the stem to the inside is termed exarch. The converse is termed endarch. Exarch development is commonly found in primitive land plants, e.g. lycophytes and endarch development is common in seed plants. In plants such as *Rhynia gwynne-*

vaughanii, and other rhyniophytes, which have a single solid central strand of xylem cells, development is inferred as centrarch and the xylem matures from the centre of the strand outwards (Taylor *et al.* 2008). The majority of studies on vascular tissue development have been carried out on the xylem cells of seed plants such as *Zinia elegans* and *Arabidopsis thaliana* (Carland *et al.* 1999; Carland *et al.* 2002; Motose *et al.* 2004), and a number of biochemical pathways have be shown to control the development of xylem cells.

Vascular tissue formation, like plant development in general, is a dynamic process and difficult to study in plant fossils where multiple stages of development need to be preserved to infer any developmental pattern. All information here on tracheid development in *Rhynia gwynne-vaughanii* is taken from multiple petrographic slides containing vegetative propagules as described previously or mature axes, allowing for a concise evaluation of the formation of tracheids, from early stages in the development of the shoot to its formation in new branches on mature axes. Synchrotron tomographic data is primarily used to emphasise the morphology of the tracheids.

At the Late Globular Stage III, ovoid, pitted cells develop in the basal zone of the propagule and have a similar morphology to water-conducting cells seen in *Aglaophyton major* (figure 3.12C and D). In propagules with elongation of the shoot axis (latest Stage III), these cells remain conspicuous at the base of the propagule. Similar cells are also seen near the base of branches, i.e., where the branch connects to the primary shoot axis. Elongate tracheid cells are only found around at the basal end of Stage III propagules, which have commenced shoot elongation. Tracheids at the basal end of these propagules are relatively short and have a wider diameter than those seen at the apical end of the propagule, (e.g. specimen RTK 4, Figure 3.5C). The disparity of tracheid morphology in different parts of the shoot is indicative of their respective functions during specific periods in development. Longer tracheids at the early stages of shoot or branch formation would be functionally redundant, as essential moisture needs to travel just a short distance to the apical regions. More elongate tracheids or water conducting cells are only necessary once the shoot elongates and function in transporting moisture up the shoot. The development of secondary walls is a primary indicator of a significantly higher water-transport capacity and act as protection against the collapse of the cells under the negative pressure created by the capillary action of water being pulled up through the cells, a problem aided by the drag created by the evaporation of water from the shoot via the stomata.

Although difficult to directly corroborate it is proposed that, based on the data shown here, tracheid development in *Rhynia gwynne-vaughanii* followed a developmental path essentially similar to that of xylem cells in extant vascular plants and is linked to the initiation of shoot elongation. In this case xylem cells are derived from xylem precursor cells, derivatives of the procambium, which

contains all the cells destined to form vascular tissues. Figure 4.8 shows diagrammatically the proposed path of tracheid development in *Rhynia gwynne-vaughanii*. Procambial cells derived from the meristem initially form the ovoid, pitted cells within the Late Globular Stage III propagule, and probably functioned in the distribution of water throughout the still relatively small plant. Once shoot elongation commences the cells forming water conducting cells also elongate (along with epidermal cells) and cell fates are assigned, with some cells forming the tracheids and others the phloem cells. Following cell elongation, pits in cells are arranged in a linear fashion marking the position of secondary wall deposition and lignin production (figure 3.13E and F). The tracheids develop from the centre outwards as a single central strand of cells, and longer and narrower tracheid cells, with strongly defined secondary wall tissues, develop the further from the basal region of the propagule the apical meristem moves. Modern studies of xylem formation show that the acropetal movement of xylogen from the maturing xylem cells towards the immature procambial cells prompts the formation of more xylem cells, (figure 4.9), (Motose *et al.* 2004). This action results in a continuous maturity of the xylem, with the most mature xylem cells available the more basal regions of the plant shoot available to direct water up through the shoot. Figure 3.13E and F shows secondary walls that are better defined towards the basal end of the propagule (seen in figure 2.5), supporting the hypothesis that tracheid formation in *Rhynia gwynne-vaughanii* proceeded from the apical areas and matured basipetally.

Table 4.1 highlights the main biochemical and genetic factors that control xylem and overall shoot development (Aloni 1987; Carland *et al.* 1999; Fukuda 1997, 2004; Carland *et al.* 2002; Ye 2002; Motose *et al.* 2004; Carlsbecker and Helariutta 2005, reviewed in Baucher *et al.* 2007). Several phytohormones involved in the development of vascular tissues, e.g. auxin and cytokinin, (Fukuda 2004) are also involved in the development of the shoot axis (see section 4.1.2), linking vascular-tissue development to the overall elongation and growth of the sporophyte. Given the similarities in structure between early tracheid development in *Rhynia gwynne-vaughanii* and the water-conducting tissues in the protracheophytes, the development of tracheids in *Rhynia gwynne-vaughanii* is based on an overall pattern which was already established in the non-vascular polysporangiates with the formation of lignin-rich secondary walls the defining factor that distinguishes the vascular plants.

Figure 4.1

A: Orientation of embryo with respect to the archegonial neck. Modified from Johnson and Renzaglia (2009).

B: Development of the sporophyte shoot in bryophytes. Modified from Niklas (2008).

Figure 4.2

Comparative developmental anatomy: Sporophyte development in lycophytes and monilophytes. Modified from Niklas (2008).

Figure 4.3

A: Sporophyte development in gymnosperms. Modified from von Arnold *et al.* (2002).

B: Sporophyte development in angiosperms. Modified from Niklas (2008).

Figure 4.4

Archegonium of *Lyonophyton rhyniensis* with possible early stage *Aglaophyton* development. Orientation of embryo not clear. Scale 50 μm.

Figure 4.5

Comparison of the structure of the SAM of *Aglaophyton major* and *Rhynia gwynne-vaughanii* with those of extant vascular land plants. Constructed from data in: Gifford and Corson (1971), Gifford (1983), Imaichi (2008), and references to SAM structure pp. 89–91.

Figure 4.6

Stomatal ontogeny in extant land plants. Development in *Rhynia gwynne-vaughanii* resembles mesoperigenous ranunculous. Modified from Fryns-Claessens and Van Cotthem (1973).

Figure 4.7

Development of the *Rhynia gwynne-vaughanii* sporophyte shoot from asexual reproduction.

Figure 4.8

Structure of secondary walls in early tracheids of early vascular land plants. Modified from Edwards (2003b).

Figure 4.9

Xylem development in extant land plants. Brassinosteroid-induced tracheary element formation through expression of HD-ZIP III genes that promote secondary wall production and programmed cell death (PCD). The vacuole accumulates several PCD-specific hydrolytic enzymes (ZEN1, ZRNas, ZCP4), enlarging the vacuole and dissolving it and releasing the enzymes into the cytoplasm, causing the autolysis of the cell organelles. Perforation of the cells walls is the final process, with all contents lost and water allowed to move freely between tracheary cells. Modified from Fukuda (2004).

Figure 4.1

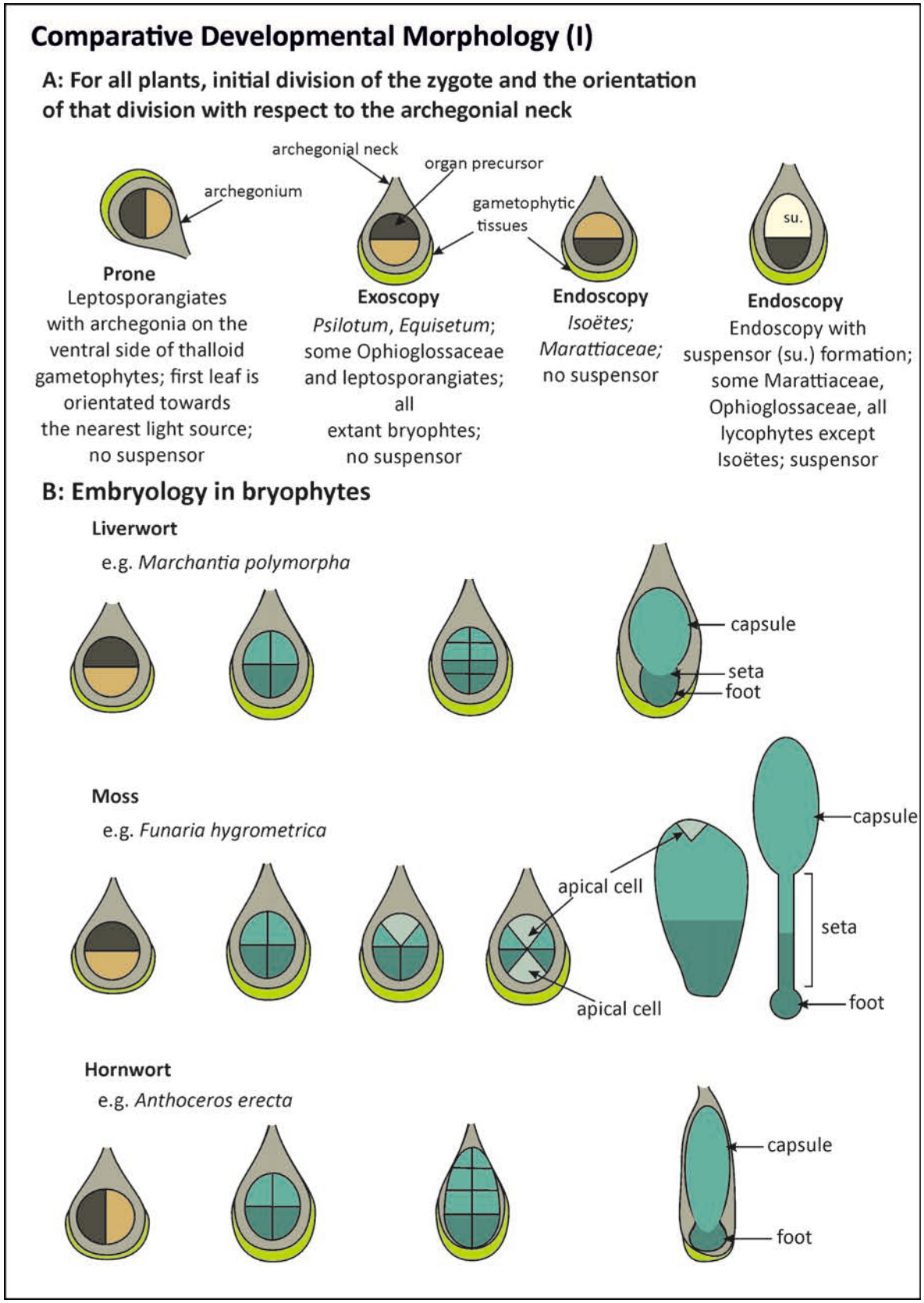

Comparative Developmental Morphology (I)
A: For all plants, initial division of the zygote and the orientation of that division with respect to the archegonial neck
archegonial neck
organ precursor
archegonium
gametophytic tissues
su.
Prone
Leptosporangiates with archegonia on the ventral side of thalloid gametophytes; first leaf is orientated towards the nearest light source; no suspensor
Exoscopy
Psilotum, Equisetum; some Ophioglossaceae and leptosporangiates; all extant bryophtes; no suspensor
Endoscopy
Isoëtes; Marattiaceae; no suspensor
Endoscopy
Endoscopy with suspensor (su.) formation; some Marattiaceae, Ophioglossaceae, all lycophytes except Isoëtes; suspensor
B: Embryology in bryophytes
Liverwort
e.g. Marchantia polymorpha
capsule
seta
foot
Moss
e.g. Funaria hygrometrica
apical cell
apical cell
capsule
seta
foot
Hornwort
e.g. Anthoceros erecta
capsule
foot

Figure 4.2

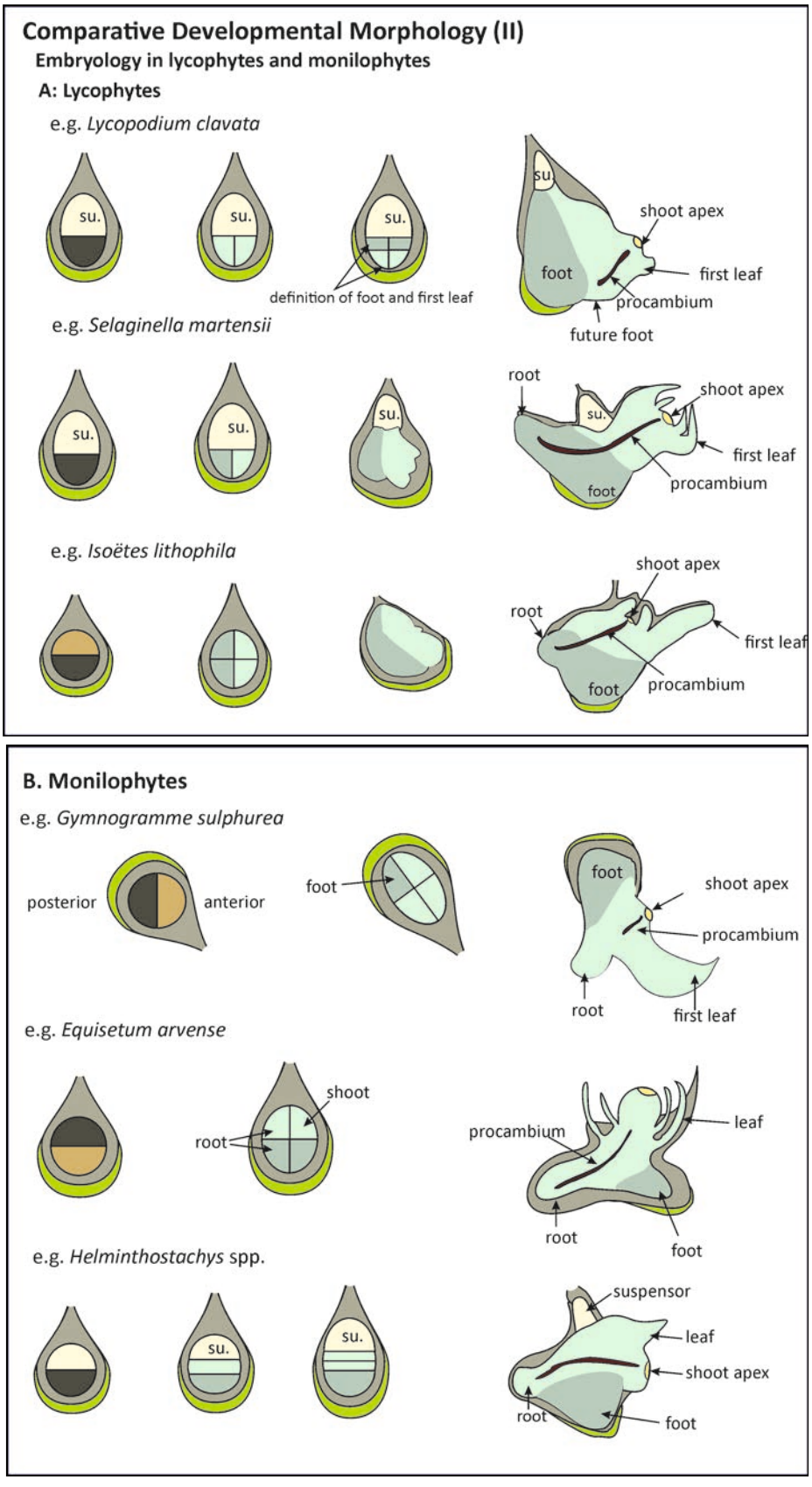

Comparative Developmental Morphology (II)
Embryology in lycophytes and monilophytes
A: Lycophytes
e.g. Lycopodium clavata
su.
su.
su.
definition of foot and first leaf
su
shoot apex
foot
first leaf
procambium
future foot
e.g. Selaginella martensii
su.
su.
su.
root
su.
shoot apex
first leaf
procambium
foot
e.g. Isoëtes lithophila
shoot apex
root
first leaf
foot
procambium
B. Monilophytes
e.g. Gymnogramme sulphurea
posterior
anterior
foot
foot
shoot apex
procambium
root
first leaf
e.g. Equisetum arvense
shoot
root
procambium
leaf
root
foot
e.g. Helminthostachys spp.
su.
su.
suspensor
leaf
shoot apex
root
foot

Figure 4.3

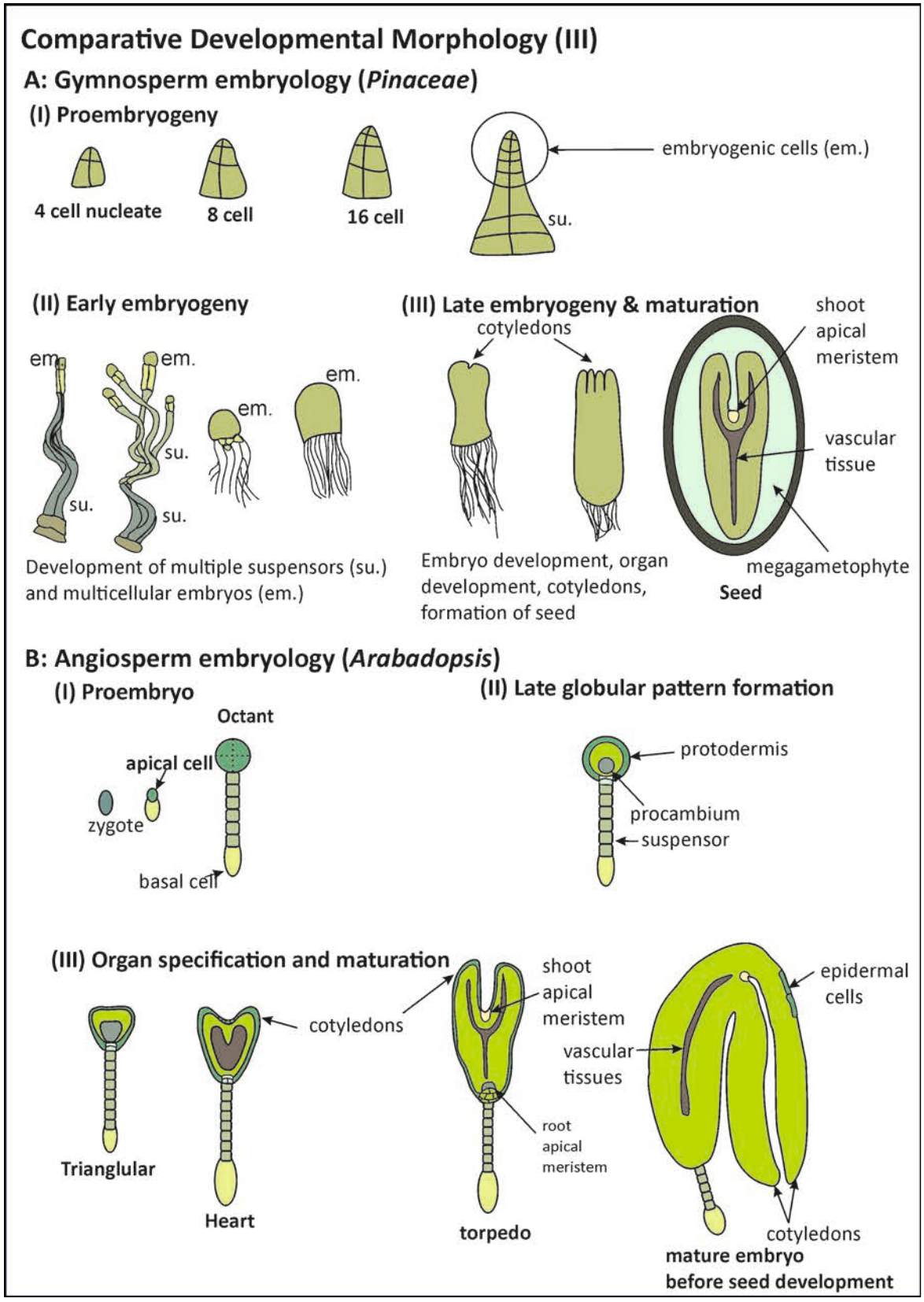
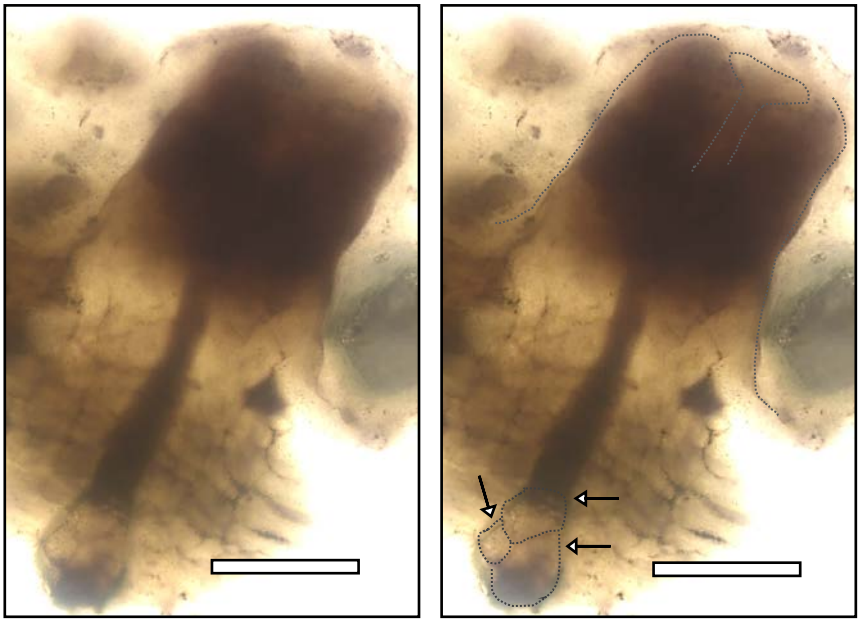

Comparative Developmental Morphology (III)
A: Gymnosperm embryology (Pinaceae)
(I) Proembryogeny
4 cell nucleate
8 cell
16 cell
embryogenic cells (em.)
su.
(II) Early embryogeny
em.
su.
Development of multiple suspensors (su.) and multicellular embryos (em.)
(III) Late embryogeny & maturation
cotyledons
Embryo development, organ development, cotyledons, formation of seed
shoot apical meristem
vascular tissue
megagametophyte
Seed
B: Angiosperm embryology (Arabadopsis)
(I) Proembryo
Octant
apical cell
zygote
basal cell
(II) Late globular pattern formation
protodermis
procambium
suspensor
(III) Organ specification and maturation
cotyledons
Triangular
Heart
shoot apical meristem
vascular tissues
root apical meristem
torpedo
epidermal cells
cotyledons
mature embryo before seed development

Figure 4.4



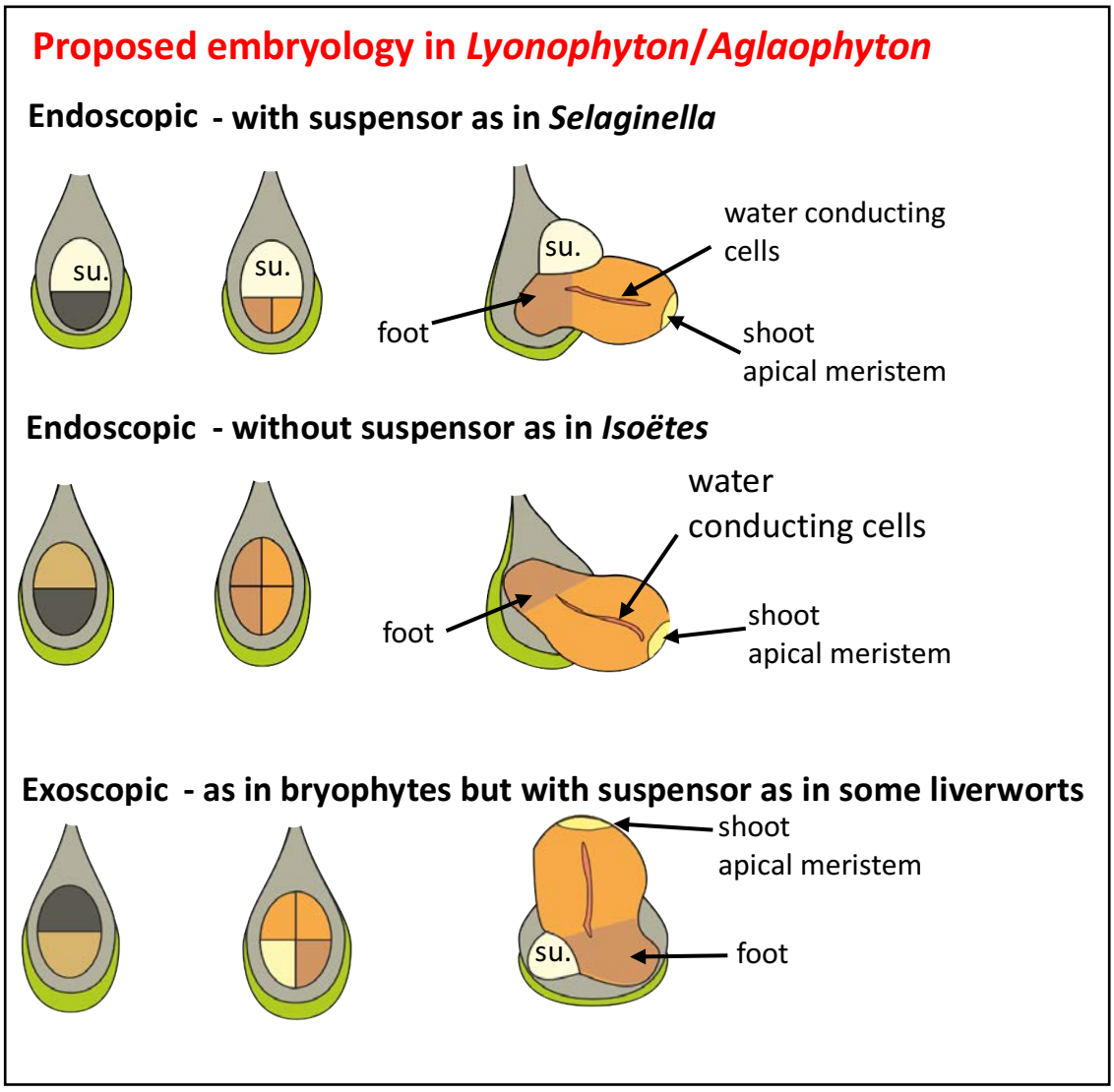

Proposed embryology in Lyonophyton/Aglaophyton
Endoscopic - with suspensor as in Selaginella
su.
su.
su.
water conducting cells
foot
shoot apical meristem
Endoscopic - without suspensor as in Isoëtes
water conducting cells
foot
shoot apical meristem
Exoscopic - as in bryophytes but with suspensor as in some liverworts
shoot apical meristem
su.
foot

Figure 4.5

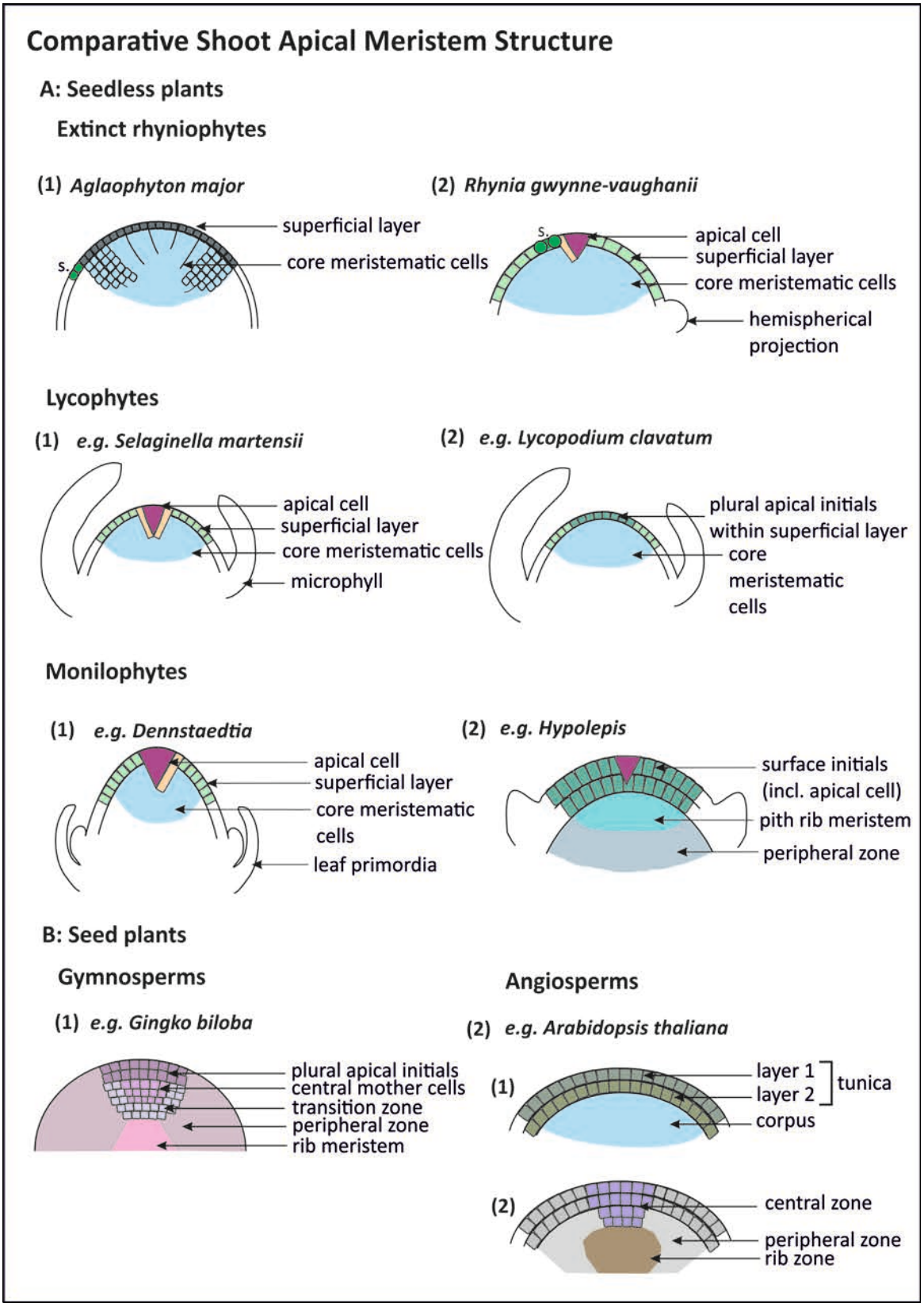

Comparative Shoot Apical Meristem Structure
A: Seedless plants
Extinct rhyniophytes
(1) Aglaophyton major
superficial layer
core meristematic cells
S.
(2) Rhynia gwynne-vaughanii
S.
apical cell
superficial layer
core meristematic cells
hemispherical projection
Lycophytes
(1) e.g. Selaginella martensii
apical cell
superficial layer
core meristematic cells
microphyll
(2) e.g. Lycopodium clavatum
plural apical initials within superficial layer
core meristematic cells
Monilophytes
(1) e.g. Dennstaedtia
apical cell
superficial layer
core meristematic cells
leaf primordia
(2) e.g. Hypolepis
surface initials (incl. apical cell)
pith rib meristem
peripheral zone
B: Seed plants
Gymnosperms
(1) e.g. Gingko biloba
plural apical initials
central mother cells
transition zone
peripheral zone
rib meristem
Angiosperms
(2) e.g. Arabidopsis thaliana
(1)
layer 1
layer 2
tunica
corpus
(2)
central zone
peripheral zone
rib zone

Figure 4.6

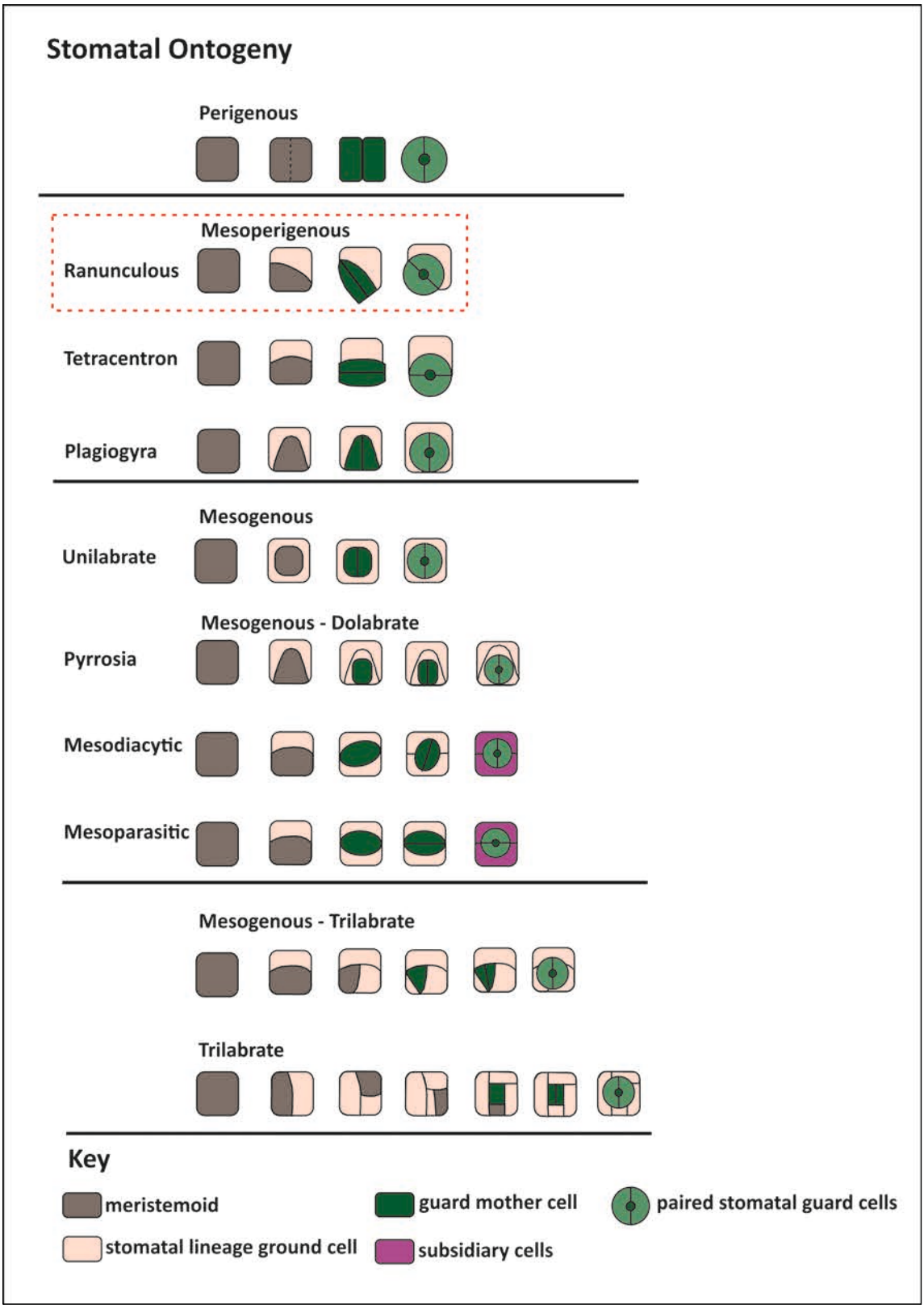

Stomatal Ontogeny
Perigenous
Mesoperigenous
Ranunculous
Tetracentron
Plagiogyra
Mesogenous
Unilabrate
Mesogenous - Dolabrate
Pyrrosia
Mesodiacytic
Mesoparasitic
Mesogenous - Trilabrate
Trilabrate
Key
meristemoid
stomatal lineage ground cell
guard mother cell
subsidiary cells
paired stomatal guard cells

Figure 4.7

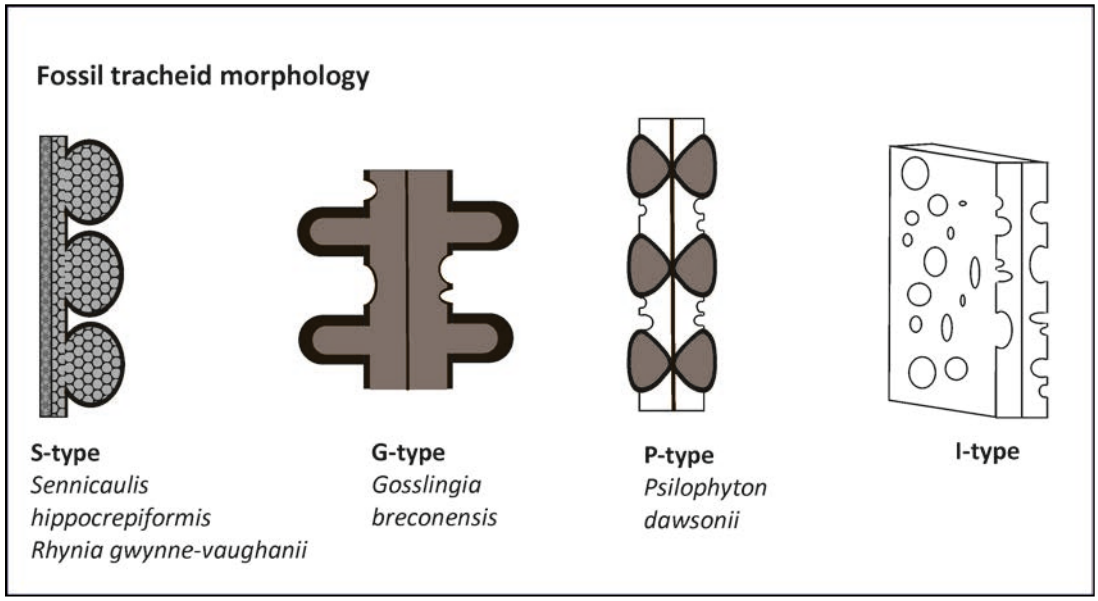

Fossil tracheid morphology
S-type
Sennicaulis
hippocrepiformis
Rhynia gwynne-vaughanii
G-type
Gosslingia
breconensis
P-type
Psilophyton
dawsonii
I-type

Figure 4.8

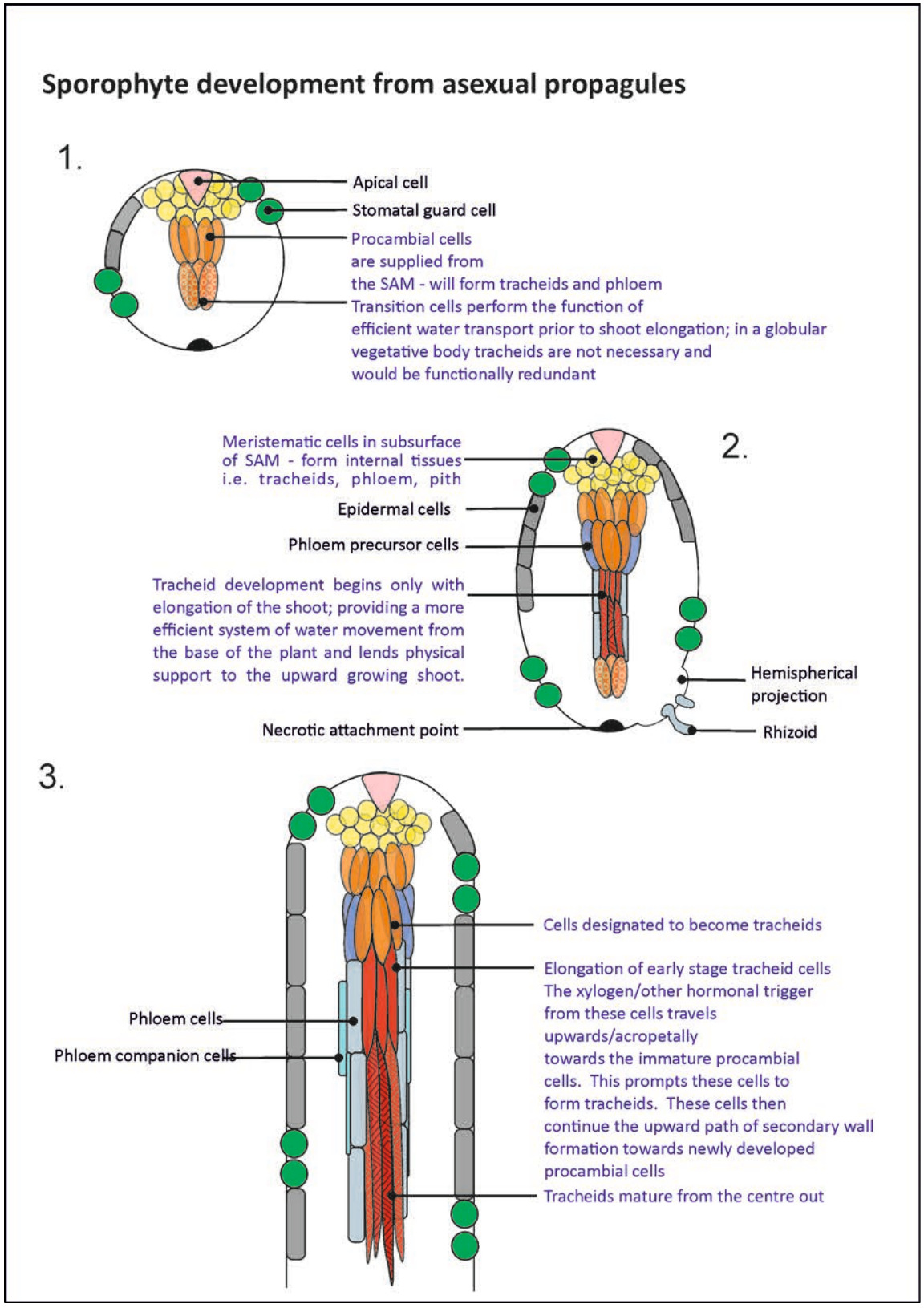

Sporophyte development from asexual propagules
1.
Apical cell
Stomatal guard cell
Procambial cells
are supplied from
the SAM - will form tracheids and phloem
Transition cells perform the function of
efficient water transport prior to shoot elongation; in a globular
vegetative body tracheids are not necessary and
would be functionally redundant
2.
Meristematic cells in subsurface
of SAM - form internal tissues
i.e. tracheids, phloem, pith
Epidermal cells
Phloem precursor cells
Tracheid development begins only with
elongation of the shoot; providing a more
efficient system of water movement from
the base of the plant and lends physical
support to the upward growing shoot.
Hemispherical
projection
Necrotic attachment point
Rhizoid
3.
Cells designated to become tracheids
Elongation of early stage tracheid cells
The xylogen/other hormonal trigger
from these cells travels
upwards/acropetally
towards the immature procambial
cells. This prompts these cells to
form tracheids. These cells then
continue the upward path of secondary wall
formation towards newly developed
procambial cells
Tracheids mature from the centre out
Phloem cells
Phloem companion cells

Figure 4.9

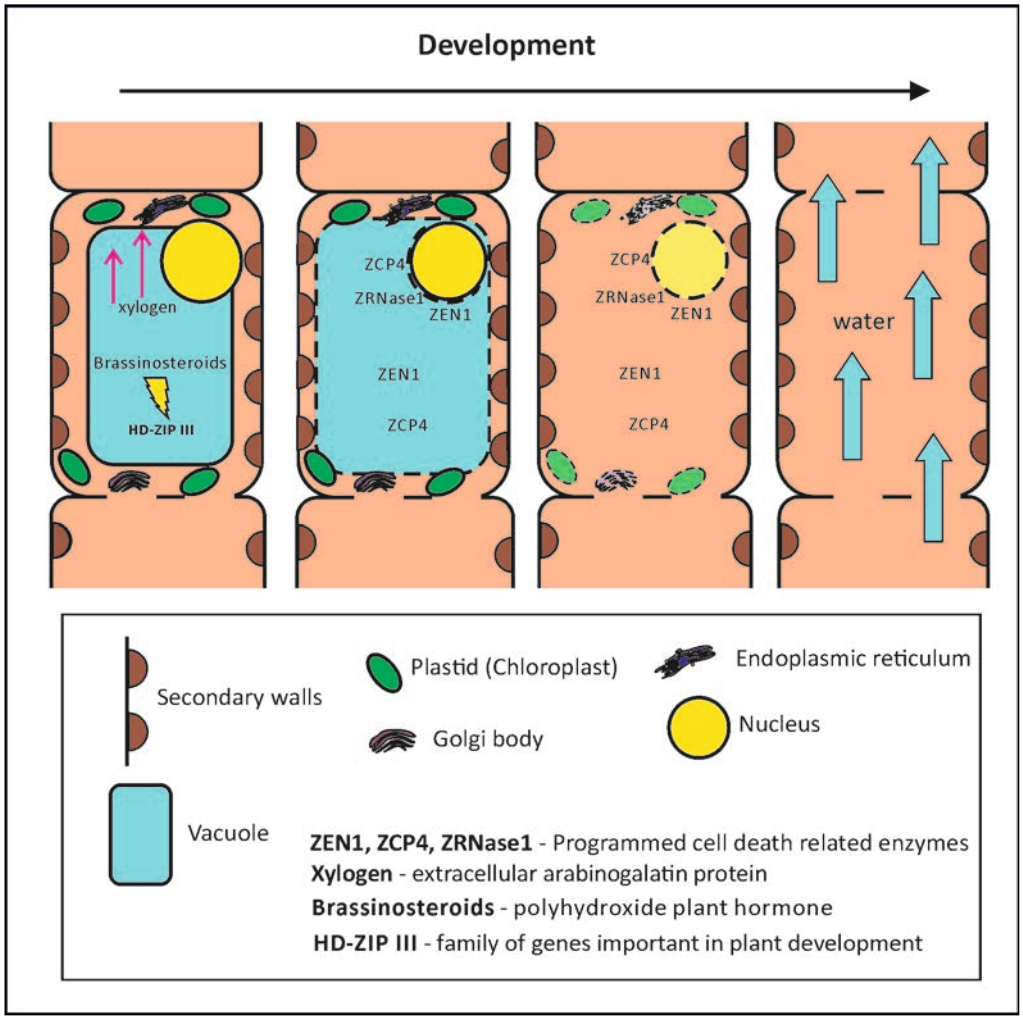

Development
xylogen
Brassinosteroids
HD-ZIP III
ZCP4
ZRNase1
ZEN1
ZEN1
ZCP4
ZCP4
ZRNase1
ZEN1
ZEN1
ZCP4
water
Secondary walls
Plastid (Chloroplast)
Endoplasmic reticulum
Golgi body
Nucleus
Vacuole
ZEN1, ZCP4, ZRNase1 - Programmed cell death related enzymes
Xylogen - extracellular arabinogalatin protein
Brassinosteroids - polyhydroxide plant hormone
HD-ZIP III - family of genes important in plant development

Table 4.1

Genes and function	Encoding genes and function
Class III HD Zip Genes leaf polarity; initiation and maintenance of SAM, axillary SAMs, patterning and development of vascular tissues, adaxial identification and growth of leaves and other leaf-derived organs in *Arabidopsis* thaliana	*Arabidopsis thaliana*: AtHB8, CORONA/AtHB15,PHABULOSA (PHB), PHAVULOTA (PHV), REVOLUTA (REV)
KANADI Genes Promotes abaxial cell fate in leaves, lateral organ, integument development; vascular bundles (phloem). Mutually antagonistic activity with Class II HD-Zip genes (gain of function alleles of Class II HD-Zip genes resemble phenotypically the loss of function alleles of KANADI and vice versa)	*Arabidopsis thaliana*: 50 members but just KAN1, KAN2, KAN3, KAN4 promote abaxial fate.
Class I KNOX Genes Meristem formation/maintenance, internode elongation, pedicel development, positioning of lateral organ primordia, regulate and regulated by phytohormones, promoting cytokinin synthesis and down regulation of gibberellic acid to promote meristematic activity	*Arabidopsis thaliana*: SHOOT MERISTEMLESS (STM), KNOTTED-1 (KN1) expressed in shoot meristem. BREVIPEDCELUS (BP) in *Arabidopsis* and OSH15 in rice Present in conifers, monilophytes, *Selaginella*, *Physcomitrella*
MADS-box Genes Reproductive organ development	46 MIKC MADS-box genes, 38 MIKC^{c}, PETALA1, APETALA3, PISTILLATA, AGAMOUS, SEPALLATA; 8 MIKC*
APETALA2 Genes Stem cell maintenance in the SAM, seed coat development, seed size, flowering time, cell division in lateral organs	APETALA2 (APT2), AINTEGUMENTA (ANT)
LEAFY, YABBA, TCP, GRAS	**LEAFY**: Highly conserved from mosses to angiosperms, specification of floral meristem, vegetative to reproductive transition, but mosses LEAFY is an orthologue rather than a homolog. **YABBA**: differentiation of abaxial tissue, promote lamina growth, down-regulation of some Class I KNOX genes. Not detected in *Selaginella* or *Physcomitrella*. Possible role in megaphyll evolution. **TCP**: leaf development, shape, repression of cell division or meristem activity. **GRAS**: SAM development, root patterning, response to gibberillic acid in angiosperms, HAIRY MERISTEM (HAM); SHORT ROOT (SHR) and SCARECROW (SCR); DELLA; LATERAL SUPRESSOR (LS).
Auxin-related Genes Auxin: patterning of angiosperm tissue, apical-basal polarity, root meristem establishment, vascular tissue patterning, control of apical dominance	**PIN-FORMED (PIN**) auxin transporters, *Selaginella*, *Arabidopsis*. PIN-like in *Physcomitrella*.
CUC, NAC, WUS, CLV, DVL/ROT4	**CUC(cup–shaped cotyledons)**: organ separation, meristem development, **NAC Family = NAM (no apical meristem) + ATAF *Arabidopsis* transcription activation) + CUC**: plant specific, uncertain functions, possibly SAM development, flowers, lateral shoots, PCD. **WUS**: maintenance of central cell zone of the SAM (*Arabidopsis*) by repressing **CLAVATA (CLV)** signalling, encodes for WOX class of genes **DVL/ROT4**: DEVIL/ROTUNDIFOLIA; role generally unknown, possibly leaf development.

Chapter 5

Conclusions, critique and future work

5.0 Conclusions and critique

The primary objective of the studies described in this volume was to establish the developmental morphology of *Rhynia gwynne-vaughanii*, one of the earliest and best preserved vascular land plants in the fossil record. Ultimately it was possible to attempt this type of research because of the unique preservation quality of the Rhynie chert and the large quantity of *Rhynia gwynne-vaughanii* available. The overall development of *Rhynia gwynne-vaughanii* bears more of a resemblance to that of the lycophytes than the bryophytes, although basic development, consisting of a globular phase, followed by an extended period of shoot elongation, is reminiscent of sporophyte development in the bryophytes. However, the same could be said for the lycophytes and the monilophytes, which also have a short globular stage where the specialised organs (foot, first root, shoot and first leaf) are defined within the embryo. Further development involves shoot elongation and the further formation of leaves and roots. The principle difference between *Rhynia gwynne-vaughanii* and the extant members of the early divergent groups both extant is the development of specialised organs such as enations, leaves, and roots.

In *Rhynia gwynne-vaughanii* the developmental patterns of stomata, rhizoids and tracheids fit within established classification schemes for the same in extant land plants. This indicates that the developmental pathways governing their formation were set down at a much earlier stage in plant evolution, as is evidenced, with the exception of tracheids, by their occurrence in the bryophytes. The formation of tracheids follows a developmental pathway set down within at least the non-

vascular protracheophyte lineage, with the formation of lignified tracheids the defining feature within the vascular plants, where water transport is concerned.

By controlling shoot development, the evolution of the shoot apical meristem (SAM) undoubtedly represents a major innovation in land plant morphological development. The SAM of *Rhynia gwynne-vaughanii* does not resemble that of *Aglaophyton major*, and is histologically more defined, with a confirmed single apical initial cell. The structural modification of the SAM within the vascular land plant lineage is likely the main factor affecting the difference in morphological complexity seen between *Rhynia gwynne-vaughanii* and *Aglaophyton major*, two plants which for much of the history of Rhynie chert study were considered so similar that they shared a genus.

Aglaophyton major and *Rhynia gwynne-vaughanii* are representative of the trend toward increased morphological complexity at a very early point in the radiation of land plants, driven primarily by an increase in SAM organisation, complexity and structure. As part of this increase in SAM organisation and complexity, the hemispherical projections on the shoot of *Rhynia gwynne-vaughanii*, which developed as part of the overall development of the sporophyte shoot, represent the initial evolution of specialised lateral structures in land plants. The evolution of complex land plant morphology and physiology has been directed towards becoming increasingly better adapted to the terrestrial environment. Competition for light, space, water and nutrients probably played a large role in the evolution of variation in land plant biology. These studies do not affect the established phylogenetic relationships among the earliest land plants, however the divergence of the vascular land plant groups is probably much deeper than generally appreciated. Plant fossils such as *Asteroxylon mackiei* and *Baragwanathia longifolia* (Hueber 1983) are evidence of stem lycophytes by the late Silurian and Early Devonian, and plants with the level of organisation seen in *Rhynia gwynne-vaughanii* are probably representative of the initial radiation of vascular plants in perhaps the early-middle Silurian.

5.1 SXRTM and the Rhynie chert

As these studies performed the first extensive testing of SXRTM on Rhynie chert material, it is necessary to make an overall assessment of the usefulness of this technique and the possibility for future similar work. Although SXRTM allowed for the reconstruction of specialised tissues and cells, overall the results from SXRTM were mixed. Poor preservation and strong banded pattern of the silica contributed to many specimens providing almost no usable data. With traditional thin sections, all of these factors can cause problems but do not necessarily impact the usable data that can be extracted. Where SXRTM succeeded was when specific questions regarding morphology were asked, i.e. in this study, what was the structure of the SAM, did it have a single apical cell, or

multiple, and if just a single then what was its three dimensional shape? Since the morphology of the specialised tissues on the *Rhynia gwynne-vaughanii* shoot are already well documented and described, future questions regarding function of these specialised structures may be answerable using SXRTM, e.g. calculating flow rates through the tracheids. For the foreseeable future traditional thin-sectioning will be the primary method of Rhynie chert study and even where SXRTM may prove useful it is in any case necessary to make thin sections beforehand.

5.2 The Rhynie chert and role in future work on early land plant evolution

Whereas some questions regarding early land plant development have been answered in this volume, the results here only scratch the surface. Rhynie chert plants such as *Aglaophyton major*, *Nothia aphylla*, and *Asteroxylon mackiei* all offer excellent potential for similar future studies. Each of these plants represents a separate plant group spanning the evolution of the polysporangiates and vascular land plants. Although thin sectioning will be quite possible for these plants, when conducting research on the Rhynie chert a certain amount of luck can dictate whether the specimens chosen will provide the data needed. Serial thin sectioning, as was carried out here for *Rhynia gwynne-vaughanii*, offers the best chance of extracting useful data from specimens chosen for study. In addition, other Rhynie chert plants do not occur in as large a quantity as *Rhynia gwynne-vaughanii*, and selection of specimens will need to be done with great care. A further issue is the preparation of samples, other than *Rhynia gwynne-vaughanii*, for scanning using SXRTM. For studies using SXRTM it is necessary to prepare new specimens, in relatively thick slides, preserving as much of the three-dimensional structure as possible. While the simple morphology of *Aglaophyton major* may allow for it to be prepared in a similar manner to *Rhynia gwynne-vaughanii*, both *Asteroxylon mackiei* and *Nothia aphylla* have lateral organs, i.e. enations and sporangia, which could make preparation more difficult. All of these plants are also larger than *Rhynia gwynne-vaughanii* meaning larger samples, which will need to be scanned in multiple sessions, as was done here for specimen RTK4.

No matter how essential the Rhynie chert is shown to be regarding our understanding of the biology, physiology and development of early land plants, it is important to realise that the Rhynie chert represents just a single ecosystem and six plant species (the Windyfield chert providing one extra species). We cannot rely too heavily on these species of early land plant to close major gaps in our understanding of early land plant evolution; the accumulation of data across several lines of evidence from all fields of plant research is necessary. This is especially important where broad questions regarding origins of land plants are concerned. From a palaeobiological point of view the origin of land plants is relatively sudden within the fossil record and even though some molecular

data supports a much earlier origin (Clarke *et al.* 2011) more are more consistant with data from the fossil record. A Neoproterozoic origin for land plants, if accurate (Clarke *et al.* 2011), would require a better understanding of the palaeoenvironmental and palaeoclimatic factors in operation during the Precambrian to Ordovician that may have impacted and delayed the radiation of land plants, e.g. 'Snowball Earth' 750-580 Ma, (Harland 1964; Kirschvink 1992; Hoffman *et al.* 1998; Allen and Etienne 2008); fragmentation of the supercontinent Rodinia during the mid-Proterozoic (750 Ma); further glaciation events at the end of the Ordovician, including lowering of CO_2 levels and an expanding ice sheet over Gondwana (Raup and Sepkowski 1982, 1984; Hoffman 1999; Schrag *et al.* 2002; Lewis *et al.* 2004; Donnadieu *et al.* 2004; Finnegan *et al.* 2011). It is necessary to accept that the early land plant fossils we have presently represent a radiation event, and are primarily useful to understand the evolution of form and function within land plants rather than deeper questions about plant origins.

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Appendix

Synchrotron tomographic microscopy

***Rhynia gwynne-vaughanii* specimens that returned poor results**

Table of specimens with synchrotron scanning settings

***Aglaophyton major* apices**

RTK6 and RTK 6b (Figure 1–3)

Partial hemispherical projection with rhizoids. In RTK 6 resolution of the cells is very good but no new data regarding rhizoid development or morphology was apparent. RTK 6b had comparatively poorer cell resolution.

RTK 7 (Figure 4)

Incomplete propagule, apex missing, banded silica mineralogy shows strong resolution obscuring cells.

RTK 9 (Figure 5)

Full globular propagule, but with broad patches of cell decay. Poor resolution of the cells in isosurface.

RTK 11 (Figure 6–8)

Globular propagule with on exposed edge, the remainder of the propagule is embedded in the chert. Exposed section returned excellent detail in isosurface, however internally the resolution of the cells is poor with cell walls only faintly visible in some cases and in other case not visible at all.

RTK 12 (Figure 9 and 10)

Globular propagule with very poor preservation quality and strong mineralogy patterning. The propagule is characterised by several internal spaces.

RTK 13 (Figure 11and 12)

Globular propagule with slight elongation at the apical end. Poor preservation quality, seen in both the tomographic slices and in isosurface returning low resolution of cells. Edges of the propagule show some damage.

RTK 14 (Figure 13 and 14)

One globular propagule and one fragmentary plant axis, but no apical areas. Preservation relatively good but cell resolution in tomographic sections poor. Some cell walls and basic shape of propagule visible in isosurface.

RTK 16 (Figure 15 and 16)

Partial globular propagule with large patch of cell decay. Cell resolution relatively poor in tomographic sections and cell walls poorly rendered in isosurface.

RTK 19 (Figure 17 and 18)

Isolated *Rhynia gwynne-vaughanii* axis with intact apex. Figure 18 shows the apex prepared in thin section and ground down to show the poor resolution of the cells in the apical area, as well as damage and decay.

RTK 20 (Figure 19)

Poorly preserved globular propagule, with poor internal resolution of cells. Identified apical area shows extensive damage.

RTK21 (Figure 20 and 21)

Well-preserved globular propagule but with poor resolution of cells in tomographic sections.

Figures 22, 23, 24

Propagules embedded in chert. Scanned for SRXTM. Scales 100 μm.

Figure 25

Apex of *Aglaophyton major*, with dichotomy. Slide 2234. Scale 1 mm.

Figure 26

Surface of apex of *Aglaophyton major*. Slide Scale1993. 250 μm.

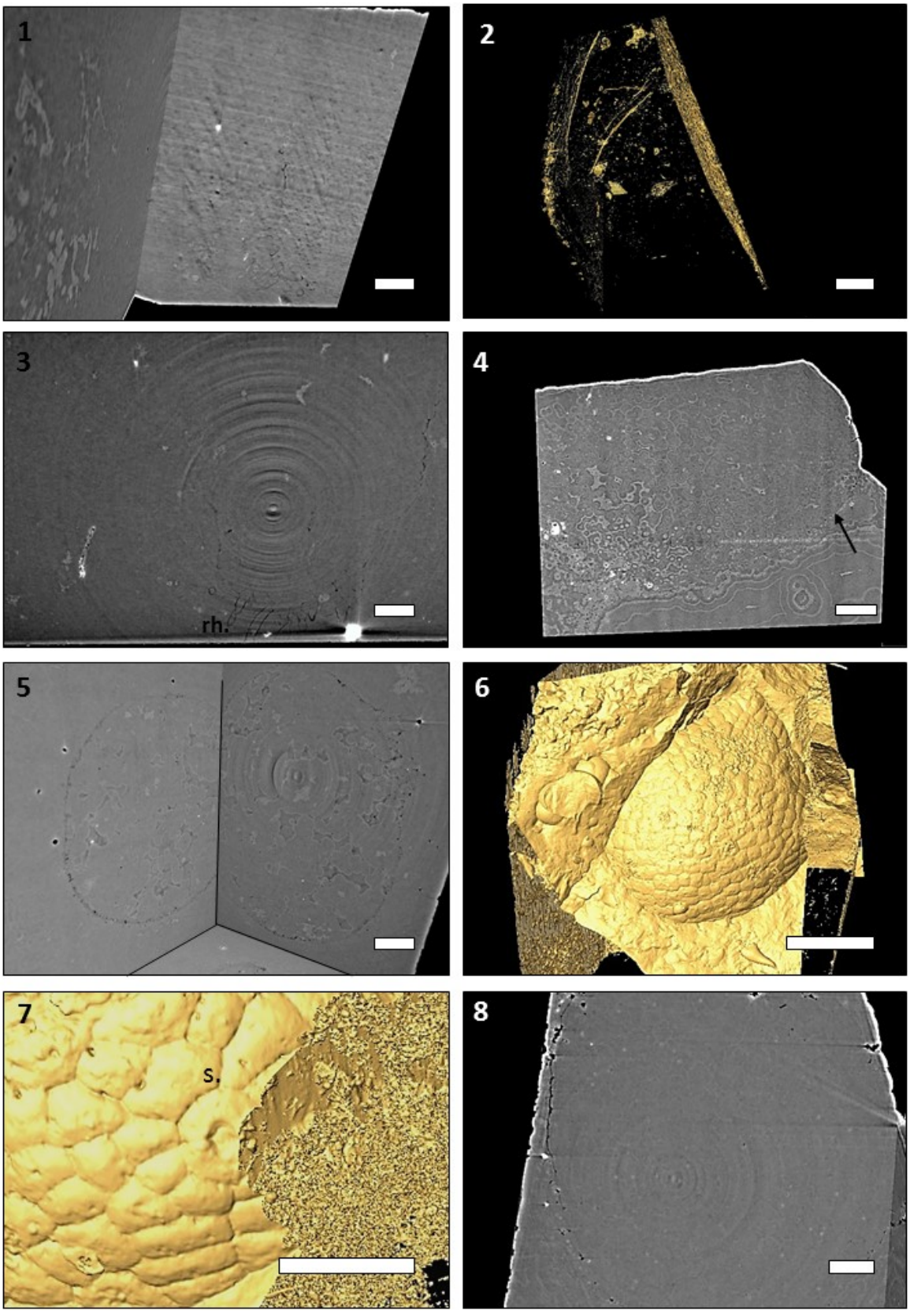
Figure 27

Two shoots of *Aglaophyton major*. Slide 2084. Scale 1 mm.

Figure 28

Palaeoblasocladia milleri fungi found commonly on the axes of *Aglaophyton major*.

Slide 2005. Scale 250 μm.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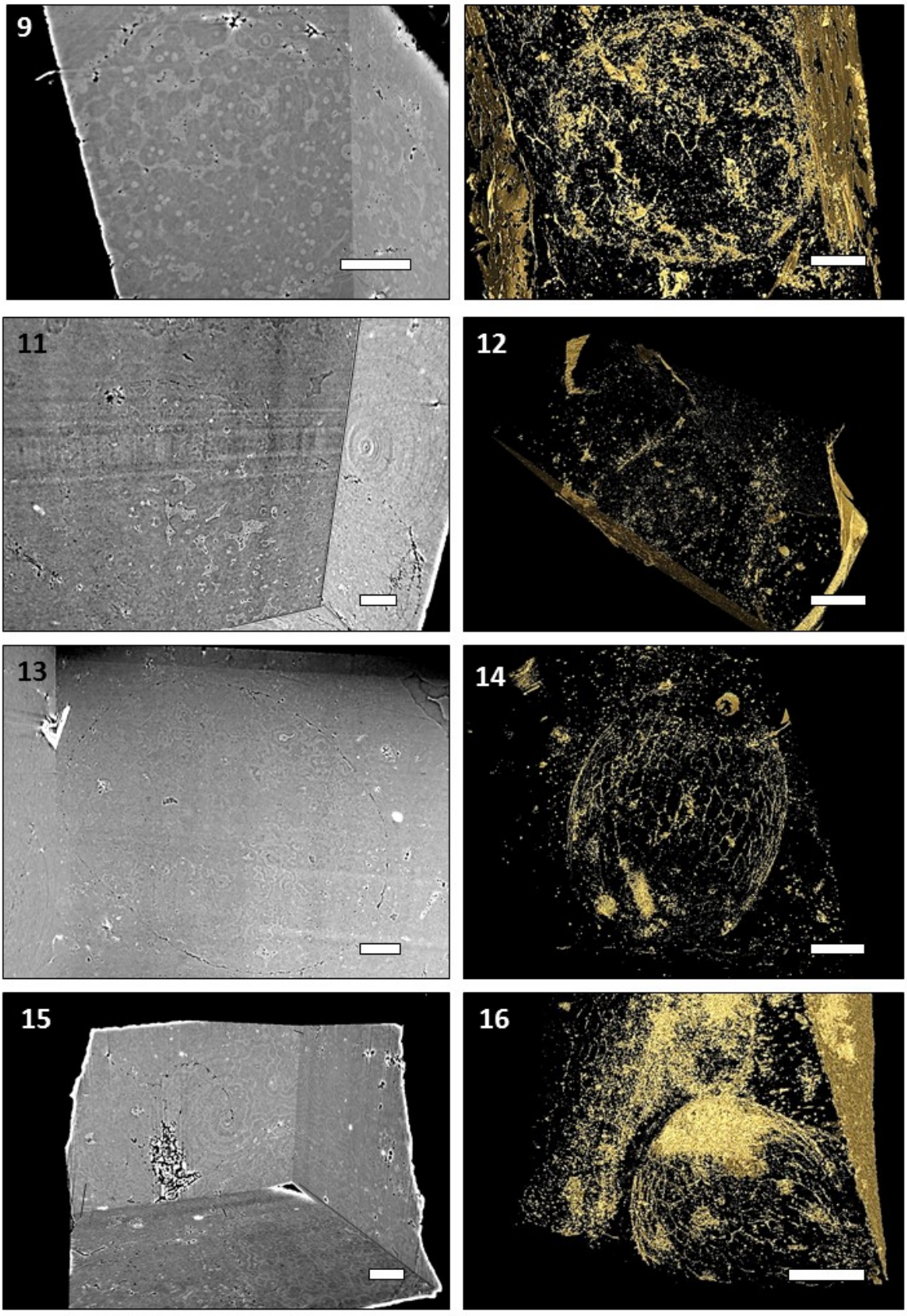
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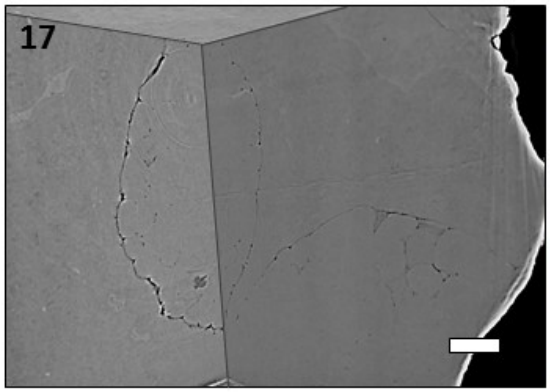
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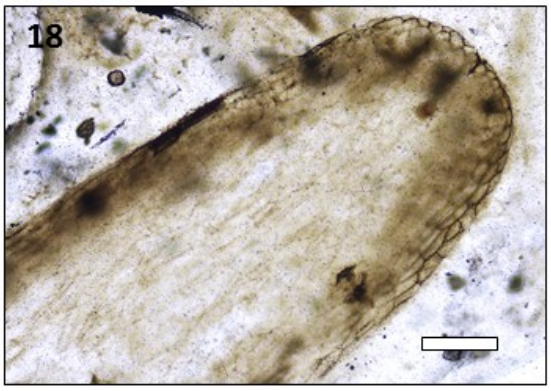
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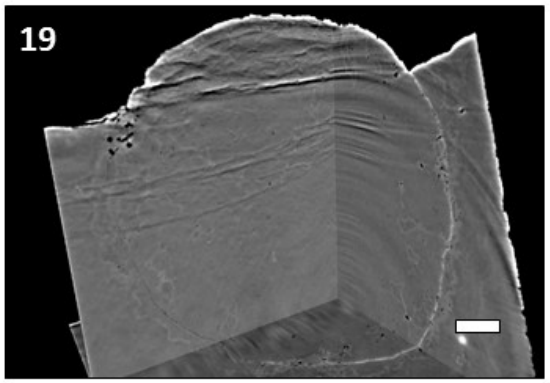
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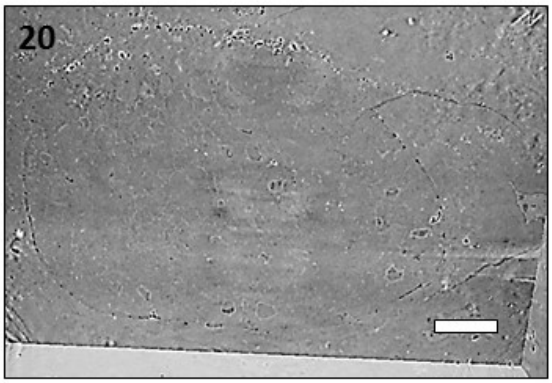
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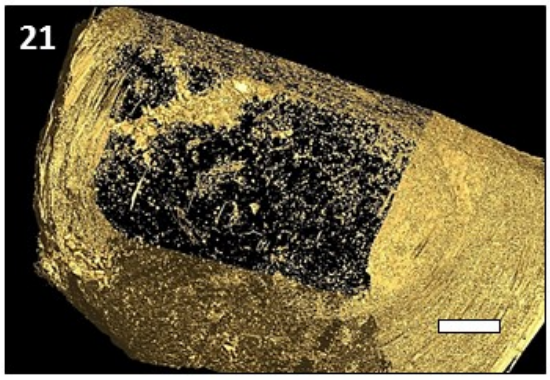
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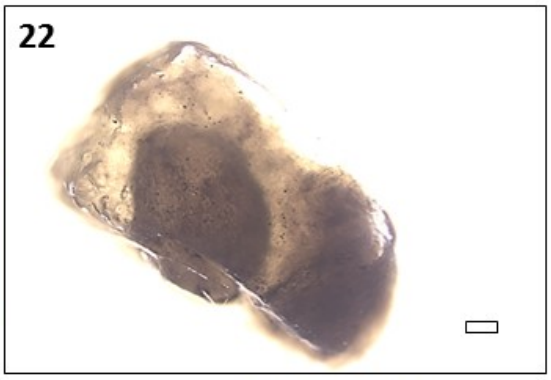
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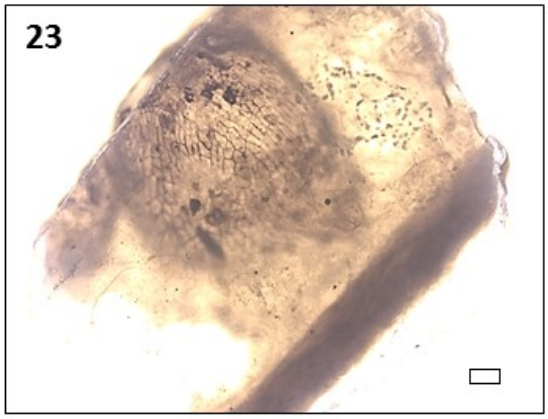
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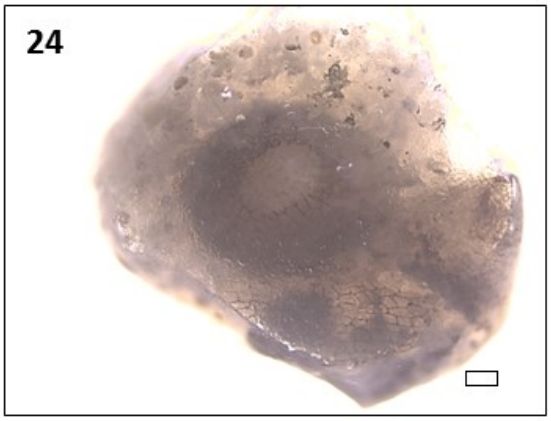
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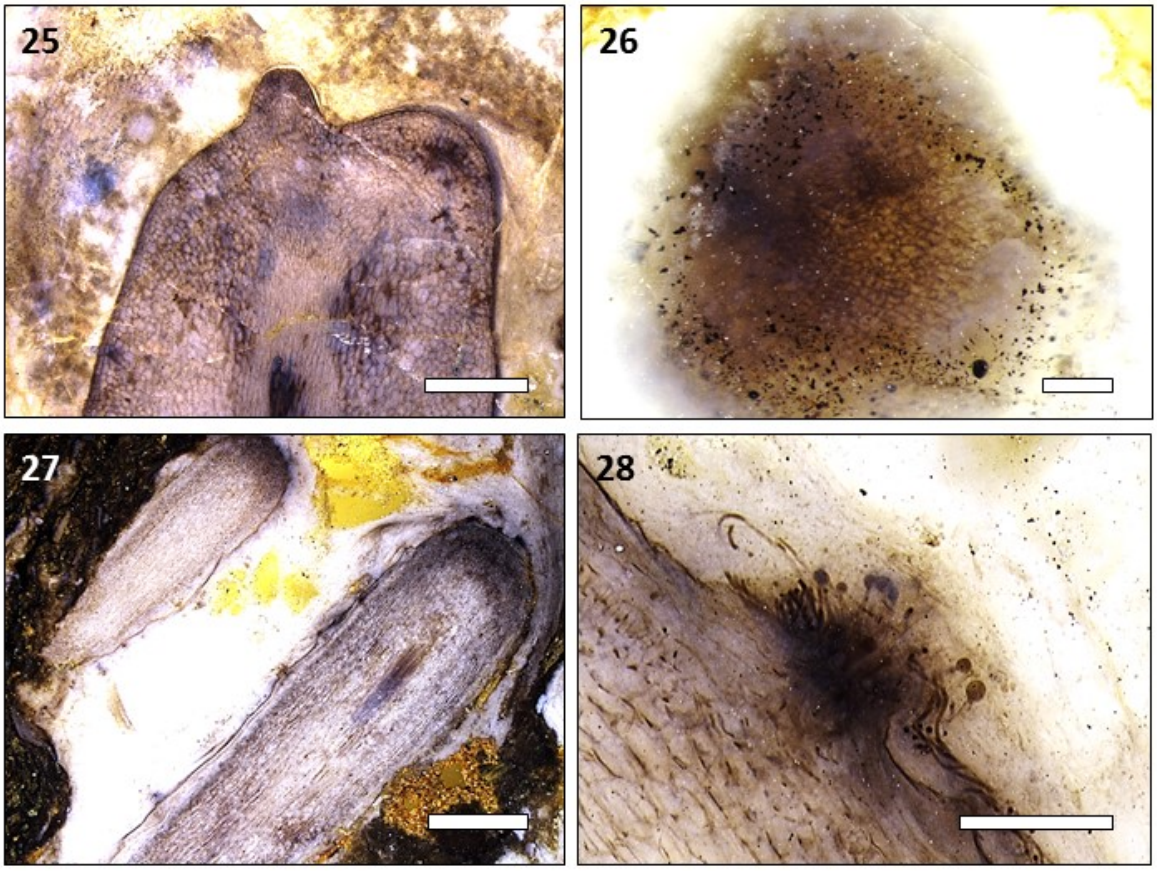
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Specimen/File Number	Slide Number	Specimen Description	Sample Number	keV	Projections	Exposure	Scintillator	Objective	Comments
RTK 1	Rhynie chert, plant <1mm	Test	-	20	1501	-	LuAG-20um	x10	OK
RTK 2	Rhynie chert, plant <1mm	Test	-	20	1501	-	LuAG-20um	x10	-
RTK 3	-	*Rhynia* complete, but isolated apex	-	20	1501	83	LuAG-20um	x10	OK phase contrast
RTK 4	-	*Rhynia*, vascular tissue with projections, propagule	-	20	1501	83	LuAG-20um	x10	OK phase contrast
RTK 4B	-	*Rhynia*, bottom part of RTK 4 vascular	-	20	1501	83	LuAG-20um	x10	OK phase contrast
RTK 5	-	*Rhynia*, apical growth on propagule	-	20	1501	83	LuAG-20um	x10	OK phase contrast
RTK 6_	RA 14.2	*Rhynia* 1. slide 14.2 propagule vasc	-	20	1501	120	LuAG-20um	x10	phase, 30 mm dist. Different part of RTK6_
RTK 6b	RA 14.2	*Rhynia* 1. slide 14.2 propagule vasc	-	20	1501	120	LuAG-20um	x10	phase, 30 mm dist.
RTK 7_	RA 2	*Rhynia* 2.slide 2.11 propagule, apical growth	-	20	1501	120	LuAG-20um	x10	phase, 30 mm dist.
RTK 8_	RA 9.5	Propagule with apex	-	20	1501	120	LuAG-20um	x10	phase, 30 mm dist.
RTK 86a	RA 9.5	Propagule with apex	-	15	1503	100	LuAG-20um	x20	OK
RTK 86b	RA 9.5	Propagule with apex	-	15	1503	100	LuAG-20um	x20	OK
RTK 8t	RA 9.5	Propagule with apex	-	25	1201	1000	Lag:Ce 20 mu	x20	no rec/experiment in water, not fine
RTK 8t_b	RA 9.5	Propagule with apex	-	25	1201	1000	Lag:Ce 20 mu	x20	no rec/experiment in water, not fine
RTK 8t_c	RA 9.5	Propagule with apex	-	-	-	-	-	-	-

Specimen/File Number	Slide Number	Specimen Description	Sample Number	keV	Projections	Exposure	Scintillator	Objective	Comments
RTK8t_d	RA 9.5	Propagule with apex	-	-	-	-	-	-	-
RTK 8u_1_	RA 9.10	Propagule with apex	-	15	1501	200	Lag:Ce 20 mu	x20	not reconstructed
RTK 8u_2_	RA 9.11	Propagule with apex	-	15	1501	200	Lag:Ce 20 mu	x20	OK 15mm distance to scin
RTK 8xaFusion	-	Propagule with apex	-		1501	200	Lag:Ce 20 mu	x20	OK
RTK 9_	RA22.5(i)	Growing propagules	-	20	1501	120	LuAG-20um	x10	OK phase, 30 mm dist.
RTK 10_	RA22.5(i)	Growing propagules	-	20	1501	120	LuAG-20um	x10	OK phase, 30 mm dist.
RTK 11_	RA3a.4	Propagule	-	20	1501	120	LuAG-20um	x10	OK phase, 30 mm dist.
RTK 11b_	RA3a.5	offset	-	20	1501	120	LuAG-20um	x10	OK phase, 30 mm dist.
RTK 12(a)	RA3a.2	Three propagules/centre propagule (full)/others partial	1	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 13(a)	RA3b.1	Two full propagules, parts exposed but apex embedded in chert	2	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 14(a)	RA22.2	Full propagule with other plant material - *Rhynia* axis	3	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 15(a)	RA20.1	Apex mature, apex embedded in matrix, rest exposed	4	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 16(a)	RA? Slide Unknown	Full propagule, small bit missing	5	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 17(a)	RA18.0	Germinating spore	6	21	1501	190	Lag:Ce 20 mu	x10	OK
RTK 17a		*Rhynia* apex 1		20	1501	230	LAG:Ce 20um	x10	not reconstructed

Specimen/File Number	Slide Number	Specimen Description	Sample Number	keV	Projections	Exposure	Scintillator	Objective	Comments
RTK 17b	-	*Rhynia* apex 1	-	20	1501	230	LAG:Ce 20um	x10	OK phase
RTK 18a	-	*Rhynia* mature apex 3	-	20	1501	230	LAG:Ce 20um	x10	OK phase
RTK 19a	-	*Rhynia* mature apex 5	-	20	1501	230	LAG:Ce 20um	x10	OK phase
RTK 19b	-	*Rhynia* mature apex 5	-	20	1501	230	LAG:Ce 20um	x10	phase, Federica will reconstruct
RTK 20a	-	*Rhynia* globular propagule 4	-	20	1501	230	LAG:Ce 20um	x10	OK phase
RTK 21a	-	*Rhynia* globular propagule 6	-	20	1501	230	LAG:Ce 20um	x10	OK phase