Current Biology

CellPress

Review

The origin and evolution of stomata

James W. Clark[1,4,*], Brogan J. Harris[1,4], Alexander J. Hetherington[2], Natalia Hurtado-Castano[3], Robert A. Brench[3], Stuart Casson[3], Tom A. Williams[1], Julie E. Gray[3], and Alistair M. Hetherington[1]

[1]School of Biological Sciences, University of Bristol, Life Sciences Building, Tyndall Avenue, Bristol BS8 1TQ, UK
[2]Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Max Born Crescent, Edinburgh EH9 3BF, UK
[3]Plants, Photosynthesis and Soils, School of Biosciences, University of Sheffield, Sheffield S10 2TN, UK
[4]These authors contributed equally
*Correspondence: james.clark@bristol.ac.uk
https://doi.org/10.1016/j.cub.2022.04.040

SUMMARY

The acquisition of stomata is one of the key innovations that led to the colonisation of the terrestrial environment by the earliest land plants. However, our understanding of the origin, evolution and the ancestral function of stomata is incomplete. Phylogenomic analyses indicate that, firstly, stomata are ancient structures, present in the common ancestor of land plants, prior to the divergence of bryophytes and tracheophytes and, secondly, there has been reductive stomatal evolution, especially in the bryophytes (with complete loss in the liverworts). From a review of the evidence, we conclude that the capacity of stomata to open and close in response to signals such as ABA, CO_2 and light (hydroactive movement) is an ancestral state, is present in all lineages and likely predates the divergence of the bryophytes and tracheophytes. We reject the hypothesis that hydroactive movement was acquired with the emergence of the gymnosperms. We also conclude that the role of stomata in the earliest land plants was to optimise carbon gain per unit water loss. There remain many other unanswered questions concerning the evolution and especially the origin of stomata. To address these questions, it will be necessary to: find more fossils representing the earliest land plants, revisit the existing early land plant fossil record in the light of novel phylogenomic hypotheses and carry out more functional studies that include both tracheophytes and bryophytes.

Introduction

Stomata are pores bordered by guard cells on the epidermal surfaces of almost all extant land plants (embryophytes). They are present in the vascular plants and two of the three lineages of bryophytes (mosses and hornworts; Box 1)[1]. Extant liverworts (the third lineage of bryophytes) lack stomata, although this is believed to reflect a loss of these structures during evolution[2]. Similarly, while most extant mosses and hornworts possess stomata, there are examples of where they have been lost during evolution[3,4]. Stomata function as microscopic, valve-like structures which, through opening and closing, regulate the loss of water vapour from, and the uptake of CO_2 into, the leaf[5–7]. The acquisition of stomata — together with a waxy cuticle, sub-stomatal air spaces and an internal system for moving water and nutrients, from their sites of uptake, throughout the plant — are key steps that allowed early plants to adapt to, and thereby spread through, ancient terrestrial environments[4,8].

To understand why stomata are among the key innovations that facilitated the radiation and success of the early terrestrial flora, it is helpful to consider the roles they play in living species. Most studies have focussed on the function of stomata in angiosperms, and this in turn has coloured our understanding of the roles these structures play in the earliest plants. Except for the submerged leaves of aquatic angiosperms, which lack or have a greatly reduced cuticle[9,10], the presence of the cuticle renders the leaf surface largely impermeable to CO_2. This means that stomata are the predominant sites of CO_2 uptake. Stomata also control the loss of water vapour from the plant to the atmosphere. This process, called evapotranspiration, provides the driving force for the uptake and subsequent movement of water and mineral nutrients throughout the plant, and affords the plant limited cooling capacity. Together, evapotranspiration and the uptake of CO_2 are referred to as 'leaf gas exchange'. Puncturing the epidermis with pores provides an opportunity for excessive water loss and for pathogens to gain access to the plant body. To counter this, plants developed strategies — including stomatal closure — to reduce the chances of desiccation or infection[11,12]. In addition, stomata have a specialised role in some mosses, where they are localised to a reproductive structure known as the capsule located on the sporophyte. To the best of our knowledge, the first to speculate on the function of stomata in mosses was the English botanist William Valentine in 1838. He concluded that their function was associated with the drying of spores prior to dispersal[13]. This was subsequently revisited with the conclusion that moss stomata are involved in the drying out and shrinkage of the capsule leading to spore dispersal[14,15]. More recently it has also been suggested that moss capsule stomata facilitate the uptake of CO_2[16,17].

Both the aperture of the stomatal pore and the number of stomata that develop on the surface of the leaf are controlled by signals from the environment — light (quality and quantity), atmospheric CO_2 concentration, relative humidity (vapour pressure deficit) and endogenous signals such as the plant hormone abscisic acid (ABA) that builds up during reduced soil water availability[5–7]. The ability to control stomatal aperture and density provides plants with the capacity to control water loss and

Box 1. Glossary of terms.

Angiosperms – flowering plants.
Assemblages – groups of fossils occurring together in the same stratum.
Bryophytes – non-vascular plants consisting of hornworts, mosses and liverworts.
Embryophytes – land plants, a monophyletic lineage derived from an algal ancestor.
Euphyllophytes – a monophyletic lineage consisting of ferns and their allies, gymnosperms and angiosperms.
Gametophyte – the haploid multicellular generation of land plants.
Holoparasitic – parasitic plants that obtain all their water and nutrients from their host.
Monophyletic – a group of organisms including the common ancestor and all descendent species.
Orthologues – the same gene in different species, which are derived via a speciation event.
Paralogues – genes derived from a duplication event.
Paraphyletic – a group of organisms descended from a common evolutionary ancestor, but not including all the descendent groups.
Poikilohydric – organisms lacking the ability to regulate their cell and tissue water content.
Polysporangiates – a lineage of extant and extinct plants defined by a branched sporophyte.
Setaphytes – a bryophyte lineage consisting of mosses and liverworts.
Sporophyte – the diploid multicellular generation of land plants.
Tracheophytes – vascular plants including lycophytes, ferns, gymnosperms and angiosperms.

CO_2 uptake in the short and long term. This plays out as changes in water use efficiency (WUE, the amount of water used to produce a unit of biomass) and thereby contributes to the capacity of a plant to adapt to changing environmental conditions and their ability to colonise drier regions of the Earth.

In this review, we will highlight how palaeontological, phylogenomic, molecular and physiological data provide insights into the origin and evolution of stomata, with particular attention being paid to the evolution of stomatal function. We will also discuss how these data reveal gaps in our knowledge and identify opportunities for future research.

What can the fossil record tell us about the origin and evolution of stomata?

The fossil record is a good place to start when seeking evidence to inform our understanding of the origin and evolution of stomata. Recent studies demonstrate that it can have a profound influence on our understanding of key plant traits and stomata are no exception[18]. The stomata of fossil plants have been studied in a variety of contexts[19]. The well-documented relationship between stomatal characteristics — such as stomatal size and density — and the environment have made fossil stomata important to the study of past climates[20]. The distinctive morphology of the stomatal complex has meant that they have been used to distinguish, and occasionally define, extinct lineages[21–23]. In addition, guard cell size has been used as a proxy to infer genome sizes and ploidy levels in fossil plants[24–26].

The earliest unequivocal evidence for land plants is the presence of isolated spores in the Ordovician approximately 470 million years ago (mya)[27,28] and slightly younger fragments of sporangia (spore-bearing organs) around 450 mya[1,29]. These fragments show that plants were on land by the Ordovician but provide no evidence of stomata. The earliest putative evidence for stomata also comes from the Ordovician but from a slightly younger deposit from Zbrza, Poland, 445 mya[30]. These fossils consist of small cylindrical, dichotomously branched, leafless axes up to a couple of millimetres in length with terminal structures interpreted as sporangia. The bodies of these early land plants were composed of cylindrical branches termed 'axes'. In this context, the term axes (plural), or axis (singular), is used because, at this time, the organs that we recognize as shoots, leaves and roots had not evolved. A single axis was preserved with a possible stoma, composed of two poorly preserved kidney-shaped structures interpreted as guard cells. Given the poor preservation of the specimen, it is difficult to be confident about the interpretation of this as a stoma; however, the size of the putative stomatal complex (29 μm long x 21 μm wide) does fall within the known range of stomata from later in the geological record[8]. Despite evidence for plants being on land in the Ordovician, there are currently no structures that can be unequivocally identified as stomata from this time period.

Towards the end of the Silurian (c. 425 mya), we find the first evidence for abundant plant life on land, with assemblages of vascular plants including examples of genera such as *Cooksonia.* This is an extinct genus of vascular plants with thin bifurcating axes and terminal sporangia. Taxa of this genus do not form a monophyletic group, and sit on the land plant phylogenetic tree around the divergence of lycophytes and euphyllophytes[31,32] (see Figure 1 for further details). These earliest records unfortunately lack the degree of preservation required to seek evidence for the presence of stomata. The first unequivocal stomata were described from 420 myo plant axes, indicating a minimum age for stomata at the end of the Silurian[8].

The Devonian (419–358 mya) was a period characterised by a strong radiation of land plants with abundant evidence for stomata. Fossils from the lowermost Devonian (c. 415 mya) have stomata and already display significant variation in stomatal form[8]. *Cooksonia* had stomata distributed on axes and sporangia[8,33], as did the eophytes, a group of unclear taxonomic affinity preserved based on fragments of tiny sporophytic axes and terminal sporangia[34,35]. Spores produced by eophytes are called cryptospores[36]. Cryptospores are observed from the middle Cambrian and superficially resemble the earliest land plant spores from the uppermost Ordovician, but lack a clear tetrad mark. Cryptospores provide clear evidence for plant life on

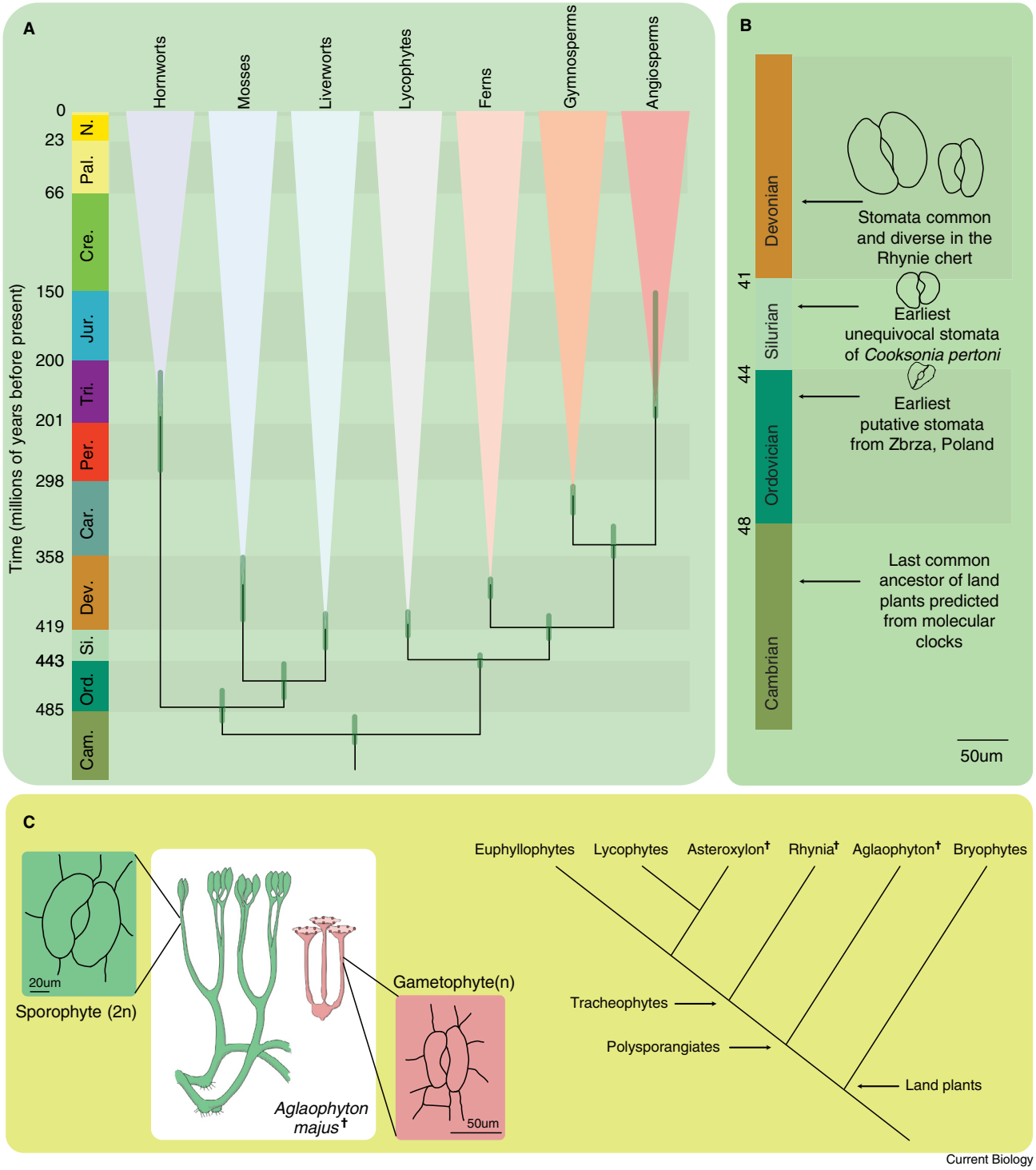

Figure 1. The phylogenetic context for stomatal origins and evolution.
(A) A time-calibrated phylogeny of land plants showing the ages of the major lineages and the evolutionary relationships among them. The depicted relationships are based on a body of recent literature (see text for details). Molecular clock estimates of lineage age are uncertain, as depicted by the vertical error bars. (B) A timeline of stomatal evolution in the fossil record. Stomata of several key early fossils are depicted alongside the geological age at which they are found. (C) The phylogenetic position and stomatal morphology of *Aglaophyton majus*, a species found in the Early Devonian Rhynie chert. Stomata are found on each generation with comparable morphology. Line drawings are after drawings in the following papers[8,25,118,119].

land, but it is not until the Devonian that the earliest unequivocal proof of their producers becomes available. Devonian fossils of *Cooksonia* and eophytes provide evidence of stomata on both axes and sporangia in groups of plants, with fossil records that extend much earlier than the Devonian.

Much of what is known about the anatomy of Early Devonian plants is from a site of exceptional preservation near the village of Rhynie in Scotland[37]. The Early Devonian Rhynie chert represents a hot-spring ecosystem[38], containing a variety of species, each with cellular-level preservation. Most of these plants were only a few tens of centimetres high with branched photosynthetic axes, terminal or lateral sporangia. Due to their branched sporophyte axes these plants are all termed polysporangiophytes (Figure 1). Today, polysporangiophytes encompass all living vascular plants, including both lycophytes and euphyllophytes. However, most plants in the Rhynie chert diverged before, or around the time of, the split between living lycophytes and euphyllophytes. Therefore, they provide a key insight into plant evolution in the first vascular plants. Stomata, with kidney-shaped guard cells, are present in all well-described species, and the morphology of stomata varies extensively between species, indicating that stomata had diversified by the Early Devonian[8]. The shape and size vary from large, elongate stomata observed in *Horneophyton ligneri*[39], to the rounded and small stomata in *Nothia aphylla* (possibly an early diverging lycophyte)[40]. The distribution of stomata across tissues is also variable but generally extensive, with stomata in the extinct lycopsid, *Asteroxylon mackiei*, present on all but rooting axes[41], while others, such as *Rhynia gwynne-vaughnii* (an extinct vascular plant), possess scattered stomata on all regions of axes including rhizomes and sporangia[8]. The substomatal cavities associated with the stomata in each species also vary considerably. In *Aglaophyton majus* (a non-vascular plant) and *Rhynia,* they consist of a channel below the pore, formed by epidermal and hypodermal cells, leading to a substomatal chamber, cutinised in *Aglaophyton* but not in *Rhynia*[8], while in *Horneophyton* the surrounding epidermal cells partially subtend the guard cells, creating a funnel-shaped chamber[8]. *Nothia* is an exception within the Rhynie chert, with guard cells opening directly over the substomatal chamber[40]. The plants in the Rhynie chert therefore display a great variety of sizes, distributions and associated substomatal cavities, demonstrating the diversity present in the Early Devonian.

There is evidence suggesting that there was diversity in opening and closure mechanisms in the Early Devonian, at least in terms of cell wall mechanics. Some species, such as *Zosterophyllum myretonianum* (a zosterophyll, an extinct early diverging lineage of lycophytes)[42], show heavily cutinized walls in adjacent epidermal cells, suggesting that lateral movement of the guard cells was impossible[8]. Instead, the flexible thinner periclinal walls would have allowed opening of the pore[43]. This mechanism is also seen in some extant plants, including mosses and the lycophyte *Huperzia*[44]. *Nothia*, however, possessed thickened periclinal and anticlinal walls and so the form of stomatal movement proposed for other Rhynie chert plants was likely not the case in this species. The stomata in early land plants always lack differentiated subsidiary cells[8] and so the elaboration of the complex and the development of subsidiary cells likely arose later during vascular plant evolution[21].

Despite their overall apparent morphological similarity to the stomata of extant plants, stomata in the Rhynie flora also demonstrate unique traits, such as development on the gametophyte. Gametophytes of several species have been identified based on shared anatomy, co-occurrence and development from spores. Gametophytes, such as *Lyonophyton rhyniense*, the gametophyte of *Aglaophyton*, tend to be of similar morphology and anatomy to the sporophyte generation, indicating two free-living generations[44,45]. In extant land plants, stomata are confined to the sporophyte, even in bryophytes where the gametophyte is larger, free-living, photosynthetic and the sporophyte is greatly reduced. The presence of stomata on the gametophyte generation of plants in the Rhynie chert is therefore a novel characteristic of these early land plants. Stomatal densities and morphologies are similar in the gametophytes and sporophytes of Rhynie chert plants[45–49] and so it is possible that they performed the same role in both generations. This suggests that a comparatively complex gametophyte is ancestral to vascular plants[45,46,48,49] and that gametophytic stomata have since been lost with the overall reduction in size and complexity of the tracheophyte gametophyte.

Assigning a role to stomata in these early plants is difficult, for two main reasons. Firstly, the function of stomata in the two major groups of living land plants, bryophytes and tracheophytes, is predicted to be different. In extant bryophytes, stomata only occur on the sporangium, where they are believed to play a role in sporangium drying, the release of spores[14,15], and CO_2 uptake[16]. In contrast, in living tracheophytes, stomata predominantly occur on leaves and both vegetative and reproductive axes, and function primarily in the control of gas exchange (loss of water vapour and CO_2 uptake). The early fossil record suggests evidence for both character states being present by the Early Devonian. For example, in *Sporogonites*, an extinct unbranched species with a terminal sporangium[50,51], stomata occur on terminal sporangia, whereas in fossil lycopsids such as *Asteroxylon* they are only recorded on axes, including rhizomes, and not on sporangia. However, many fossil species possess a mosaic of traits that are typical of both bryophytes and tracheophytes, a condition which also occurs in species from the Rhynie chert[8]. For example, among the eophytes, stomata are found on both the sporangium and the axes[34,35], a condition which also occurs in *Aglaophyton*[52], *Horneophyton*[8] and *Nothia*[40] from the Rhynie chert.

The second reason why assigning a role for stomata in early land plants is difficult is due to the uncertainty in the placement of early fossils on the land plant phylogeny and their fragmentary preservation[31,53–55]. Some of the most intriguing extinct species, such as *Sporogonites*, are unresolved phylogenetically yet may inform the evolution of stomata in bryophytes. *Sporogonites* bears a superficial resemblance to modern bryophytes[56] and may, therefore, should taxonomic placement be concluded, help inform early bryophyte evolution[56,57]. *Sporogonites,* like many of the fossils from the Early Devonian, are known only from small, isolated fragments, meaning we still lack a clear understanding of their overall form and lifecycle. To address whether *Sporogonites* was reliant on stomata requires exceptionally well-preserved fossils. In summary, the presence of stomata on axes, including rhizomes and the gametophyte generation in the earliest land plant fossils, does not support the

hypothesis that stomata functioned solely to facilitate sporangium drying and the release of spores. Rather, it suggests that stomata may have evolved to facilitate control over both water loss, CO_2 uptake and sporangial drying.

The function of stomata in some of these species remains a puzzle. For example, *Electorotheca enigmatica* possessed stomata on sterile appendages overlying the sporangium[55]. As there is no evidence for vascular tissue in this species, if the stomata facilitate transpiration, then they do so without the anatomical innovations (xylem and sub-stomatal cavities) found in tracheophytes. In addition, the density of stomata on the sporangium is so low that it is unlikely to provide much assistance with drying the sporangial walls or gas exchange in photosynthetic tissue. Research on the extinct eophytes has resulted in them being tentatively placed on the vascular plant stem lineage[34,35]. However, it is possible that they represent a member of the broader, more inclusive embryophyte stem lineage. If this were the case, it would suggest that an ancestral land plant may have possessed stomata on both the sporangium and axes, each respectively retained in non-vascular and vascular descendants.

The review of the earliest fossil record of stomata reveals four important insights: first, despite fragmentary evidence of plants being preserved back to 470 mya, unequivocal evidence of stomata is only found in c. 420 mya old fossils, leaving a 50-million-year gap in our understanding of early stomatal evolution. Second, when stomata are first found in the fossil record, they are common features in most early well-preserved fossils. Third, the presence of stomata on sporangia, axes and gametophytes indicates that by the Early Devonian the primary function of stomata was not restricted to spore release. Finally, the early fossil record provides no evidence of lineages that we can confidently infer to lack stomata ancestrally, or lineages with a gradual acquisition of stomatal characters. However, the abundant stomatal fossils from the Lower Devonian allow us to investigate stomatal diversity at this key time point. To date, no fossils have been discovered that possess an intermediate form that would help explain how stomata first developed from epidermal cells. Is this because stomatal progenitors and intermediate forms have not been recognized by palaeobotanists? This is of course possible because it is difficult to predict what such structures would have looked like. It is also made more difficult by the incompleteness of the fossil record. For example, the oldest undisputable bryophyte fossils date from c. 385 mya[58] yet lack the details required to infer the presence, absence or nature of their stomata. In this context, while similarities between extant plants and the earliest records of stomata in the fossil record have led to predictions that stomata have remained conserved in morphology and function for hundreds of millions of years, we now know that by the earliest Devonian (c. 395–419 mya), stomata were diverse in terms of form, distribution and potentially function.

In summary, from the fossil record we learn that stomata are ancient structures that had diversified by the Early Devonian. Their presence on the gametophytes, axes, rhizomes and sporophytes of the predicted common ancestor of vascular plants suggests that their role was not restricted to facilitating spore dispersal. However, until new fossils are discovered the origin and ancestral functions of stomata remain a mystery. For this reason, we will next examine what phylogenomics has to contribute towards solving the puzzle of the origin and evolution of stomata.

Insights into stomatal evolution gained from phylogenomic research

The results of recent phylogenomic analyses support two major monophyletic land plant lineages: Tracheophyta and Bryophyta (Figure 1A)[2,59–61]. Within the bryophytes, liverworts and mosses are also consistently found as sister lineages forming a group termed the setaphytes. The sister relationship between liverworts and mosses is important because it means that the absence of stomata in liverworts is likely the result of secondary loss, rather than ancestral absence. It also implies an origin of stomata prior to the divergence of tracheophytes and bryophytes (c. 495–515 mya[62]) and therefore the presence of stomata in the common ancestor of all living land plants[2]. Stomata are the rule in tracheophytes, and are only lost in species that have secondarily evolved to become aquatic, poikilohydric or holoparasitic[4,63]. In bryophytes, stomata are absent in all liverworts as well as several genera of mosses, including the earliest diverging lineages of mosses and certain genera of hornworts. A recent survey of the presence of stomata in mosses proposed over 60 independent losses[3]. The loss of stomata in many lineages of mosses and hornworts highlights that stomatal loss is an ongoing process. Stomata appear to be an example of an adaptive loss of function and yet the parallels in the loss of stomata between various bryophytes and tracheophytes have not yet been explored.

In extant dicots, we have a good understanding of stomatal development. In a simplified overview, the process is initiated by the asymmetric cell division of a meristemoid mother cell (MMC). This produces a meristemoid and a larger stomatal lineage ground cell (SLGC). MMCs go through a series of amplifying asymmetric divisions until a guard mother cell (GMC) is produced. This then divides symmetrically to produce a pair of guard cells. Important molecular master regulators in this process are the basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE and FAMA. SPCH is required for meristemoid development, MUTE promotes the development of the GMC, while FAMA regulates the symmetric division that produces the two guard cells. A mitogen-activated protein kinase (MAPK) cascade, which is controlled by peptides known as epidermal patterning factors (EPFs), acts to regulate all steps of stomatal development, though this has only been directly demonstrated for SPCH and MUTE. The developmental sequence is influenced by environmental factors and is subject to rules, including the 'one spacing' rule that prevents the co-occurrence of adjacent stomata. Stomatal development in *Arabidopsis*[64–67] and grasses[68] has been recently and authoritatively reviewed.

In the light of species relationships, phylogenetic analysis of individual gene families can reveal instances of gene duplication, loss and functional divergence. Gene families characterised in *Arabidopsis thaliana* have revealed the evolutionary history of the genetic toolkit underpinning stomatal development[2,15,69–73]. The phylogenetic history of SPCH, MUTE and FAMA showed that they are paralogues (Figure 2B) and are present in most angiosperms. However, only two paralogues are

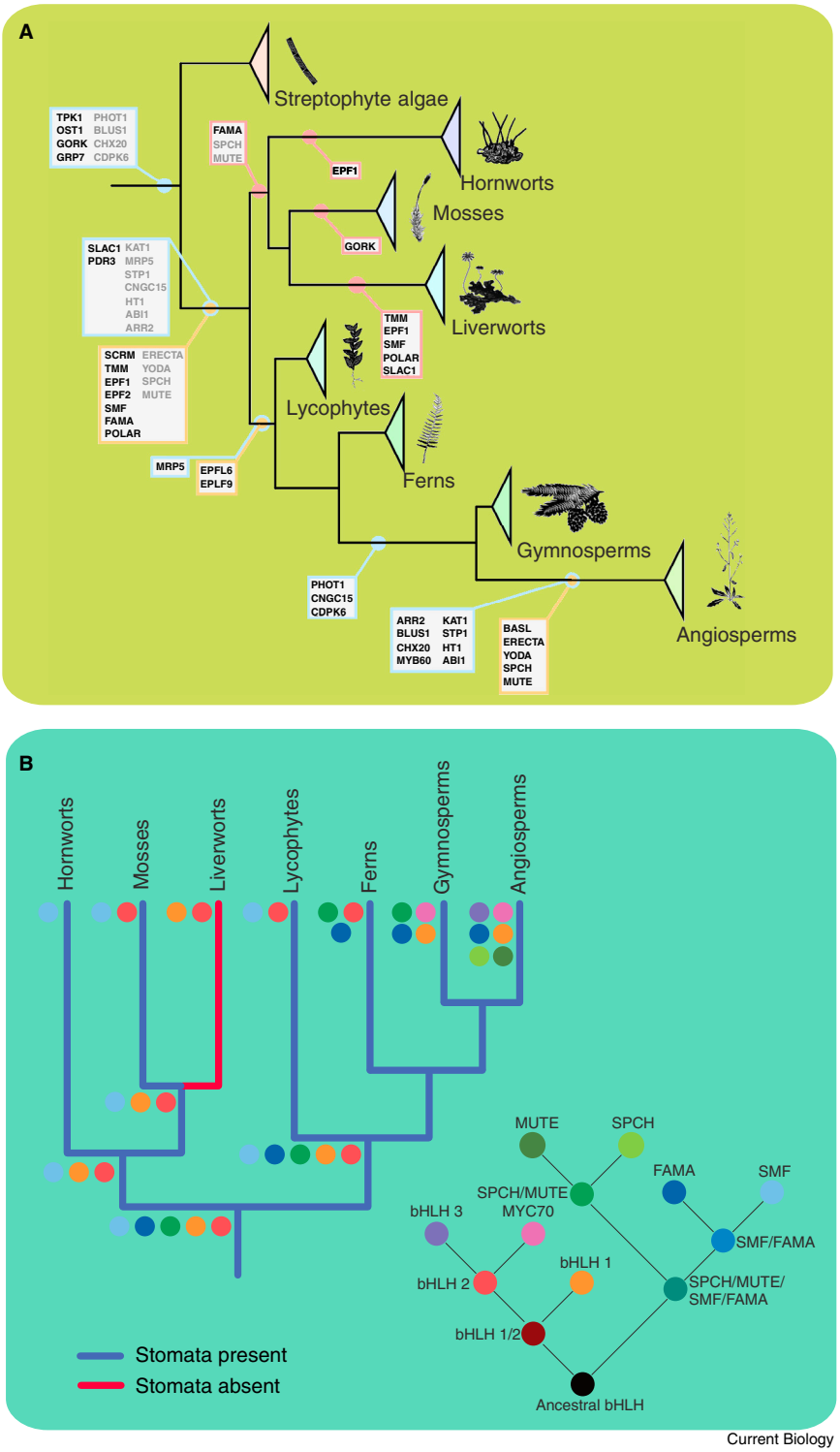

Figure 2. Stomatal gene family evolution across land plants.

(A) Each major lineage is represented. At each node of the phylogeny, the origins of genes are shown in blue, gene duplications in yellow and gene losses in red. Genes names shown in grey represent pre-duplication copies of the gene. (B) The diversification of the bHLH stomatal development genes. The presence of individual genes is represented by coloured dots, with present genes shown next to each extant lineage and at the ancestral nodes. Lines of the phylogeny are coloured according to the presence or absence of stomata, reflecting the likely hypothesis that stomata were present in the ancestral land plant.

present in the moss *Physcomitrium patens* (previously named *Physcomitrella patens*)[70,74]. Further functional and phylogenetic analyses in *Physcomitrium* identified an orthologue of SPCH, MUTE and FAMA, referred to as SMF, that is required for stomatal development[15,72]. Moss lacking the PpSMF1 gene failed to form any stomata and had delayed capsule opening and spore dispersal[15]. Subsequent work showed that FAMA, SMF and a gene resembling both SPCH and MUTE were present in the common ancestor of all embryophytes[2]. This suggests that FAMA and SPCH/MUTE were lost in the bryophyte stem lineage. An orthologue of SMF was identified in lycophytes[2], which further supports an origin for SMF prior to the divergence of bryophytes and tracheophytes. The conclusion of these analyses is that the ancestral stomatal development pathway consisted of FAMA, SMF and SPCH/MUTE. Extant lineages have retained only one of either SMF or FAMA, suggesting a degree of functional redundancy between SMF and FAMA in early land plants[75].

Phylogenetic analyses of the EPFs have identified orthologs in both bryophytes and tracheophytes[2,15,69,72,73,76]. As with the bHLHs, the EPFs that underpin stomatal development were present in the ancestral embryophyte. Harris *et al.*[2] identified seven stomatal development genes (TMM, EPF1, EPF2, SCRM2, SMF, FAMA and POLAR) that mapped back to the ancestor of all embryophytes, which has since been corroborated by a denser sampling approach[69]. To demonstrate functionality, Caine and colleagues[72] knocked out the *Physcomitrium* homologues of *Arabidopsis EPF1* and *2*, resulting in sporophytes with increased numbers of stomata. The deep origins of many genes involved in stomatal development suggests that, in the first land plants, the signalling pathways underlying stomatal development were probably more complex than those found in extant bryophytes. It seems likely that in terms of stomatal development, extant lineages of bryophytes evolved by reduction from a more complex ancestor[2,77].

Phylogenetic analyses of the genes involved in *Arabidopsis* stomatal opening and closure reveal that many have orthologues

in streptophyte algae. These data suggest that the origin of the stomatal signalling pathway predates the appearance of land plants[2,78–80]. The protein kinase *OPEN STOMATA 1* (*OST1* also known as *SnRK2e*) is an important component in the *Arabidopsis* stomatal closure intracellular signalling network. Deletion of a *Physcomitrium* orthologue of *OST1* results in stomata that exhibit reduced ABA-induced stomatal closure, confirming that this gene product plays a role in pore closure in moss[81]. *OST1* orthologues from the alga *Klebsormidium nitens*, the liverwort *Marchantia polymorpha*, *Physcomitrium* and the lycophyte *Selaginella uncinata* have been used to genetically complement, and thereby restore, ABA-induced stomatal closure in the *Arabidopsis ost1* mutant[79,81–83]. The observation that deletion of the moss orthologue results in compromised ABA-induced closure[83] argues against the suggestion that *OST1* functioned in a different role in the ancestral embryophyte and was co-opted into stomatal signalling later in embryophyte evolution[83].

A suite of genes known to be involved in the control of stomatal movement in *Arabidopsis* were mapped to the ancestor of all embryophytes[2,69]. Eleven out of the eighteen genes assessed, including *OST1*, were predicted to have been present prior to the split between bryophytes and tracheophytes. This suggests that a significant portion of genes required for stomatal movement in *Arabidopsis* were present in the ancestral embryophyte. Of these, a number were secondarily lost in bryophytes in a situation reminiscent of the genes controlling stomatal development[2]. It was also shown that all the mosses sampled had lost the key voltage-gated ion channel GORK, and all liverworts had lost SLAC1, both of which are well known components of the *Arabidopsis* stomatal closure pathway[2]. However, the most prominent losses in bryophytes were associated with stomatal development genes. We hypothesise that if there is evolutionary pressure to lose stomata, the loss of developmental genes is the most efficient way to accomplish this change, rather than incremental functional reductions. These findings are summarised in Figure 2.

There are two insights that emerge from the phylogenomic work. The first is the establishment of the land plant phylogeny, which leads to the conclusion that stomata were present in the common ancestor of all living land plants. During evolution they have been secondarily lost on many occasions within the bryophytes, including a complete loss in the common ancestor of the liverworts. The alternative to this hypothesis is that stomata have multiple, independent origins across land plants and that their similarities have resulted from convergence[84]. This possibility is not supported by the phylogenomic data. The second insight is that the data support a more gene-rich origin for stomata in the last common ancestor of the land plants than is found in present-day bryophytes. However, it should be emphasised that phylogenetic inferences reflect both the data available and the analytical methods used[85]. As more genomes become available, the phylogeny of the land plants will become clearer and this may have a bearing on our understanding of stomatal evolution. However, given the support for the monophyly of bryophytes and particularly for a clade of liverworts and mosses, the most straightforward conclusion is that stomata evolved once in the last common ancestor of the land plants, and there seem to be no compelling reasons to invoke multiple later convergent origins of stomata.

The evolution of stomatal opening and closure: insights from extant species

In *Arabidopsis*, changes in environmental conditions are either detected directly by receptors in or at the surface of the guard cell or generate increases in the concentration of hormones, such as abscisic acid (ABA), which are perceived by receptors in the guard cells. Once the environmental signal has been perceived, guard cell intracellular signalling networks couple the stimuli to their final targets, typically ion channels. Changes in ion-channel activity result in either the loss of salt, followed by water and turgor loss from the guard cell that results in stomatal closure, or the accumulation of salt and water in the guard cell that leads to stomatal opening (Figure 3). The transport of sugars and organic acids is also involved in the determination of stomatal aperture. This topic has been recently been reviewed in depth elsewhere[5–7].

Our knowledge of stomatal function in extant angiosperms motivates a series of evolutionary questions: firstly, when did stomata acquire the ability to open and close in response to changes in the environment? Secondly, was the capacity to respond to different signals acquired at different points during evolution? Finally, has stomatal behaviour, such as speed of response, changed during evolution? Before attempting to answer these questions, it is important to be aware of the inherent limitations and uncertainties associated with using the responses of extant species to predict the behaviour of extinct taxa.

Firstly, much of what we know about guard cell function stems from work in *Arabidopsis*. In the future, it will be very important to investigate stomatal behaviour in other species. This will make it possible to establish whether the model we have built is representative of all groups, or whether there is diversity in the cellular and molecular mechanisms that underpin stomatal movements. We already know that there are some differences in stomatal behaviour, such as in CAM plants, where in contrast to C3 species, stomata open during the night[86]. It is also important to recognize that the species we are looking at today represent the results of hundreds of millions of years of evolution — and the process is ongoing. In addition, the environmental conditions under which the extinct ancestors of today's plants thrived were, in many cases, significantly different to the conditions experienced by species living today. A case-in-point would be the atmospheric CO_2 concentration, which is greatly reduced today compared with the levels experienced by the earliest plants[87]. Finally, but significantly, we have the phylogenomic evidence[2,59–61] that supports the monophyly of stomata with an origin before the divergence of the tracheophytes and bryophytes. This means that these two lineages, as well as the major lineages within each, have been proceeding on independent and separate evolutionary trajectories for hundreds of millions of years. These factors mean that we need to be cautious when drawing conclusions about the evolution of stomatal function based on data from extant species.

There are conflicting views concerning the evolution of stomatal function. In 2011, McAdam and Brodribb[88] proposed that bryophyte, lycophyte and fern stomata were unable to close in response to ABA and elevated concentrations of CO_2. They suggested that changes in stomatal aperture in these genera took place by adjustment of guard cell turgor as a result of variation

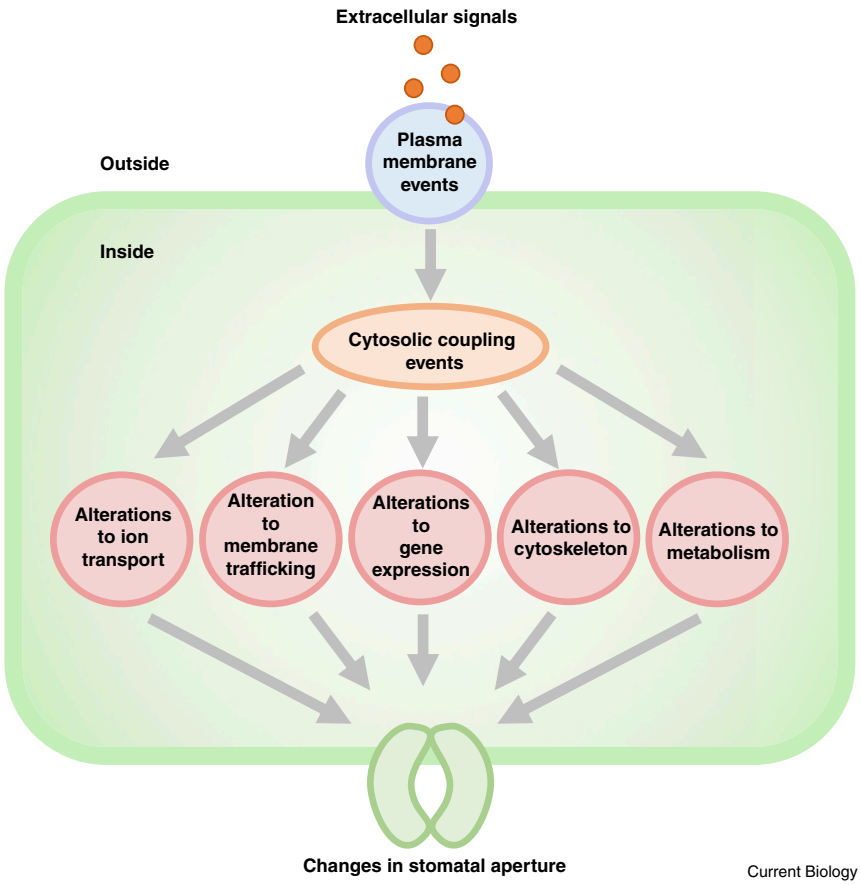

Figure 3. Guard cell stimulus–response coupling.
Extracellular signals are perceived by intracellular receptors or at the plasma membrane. These activate cytosolic coupling events such as increases in the concentration of intracellular messengers such as Ca^{2+} and reactive oxygen species (ROS) and enzymes, particularly protein kinases and phosphoprotein phosphatases. These in turn result in the coordinated regulation of metabolic reactions, and changes to the cytoskeleton, ion transport and gene expression. The net result of these processes is alterations to guard cell turgor leading to changes in stomatal aperture. These events need to be coordinated both in space (the appropriate cellular compartment) and in time (occurring in the correct sequence). For full details see the following reviews[5–7].

in leaf apoplastic water potential (hydropassive mechanisms)[88]. That is without recourse to the signal transduction networks involving adjustment of guard cell turgor through the uptake or release of ions, or synthesis of organic solutes in guard cells (hydroactive mechanisms)[83,88]. Their contention was that hydroactive movement only evolved with the emergence of gymnosperms and angiosperms[88,89]. The counterview is that changes in stomatal aperture in the earliest diverging lineages took place through hydroactive means. This has led, by extension, to the suggestion that hydroactive stomatal movement was already present in the earliest land plants[81,82].

Figure 4 shows that, within extant bryophytes, lycophytes and ferns, there are examples of species that close their stomata in response to ABA and CO_2 and those that do not. The same is true for blue-light-induced opening. These data do not support the suggestion that hydroactive responses first evolved with the emergence of the gymnosperms[88,89]. The gold standard approach in establishing whether stomata move through hydroactive responses is to knock out genes proposed to be involved in, for example, the guard cell ABA signalling pathway. Unfortunately, the inability to conduct, on a routine basis, stable genetic transformation in most species means that this approach cannot be brought to bear on the question of hydroactive or hydropassive responses. However, there is an exception. In *Arabidopsis* the protein kinase OST1 is required for ABA-induced stomatal closure[90]. Deletion of the moss *Physcomitrium* orthologue of OST1 interferes with the ability of ABA to induce stomatal closure[81]. These data provide compelling evidence to support the presence of hydroactive closure outside the gymnosperm and angiosperm clade. In the fern *Ceratopteris richardii gaia1* mutant (a homologue of *OST1*), a reduction in vapour pressure deficit or dehydration, induced by leaf excision, caused stomatal conductance decrease to the same extent as in wild type, suggesting that this gene was not involved in these responses[91]. However, more recent work showed that there are additional *OST1* homologues in the *C. richardii* transcriptome. This means that the failure to interfere with closure in *gaia1* may be because other *C. richardii OST1* homologues are able to compensate for the loss of GAIA[92]. In the same paper, it was shown that stomata in this fern can and do respond directly to ABA[92]. The evidence for hydroactive stomatal opening is presented in Figure 4 and Table S1, and is complemented by experimental work using fusicoccin, a compound that constitutively activates the plasma membrane H^+-ATPase. Addition of fusicoccin to two mosses and a lycophyte induced stomatal opening[81,82].

The key message to emerge from Figure 4 and Table S1 is that stomata from all lineages have the capacity to respond to all or some of the following signals: ABA, CO_2, blue or white light. In the context of evolution, it is not how much they respond — rather a positive response shows that the required intracellular signalling network is present and in operation. Research in the lycophyte *Selaginella uncinata* shows a dose response relationship to ABA extending from 1–25 μM[82], while 50 μM ABA elicits significant closure in *Physcomitrium patens*[81]. In the case of CO_2, significant closure is observed in *P. patens* in response to an increase from 100 to 400 ppm[81] and in *S. uncinata* from 425 to 700 ppm[82]. Both ABA and CO_2 concentrations are well within the range over which these stimuli operate in angiosperms. In the future, it will be important to measure the affinities of the bryophyte, lycophyte and fern guard cell receptors for ABA, because this value, rather than bulk tissue levels, dictates the stomatal response.

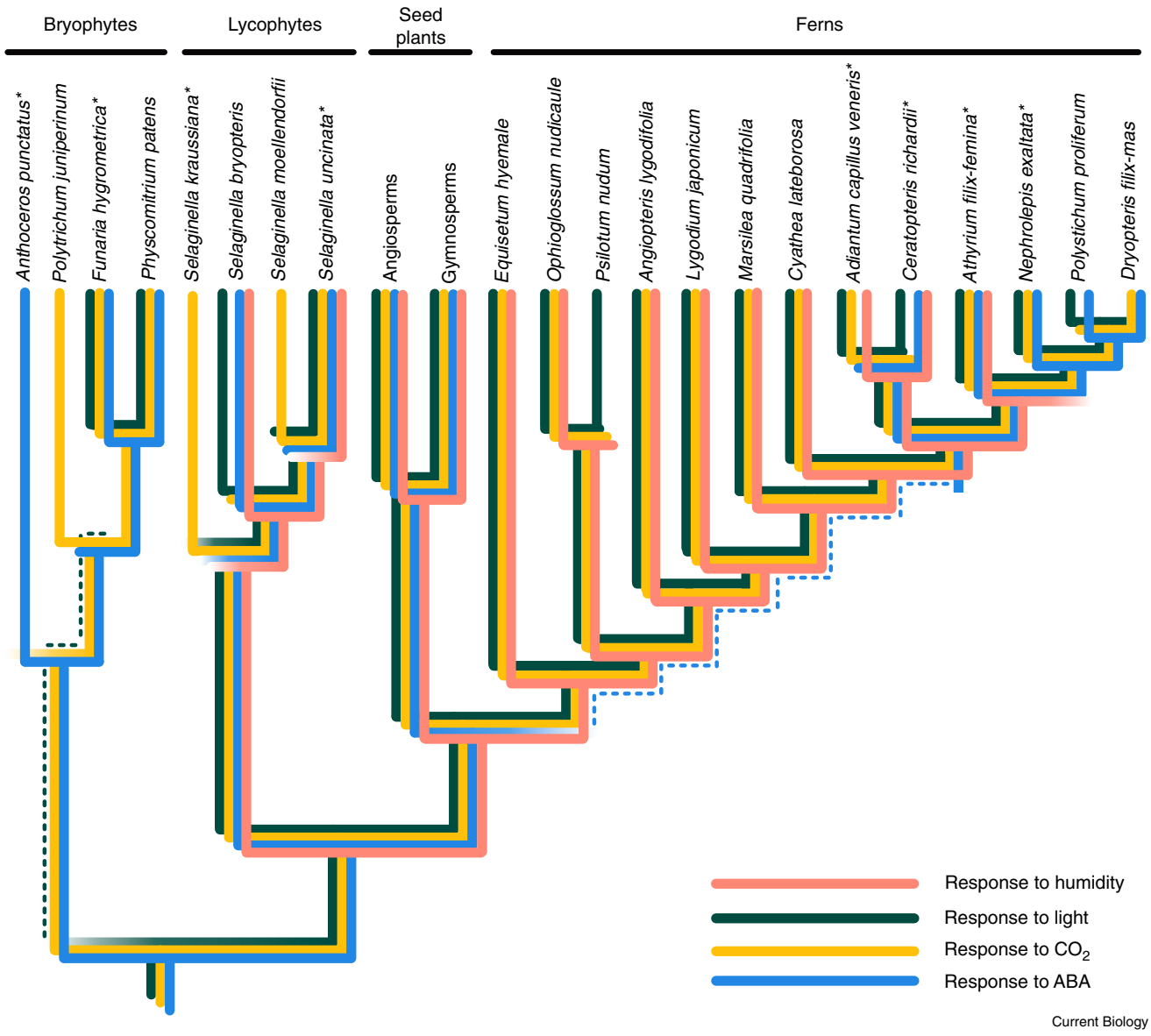

Figure 4. The diversity of stomatal responses among land plants.
Experimental evidence for stomatal responses to humidity, light, CO_2 and ABA are mapped onto the land plant phylogeny for species selected to represent a diversity of stomatal responses to various stimuli (data for all available species and relevant references are found in Table S1). This shows the possibility that stomatal responses to environmental cues are widely distributed among and possibly ancestral to land plants. Note that in many cases, the absence of a response does not indicate loss, but that the response has not been determined. Species where conflicting responses have been determined are marked with an asterisk. The dotted lines represent a part of the tree where the evolution of the ABA response in ferns and light response in bryophytes remains uncertain and warrants further investigation.

The clear evidence that stomata open and close using hydroactive mechanisms in some species of all extant genera, from angiosperms to bryophytes, allows the rejection of the hypothesis that hydroactive responses are only present in gymnosperms and angiosperms[88,89]. What might the behaviour of stomata in extant genera tell us about the evolution of stomatal function? Bearing in mind the caveats mentioned above, one interpretation of the extant data would be that stomata in the earliest embryophytes responded actively; however, during the course of millions of years of evolution this has been lost, or masked, in some extant genera and species. Alternatively, the ability to respond actively has evolved independently, on multiple occasions, in multiple genera over evolutionary time and this gives rise to the pattern of hydroactive and hydropassive responses seen in Figure 4. There is also a further possibility. Regulation of leaf gas exchange can be achieved by either controlling stomatal aperture or stomatal density or both. There is evidence that species that do not close their stomata in response to elevated CO_2 are more likely to show a significant reduction in stomatal density in response to this same signal. In contrast, species with a strong closure response to elevated CO_2 are less likely to have a significant developmental response to CO_2[93].

Failure to detect a hydroactive response in extant species can also be explained by conditionality — that is, that a response only occurs under certain growth conditions. In stomatal biology, this phenomenon is known from work on species, including *Arabidopsis thaliana*, where the ability of stomata to respond to ABA is dictated by the relative humidity of the atmosphere[94]. Might a similar failure to respond also result from conditionality in early diverging lineages? There are data suggesting that this is the case. Hörak *et al.*[95] showed that in some fern species the ability to close stomata in response to ABA and CO_2 is conditional and dependent upon the relative humidity of their environment. Recently, the issue of conditionality was investigated in the fern *Ceratopteris richardii*. Plackett *et al.*[92] showed that stomata of *C. richardii* close in response to either low relative humidity or ABA, but that the ability to respond is dependent on a prior exposure to either ABA or reduced atmospheric relative humidity. Data from RNA sequencing experiments suggested that exposure to ABA or reduced relative humidity acted to prime the closure signalling pathway such that it operates at a lower threshold. The results of these experiments provide an explanation as to why stomatal responses to extracellular signals in all lineages might sometimes be absent.

If the ability of stomata to respond to extracellular signals is the ancestral state, which selective pressures resulted in the loss, or lack of retention, of this important trait? At this stage it is only possible to speculate; however, the most plausible explanation is that losses might have occurred during adaptation to life in habitats where the ability to, for example, close in response to ABA is of no selective advantage. Such a situation might arise in habitats characterised by low vapour pressure deficit (high atmospheric relative humidity), especially when coupled with a low stature.

Evolution of stomatal size and speed of response

Optimisation of carbon uptake while controlling the loss of water has resulted in a diversity of stomatal sizes, densities and morphologies[96]. Size and density together determine the maximum diffusive conductance to CO_2 and water vapour[96]. Smaller, and more densely distributed, stomatal pores achieve higher rates of conductance due to the shorter diffusion distance associated with the reduced depth of their guard cells[97]. This has led to the hypothesis that decreasing atmospheric CO_2 concentrations after the emergence of vascular plants favoured those with smaller stomata at high densities, and indeed fossil evidence supports positive and negative correlations between stomatal size and density, respectively, with atmospheric CO_2 concentration over the past 400 million years of land plant evolution[97,98]. Indeed, the increases in anthropomorphic atmospheric CO_2 emissions over the last 200 years coincide with decreased stomatal densities across many species[99].

Increases and decreases in photosynthetic assimilation capacity can occur an order of magnitude faster than adjustments in stomatal conductance, limiting carbon uptake during opening and causing superfluous water loss during stomatal closing. Together these two parameters have a detrimental effect on water use efficiency[7,100] meaning that faster acting stomata could confer advantages, particularly in fluctuating environments when water is restricted[101,102].

Apart from angiosperms, other tracheophyte clades, such as ferns, are generally believed to have relatively slow stomatal responses. However, some ferns have stomatal response speeds that are comparable to those of angiosperms. Recent work has revealed that Polypodiales, a relatively modern and species-rich order of leptosporangiate ferns, have faster responses to blue light than, for example, the more ancient eurosporangiate fern *Angiopteris evata* (Marattiales) and *Arabidopsis*[103]. This impressive stomatal speed may have enabled later-diverging fern species to occupy the shady understory beneath an increasingly angiosperm dominated canopy[104].

There are two major stomatal morphologies — those with kidney-shaped and those with dumbbell-shaped guard cells. The kidney shape was the earlier to evolve, and these are found extensively in early fossil land plants (Figure 1). Dumbbell-shaped cells appear only in grasses and their relatives during this time[1,105]. The dumbbell innovation is associated with increased stomatal operating efficiency, achieving a larger change in pore area for a given change in guard cell turgor and a more rapid response to changes in light[96,106]. The increased efficiency of dumbbell-shaped guard cells is thought to have enabled grasses to expand from the tropical understory to dryer niches during a time of global aridification[1]. The dedicated subsidiary cells associated with dumbbell stomata contribute to their efficiency. Mutants lacking such subsidiary cells have impaired opening and closing, and change pore width more slowly than wild type[107]. The enhanced speed of grass stomata has also been linked to their smaller size and larger surface area to volume ratio for ionic exchange[1,97,108]. In closely related kidney-shaped species of the genus *Banksia*, stomatal size was negatively correlated with opening speed[109]. However, this is not always the case[106], and stomatal response speeds appear to be more correlated with conditions during diversification, with species diversifying in periods of low or declining atmospheric CO_2 being able to close their stomata most quickly[110], as proposed many years ago[111]. Are there other underlying explanations to account for the ability of grass stomata to adjust their pore apertures rapidly? It is probable that these faster guard cells also have enhanced signal transduction responses and increased capacity to exchange ions with the apoplast or surrounding subsidiary cells[112,113], and that their morphology and/or cell wall composition support faster aperture changes[114]. However, these properties remain to be empirically determined. Further, subsidiary cells of different morphologies are widespread among land plants and yet their function in relation to stomatal movement remains unclear.

The evolution of stomatal developmental responses to changes in atmospheric CO_2

In a pioneering study Woodward showed there was a negative correlation between the concentration of atmospheric CO_2 and stomatal density[99], which extended over 200 years. This relationship was subsequently extended into geological time by palaeobotanists (for an example, see McElwain *et al.*[115]) and stomatal density has been widely used since as a proxy for estimating palaeoatmospheric CO_2[25,87,116]. In present-day angiosperms stomatal density is affected by changes in development, which in turn is regulated by the atmospheric CO_2 concentration, with over ambient concentrations generally, but not

exclusively, promoting decreases in stomatal density[117]. The underlying signal transduction pathway responsible for coupling the perception of changes in atmospheric CO_2 to changes in stomatal development and, therefore, density is beginning to be resolved, largely from work on *Arabidopsis*[6]. While it is impossible to test whether the relationship between atmospheric CO_2 concentration and stomatal development is causal in extinct genera, it has been possible to compare stomatal density in the Ginkgoales, the group that includes the single extant species *Ginkgo biloba*, and has a fossil record dating back to the Permian[118]. The results show that, in extant *Ginkgo biloba*, growth in increased CO_2 results in a decrease in stomatal density compared with growth at current ambient levels of the gas[119]. These studies were extended to include fossil material from three extinct species, assigned to *Ginkgo* or *Ginkgoites*[118,119], and which spanned the Triassic and Jurassic periods. At this time, CO_2 levels were inferred to be higher than they are today. When the stomatal densities were measured in the fossil material, it was found that they were consistently lower than seen in *G. biloba* growing at current ambient CO_2[119]. If *Ginkgo* had the ability to respond to changes in CO_2 by controlling stomatal development, this implies that the underlying signalling pathway was functional in the Triassic. In extant genera such as *Arabidopsis*, stomatal development is controlled by other environmental signals, including light. Given that genes in the signalling network responsible for the light-modulated control of stomatal development are being discovered[120,121], it will be interesting to use phylogenomic approaches to test whether this might also be an evolutionarily ancient response.

What was the role of stomata in early plants?

Raven, in his review on the selection pressures on stomatal evolution[4], considered this question and concluded that the most likely role of stomata in the first land plants was to optimise carbon gain per unit water lost under fluctuating environmental conditions. In reaching this conclusion, he took into consideration the fact that these very early plants, including those of the Rhynie chert, would have experienced an atmospheric concentration of CO_2 that was likely 10x higher than it is today. His position was also influenced by the phylogeny prevailing in 2002. As discussed above, the case for bryophyte monophyly has strengthened since then and phylogenomic evidence suggests that the emergence of bryophytes was associated with the loss of many genes, including those associated with stomatal development and function (Figure 2). This resulted in the loss of stomata in liverworts and in the ongoing loss of stomata in some mosses and hornworts[3,15]. As bryophyte stomata can be regarded as derived, to understand the role of stomata in early plants we need to focus on early embryophytes that preceded the divergence of tracheophytes and bryophytes. From phylogenomic evidence we know that, in terms of stomatal genes, these plants were more complex than extant bryophytes. If the emergence of bryophytes involved the loss of genes that contribute to endo- or homiohydry, then it is quite possible that the stomata in extinct early plants may have fulfilled multiple roles, including aiding the dispersal of spores as shown in extant bryophytes[14,15]. However, whether or not this is the case will depend on identifying new fossils and phylogenomic evidence pointing to the presence of a water-conducting system and air spaces in the earliest plants. Based on current evidence there are no compelling arguments that would, at this stage, suggest a divergence from Raven's conclusion[4] that the role of stomata in the earliest land plants was to optimise carbon gain per unit water loss, or expressed another way, was to optimise water use efficiency in the face of changing environmental conditions.

Unanswered questions in stomatal evolution

Phylogenomic evidence suggests that the common ancestor of tracheophytes and bryophytes had stomata that were likely more genetically complex than those found in extant bryophytes[2] and have since elaborated in tracheophytes[122]. However, this still leaves unanswered the question of their origin. The fossil record indicates that ancestral stomata looked almost identical to stomata in living species[33] and developed in what appears to be a similar fashion and, as such, provide no clues to their origin. Equally perplexing is the observation that stomata of early plants were sparse, at least when compared with modern angiosperms[8]. This suggests, in the environmental conditions which prevailed at that time, that gas exchange requirements of these diminutive plants were met by what we would regard today as a very low number of stomata. Until new fossils from species that predate the divergence of bryophytes and tracheophytes are identified, finding answers to the question of the origin of stomata remains problematic.

Another question is prompted by the occurrence of stomata on gametophytes of vascular plants from the Rhynie chert[45,123]. This observation is interesting as it suggests that stomata were a feature of the gametophyte generation of the common ancestor of vascular plants. This is critical for two key reasons. Firstly, it indicates that in the gametophyte generation stomata were not associated with spore dispersal. Secondly, it raises the possibility that stomata may have evolved first in the gametophyte generation of land plants. Eophyte axes are almost always less than 1 mm in diameter, many even less than 0.2 mm. Even when accounting for shrinkage of axes during fossilisation these mean axis diameters are very small. Although fossils are found as fragments and the maximum height is unknown, the plants are not inferred to have exceed a couple of centimetres[34,35]. It has been postulated that these plants were so small that they must have been dependent on currently unpreserved and therefore unknown gametophytes[124]. Furthermore, the diminutive stature of sporophytic axes has led to the hypothesis that stomata would not have played a significant role in transpiration[34,35]. It has been suggested that the primary role of stomata in sporophytes of the earliest land plants may be more analogous to some living bryophytes, where they are involved in sporangia drying, spore release[14,15] and CO_2 uptake[16,17]. However, the presence of stomata on the gametophytes of the Rhynie chert plants suggests another hypothesis. As stomata are characteristics of the gametophyte of species that phylogenetically span the origin of vascular plants, this suggests they were a characteristic of the gametophyte of the common ancestor of vascular plants. In turn this prompts the suggestion that they may have also been characteristics of the gametophyte stage of the earliest land plants (currently only known from their sporophyte stage). It is therefore a possibility that stomata may have played a role in gas exchange in the photosynthetic gametophyte, leading to the suggestion that stomata evolved first in the gametophyte.

This is a hypothesis that could be tested in the future when the gametophytes of the earliest eophytes and *Cooksonia*-like plants are discovered. If these early gametophytes are found to display stomata, it may suggest that the original function of stomata was in the gametophyte generation and was associated with gas exchange.

Another subject requiring further investigation is the evolutionary relationship between the cuticle and stomata. To the best of our knowledge there are no examples in the fossil record, or in extant genera, of cuticle-less land plants that possess stomata. However, there are examples of cuticle bearing plants that lack stomata in fossil and extant genera[2,8]. This might suggest that the cuticle evolved first or that the two structures evolved at the same time. Intriguingly, research in *Arabidopsis* has revealed that some genes involved in cuticular wax and cutin biosynthesis are also involved in the control of stomatal development[125–127], permitting co-regulation of stomatal development and cuticular properties.

Conclusions

Although there are major unresolved issues concerning the evolution and origin of stomata, phylogenetic and phylogenomic data have provided new insights into stomatal evolution. In particular, the establishment of a robust phylogeny, supporting stomatal monophyly, provides a solid framework for understanding stomatal evolution. The picture emerging suggests that stomata are ancient structures present in the earliest land plants and predate the divergence of the bryophytes and the tracheophytes. Phylogenomic data also support a loss of stomatal genes in bryophytes that took place after the divergence of bryophytes and tracheophytes. These data do not support the suggestion that stomata evolved on multiple occasions in multiple genera; instead, a single point of origin is supported.

There are inherent problems in making assumptions about stomatal function and roles in early plants, based on the behaviour of extant species that are separated from their ancestors by millions of years of evolution. Evolution is an ongoing process and it is striking that many extant bryophyte lineages are characterised by a complete loss, or ongoing loss, of stomata. In the angiosperms, the same is true in the case of seagrasses and in the pre-emergent leaves of aquatic plants[9,10,63]. The data in Figure 4 reveal that there are examples of species that fail to show hydroactive stomatal movement under the conditions tested. However, there are numerous independent reports from diverse genera showing hydroactive stomatal opening and stomatal closure. Based on these data, we reject the suggestion[80,81] that hydroactive stomatal responses evolved with the emergence of gymnosperms. Instead, we conclude that hydroactive stomatal behaviour is the ancestral state present in all lineages and likely predates the emergence of the bryophytes and tracheophytes. We suggest that lack of response is either due to secondary loss of function or conditional behaviour. Presumably, loss of stomata and/or functionality reflects evolutionary adaptation to particular environmental niches where retention of stomata offers no selective advantage. It is also clear that stomata are evolving: for example, the evolution of night-opening stomata in CAM plants[78] or in ferns where relatively recent (200 mya), in evolutionary terms, leptosporangiate ferns have, compared with the ancestral ferns, evolved to open very rapidly in response to blue light, a trait that confers selective advantages in the understory, shaded habitats in which they live[88].

Phylogenomics will continue to provide insights into stomatal evolution. To extrapolate evolutionary conclusions, physiological and functional studies will need to sample the diversity of stomata across different lineages of land plants. Characterisation of the diversity of molecular mechanisms underlying stomatal function will, in turn, contribute to our understanding of stomatal evolution. The biggest question concerning stomata remains their origin. There is a pressing need to uncover more fossils predating the divergence of bryophytes and tracheophytes, and to re-examine existing fossils. These together with fossils representing the earliest stages of bryophyte evolution should help to shed light on the origins of the stomatal pore.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online with this article at https://doi.org/10.1016/j.cub.2022.04.040.

ACKNOWLEDGEMENTS

The authors wish to acknowledge support from the Leverhulme trust (RPG-2019-004). B.J.H. and R.A.B. are grateful, respectively, to the New Phytol. Foundation and the UK BBSRC (BB/M011151/1) for the award of postgraduate studentships. T.A.W. and J.E.G. are supported by Royal Society University Research and Leverhulme Trust Senior Research Fellowships (URF/R/201024 and SRF\R1\21000149). A.J.H. was funded by a UK Research and Innovation Future Leaders Fellowship (MR/T018585/1). The authors are grateful to three anonymous reviewers who provided constructive and authoritative comments during the peer review process.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Hetherington, A.M., and Woodward, F.I. (2003). The role of stomata in sensing and driving environmental change. Nature *424*, 901–908.
2. Harris, B.J., Harrison, C.J., Hetherington, A.M., and Williams, T.A. (2020). Phylogenomic evidence for the monophyly of bryophytes and the reductive evolution of stomata. Curr. Biol. *30*, 2001–2012.
3. Renzaglia, K.S., Browning, W.B., and Merced, A. (2020). With 60 independent losses, stomata are expendable in mosses. Front. Plant Sci. *11*, 567.
4. Raven, J.A. (2002). Selection pressures on stomatal evolution. New Phytol. *153*, 371–386.
5. Hsu, P.-K., Dubeaux, G., Takahashi, Y., and Schroeder, J.I. (2021). Signalling mechanisms in abscisic acid-mediated stomatal closure. Plant J. *105*, 307–321.
6. Assmann, S.M., and Jegla, T. (2016). Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO2. Curr. Opin. Plant Biol. *33*, 157–167.
7. Lawson, T., and Matthews, J. (2020). Guard cell metabolism and stomatal function. Annu. Rev. Plant Biol. *71*, 273–302.
8. Edwards, D., Kerp, H., and Hass, H. (1998). Stomata in early land plants: an anatomical and ecophysiological approach. J. Exp. Bot. *49*, 255–278.
9. Kim, J., Joo, Y., Kyung, J., Jeon, M., Park, J.Y., Lee, H.G., Chung, D.S., Lee, E., and Lee, I. (2018). A molecular basis behind heterophylly in an amphibious plant, *Ranunculus trichophyllus*. PLoS Genet. *14*, e1007208.

10. Koga, H., Kojima, M., Takebayashi, Y., Sakakibara, H., and Tsukaya, H. (2021). Identification of the unique molecular framework of heterophylly in the amphibious plant *Callitriche palustris* L. Plant Cell *33*, 3272–3292.

11. Melotto, M., Zhang, L., Oblessuc, P.R., and He, S.H. (2017). Stomatal defense a decade later. Plant Physiol. *174*, 561–571.

12. Ye, W., Munemasa, S., Shinya, T., Wu, We, Ma, T., Lu, J., Kinoshota, T., Kaku, H., Shibuya, N., and Murata, Y. (2020). Stomatal immunity against fungal invasion comprises not only chitin-induced stomatal closure but also chitosan-induced cell death. Proc. Natl. Acad. Sci. USA *117*, 20932–20942.

13. Valentine, W. (1839). On the existence of stomata in mosses. Trans. Linn. Soc. *18*, 239–245.

14. Duckett, J.G., Pressel, S., P'ng, K.M.Y., and Renzaglia, K.S. (2009). Exploding a myth: the capsule dehiscence mechanism and the function of pseudostomata in Sphagnum. New Phytol. *183*, 1053–1063.

15. Chater, C.C., Caine, R.S., Tomek, M., Wallace, S., Kamisugi, Y., Cuming, A.C., Lang, D., MacAlister, C.A., Casson, S., Bergmann, D.C., *et al*. (2016). Origin and function of stomata in the moss *Physcomitrella patens*. Nat. Plants *2*, 16179.

16. Kubasek, J., Hajek, T., Duckett, J., Pressel, S., and Santrucek, J. (2021). Moss stomata do not respond to light and CO_2 concentration but facilitate carbon uptake by sporophytes: a gas exchange, stomatal aperture and ^{13}C-labelling study. New Phytol. *230*, 1815–1828.

17. Kubasek, J., Hajek, T., Duckett, J., Pressel, S., and Santrucek, J. (2021). Erratum for [16]. New Phytol. *231*, 2399.

18. Hetherington, A.J., and Dolan, L. (2019). Rhynie chert fossils demonstrate the independent origin and gradual evolution of lycophyte roots. Curr. Opin. Plant Biol. *47*, 119–126.

19. McElwain, J.C., and Steinthorsdottir, M. (2017). Paleoecology, ploidy, paleoatmospheric composition, and developmental biology: A review of the multiple uses of fossil stomata. Plant Physiol. *174*, 650.

20. McElwain, J.C., and Chaloner, W.G. (1995). Stomatal density and index of fossil plants track atmospheric carbon dioxide in the Palaeozoic. Ann. Bot. *76*, 389–395.

21. Rudall, P.J., Hilton, J., and Bateman, R.M. (2013). Several developmental and morphogenetic factors govern the evolution of stomatal patterning in land plants. New Phytol. *200*, 598–614.

22. Hamshaw Thomas, H., and Bancroft, N. (1913). VI. On the cuticles of some recent and fossil cycadean fronds. Trans. Linn. Soc. Lon. *8*, 155–204.

23. Kerp, H. (1990). The study of fossil gymnosperms by means of cuticular analysis. PALAIOS *5*, 548–569.

24. Masterton, J. (1994). Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science *264*, 421–424.

25. Franks, P.J., Leitch, I.J., Ruszala, E.M., Hetherington, A.M., and Beerling, D.J. (2012). Physiological framework for adaptation of stomata to CO2 from glacial to future concentrations. Philos. Trans. R. Soc. *367*, 537–546.

26. Lomax, B.H., Hilton, J., Bateman, R.M., Upchurch, G.R., Lake, J.A., Leitch, I.J., Cromwell, A., and Knight, C.A. (2014). Reconstructing relative genome size of vascular plants through geological time. New Phytol. *201*, 636–644.

27. Strother, P.K., Al-Hajri, S., and Traverse, A. (1996). New evidence for land plants from the lower Middle Ordovician of Saudi Arabia. Geology *24*, 55–58.

28. Rubinstein, C.V., Gerrienne, P., de la Puente, G.S., Astini, R.A., and Steemans, P. (2010). Early Middle Ordovician evidence for land plants in Argentina (eastern Gondwana). New Phytol. *188*, 365–369.

29. Wellman, C.H., Osterloff, P.L., and Mohiuddin, U. (2003). Fragments of the earliest land plants. Nature *425*, 282–285.

30. Salamon, M.A., Gerrienne, P., Steemans, P., Gorzelak, P., Filipiak, P., Le Hérissé, A., Paris, F., Cascales-Miñana, B., Brachaniec, T., Misz-Kennan, M., *et al*. (2018). Putative Late Ordovician land plants. New Phytol. *218*, 1305–1309.

31. Kenrick, P., and Crane, P.R. (1997). Origin and Early Diversification of Land Plants (Smithsonian Institution Press).

32. Gonez, P., and Gerrienne, P. (2010). A new definition and a lectotypification of the genus Cooksonia Lang 1937. Int. J. Plant Sci. *171*, 199–215.

33. Edwards, D., Fanning, U., and Richardson, J.B. (1986). Stomata and sterome in early land plants. Nature *323*, 438–440.

34. Edwards, D., Morris, J.L., Axe, L., Duckett, D.G., Pressel, S., and Kenrick, P. (2021). Piecing together the eophytes - a new group of ancient plants containing cryptospores. New Phytol. *233*, 1440–1455.

35. Edwards, D., Morris, J.L., Axe, L., and Duckett, J.D. (2021). Picking up the pieces: New charcoalified plant mesofossils (eophytes) from a Lower Devonian lagerstätte in the Welsh borderland, UK. Rev. Palaeobot. Palyno. *297*, 104567.

36. Edwards, D., Morris, J.L., Richardson, J.B., and Kenrick, P. (2014). Cryptospores and cryptophytes reveal hidden diversity in early land floras. New Phytol. *202*, 50–78.

37. Edwards, D., Kenrick, P., and Dolan, L. (2018). History and contemporary significance of the Rhynie cherts—our earliest preserved terrestrial ecosystem. Philos. Trans. R. Soc. B *373*, 20160489.

38. Wellman, C.H. (2018). Palaeoecology and palaeophytogeography of the Rhynie chert plants: further evidence from integrated analysis of in situ and dispersed spores. Philos. Trans. R. Soc. B *373*, 20160491.

39. Hass, H. (1991). Die Epidermis von Horneophyton lignieri (Kidston & Lang) Barghoorn & Darrah. Neues Jahrbuch für Geologie und Paläontologie. Abhandlungen *183*, 61–85.

40. El-Saadawy, W.E.-S., and Lacey, W.S. (1979). Observations on Nothia aphylla Lyon ex Høeg. Rev. Palaeobot. Palyno. *27*, 119–147.

41. Hetherington, A.J., Bridson, S.L., Lee Jones, A., Hass, H., Kerp, H., and Dolan, L. (2021). An evidence-based 3D reconstruction of *Asteroxylon mackiei* the most complex plant preserved from the Rhynie chert. eLife *10*, e69447.

42. Lele, K.M., and Walton, J. (1961). XVII. Contributions to the knowledge of *Zosterophyllum myretonianum* Penhallow from the Lower Old Red Sandstone of Angus. T. R. Soc. Edin. Earth *64*, 469–475.

43. Edwards, D., and Axe, L. (1992). Stomata and mechanics of stomatal functioning in some early land plants. Courier Forschungsinstitut Senckenberg *147*, 59–73.

44. Edwards, D. (2003). Embryophytic sporophytes in the Rhynie and Windyfield cherts. T. R. Soc. Edin. Earth *94*, 397–410.

45. Remy, W., Gensel, P.G., and Hass, H. (1993). The gametophyte generation of some early Devonian land plants. Int. J. Plant Sci. *154*, 35–58.

46. Kerp, H., Trewin, N.H., and Hass, H. (2004). New gametophytes from the Early Devonian Rhynie chert. T. R. Soc. Edin. Earth *94*, 411–428.

47. Kerp, H. (2018). Organs and tissues of Rhynie chert plants. Philos. Trans. R. Soc. B *373*, 20160495.

48. Kenrick, P. (1994). Alternation of generations in land plants: New phylogenetic and palaeobotanical evidence. Biol. Rev. *69*, 293–330.

49. Kenrick, P. (2018). Changing expressions: a hypothesis for the origin of vascular plant life cycle. Philos. Trans. R. Soc. *373*, 20170149.

50. Halle, T.G. (1916). A fossil sporogonium from the Lower Devonian of Roragen in Norway. Botaniska Notiser *79*, 81.

51. Croft, W.N., and Lang, W.H. (1942). The Lower Devonian flora of the Senni Beds of Monmouthshire and Breconshire. Philos. Trans. R. Soc. Lond. *231*, 131–163.

52. Edwards, D.S. (1986). *Aglaophyton major*, a non-vascular land-plant from the Devonian Rhynie Chert. Bot. J. Linn. Soc. *93*, 173–204.

53. Tomescu, A.M.F. (2021). Mysteries of the bryophyte-tracheophyte transition revealed: enter the eophytes. New Phytol. *233*, 1018–1021.

54. Cascales-Miñana, B., Steemans, P., Servais, T., Lepot, K., and Gerrienne, P. (2019). An alternative model for the earliest evolution of vascular plants. Lethaia *52*, 445–453.

55. Morris, J.L., Edwards, D., and Richardson, J.B. (2018). The advantages and frustrations of a plant Lagerstätte as illustrated by a new taxon from the Lower Devonian of the Welsh Borderland, UK. In Transformative Paleobotany (Elsevier), pp. 49–67.

56. Andrews, H.N., Jr. (1958). Notes on Belgian specimens of Sporogonites. Palaeobotanist *7*, 85–89.

57. Edwards, D. (1979). A late Silurian flora from the Lower Old Red Sandstone of south-west Dyfed. Palaeontology *22*, 23–52.

58. Hernick, L., Landing, E., and Bartowski, K. (2008). Earth's oldest liverworts— *Metzgeriothallus sharonae* sp. nov. from the Middle Devonian (Givetian) of eastern New York, USA. Rev. Palaeobot. Palyno. *148*, 154–162.

59. Leebens-Mack, J.H., Barker, M.S., Carpenter, E.J., Deyholos, M.K., Gitzendanner, M.A., Graham, S.W., Grosse, I., Li, Z., Melkonian, M., Mirarab, S., *et al*. (2019). One thousand plant transcriptomes and the phylogenomics of green plants. Nature *574*, 679–685.

60. Puttick, M.N., Morris, J.L., Williams, T.A., Cox, C.J., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Schneider, H., Pisani, D., and Donoghue, P.C.J. (2018). The interrelationships of land plants and the nature of the ancestral embryophyte. Curr. Biol. *28*, 733–745.

61. de Sousa, F., Foster, P.G., Donoghue, P.C.J., Schneider, H., and Cox, C.J. (2019). Nuclear protein phylogenies support the monophyly of the three bryophyte groups (Bryophyta Schimp.). New Phytol. *222*, 565–575.

62. Morris, J.L., Puttick, M.N., Clark, J.W., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Yang, Z., Schneider, H., and Donoghue, P.C.J. (2018). The timescale of early land plant evolution. Proc. Natl. Acad. Sci. USA *115*, E2274–E2283.

63. Olsen, J.L., Rouzé, P., Verhelst, B., Lin, Y.-C., Bayer, T., Collen, J., Dattolo, E., De Paoli, E., Dittami, S., Maumus, F., *et al*. (2016). The genome of the sea grass Zostera marina reveals angiosperm adaptation to the sea. Nature *530*, 331–335.

64. Zoulias, N., Harrison, E.,L., Casson, S.,A., and Gray, J.,E. (2018). Molecular control of stomatal development. Biochem. J. *475*, 441–454.

65. Han, S.-L., Kwak, J.M., and Qi, X. (2021). Stomatal lineage control by developmental program and environmental cues. Front. Plant Sci. *12*, 751852.

66. Lee, L.R., and Bergmann, D.C. (2019). The plant stomatal lineage at a glance. J. Cell Sci. *132*, 228551.

67. Qi, X., and Torii, K.U. (2018). Hormonal and environmental signals guiding stomatal development. BMC Biol. *16*, 21.

68. Hepworth, C., Caine, R.S., Harison, E.L., Sloan, J., and Gray, J.E. (2018). Stomatal development: focusing on the grasses. Curr. Opin. Plant Biol. *41*, 1–7.

69. Bowles, A.M.C., Paps, J., and Bechtold, U. (2022). Water-related innovations in land plants evolved by different patterns of gene cooption and novelty. New Phytol. https://doi.org/10.1111/nph.17981.

70. Liu, T., Ohashi-Ito, K., and Bergmann, D.C. (2009). Orthologs of *Arabidopsis* thaliana stomatal bHLH genes and regulation of stomatal development in grasses. Development *136*, 2265–2276.

71. Peterson, K.M., Rychel, A.L., and Torii, K.U. (2012). Out of the mouths of plants: The molecular basis of the evolution and diversity of stomatal development. Plant Cell *22*, 296–306.

72. Caine, R.S., Chater, C.C., Kamisugi, Y., Cuming, A.C., Beerling, D.J., Gray, J.E., and Fleming, A.J. (2016). An ancestral stomatal patterning module revealed in the non-vascular land plant *Physcomitrella patens*. Development *143*, 3306–3314.

73. Chater, C., Caine, R.S., Fleming, A.J., and Gray, J.E. (2017). Origins and evolution of stomatal development. Plant Physiol. *174*, 624–638.

74. Rensing, S.A., Goffinet, B., Meyberg, R., Wu, S.-Z., and Bezanilla, M. (2020). The moss *Physcomitrium (Physcomitrella) patens*: A model organism for non-seed plants. Plant Cell *32*, 1361–1376.

75. MacAlister, C.A., and Bergmann, D.C. (2011). Sequence and function of basic helix-loop-helix proteins required for stomatal development in *Arabidopsis* are deeply conserved in land plants. Evol. Dev. *13*, 182–192.

76. Takata, N., Yokota, K., Ohki, S., Mori, M., Taniguchi, T., and Kurita, M. (2013). Evolutionary relationship and structural characterization of the EPF/EPFL gene family. PLoS One *8*, 65183.

77. Rich, M.K., and Delaux, P.M. (2020). Plant evolution: when *Arabidopsis* is more ancestral than *Marchantia*. Curr. Biol. *30*, 642–644.

78. Hauser, F., Waadt, R., and Schroeder, J.I. (2011). Evolution of abscisic acid signalling and signalling mechanisms. Curr. Biol. *21*, 346–355.

79. Lind, C., Dreyer, I., López-Sanjurjo, E.J., von Meyer, K., Ishizaki, K., Kohchi, T., Lang, D., Zhao, Y., Kreuzer, I., Al-Rasheid, K.A.S., *et al*. (2015). Stomatal guard cells co-opted an ancient ABA-dependent desiccation survival system to regulate stomatal closure. Curr. Biol. *25*, 928–935.

80. Isner, J.C., Olteanu, V.-A., Hetherington, A.J., Coupel-Ledru, A., Sun, P., Pridgeon, A.J., Jones, G.S., Oates, M., Williams, T.A., Maathuis, F.J.M., Kift, R., *et al*. (2019). Short- and long-term effects of UVA on *Arabidopsis* are mediated by a novel cGMP phosphodiesterase. Curr. Biol. *29*, 2580–2585.

81. Chater, C., Kamisugi, Y., Movahedi, M., Fleming, A.J., Cuming, A.C., Gray, J.E., and Beerling, D.J. (2011). Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. Curr. Biol. *21*, 1025–1036.

82. Ruszala, E.M., Beerling, D.J., Franks, P.J., Chater, C.C., Casson, S.A., Gray, J.E., and Hetherington, A.M. (2011). Land plants acquired active stomatal control early in their evolutionary history. Curr. Biol. *21*, 1030–1035.

83. Sussmilch, F.C., Schultz, J., Hedrich, R., and Roelfsma, M.R.G. (2019). Acquiring control: The evolution of stomatal signalling pathways. Trends Plant Sci. *24*, 342–351.

84. Duckett, J.G., and Pressel, S. (2018). The evolution of the stomatal apparatus: Intercellular spaces and sporophyte water relations in bryophytes—two ignored dimensions. Philos. Trans. R. Soc. B *373*, 20160498.

85. Williams, T.A., Schrempf, D., Szollosi, G.J., Cox, C.J., Foster, P.G., and Embley, T.M. (2021). Inferring the deep past from molecular data. Genome Biol. Evo. *13*, evab067.

86. Males, J., and Griffiths, H. (2017). Stomatal biology of CAM plants. Plant Physiol. *174*, 550–560.

87. McElwain, J.C. (2018). Vegetation responses to past global change. Annu. Rev. Plant Biol. *69*. 761–687.

88. Brodribb, T.J., and McAdam, S.A.M. (2011). Passive origins of stomatal control in vascular plants. Science *331*, 582–585.

89. McAdam, S.A.M., and Brodribb, T.J. (2012). Fern and lycophyte guard cells do not respond to endogenous abscisic acid. Plant Cell *24*, 1510–1521.

90. Mustilli, A.-C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, G. (2002). *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species. Plant Cell *14*, 3089–3099.

91. McAdam, S.A.M., Brodribb, T.J., Banks, J.A., Hedrich, R., Atallah, N.M., Cai, C., Geringer, M.A., Lind, C., Nichols, D.S., Stachowski, K., *et al*. (2016). Abscisic acid controlled sex before transpiration in vascular plants. Proc. Natl. Acad. USA *113*, 12862–12867.

92. Plackett, A.R.G., Emms, D., Kelly, S., Hetherington, A.M., and Langdale, J.A. (2021). Conditional stomatal closure in a fern shares molecular features with flowering plant active stomatal closure. Curr. Biol. *31*, 4560–4570.e5.

93. Haworth, M., Elliott-Kingston, C., and McElwain, J.C. (2013). Co-ordination of physiological and morphological responses of stomata to elevated [CO2] in vascular plants. Oecologia *171*, 71–82.

94. Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D., Hetherington, A.M., Muller, B., and Simonneau, T. (2013). Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. Curr. Biol. *23*, 1805–1811.

95. Hörak, H., Kollist, H., and Merilo, E. (2017). Fern stomatal responses to ABA and CO2 depend on species and growth conditions. Plant Physiol. *174*, 672–679.

96. Franks, P., and Farquhar, G. (2007). The mechanical diversity of stomata and its significance in gas-exchange control. Plant Physiol. *143*, 78–87.

97. Franks, P., and Beerling, D. (2009). Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. Proc. Natl. Acad. Sci. USA *106*, 10343–10347.

98. Beerling, D., Osborne, C., and Chaloner, W. (2001). Evolution of leaf-form in land plants linked to atmospheric CO_2 decline in the Late Palaeozoic era. Nature *410*, 352–354.

99. Woodward, F. (1987). Stomatal numbers are sensitive to increases in CO2 from pre-industrial levels. Nature *327*, 617–618.

100. Vialet-Chabrand, S.R.M., Matthews, J.S.A., McAusland, L., Blatt, M.R., Griffiths, H., and Lawson, T. (2017). Temporal dynamics of stomatal behavior: modeling and implications for photosynthesis and water use. Plant Physiol. *174*, 603–613.

101. Lawson, T., and Blatt, M. (2014). Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. Plant Physiol. *164*, 1556–1570.

102. Lawson, T., and Vialet-Chabrand, S. (2018). Speedy stomata, photosynthesis and plant water use efficiency. New Phytol. *221*, 93–98.

103. Cai, S., Huang, Y., Chen, F., Zhang, X., Sessa, E., Zhao, C., Marchant, D.B., Xue, D., Chen, G., Dai, F., *et al*. (2021). Evolution of rapid blue-light response linked to explosive diversification of ferns in angiosperm forests. New Phytol. *230*, 1201–1213.

104. Schneider, H., Schuettpelz, E., Pryer, K., Cranfill, R., Magallón, S., and Lupia, R. (2004). Ferns diversified in the shadow of angiosperms. Nature *428*, 553–557.

105. Sack, F.D. (1994). Structure of the stomatal complex of the monocot *Flagellaria indica*. Am. J. Bot. *81*, 339–344.

106. McAusland, L., Vialet-Chabrand, S., Davey, P., Baker, N., Brendel, O., and Lawson, T. (2016). Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. New Phytol. *211*, 209–1220.

107. Raissig, M., Matos, J., Anleu Gil, M., Kornfeld, A., Bettadapur, A., Abrash, E., Allison, H., Badgley, G., Vogel, J., Berry, J., and Bergmann, D. (2017). Mobile MUTE specifies subsidiary cells to build physiologically improved grass stomata. Science *355*, 215–1218.

108. Raven, J. (2014). Speedy small stomata? J. Exp. Bot. *65*, 1415–1424.

109. Drake, P., Froend, R., and Franks, P. (2013). Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. J. Exp. Bot. *64*, 495–505.

110. Elliott-Kingston, C., Haworth, M., Yearsley, J., Batke, S., Lawson, T., and McElwain, J. (2016). Does size matter? Atmospheric CO_2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO_2. Front. Plant Sci. *7*, 1253.

111. Robinson, J. (1994). Speculations on carbon dioxide starvation, Late Tertiary evolution of stomatal regulation and floristic modernization. Plant Cell Environ. *17*, 345–354.

112. Büchsenschütz, K., Marten, I., Becker, D., Philippar, K., Ache, P., and Hedrich, R. (2005). Differential expression of K+ channels between guard cells and subsidiary cells within the maize stomatal complex. Planta *222*, 968–976.

113. Roelfsema, M., and Hedrich, R. (2005). In the light of stomatal opening: new insights into 'the Watergate'. New Phytol. *167*, 665–691.

114. Gray, A., Liu, L., and Facette, M. (2020). Flanking support: How subsidiary cells contribute to stomatal form and function. Front. Plant Sci. *11*, 881.

115. McElwain, J.C., and Challoner, W.G. (1995). Stomatal density and index of fossil plants track atmospheric carbon dioxide in the palaeozoic. Ann. Bot. *76*, 389–395.

116. Franks, P.J., Adams, M.A., Amthor, J.S., Barbour, M.M., Berry, J.A., Ellsworth, D.S., Farquhar, G.D., Ghannoum, O., Lloyd, J., McDowell, N., *et al*. (2013). Sensitivity of plants to changing atmospheric CO2 concentration: from the geological past to the next century. New Phytol. *197*, 1077–1094.

117. Woodward, F.I., and Kelly, C.K. (1995). The influence of CO2 concentration on stomatal density. New Phytol. *131*, 311–327.

118. Zhou, Z.-Y. (2009). An overview of fossil Ginkgoales. Palaeoworld *18*, 1–22.

119. Beerling, D.J., McElwain, J.C., and Osborner, C.P. (1998). Stomatal responses of the "living fossil" Ginkgo biloba L. to changes in atmospheric CO_2 concentrations. J. Exp. Bot. *49*, 1603–1607.

120. Casson, S.A., Franklin, K.E., Gray, J.E., Grierson, C.S., Whitelam, G.C., and Hetherington, A.M. (2009). Phytochrome B and PIF4 regulate stomatal development in response to light. Curr. Biol. *19*, 229–234.

121. Casson, S., and Hetherington, A.M. (2014). Phytochrome B is required for light-mediated systemic control of stomatal development. Curr. Biol. *24*, 1216–1221.

122. Taylor, T.N., Kerp, H., and Hass, H. (2005). Life history biology of early land plants: Deciphering the gametophyte loss. Proc. Natl. Acad. Sci. USA *102*, 5892–5897.

123. Boyce, C.K. (2008). How green was *Cooksonia*? The importance of size in understanding the early evolution of physiology in the vascular plant lineage. Paleobiology *34*, 179–194.

124. Gray, J.E., Holroyd, G.H., van der Lee, F., Bahrami, A.R., Sijmons, P.C., Woodward, F.I., Schuch, W., and Hetherington, A.M. (2000). The HIC signalling pathway links CO_2 perception to stomatal development. Nature *408*, 713–716.

125. Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G., and Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell *16*, 2463–2480.

126. Yang, S.-L., Tran, N., Tsai, M.-Y., and Kimmy Ho, C.-M. (2021). Misregulation of MYB16 expression causes stomatal cluster formation by disrupting polarity during asymmetric cell divisions. Plant Cell *34*, 455–476.

127. Pressel, S., Renzaglia, K.S., Dicky Clymo, R.S., and Duckett, J.G. (2018). Hornwort stomata do not respond actively to exogenous and environmental cues. Ann. Bot. *122*, 45–57.