
Article:
Chater, C., Caine, R.S. orcid.org/0000-0002-6480-218X, Fleming, A.J. orcid.org/0000-0002-9703-0745 et al. (1 more author) (2017) Origins and evolution of stomatal development. Plant Physiology , 174 (2). pp. 624-638. ISSN 0032-0889

https://doi.org/10.1104/pp.17.00183

Origins and evolution of stomatal development

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Word count 7366

Author contributions

C.C., B.C., A.J.F., and J.E.G. analysed and interpreted the data and wrote the manuscript. J.E.G. and A.J.F. conceived the project.

One-sentence summary Molecular genetic comparisons and manipulations of regulators of stomatal development raise the possibility of a single origin for stomata early in land plant evolution.

Advances Box

- Stomata are crucial to plant water relations and permit the entry of CO_2 for photosynthesis across many extant land plant species. The model plant Arabidopsis continues to provide a wealth of information about how plant stomatal development and stomatal patterning are regulated.
- The patchy fossil record suggests stomata are ancient and highly conserved features of land plants, but our limited knowledge of extinct taxa and ambiguous relationships between early divergent extant lineages have hampered understanding of stomatal evolutionary development.
- The field has benefited greatly from the use of molecular genetic analyses and cross-species comparisons. Studies of model species including Arabidopsis, the moss Physcomitrella, and the grass Brachypodium have shown that the molecular

signalling pathways regulating stomatal development and patterning are similar from early to recently diverging land plant taxa, raising the possibility of a single evolutionary origin for stomata.

Outstanding Questions Box

- We now know that moss have functional SMF (orthologous to Arabidopsis *SPEECHLESS*, *MUTE* and *FAMA*), SCRM and EPF1 components, and genome sequences suggest that equivalents are also present in hornworts. Do these same regulators govern stomatal development in all stomatous species?
- The liverworts do not have stomata, yet they have genes distantly related to SMF1, SCRM and EPF1. Do their encoded proteins oversee comparable processes that evolved before the evolution of stomata (*i.e.* do they share an ancestral function) or have they been co-opted after the evolution of stomata for divergent purposes?
- How far back do SMF1, SCRM and EPF1 orthologues go? Are they present in algal ancestors, and if so, what is their function?
- Stomatal development arose very early in land plant evolution but we do not know why. Was the original function of ancestral stomata to facilitate gas exchange, aid spore dispersal, or something else?

Abstract

The fossil record suggests stomata-like pores were present on the surfaces of land plants over 400 million years ago. Whether stomata arose once or whether they arose independently across newly evolving land plant lineages has long been a matter of debate. In Arabidopsis, a genetic toolbox has been identified which tightly controls stomatal development and patterning. This includes the bHLH transcription factors *SPEECHLESS*, *MUTE*, *FAMA* and *ICE/SCREAMs* (*SCRMs*) which promote stomatal formation. These factors are regulated via a signalling cascade which includes mobile *EPIDERMAL PATTERNING FACTOR* (*EPF*) peptides to enforce stomatal spacing. Mosses and hornworts, the most ancient extant lineages to possess stomata, possess orthologues of these Arabidopsis stomatal toolbox genes and manipulation in the model bryophyte *Physcomitrella patens* has shown that the bHLH and EPF components are also required for moss stomatal development and patterning. This supports an ancient and tightly conserved genetic origin of stomata. Here, we review recent discoveries and, by interrogating newly available plant genomes, we advance the story of stomatal development and patterning across land plant evolution. Furthermore, we identify potential orthologues of the key toolbox

genes in a hornwort, further supporting a single ancient genetic origin of stomata in the ancestor to all stomatous land plants.

Introduction

Stomata, microscopic turgor-driven valves formed by guard cells, are present on the aerial surfaces of most land plants (Fig. 1A-G). The regulation of stomatal apertures controls plant water loss, promotes the uptake of carbon dioxide and in many cases assists in regulating internal temperatures (Zeiger et al., 1987; Mustilli et al., 2002; Xu et al., 2016). Stomata are also a major site of pathogen entry and plant defence (Gudesblat et al., 2009). Despite their central role in so many processes, their origins and evolutionary history have long been a matter of considerable debate (Payne, 1979; Chater et al., 2011; Pressel et al., 2014; Franks and Britton-Harper, 2016; McAdam and Brodribb, 2016). Along with root-like structures, a waxy cuticle and vasculature, stomata were a key innovation that enabled plants to conquer the land (Fig. 1A) (Berry et al., 2010). The presence of stoma-like structures on very ancient land plant fossils, the absence of stomata in liverworts, the apparent secondary losses of stomata from several basal and highly derived clades, as well as developmental, morphological and physiological variation have presented plant biologists with many quandaries when interpreting how and when stomata have evolved (Haig, 2013; Rudall et al., 2013; Pressel et al., 2014). Their presence and absence across the land plant phylogeny presents difficulties in understanding major transitions in plant evolution. Owing to the apparent conflicting evidence, the fundamental question remains as to whether stomata are monophyletic in origin. Excitingly, we are now in an era where tractable genetic plant systems and corresponding sequenced genomes are plentiful and so the definitive answer to this question is close. In this review we discuss the recent literature relating to the evolution of the signalling components that regulate stomatal development and propose what future research might be needed to shed more light on the origin and role of stomata in aiding in the terrestrialisation of life on Earth.

This update focuses on the origins and evolution of the molecular and genetic machinery involved in stomatal production on the plant epidermis. Although we discuss the origins of stomatal function in the context of these new discoveries, the evolution of guard cell signalling and stomatal behaviour has recently been reviewed (Assmann and Jegla, 2016; Chen et al., 2016; Xu et al., 2016). The complex cellular processes underpinning stomatal development, also the subject of several recent reviews (Torii, 2015; Han and Torii, 2016; Simmons and Bergmann, 2016), will be outlined briefly to provide the background to the evo-devo context.

Superficial similarities, superficial differences: lessons from across the clades

The strikingly similar morphologies of stomata across evolutionary time and across extant land plants (Fig. 1 B-G) arguably belie the often stark variation that has arisen from natural selection. This variation includes differences in ontogenetic decision-making, environmental control of patterning, and final stomatal size and shape. For example, the mature stomata of equisetum and some extinct fossil lineages possess silicified radiating ribs not seen in other taxa (Cullen and Rudall, 2016), but silicification has arisen in stomata of diverse lineages (Trembath-Reichert et al., 2015). We therefore have to carefully untangle those shared phenotypes that have come about from convergent processes and those that have a genuinely shared ancestry and shared genetic module. A clear example of this issue is the evolution of epidermal cell files and stomatal rows, as can be observed in monocots such as lilies and grasses, but also in older groups such as conifers and far more ancient groups such as equisetum. By studying the similarities and differences in stomatal development and patterning between these disparate groups, we can more clearly see the pitfalls of assigning homology (or lack of homology) based on morphology and other visible/observable characteristics alone (Rudall et al., 2013; Rudall and Knowles, 2013; Cullen and Rudall, 2016). The wealth of genomic and transcriptomic data becoming available for more species across the land plant phylogeny may now allow us to probe how deep in time such similarities reach and where novel adaptations have arisen along the way. By experimentally probing the conservation of protein function and the gene networks involved in stomatal development and patterning we can more definitively assign where homology is present.

The dicotyledonous angiosperm Arabidopsis: the 'archetypal' stomatal model

Much of what we know regarding the molecular genetic control of stomatal development comes from studies involving the genetic model species *Arabidopsis thaliana* (Fig. 1G and H). Arabidopsis was the original workbench used for studying stomatal genetics and continues to provide much insight into how stomata develop and function (Yang and Sack, 1995; Chater et al., 2015; Han and Torii, 2016). Such advances have identified many of the key genetic players responsible for permitting entry into the stomatal lineage, the formation of the meristemoid and the subsequent divisions and transitions that lead to the formation of stomata (Zhao and Sack, 1999; Ohashi-Ito and Bergmann, 2006; Hara et al., 2007; MacAlister et al., 2007; Pillitteri et al., 2007; Kanaoka et al., 2008; Hunt et al., 2010; Sugano et al., 2010). The activity of the Arabidopsis meristemoid in particular has been shown to be intricately regulated by a multitude of endogenous signalling pathways and environmental cues thereby enabling control over stomatal density and spacing during development

(Chater et al., 2014; Lau et al., 2014). Owing to an extensive knowledge base, recent studies in stomatal evolutionary development and physiology invariably call on Arabidopsis to compare and contrast systems when making evolutionary interpretations (Chater et al., 2011; MacAlister and Bergmann, 2011; Caine et al., 2016; Caine et al., 2016). Our thinking is inevitably pigeon-holed, however, because Arabidopsis is a dicot angiosperm of the Brassicaceae family, and the caveat remains that apparent "deviations" from what we observe in Arabidopsis stomata may turn out to be more appropriate models for land plants as a whole. Nevertheless, several recent stomatal evolution studies strongly support Arabidopsis's continuing role in informing our thinking (Caine et al., 2016; Chater et al., 2016; Raissig et al., 2016)

Arabidopsis stomatal development: Stomatal ontogeny spelled out in genes

Like most other land plants, stomata in Arabidopsis are comprised of a pair of guard cells which surround a central pore (Fig. 1G). A regulated series of cellular divisions ensure that once mature, each stoma is typically spaced by at least one pavement cell (Fig. 1H) (Zhao and Sack, 1999; Geisler et al., 2000; Hara et al., 2007). The development of Arabidopsis stomata begins when epidermal (protodermal) stem cells are specified via group Ia basic Helix-Loop-Helix (bHLH) transcription factor SPEECHLESS (SPCH) in a heterodimeric association with its group IIIb bHLH partners, SCREAM (SCRM) or SCRM2 (also known as INDUCER OF CBF EXPRESSION1 and 2 in some studies) (MacAlister et al., 2007; Kanaoka et al., 2008). Once specified, protodermal cells transition to meristemoid mother cells (MMCs) that then asymmetrically divide, again promoted via SPCH-SCRM/SCRM2 activity, to yield a smaller meristemoid and a larger stomatal lineage ground cell (SLGC). The meristemoid can undergo a number of self-renewing amplifying divisions via continued functioning of SPCH-SCRM/SCRM2, or can transition further into the stomatal lineage to become a guard mother cell (GMC) via the actions of MUTE (a group Ia bHLH related to SPCH) again in combination with SCRM/SCRM2 (Pillitteri et al., 2007; Kanaoka et al., 2008; Pillitteri et al., 2008). For a pair of guard cells to form, a GMC must undergo a final symmetric division which is facilitated by FAMA (a third group Ia bHLH related to SPCH and MUTE) in partnership with either of the broadly functioning SCRMs (Fig. 1H) (Ohashi-Ito and Bergmann, 2006; Kanaoka et al., 2008). Concurrently, SLGCs formed by asymmetric divisions can undergo a further asymmetric spacing division to produce a satellite meristemoid which itself can advance in the stomatal lineage to yield an additional stoma, spaced by a pavement cell (Zhao and Sack, 1999).

It has become clear in Arabidopsis that for stomatal development to be correctly integrated into other aspects of development and to prevent stomata from forming adjacent to one another, a number of extracellular and plasma membrane-bound proteins are essential to co-ordinate signals between developing stomatal and epidermal pavement cells (Yang and Sack, 1995; Shpak et al., 2005; Rychel et al., 2010; Meng et al., 2015). Some of the key players include: the Epidermal Patterning Factor (EPF) and EPF-like signalling peptides, the leucine-rich-repeat (LRR) ERECTA family of membrane receptor kinases (ERECTA, ER; ERECTA-LIKE1, ERL1 and ERECTA-LIKE2, ERL2) and the LRR membrane protein TOO MANY MOUTHS (TMM) (Fig. 1H). Of importance during early stomatal development are the negatively acting EPF2 and positively acting EPFL9 (also known as STOMAGEN) peptides which compete antagonistically for binding to ERECTA family proteins (most specifically ER), an interaction modulated by TMM (Fig. 1H) (Hara et al., 2009; Hunt and Gray, 2009; Hunt et al., 2010; Kondo et al., 2010; Sugano et al., 2010; Lee et al., 2012; Lee et al., 2015). Later in the stomatal lineage EPF1 interacts with ERECTAs (primarily ERL1), again possibly overseen by TMM, to prevent GMC transitioning (Hara et al., 2007; Lee et al., 2012; Jewaria et al., 2013; Qi et al., 2017). This prevents neighbouring cells from becoming stomata, and promotes appropriate stomatal patterning and spacing. The signals transduced via EPF2 peptides are relayed via a Mitogen-Activated Protein Kinase (MAPK) signalling cascade resulting in phosphorylation and inactivation of the nuclear residing SPCH (Wang et al., 2007; Lampard et al., 2008; Lampard et al., 2009). It is still unclear as to whether MUTE and FAMA, which act later in the lineage, are also regulated via a MAPK pathway. The development and patterning modules outlined above and in Fig. 1H involve probably hundreds, if not thousands of up and downstream components for the proper development and maturation of stomata and their neighbouring cells, and are modulated further by environmental signals and feedback from other hormone pathways (Casson et al., 2009; Chater et al., 2014; Engineer et al., 2014; Lau et al., 2014; Chater et al., 2015). Nevertheless, the available molecular evidence strongly indicates that the increasingly complex picture we are uncovering of Arabidopsis stomatal development relies on a core module of genes which was first recruited in some of the earliest land plants, well over 400 million years ago (Fig. 1A) (Peterson et al., 2010; MacAlister and Bergmann, 2011; Villagarcia et al., 2012; Chater et al., 2013; Takata et al., 2013)

Angiosperm divergence in stomatal evolution: monocots versus dicots

A topical example of the extent to which a core genetic module has been tweaked and rewired over more recent evolutionary time is in the comparison between monocot and dicot

stomatal development (Raissig et al., 2016). At first sight, monocot and dicot stomata appear distinct, but to what extent do these differences in gross morphology reflect molecular divergence? The divergence of angiosperms into monocots, with parallel leaf vasculature and rows of stomata with dumb-bell-shaped guard cells, and dicots, with reticulated venation and irregularly-positioned stomata with kidney-shaped guard cells, has long been a point of botanical interest (Zeiger et al., 1987; Rudall et al., 2013). The recent explosion in genomic resources available for grasses, and the focus on monocot model species as well as grain crop genetics, has enriched our understanding of the evolution of stomatal development pathways in monocots and provided a timely contrast with the model dicot Arabidopsis (Chen et al., 2016). These studies show that the partnership between the ICE/SCRM bHLHs and the SPCH, MUTE and FAMA-like bHLHs (referred to here as SMFs) is essential for stomatal initiation and maturation in monocots, but that their protein function and regulation differ from Arabidopsis in fundamental ways (Liu et al., 2009; Raissig et al., 2016). For example, in the grass *Brachypodium distachyon* there is specialisation of ICE1 and SCRM2 functions, whereas these proteins appear to be redundant in Arabidopsis (Kanaoka et al., 2008). Similarly, a novel SPCH duplication and neofunctionalization has occurred in Brachypodium, which suggests that ancestral grass stomatal development as a whole may have come under novel evolutionary pressures ((Chen et al., 2016) and refs therein). Indeed, *SPCH* gene duplication appears to be a common theme amongst monocots (Liu et al., 2009; Chater et al., 2016), but the extent to which this represents a divergence in gene function requires further study. Recent data from the analysis of *BdMUTE* has revealed how the acquisition of protein mobility has allowed this transcription factor to acquire a function in subsidiary cell patterning in grasses, providing insight into a novel evolutionary mechanism in stomatal evolution (Raissig et al., 2017).

One-cell spacing is tightly controlled across land plants (Hara et al., 2007; Rudall et al., 2013; Caine et al., 2016), superficially appearing even more rigidly imposed in the strict cell files of the monocots. Although to-date few studies have been published which focus on the extracellular signals involved in stomatal patterning in the grasses, it appears that *EPF*, *TMM* and *ERECTA* orthologues are present within the monocots (Caine et al., 2016). As with dicots such as Arabidopsis, the monocot EPF/L peptide family is diverse and its members probably partake in both stomatal and non-stomatal processes. The presence of putative grass orthologues of Arabidopsis EPF1, EPF2 and EPFL9 (Caine et al., 2016) suggests that they too act on the SPCH-MUTE-FAMA mediated transitions that optimise stomatal spacing. However, the functions of EPF/Ls may be subtly divergent between dicots and monocots, in line with distinct differences in their stomatal developmental ontogeny. For example, in Arabidopsis, the negatively acting EPF2 regulates asymmetric entry divisions and

subsequent meristemoid activity, thereby inhibiting amplifying divisions (Hara et al., 2009; Hunt and Gray, 2009; Caine et al., 2016). Conversely, in grasses no such amplifying divisions are apparent as the asymmetric entry division leads directly to a GMC (and a SLGC). (Liu et al., 2009; Luo et al., 2012; Raissig et al., 2016). Moreover, the function of EPF1-like peptides also appears divergent between Arabidopsis and grasses, as Arabidopsis EPF1 predominantly regulates the transition from meristemoid to GMC (Hunt and Gray, 2009; Han and Torii, 2016; Qi et al., 2017), another ontogenetic step not seen in grasses (Liu et al., 2009; Luo et al., 2012). Clearly, understanding how EPF/Ls regulate stomatal development in grasses will not only expand our understanding of stomatal developmental ontogeny, but might also provide crop researchers with invaluable new stomatal phenotypes with which to study biotic and abiotic stresses in socio-economically important species.

Evidence and counter-evidence for multiple independent origins of stomata

Raven (2002) proposed the idea of a 'monophyly' of stomata and the idea has been subsequently expanded and also repeatedly put into question as molecular phylogenies and relationships between bryophytes and other basal clades have been revised (see Fig. 1A for one example) (Qiu et al., 2006; Haig, 2013; Pressel et al., 2014; Ruhfel et al., 2014; Wickett et al., 2014; Chen et al., 2016). There are several possible scenarios of stomatal origins, as proposed by Haig (2013), Pressel et al (2014), and others. These scenarios can be reconsidered in the light of recent revisions to our understanding of the land plant phylogeny (Fig. 1A). One previous consensus view of land plant evolution considers liverworts as the basal lineage followed by the evolution of the mosses, then the hornworts and then the tracheophytes (Qiu et al., 2006; Bowman, 2011). The scenarios proposed are: (1) a single origin of stomata in the ancestor of all extant land plants, but with total loss in the ancestor of the stomataless liverwort clade (Chen et al., 2016); (2) a single origin of stomata in the ancestor of mosses, hornworts and the vascular plants, as supported by evidence of conserved guard cell signalling and function (Chater et al., 2011; Ruszala et al., 2011; Haig, 2013; Franks and Britton-Harper, 2016) and (3) independent origins of stomata in the ancestor of peristomate mosses, the ancestor of the hornworts and the ancestor of modern-day tracheophytes, based on morphological and functional differences between the stomata of different lineages (Pressel et al., 2014). This latter scenario implies multiple independent origins across land plants whereby the stomata of peristomate mosses, hornworts and vascular plants evolved convergently (Pressel et al., 2014).

One problem with respect to the single origin scenarios is the absence of stomata in the basal mosses Takakia and Andreaea, as well as the presence of so-called psuedostomata in Sphagnum (Duckett et al., 2009). The secondary 'losses' of stomata in these clades, however, could be seen to parallel the loss of stomata and stoma-associated gene networks in aquatic and semi-aquatic vascular plants, such as Isoetes (Yang and Liu, 2015) or the seagrass *Zostera marina* (Olsen et al., 2016). Furthermore, such losses appear to be a common occurrence within more derived, typically-stomatous moss lineages (Egunyomi, 1982). Similarly, as Chater et al (2016) show, the genetic ablation of stomata from the moss *P. patens* results in only apparently minor fitness consequences, suggesting that under certain environmental conditions stomata might be lost.

Further potentially confusing issues which have given rise to unnecessary contention and controversy in the stomatal evo-devo literature depend on interpretations of conservation and homologous form and function. For example, it has recently been stated that there is no evidence of homology between hornwort stomata and those of peristomate mosses and vascular plants and, instead, these structures are likely to have evolved in parallel (Pressel et al., 2014). These conclusions, based on ontogenetic differences and ultrastructural and cytological considerations such as plastid development, are perhaps a little premature in the absence of molecular studies. What is clear is that when considered in the context of their development, form and function, the stomata of hornworts and indeed mosses appear to have differences compared with those found in vascular land plants (Merced and Renzaglia, 2013; Rudall et al., 2013; Pressel et al., 2014; Chater et al., 2016; Merced and Renzaglia, 2016). Such differences in the mosses and hornworts include an absence of asymmetric entry divisions and self-renewing amplifying divisions during development and the presence in these species of initially liquid-filled sub-stomatal cavities, a trait not observed in vascular land plants (Pressel et al., 2014; Merced and Renzaglia, 2016). The loss of this fluid from the sub-stomatal cavities of hornworts and perhaps mosses coincides with sporophyte maturation, perhaps aiding dehydration, dehiscence (lysis) and subsequent spore dispersal.

Singing from the same hymn sheet: functional orthology of stomatal developmental genes between land plants

The strength of molecular evo-devo and phylogenetic approaches to understanding land plant morphological evolution has been demonstrated in studies of root development (Menand et al., 2007; Jones and Dolan, 2012; Tam et al., 2015). The production of rhizoids on moss gametophytes and the production of root hairs on the sporophytes of both monocot and dicot angiosperms have been shown to be governed by deeply conserved bHLH

orthologues despite millions of years of evolutionary divergence. However, unlike with rhizoids and root hairs where deeply conserved homologous genes have been co-opted from gametophyte to sporophyte in extant land plants, stomata only feature on sporophytes.

Two recent studies indicate that there could be strong conservation in the fundamental mechanisms by which all land plants form stomata. Caine et al (2016) and Chater et al (Chater et al., 2016) show that in the moss *P. patens* (Fig. 1C), which belongs to one of the most anciently diverging land plant lineages possessing stomata (Fig. 1A), the core molecular machinery required to instigate and pattern stomata is derived from the same common ancestor as Arabidopsis. Specifically, for moss stomata to form, orthologues of a FAMA-like gene, *PpSMF1*, and an *ICE/SCRM* like gene, *PpSCRM1*, must be present; mirroring the key regulatory steps in Arabidopsis stomatal development (Chater et al., 2016). Strikingly, when either *PpSMF1* or *PpSCRM1* genes are knocked-out, moss plants fail to produce stomata. Moreover, and again similar to Arabidopsis, for moss stomata to be correctly spaced and develop properly a functioning EPF-ERECTA-TMM patterning module must be in operation (Caine et al., 2016). This molecular evidence demonstrates the conservation of a stomatal developmental toolkit between taxa separated by over 400 million years of evolution and imply a possible universality in stomata across land plants. As with rhizoids and root hairs (Jones and Dolan, 2012), the conservation of core stomatal development and patterning modules across the land plant phylogeny does not imply the absence of selective pressures during the course of evolution.

The stomatal evolution model of bHLH gene duplication and specialisation proposed by McAlister and Bergmann (2011) and evidenced by Davies et al (2014), neatly describes the ways a relatively basic form of stomatal development can give rise to the variation and complexity observed in different extant land plant lineages. This simple model, informed by the stomatal development work in *P. patens* (MacAlister and Bergmann, 2011; Caine et al., 2016; Chater et al., 2016), is invaluable for our interpretation of the divergence of stomatal form and physiology in land plants. Moreover, the confirmation of gene function in *P. patens* stomatal development gives us confidence in predicting the presence or absence of genes in as-yet unstudied lineages of plants that have stomata (Caine et al., 2016; Chater et al., 2016). Whilst we now know that *P. patens* uses orthologous development and patterning genes to set out stomata on its epidermis the exact mechanisms that enable this to happen remain elusive. For example, we know that *PpSMF1* and *PpSCRM1* are required for stomatal formation but how are these genes regulated and at what developmental stage does this occur? Do PpEPF1, PpTMM and PpERECTAs contribute to bHLH regulation using a MAPK pathway akin to vascular land plant regulation of SPCH and does this regulation occur on stomatal lineage cells prior to and or after the formation of GMC cells? Perhaps

once these questions are answered we may truly begin to understand how the described genes enable stomatal development to occur in moss.

Does stomatal patterning assist stomatal function in mosses?

In Arabidopsis, the control of stomatal patterning has been shown to directly influence plant gas-exchange, photosynthetic function, and productivity (Dow and Bergmann, 2014; Dow et al., 2014; Franks and Casson, 2014; Franks et al., 2015; Lehmann and Or, 2015). In particular, correct spacing via alterations to stomatal size and density ensures optimal guard cell pore control and faster responses to environmental cues (Dow et al., 2014). In bryophytes, stomatal spacing appears to be controlled by a less refined system involving fewer regulatory checkpoints than in vascular plants and stomatal clustering is frequently observed (Paton and Pearce, 1957; Pressel et al., 2014). Nonetheless, the conservation of the one-cell-spacing mechanism and associated EPF signalling system in mosses demonstrates a requirement for stomatal spacing, although the evolutionary drivers for a spacing mechanism are unknown. The position of moss stomata above spongy photosynthetic tissue and active stomatal aperture control suggests that moss stomatal patterning might be governed by the same evolutionary pressures as those in angiosperms, i.e. efficient gas exchange and regulation of water loss (Garner and Paolillo, 1973; Chater et al., 2011; Merced and Renzaglia, 2014). Alternatively (but not exclusively), the correct spacing of stomata around the moss sporophyte base may be important in making sporophyte capsules less vulnerable to invasion by pathogens, or in enabling efficient spore dehiscence (Paton and Pearce, 1957; Caine et al., 2016). The function(s) of moss stomata remain largely untested because of the technical difficulties associated with the small size of spore capsules. However, recently evidence to support a role for stomata in moss spore dehiscence has emerged from experiments to knock out SMF gene expression in Physcomitrella. The resulting spore capsules lacking this key regulator fail to produce stomata and show delayed spore dehiscence.

Arabidopsis adjusts stomatal density in response to sub-ambient or elevated CO_2, by modulation of EPF2 peptide levels (Engineer et al., 2014). Fossilised plant cuticles indicate that early land plants could probably respond to changes in atmospheric CO_2 concentration by altering stomatal size and density, suggesting that developmental responses to environmental cues such as CO_2 are ancient (McElwain and Chaloner, 1995; Franks and Beerling, 2009). Thus it is possible that *P. patens* uses its single orthologous EPF gene to regulate CO_2-responsive stomatal patterning in a similar way although recent studies suggest that at several moss species do not alter stomatal density (or size) in response to

CO_2 (Baars and Edwards, 2008; Field et al., 2015). The moss *PpEPF1* cannot restore stomatal spacing when expressed in Arabidopsis *epf1* (Caine et al., 2016), and it seems likely that the EPF gene family underwent a duplication in vascular land plants, and that functions diverged to allow more sophisticated and improved regulation of stomatal spacing.

Ancient stomata and associated pores

Extant plants provide extensive examples of variation in stomatal form and function, whereas the fossil record is more limited with regard to stomatal evolution. This is especially true of the bryophytes and their stomata, which are absent from the ancient land plant fossil record, although, ancient bryophyte-like plants with branching sporophytes and stomata have been recently been identified (Edwards et al., 2014). The oldest fossilised plants discovered with stomata belong to the early vascular plant Cooksonia (Edwards et al., 1992) which diverged sometime after the ancestors of the bryophytes diverged from the common land plant lineage (Fig. 1A). Intriguingly, there is fossil evidence of early land plant gametophyte stomata which may, by the authors' own interpretation, have pre-dated the emergence of extant bryophyte lineages (Remy et al., 1993). Such findings imply that stomata may have first evolved on the gametophyte and subsequently been co-opted by the sporophyte in a similar manner by which root hairs evolved from rhizoids (Jones and Dolan, 2012). However, the interpretation of Remy and colleagues (1993) is one of a number proposed and requires the characterisation of further fossils to support.

Whilst stomata are absent from extant bryophyte gametophytes, there are similar structures present on the gametophytes of extant hornworts and liverworts. These include mucilage clefts and air pores (Fig. 1B), which have at times been suggested to share homology to stomata (Zeiger et al., 1987; Villarreal and Renzaglia, 2006; Rudall et al., 2013; Villarreal and Renzaglia, 2015; Shimamura, 2016). Whilst nothing is known about the genes underpinning hornwort mucilage clefts, recent work shows that *Marchantia* liverwort pore development is controlled by genes not previously linked with stomatal differentiation (Ishizaki et al., 2013; Jones and Dolan, 2017). These include *NOPPERABO1*, a Plant U-box (PUB) E3 ubiquitin ligase, which is required for pore formation, and *MpWIP* which encodes a zinc finger protein that regulates nascent pore morphogenesis. Neither of these genes appears orthologous to those involved in stomatal development, which further supports the view that air pores and stomata are not homologous structures (Rudall et al., 2013). To date, it is unclear whether the canonical genes associated with stomatal development are present

in liverworts and hornworts. Clearly, before a definitive theory can be proposed relating to the origins of stomata in land plants, improved molecular data for basal plant taxa as well as further fossil evidence are required.

New phylogenies relating to stomatal development genes support a conservation of a core genetic module in stomatous land plants

In light of the recent findings in Physcomitrella (Caine et al., 2016; Chater et al., 2016) and following on from MacAlister and Bergmann (2011) and Ran et al (2013), we can now trace the ancestry of genes involved in the core stomatal developmental bHLH module across the plant kingdom (Fig. 2).

Using the hornwort *Anthoceros punctatus* and pseudostomate *Sphagnum fallax* genomes (Szovenyi et al., 2015; Shaw et al., 2016) and the pre-release of the liverwort *Marchantia polymorpha* genome on Phytozome V11 (Goodstein et al., 2012) we can begin to identify whether genes required for stomatal development are present in unexplored taxa and plant groups which lack stomata. Strikingly, our analyses indicate that the stomatous hornwort *A. punctatus* possesses genes closely related to both *PpSMF1* and *PpSCRM1* (Fig. 2A and D) (*N.B.*, *PpSMF2* is a *P. patens* in-paralogue and has no discernible function during stomatal development (Chater et al., 2016)). Observations of key amino acid residues in the bHLH binding domains and coiled-coil domains of the putative *A. punctatus* SMF1 and SCRM1 re-affirms that the sequences of these peptides share a very high degree of homology with both moss and other land plant orthologues (Fig. 2 B,C,E and F). This is particularly evident in the DNA binding domains, with ApSMF1 and ApSCRM1 sharing identical residues to almost all FAMA and SCRM/2 sequences identified in the other species analysed (Fig. 2B and E).

Assessment of putative stomatal associated bHLH orthologues in *M. polymorpha* and *S. fallax* revealed only genes sister to *SMF*, although orthologues of *SCRM* genes may be present. These sister *SMF* genes show clear divergence in their bHLH regions, strongly suggesting that they do not play a role in stomatal development in these species (Fig. 2B and C). The presence of air pores in *M. polymorpha* and pseudostomata in *S. fallax* invites us to speculate that these sister bHLHs may have evolved from genes that once initiated stomata in the ancestors of liverworts and sphagnum, respectively. Sequencing of more liverwort and basal moss taxa, combined with gene-function studies, could shed further light on the molecular evolution of these stoma-like structures as currently only a limited amount is known relating to the genetics underpinning air pores (Ishizaki et al., 2013; Jones and Dolan, 2017) and nothing is known about the genes underpinning pseudostomata

development. Furthermore, phylogenetic studies of genes involved in guard cell function might provide further clues as to the level of homology between gametophyte pores, pseudostomata and stomata themselves.

Assessing *SMF* gene family function in non-vascular and vascular land plant representatives

MacAlister and Bergmann (2011) and Davies and Bergmann (2014) have neatly set out a framework by which vascular land plants might have increased the complexity of their stomatal developmental modules over evolutionary time. It is hypothesised that an ancestral FAMA-like bHLH governed GMC formation (with a role akin to that of MUTE in Arabidopsis) as well as the subsequent production of guard cells (akin to FAMA) in early land plants. Subsequently, this multi-functional bHLH underwent a gene duplication resulting in a MUTE-like gene product and specialisation of the two distinct functions. A subsequent duplication event occurred in the ancestral angiosperms which led to a third SMF gene, *SPCH,* and further specialisation (Fig. 2A) (MacAlister and Bergmann, 2011; Ran et al., 2013). In grasses, an additional duplication resulted in two *SPCH*s, further partitioning the stomatal developmental program (Fig. 2A) (Liu et al., 2009; Ran et al., 2013; Raissig et al., 2016). This neofunctionalisation of the SMFs and the subsequent divergence of stomatal ontogenetic control can be seen in the comparison of moss, lycophyte, grass and dicot SMF protein domain structures (Fig. 3) (MacAlister and Bergmann, 2011; Davies and Bergmann, 2014; Raissig et al., 2016).

Arabidopsis SMF bHLHs are becoming well characterised, with key domains and motifs linked directly to protein function (Lampard et al., 2009; Davies and Bergmann, 2014; Yang et al., 2014). As expected for a transcription factor, DNA binding is critical to FAMA's role in guard cell formation. A bHLH DNA binding domain can be observed across moss, lycophyte, grass and dicot FAMA variants (Fig. 3A-D). An adjacent SQR motif may function as a phosphorylation site for a protein kinase C, and could represent regulatory point shared across all FAMA orthologues. The analysis of the domain structure of these bHLHs provides some evidence for an ancestral multifunctional bHLH (Fig. 3). New gene models suggest that *P. patens* and *S. moellendorffii* possess FAMA-like orthologues, and reveal the presence of extensive N-terminal regions which are absent from vascular land plant FAMAs (compare 3A and 3B with 3C and 3D).

The Arabidopsis SPCH MAPK target domain is C-terminal to the bHLH region. Mutations of residues within this domain lead to incorrect regulation of stomatal entry divisions (Lampard

et al., 2009; Yang et al., 2014). In *P. patens,* there is sparse evidence for this MAPK domain although one SP motif is present (Fig. 3A). *S. moellendorffii* contains SP/TP motifs in all three SmSMFs, although their lower number compared to angiosperms suggests a more restricted domain with perhaps less regulatory control (Fig. 3B, C and D). Interestingly, the presence of SP/TP motifs in BdMUTE suggests novel functionality in the grass MUTEs compared to the dicot Arabidopsis (Fig. 3C and D) and may offer insights into potential SPCH-like capabilities that have been proposed for rice OsMUTE (Liu et al., 2009).

In addition to MAPK regulation, PEST domains involved in protein degradation are important for SPCH (and possibly SCRM) regulation in Arabidopsis (Fig. 3D) (Raissig et al., 2016). Although Brachypodium SPCH proteins possess only weak conservation of PEST target sites, their presence in earlier diverging homologues suggests a regulatory mechanism that had evolved prior to the lycophytes splitting from the ancestral lineage (Fig. 3B). The *S. moellendorffii* SmSMFs could be seen as evolutionary intermediates, with putative PEST domains and MAPK target sites suggesting SPCH-like functionality, in combination with bHLH and DNA binding domains reminiscent of FAMA (Fig. 2A). In the moss PpSMF1, SPCH-like signature S/T-P motifs are very limited, and no clear PEST domains are clearly apparent yet there is clear conservation of the SQR motif and E-box DNA binding domains, suggesting that this protein is more like FAMA than SPCH (or MUTE). Clearly, functional analyses of additional non-vascular and vascular plant bHLHs are required to further understand the evolution of the SMFs and stomatal developmental ontogeny during land plant evolution.

Further evidence for the conservation of stomata via analysis of stomatal patterning genes

Intercellular signalling components that regulate the SMF/SCRM transcriptional control module, namely EPF, TMM and ERECTA, are also deeply conserved and, in the case of the EPF/Ls, have undergone considerable expansion across land plant evolution (Takata et al., 2013; Caine et al., 2016). Analysis of stomatal patterning-associated EPF peptide sequences can further inform our understanding of the origins of stomata (Fig. 4A). For example, the hornwort *A. punctatus* ApEPF1 is closely related to PpEPF1 and other stomatal acting EPFs from later diverging lineages. In contrast, the astomatous *M. polymorpha* appears to possess only a single more distantly related gene, and the pseudostomatous *S. fallax* only the EPFL4/5/6-like subgroup of the EPF peptide family. A likely interpretation of these results is that stoma-associated EPFs have been lost in the liverwort pseudostomatous basal moss lineages, but conserved in hornworts, mosses and

vascular plants. Taken together with the SMF/SCRM analysis set out in Figure 2, these observations suggest that whilst the complexity of stomatal development mechanisms has exploded in vascular plants, a more limited basic module has been retained by stomatous non-vascular land plants (Caine et al., 2016; Chater et al., 2016).

Integrating empirical and phylogenetic data to predict a model for stomatal development in the earliest land plants

The recent studies of stomatal development in *P. patens* (Caine et al., 2016; Chater et al., 2016) combined with newly available genomic data in other early diverging lineages (Fig. 2 and 4) provide a window into the very earliest mechanisms that may have been used by the extinct common ancestor of modern plants to build stomata (Fig. 4B). The production of stomata on the sporophytes of mosses and hornworts appears to require much simpler cellular processes than that of dicots (Pressel et al., 2014; Merced and Renzaglia, 2016). For example, there is no evidence for asymmetric cell divisions in either stomatal lineage. It is probable that the earliest evolving stomatal development mechanisms were also relatively uncomplicated and did not require the production of a meristemoid through an asymmetric division. These early mechanisms may have been initiated in the expanding sporophyte via the actions of an ancestral heterodimeric bHLH complex consisting of SMF and SCRM orthologues, regulating transcriptional activity in specific protodermal cells and promoting GMC and stomatal fate. To enforce stomatal patterning by cell-cell signalling prior to (and perhaps during) GMC formation, an ancestral EPF, TMM and ERECTA module arose or was co-opted. Once formed, GMCs could then undergo differentiation and finally a symmetric division to form a pair of guard cells. The same ancestral SMF/SCRM bHLH heterodimers responsible for lineage initiation may have also orchestrated the lineage conclusion. We propose that the richness and complexity that now governs plant epidermal development arose from this relatively simple program.

Conclusions and future directions

Occam's razor is a powerful tool to guide research into the origins of stomatal form and function. A single origin of a core genetic module for stomatal development in the common ancestor to hornworts, mosses, and vascular plants is arguably the most parsimonious explanation for the wealth of evidence from the fossil record and from the taxonomic, genomic, transcriptomic, and morphological data amassing from across extant land plants.

The Arabidopsis model has provided copious insight into dicot stomatal development and patterning. By applying this knowledge to outstanding evolutionary questions we are reaping the rewards of decades of molecular and genetic Arabidopsis research. These insights, from the base of the land plant tree to the most recently divergent taxa, are testament to the power of this approach. We will improve our understanding of the origins and evolutionary development of stomata as we obtain better resolution of the early land plant phylogeny and expand the range of genetic models available (see Outstanding Questions). The development of molecular genetic techniques for the liverwort Marchantia (Ishizaki et al., 2008) and the hornwort Anthoceros (Szovenyi et al., 2015) will permit a greater understanding of the relationships between ancestral clades and the acquisition of those traits that permitted the colonisation of the land. With the identification of new genes that potentially act on stomatal development, we now have an updated roadmap with which to interrogate some of the unanswered questions relating to the evolution of stomata.

Based on the current land plant phylogeny, developmental studies and phylogenies of the key genes involved in stomatal development and pattering, it would seem that the core regulatory network overseeing these processes first evolved prior to the divergence of the hornworts from the ancestral lineage. This appraisal, based on the current phylogeny, points to a single origin of stomata in land plants with subsequent losses in the liverworts and early diverging mosses. Exciting times lie ahead in truly understanding from where stomata arose nearly half a million years ago.

Acknowledgements

We are grateful to Professor J. Langdale, Dr. Eftychios Frangedakis and Dr. Steve Kelly, University of Oxford, for kindly providing access to *A. punctatus* sequence data prior to publication (these data are now available at https://www.ncbi.nlm.nih.gov/sra); and to Joanna Landymore and Jessica Dunn, University of Sheffield, for providing some of the stomatal images used in Figure 1. Our work was funded by the BBSRC and the Newton Fund-Mexican Academy of Sciences-CONACyT.

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