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1 **Origins and evolution of stomatal development**

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14 **Author contributions**

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

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

20 **Advances Box**

- 21
- 22 • Stomata are crucial to plant water relations and permit the entry of CO<sub>2</sub> for  
23 photosynthesis across many extant land plant species. The model plant Arabidopsis  
24 continues to provide a wealth of information about how plant stomatal development  
25 and stomatal patterning are regulated.
  - 26 • The patchy fossil record suggests stomata are ancient and highly conserved features  
27 of land plants, but our limited knowledge of extinct taxa and ambiguous relationships  
28 between early divergent extant lineages have hampered understanding of stomatal  
29 evolutionary development.
  - 30 • The field has benefited greatly from the use of molecular genetic analyses and cross-  
31 species comparisons. Studies of model species including Arabidopsis, the moss  
Physcomitrella, and the grass Brachypodium have shown that the molecular

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

### 35 **Outstanding Questions Box**

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

49

### 50 **Abstract**

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

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

68

## 69 **Introduction**

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

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

101 **Superficial similarities, superficial differences: lessons from across the clades**

102 The strikingly similar morphologies of stomata across evolutionary time and across extant  
103 land plants (Fig. 1 B-G) arguably belie the often stark variation that has arisen from natural  
104 selection. This variation includes differences in ontogenetic decision-making, environmental  
105 control of patterning, and final stomatal size and shape. For example, the mature stomata of  
106 equisetum and some extinct fossil lineages possess silicified radiating ribs not seen in other  
107 taxa (Cullen and Rudall, 2016), but silicification has arisen in stomata of diverse lineages  
108 (Trembath-Reichert et al., 2015). We therefore have to carefully untangle those shared  
109 phenotypes that have come about from convergent processes and those that have a  
110 genuinely shared ancestry and shared genetic module. A clear example of this issue is the  
111 evolution of epidermal cell files and stomatal rows, as can be observed in monocots such as  
112 lilies and grasses, but also in older groups such as conifers and far more ancient groups  
113 such as equisetum. By studying the similarities and differences in stomatal development and  
114 patterning between these disparate groups, we can more clearly see the pitfalls of assigning  
115 homology (or lack of homology) based on morphology and other visible/observable  
116 characteristics alone (Rudall et al., 2013; Rudall and Knowles, 2013; Cullen and Rudall,  
117 2016). The wealth of genomic and transcriptomic data becoming available for more species  
118 across the land plant phylogeny may now allow us to probe how deep in time such  
119 similarities reach and where novel adaptations have arisen along the way. By experimentally  
120 probing the conservation of protein function and the gene networks involved in stomatal  
121 development and patterning we can more definitively assign where homology is present.

122

123 **The dicotyledonous angiosperm *Arabidopsis*: the 'archetypal' stomatal model**

124 Much of what we know regarding the molecular genetic control of stomatal development  
125 comes from studies involving the genetic model species *Arabidopsis thaliana* (Fig. 1G and  
126 H). *Arabidopsis* was the original workbench used for studying stomatal genetics and  
127 continues to provide much insight into how stomata develop and function (Yang and Sack,  
128 1995; Chater et al., 2015; Han and Torii, 2016). Such advances have identified many of the  
129 key genetic players responsible for permitting entry into the stomatal lineage, the formation  
130 of the meristemoid and the subsequent divisions and transitions that lead to the formation of  
131 stomata (Zhao and Sack, 1999; Ohashi-Ito and Bergmann, 2006; Hara et al., 2007;  
132 MacAlister et al., 2007; Pillitteri et al., 2007; Kanaoka et al., 2008; Hunt et al., 2010; Sugano  
133 et al., 2010). The activity of the *Arabidopsis* meristemoid in particular has been shown to be  
134 intricately regulated by a multitude of endogenous signalling pathways and environmental  
135 cues thereby enabling control over stomatal density and spacing during development

136 (Chater et al., 2014; Lau et al., 2014). Owing to an extensive knowledge base, recent studies  
137 in stomatal evolutionary development and physiology invariably call on *Arabidopsis* to  
138 compare and contrast systems when making evolutionary interpretations (Chater et al.,  
139 2011; MacAlister and Bergmann, 2011; Caine et al., 2016; Caine et al., 2016). Our thinking  
140 is inevitably pigeon-holed, however, because *Arabidopsis* is a dicot angiosperm of the  
141 Brassicaceae family, and the caveat remains that apparent “deviations” from what we  
142 observe in *Arabidopsis* stomata may turn out to be more appropriate models for land plants  
143 as a whole. Nevertheless, several recent stomatal evolution studies strongly support  
144 *Arabidopsis*’s continuing role in informing our thinking (Caine et al., 2016; Chater et al.,  
145 2016; Raissig et al., 2016)

146

### 147 ***Arabidopsis* stomatal development: Stomatal ontogeny spelled out in genes**

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

170 It has become clear in Arabidopsis that for stomatal development to be correctly integrated  
171 into other aspects of development and to prevent stomata from forming adjacent to one  
172 another, a number of extracellular and plasma membrane-bound proteins are essential to  
173 co-ordinate signals between developing stomatal and epidermal pavement cells (Yang and  
174 Sack, 1995; Shpak et al., 2005; Rychel et al., 2010; Meng et al., 2015). Some of the key  
175 players include: the Epidermal Patterning Factor (EPF) and EPF-like signalling peptides, the  
176 leucine-rich-repeat (LRR) ERECTA family of membrane receptor kinases (ERECTA, ER;  
177 ERECTA-LIKE1, ERL1 and ERECTA-LIKE2, ERL2) and the LRR membrane protein TOO  
178 MANY MOUTHS (TMM) (Fig. 1H). Of importance during early stomatal development are the  
179 negatively acting EPF2 and positively acting EPFL9 (also known as STOMAGEN) peptides  
180 which compete antagonistically for binding to ERECTA family proteins (most specifically  
181 ER), an interaction modulated by TMM (Fig. 1H) (Hara et al., 2009; Hunt and Gray, 2009;  
182 Hunt et al., 2010; Kondo et al., 2010; Sugano et al., 2010; Lee et al., 2012; Lee et al., 2015).  
183 Later in the stomatal lineage EPF1 interacts with ERECTAs (primarily ERL1), again possibly  
184 overseen by TMM, to prevent GMC transition (Hara et al., 2007; Lee et al., 2012; Jewaria  
185 et al., 2013; Qi et al., 2017). This prevents neighbouring cells from becoming stomata, and  
186 promotes appropriate stomatal patterning and spacing. The signals transduced via EPF2  
187 peptides are relayed via a Mitogen-Activated Protein Kinase (MAPK) signalling cascade  
188 resulting in phosphorylation and inactivation of the nuclear residing SPCH (Wang et al.,  
189 2007; Lampard et al., 2008; Lampard et al., 2009). It is still unclear as to whether MUTE and  
190 FAMA, which act later in the lineage, are also regulated via a MAPK pathway. The  
191 development and patterning modules outlined above and in Fig. 1H involve probably  
192 hundreds, if not thousands of up and downstream components for the proper development  
193 and maturation of stomata and their neighbouring cells, and are modulated further by  
194 environmental signals and feedback from other hormone pathways (Casson et al., 2009;  
195 Chater et al., 2014; Engineer et al., 2014; Lau et al., 2014; Chater et al., 2015).  
196 Nevertheless, the available molecular evidence strongly indicates that the increasingly  
197 complex picture we are uncovering of Arabidopsis stomatal development relies on a core  
198 module of genes which was first recruited in some of the earliest land plants, well over 400  
199 million years ago (Fig. 1A) (Peterson et al., 2010; MacAlister and Bergmann, 2011;  
200 Villagarcia et al., 2012; Chater et al., 2013; Takata et al., 2013)

201

## 202 **Angiosperm divergence in stomatal evolution: monocots versus dicots**

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

205 stomatal development (Raissig et al., 2016). At first sight, monocot and dicot stomata appear  
206 distinct, but to what extent do these differences in gross morphology reflect molecular  
207 divergence? The divergence of angiosperms into monocots, with parallel leaf vasculature  
208 and rows of stomata with dumb-bell-shaped guard cells, and dicots, with reticulated venation  
209 and irregularly-positioned stomata with kidney-shaped guard cells, has long been a point of  
210 botanical interest (Zeiger et al., 1987; Rudall et al., 2013). The recent explosion in genomic  
211 resources available for grasses, and the focus on monocot model species as well as grain  
212 crop genetics, has enriched our understanding of the evolution of stomatal development  
213 pathways in monocots and provided a timely contrast with the model dicot *Arabidopsis*  
214 (Chen et al., 2016). These studies show that the partnership between the ICE/SCRM bHLHs  
215 and the SPCH, MUTE and FAMA-like bHLHs (referred to here as SMFs) is essential for  
216 stomatal initiation and maturation in monocots, but that their protein function and regulation  
217 differ from *Arabidopsis* in fundamental ways (Liu et al., 2009; Raissig et al., 2016). For  
218 example, in the grass *Brachypodium distachyon* there is specialisation of ICE1 and SCRM2  
219 functions, whereas these proteins appear to be redundant in *Arabidopsis* (Kanaoka et al.,  
220 2008). Similarly, a novel SPCH duplication and neofunctionalization has occurred in  
221 *Brachypodium*, which suggests that ancestral grass stomatal development as a whole may  
222 have come under novel evolutionary pressures ((Chen et al., 2016) and refs therein).  
223 Indeed, *SPCH* gene duplication appears to be a common theme amongst monocots (Liu et  
224 al., 2009; Chater et al., 2016), but the extent to which this represents a divergence in gene  
225 function requires further study. Recent data from the analysis of *BdMUTE* has revealed how  
226 the acquisition of protein mobility has allowed this transcription factor to acquire a function in  
227 subsidiary cell patterning in grasses, providing insight into a novel evolutionary mechanism  
228 in stomatal evolution (Raissig et al., 2017).

229 One-cell spacing is tightly controlled across land plants (Hara et al., 2007; Rudall et al.,  
230 2013; Caine et al., 2016), superficially appearing even more rigidly imposed in the strict cell  
231 files of the monocots. Although to-date few studies have been published which focus on the  
232 extracellular signals involved in stomatal patterning in the grasses, it appears that *EPF*, *TMM*  
233 and *ERECTA* orthologues are present within the monocots (Caine et al., 2016). As with  
234 dicots such as *Arabidopsis*, the monocot EPF/L peptide family is diverse and its members  
235 probably partake in both stomatal and non-stomatal processes. The presence of putative  
236 grass orthologues of *Arabidopsis* EPF1, EPF2 and EPFL9 (Caine et al., 2016) suggests that  
237 they too act on the SPCH-MUTE-FAMA mediated transitions that optimise stomatal spacing.  
238 However, the functions of EPF/Ls may be subtly divergent between dicots and monocots, in  
239 line with distinct differences in their stomatal developmental ontogeny. For example, in  
240 *Arabidopsis*, the negatively acting EPF2 regulates asymmetric entry divisions and

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

253

#### 254 **Evidence and counter-evidence for multiple independent origins of stomata**

255 Raven (2002) proposed the idea of a ‘monophyly’ of stomata and the idea has been  
256 subsequently expanded and also repeatedly put into question as molecular phylogenies and  
257 relationships between bryophytes and other basal clades have been revised (see Fig. 1A for  
258 one example) (Qiu et al., 2006; Haig, 2013; Pressel et al., 2014; Ruhfel et al., 2014; Wickett  
259 et al., 2014; Chen et al., 2016). There are several possible scenarios of stomatal origins, as  
260 proposed by Haig (2013), Pressel et al (2014), and others. These scenarios can be  
261 reconsidered in the light of recent revisions to our understanding of the land plant phylogeny  
262 (Fig. 1A). One previous consensus view of land plant evolution considers liverworts as the  
263 basal lineage followed by the evolution of the mosses, then the hornworts and then the  
264 tracheophytes (Qiu et al., 2006; Bowman, 2011). The scenarios proposed are: (1) a single  
265 origin of stomata in the ancestor of all extant land plants, but with total loss in the ancestor of  
266 the stomataless liverwort clade (Chen et al., 2016); (2) a single origin of stomata in the  
267 ancestor of mosses, hornworts and the vascular plants, as supported by evidence of  
268 conserved guard cell signalling and function (Chater et al., 2011; Ruzsala et al., 2011; Haig,  
269 2013; Franks and Britton-Harper, 2016) and (3) independent origins of stomata in the  
270 ancestor of peristomate mosses, the ancestor of the hornworts and the ancestor of modern-  
271 day tracheophytes, based on morphological and functional differences between the stomata  
272 of different lineages (Pressel et al., 2014). This latter scenario implies multiple independent  
273 origins across land plants whereby the stomata of peristomate mosses, hornworts and  
274 vascular plants evolved convergently (Pressel et al., 2014).

275 One problem with respect to the single origin scenarios is the absence of stomata in the  
276 basal mosses Takakia and Andreaea, as well as the presence of so-called pseudostomata in  
277 Sphagnum (Duckett et al., 2009). The secondary 'losses' of stomata in these clades,  
278 however, could be seen to parallel the loss of stomata and stoma-associated gene networks  
279 in aquatic and semi-aquatic vascular plants, such as Isoetes (Yang and Liu, 2015) or the  
280 seagrass *Zostera marina* (Olsen et al., 2016). Furthermore, such losses appear to be a  
281 common occurrence within more derived, typically-stomatous moss lineages (Egunyomi,  
282 1982). Similarly, as Chater et al (2016) show, the genetic ablation of stomata from the moss  
283 *P. patens* results in only apparently minor fitness consequences, suggesting that under  
284 certain environmental conditions stomata might be lost.

285 Further potentially confusing issues which have given rise to unnecessary contention and  
286 controversy in the stomatal evo-devo literature depend on interpretations of conservation  
287 and homologous form and function. For example, it has recently been stated that there is no  
288 evidence of homology between hornwort stomata and those of peristomate mosses and  
289 vascular plants and, instead, these structures are likely to have evolved in parallel (Pressel  
290 et al., 2014). These conclusions, based on ontogenetic differences and ultrastructural and  
291 cytological considerations such as plastid development, are perhaps a little premature in the  
292 absence of molecular studies. What is clear is that when considered in the context of their  
293 development, form and function, the stomata of hornworts and indeed mosses appear to  
294 have differences compared with those found in vascular land plants (Merced and Renzaglia,  
295 2013; Rudall et al., 2013; Pressel et al., 2014; Chater et al., 2016; Merced and Renzaglia,  
296 2016). Such differences in the mosses and hornworts include an absence of asymmetric  
297 entry divisions and self-renewing amplifying divisions during development and the presence  
298 in these species of initially liquid-filled sub-stomatal cavities, a trait not observed in vascular  
299 land plants (Pressel et al., 2014; Merced and Renzaglia, 2016). The loss of this fluid from the  
300 sub-stomatal cavities of hornworts and perhaps mosses coincides with sporophyte  
301 maturation, perhaps aiding dehydration, dehiscence (lysis) and subsequent spore dispersal.

302

### 303 **Singing from the same hymn sheet: functional orthology of stomatal developmental** 304 **genes between land plants**

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

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

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

330 The stomatal evolution model of bHLH gene duplication and specialisation proposed by  
331 McAlister and Bergmann (2011) and evidenced by Davies et al (2014), neatly describes the  
332 ways a relatively basic form of stomatal development can give rise to the variation and  
333 complexity observed in different extant land plant lineages. This simple model, informed by  
334 the stomatal development work in *P. patens* (MacAlister and Bergmann, 2011; Caine et al.,  
335 2016; Chater et al., 2016), is invaluable for our interpretation of the divergence of stomatal  
336 form and physiology in land plants. Moreover, the confirmation of gene function in *P. patens*  
337 stomatal development gives us confidence in predicting the presence or absence of genes in  
338 as-yet unstudied lineages of plants that have stomata (Caine et al., 2016; Chater et al.,  
339 2016). Whilst we now know that *P. patens* uses orthologous development and patterning  
340 genes to set out stomata on its epidermis the exact mechanisms that enable this to happen  
341 remain elusive. For example, we know that *PpSMF1* and *PpSCRM1* are required for  
342 stomatal formation but how are these genes regulated and at what developmental stage  
343 does this occur? Do PpEPF1, PpTMM and PpERECTAs contribute to bHLH regulation using  
344 a MAPK pathway akin to vascular land plant regulation of SPCH and does this regulation  
345 occur on stomatal lineage cells prior to and or after the formation of GMC cells? Perhaps

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

348

### 349 **Does stomatal patterning assist stomatal function in mosses?**

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

373 *Arabidopsis* adjusts stomatal density in response to sub-ambient or elevated CO<sub>2</sub>, by  
374 modulation of EPF2 peptide levels (Engineer et al., 2014). Fossilised plant cuticles indicate  
375 that early land plants could probably respond to changes in atmospheric CO<sub>2</sub> concentration  
376 by altering stomatal size and density, suggesting that developmental responses to  
377 environmental cues such as CO<sub>2</sub> are ancient (McElwain and Chaloner, 1995; Franks and  
378 Beerling, 2009). Thus it is possible that *P. patens* uses its single orthologous EPF gene to  
379 regulate CO<sub>2</sub>-responsive stomatal patterning in a similar way although recent studies  
380 suggest that at several moss species do not alter stomatal density (or size) in response to

381 CO<sub>2</sub> (Baars and Edwards, 2008; Field et al., 2015). The moss *PpEPF1* cannot restore  
382 stomatal spacing when expressed in *Arabidopsis epf1* (Caine et al., 2016), and it seems  
383 likely that the EPF gene family underwent a duplication in vascular land plants, and that  
384 functions diverged to allow more sophisticated and improved regulation of stomatal spacing.

385

386

### 387 **Ancient stomata and associated pores**

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

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

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

418

### 419 **New phylogenies relating to stomatal development genes support a conservation of a** 420 **core genetic module in stomatous land plants**

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

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

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

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

452

### 453 **Assessing *SMF* gene family function in non-vascular and vascular land plant** 454 **representatives**

455 MacAlister and Bergmann (2011) and Davies and Bergmann (2014) have neatly set out a  
456 framework by which vascular land plants might have increased the complexity of their  
457 stomatal developmental modules over evolutionary time. It is hypothesised that an ancestral  
458 FAMA-like bHLH governed GMC formation (with a role akin to that of MUTE in Arabidopsis)  
459 as well as the subsequent production of guard cells (akin to FAMA) in early land plants.  
460 Subsequently, this multi-functional bHLH underwent a gene duplication resulting in a MUTE-  
461 like gene product and specialisation of the two distinct functions. A subsequent duplication  
462 event occurred in the ancestral angiosperms which led to a third SMF gene, *SPCH*, and  
463 further specialisation (Fig. 2A) (MacAlister and Bergmann, 2011; Ran et al., 2013). In  
464 grasses, an additional duplication resulted in two *SPCHs*, further partitioning the stomatal  
465 developmental program (Fig. 2A) (Liu et al., 2009; Ran et al., 2013; Raissig et al., 2016).  
466 This neofunctionalisation of the SMFs and the subsequent divergence of stomatal  
467 ontogenetic control can be seen in the comparison of moss, lycophyte, grass and dicot SMF  
468 protein domain structures (Fig. 3) (MacAlister and Bergmann, 2011; Davies and Bergmann,  
469 2014; Raissig et al., 2016).

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

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

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

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

504

#### 505 **Further evidence for the conservation of stomata via analysis of stomatal patterning** 506 **genes**

507 Intercellular signalling components that regulate the SMF/SCRM transcriptional control  
508 module, namely EPF, TMM and ERECTA, are also deeply conserved and, in the case of the  
509 EPF/Ls, have undergone considerable expansion across land plant evolution (Takata et al.,  
510 2013; Caine et al., 2016). Analysis of stomatal patterning-associated EPF peptide  
511 sequences can further inform our understanding of the origins of stomata (Fig. 4A). For  
512 example, the hornwort *A. punctatus* ApEPF1 is closely related to PpEPF1 and other  
513 stomatal acting EPFs from later diverging lineages. In contrast, the astomatous *M.*  
514 *polymorpha* appears to possess only a single more distantly related gene, and the  
515 pseudostomatous *S. fallax* only the EPFL4/5/6-like subgroup of the EPF peptide family. A  
516 likely interpretation of these results is that stoma-associated EPFs have been lost in the  
517 liverwort pseudostomatous basal moss lineages, but conserved in hornworts, mosses and

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

522

### 523 **Integrating empirical and phylogenetic data to predict a model for stomatal** 524 **development in the earliest land plants**

525 The recent studies of stomatal development in *P. patens* (Caine et al., 2016; Chater et al.,  
526 2016) combined with newly available genomic data in other early diverging lineages (Fig. 2  
527 and 4) provide a window into the very earliest mechanisms that may have been used by the  
528 extinct common ancestor of modern plants to build stomata (Fig. 4B). The production of  
529 stomata on the sporophytes of mosses and hornworts appears to require much simpler  
530 cellular processes than that of dicots (Pressel et al., 2014; Merced and Renzaglia, 2016).  
531 For example, there is no evidence for asymmetric cell divisions in either stomatal lineage. It  
532 is probable that the earliest evolving stomatal development mechanisms were also relatively  
533 uncomplicated and did not require the production of a meristemoid through an asymmetric  
534 division. These early mechanisms may have been initiated in the expanding sporophyte via  
535 the actions of an ancestral heterodimeric bHLH complex consisting of SMF and SCRM  
536 orthologues, regulating transcriptional activity in specific protodermal cells and promoting  
537 GMC and stomatal fate. To enforce stomatal patterning by cell-cell signalling prior to (and  
538 perhaps during) GMC formation, an ancestral EPF, TMM and ERECTA module arose or was  
539 co-opted. Once formed, GMCs could then undergo differentiation and finally a symmetric  
540 division to form a pair of guard cells. The same ancestral SMF/SCRM bHLH heterodimers  
541 responsible for lineage initiation may have also orchestrated the lineage conclusion. We  
542 propose that the richness and complexity that now governs plant epidermal development  
543 arose from this relatively simple program.

544

### 545 **Conclusions and future directions**

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

551 The Arabidopsis model has provided copious insight into dicot stomatal development and  
552 patterning. By applying this knowledge to outstanding evolutionary questions we are reaping  
553 the rewards of decades of molecular and genetic Arabidopsis research. These insights, from  
554 the base of the land plant tree to the most recently divergent taxa, are testament to the  
555 power of this approach. We will improve our understanding of the origins and evolutionary  
556 development of stomata as we obtain better resolution of the early land plant phylogeny and  
557 expand the range of genetic models available (see Outstanding Questions). The  
558 development of molecular genetic techniques for the liverwort *Marchantia* (Ishizaki et al.,  
559 2008) and the hornwort *Anthoceros* (Szovenyi et al., 2015) will permit a greater  
560 understanding of the relationships between ancestral clades and the acquisition of those  
561 traits that permitted the colonisation of the land. With the identification of new genes that  
562 potentially act on stomatal development, we now have an updated roadmap with which to  
563 interrogate some of the unanswered questions relating to the evolution of stomata.

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

571

572

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### 580 **References**

- 581 **Assmann SM, Jegla T** (2016) Guard cell sensory systems: recent insights on stomatal responses to  
582 light, abscisic acid, and CO<sub>2</sub>. *Current Opinion in Plant Biology* **33**: 157-167  
583 **Baars C, Edwards D** (2008) Effects of elevated atmospheric CO<sub>2</sub> on spore capsules of the moss  
584 *Leptobryum pyriforme*. *Journal of Bryology* **30**: 36-40

585 **Berry JA, Beerling DJ, Franks PJ** (2010) Stomata: key players in the earth system, past and present.  
586 *Current Opinion in Plant Biology* **13**: 233-240

587 **Bowman JL** (2011) Stomata: Active Portals for Flourishing on Land. *Current Biology* **21**: R540-R541

588 **Caine R, Chater CC, Kamisugi Y, Cuming AC, Beerling DJ, Gray JE, Fleming AJ** (2016) An ancestral  
589 stomatal patterning module revealed in the non-vascular land plant *Physcomitrella patens*.  
590 *Development*

591 **Caine RS, Chater CC, Kamisugi Y, Cuming AC, Beerling DJ, Gray JE, Fleming AJ** (2016) An ancestral  
592 stomatal patterning module revealed in the non-vascular land plant *Physcomitrella patens*.  
593 *Development (Cambridge, England)* **143**: 3306-3314

594 **Casson SA, Franklin KA, Gray JE, Grierson CS, Whitelam GC, Hetherington AM** (2009) phytochrome  
595 B and PIF4 Regulate Stomatal Development in Response to Light Quantity. *Current Biology*  
596 **19**: 229-234

597 **Chater C, Gray JE, Beerling DJ** (2013) Early evolutionary acquisition of stomatal control and  
598 development gene signalling networks. *Current Opinion in Plant Biology* **16**: 638-646

599 **Chater C, Kamisugi Y, Movahedi M, Fleming A, Cuming AC, Gray JE, Beerling DJ** (2011) Regulatory  
600 Mechanism Controlling Stomatal Behavior Conserved across 400 Million Years of Land Plant  
601 Evolution. *Current Biology* **21**: 1025-1029

602 **Chater C, Peng K, Movahedi M, Dunn JA, Walker HJ, Liang YK, McLachlan DH, Casson S, Isner JC,  
603 Wilson I, Neill SJ, Hedrich R, Gray JE, Hetherington AM** (2015) Elevated CO<sub>2</sub>-Induced  
604 Responses in Stomata Require ABA and ABA Signaling. *Current Biology* **25**: 2709-2716

605 **Chater CC, Caine RS, Tomek M, Wallace S, Kamisugi Y, Cuming AC, Lang D, MacAlister CA, Casson S,  
606 Bergmann DC, Decker EL, Frank W, Gray JE, Fleming A, Reski R, Beerling DJ** (2016) Origin  
607 and function of stomata in the moss *Physcomitrella patens*. *Nature plants* **2**: 16179-16179

608 **Chater CCC, Oliver J, Casson S, Gray JE** (2014) Putting the brakes on: abscisic acid as a central  
609 environmental regulator of stomatal development. *New Phytologist* **202**: 376-391

610 **Chen Z-H, Chen G, Dai F, Wang Y, Hills A, Ruan Y-L, Zhang G, Franks PJ, Nevo E, Blatt MR** (2016)  
611 Molecular Evolution of Grass Stomata. *Trends in plant science*

612 **Cullen E, Rudall PJ** (2016) The remarkable stomata of horsetails (*Equisetum*): patterning,  
613 ultrastructure and development. *Annals of Botany* **118**: 207-218

614 **Davies KA, Bergmann DC** (2014) Functional specialization of stomatal bHLHs through modification of  
615 DNA-binding and phosphoregulation potential. *Proceedings of the National Academy of  
616 Sciences of the United States of America* **111**: 15585-15590

617 **Dow GJ, Bergmann DC** (2014) Patterning and processes: how stomatal development defines  
618 physiological potential. *Curr Opin Plant Biol* **21**: 67-74

619 **Dow GJ, Bergmann DC, Berry JA** (2014) An integrated model of stomatal development and leaf  
620 physiology. *New Phytologist* **201**: 1218-1226

621 **Duckett JG, Pressel S, P'ng KMY, Renzaglia KS** (2009) Exploding a myth: the capsule dehiscence  
622 mechanism and the function of pseudostomata in *Sphagnum*. *New Phytologist* **183**: 1053-  
623 1063

624 **Edwards D, Davies KL, Axe L** (1992) A vascular conducting strand in the early land plant *Cooksonia*.  
625 *Nature* **357**: 683-685

626 **Edwards D, Morris JL, Richardson JB, Kenrick P** (2014) Cryptospores and cryptophytes reveal hidden  
627 diversity in early land floras. *New Phytologist* **202**: 50-78

628 **Egunyomi A** (1982) On the stomata of some tropical african mosses. *Lindbergia* **8**: 121-124

629 **Engineer CB, Ghassemian M, Anderson JC, Peck SC, Hu HH, Schroeder JI** (2014) Carbonic  
630 anhydrases, EPF2 and a novel protease mediate CO<sub>2</sub> control of stomatal development.  
631 *Nature* **513**: 246+

632 **Field KJ, Duckett JG, Cameron DD, Pressel S** (2015) Stomatal density and aperture in non-vascular  
633 land plants are non-responsive to above-ambient atmospheric CO<sub>2</sub> concentrations. *Annals  
634 of Botany* **115**: 915-922

635 **Franks PJ, Beerling DJ** (2009) Maximum leaf conductance driven by CO<sub>2</sub> effects on stomatal size and  
636 density over geologic time. *Proceedings of the National Academy of Sciences of the United*  
637 *States of America* **106**: 10343-10347

638 **Franks PJ, Britton-Harper ZJ** (2016) No evidence of general CO<sub>2</sub> insensitivity in ferns: one stomatal  
639 control mechanism for all land plants? *New Phytologist* **211**: 819-827

640 **Franks PJ, Casson S** (2014) Connecting stomatal development and physiology. *New Phytologist* **201**:  
641 1079-1082

642 **Franks PJ, Doheny-Adams TW, Britton-Harper ZJ, Gray JE** (2015) Increasing water-use efficiency  
643 directly through genetic manipulation of stomatal density. *New Phytologist* **207**: 188-195

644 **Garner DLB, Paolillo DJ** (1973) On the functioning of stomates in *Funaria*. *Bryologist* **76**: 423-427

645 **Geisler M, Nadeau J, Sack FD** (2000) Oriented asymmetric divisions that generate the stomatal  
646 spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. *Plant Cell*  
647 **12**: 2075-2086

648 **Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U,**  
649 **Putnam N, Rokhsar DS** (2012) Phytozome: a comparative platform for green plant genomics.  
650 *Nucleic Acids Res* **40**

651 **Gudesblat GE, Torres PS, Vojnov AA** (2009) Stomata and pathogens: Warfare at the gates. *Plant*  
652 *signaling & behavior* **4**: 1114-1116

653 **Haig D** (2013) Filial mistletoes: the functional morphology of moss sporophytes. *Annals of Botany*  
654 **111**: 337-345

655 **Han S-K, Torii KU** (2016) Lineage-specific stem cells, signals and asymmetries during stomatal  
656 development. *Development (Cambridge, England)* **143**: 1259-1270

657 **Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T** (2007) The secretory peptide gene EPF1  
658 enforces the stomatal one-cell-spacing rule. *Genes & Development* **21**: 1720-1725

659 **Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T** (2009) Epidermal  
660 Cell Density is Autoregulated via a Secretory Peptide, EPIDERMAL PATTERNING FACTOR 2 in  
661 *Arabidopsis* Leaves. *Plant and Cell Physiology* **50**: 1019-1031

662 **Hunt L, Bailey KJ, Gray JE** (2010) The signalling peptide EPFL9 is a positive regulator of stomatal  
663 development. *New Phytologist* **186**: 609-614

664 **Hunt L, Gray JE** (2009) The Signaling Peptide EPF2 Controls Asymmetric Cell Divisions during  
665 Stomatal Development. *Current Biology* **19**: 864-869

666 **Ishizaki K, Chiyoda S, Yamato KT, Kohchi T** (2008) Agrobacterium-mediated transformation of the  
667 haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell*  
668 *Physiol* **49**: 1084-1091

669 **Ishizaki K, Mizutani M, Shimamura M, Masuda A, Nishihama R, Kohchi T** (2013) Essential Role of  
670 the E3 Ubiquitin Ligase NOPPERABO1 in Schizogenous Intercellular Space Formation in the  
671 Liverwort *Marchantia polymorpha*. *The Plant Cell* **25**: 4075-4084

672 **Jewaria PK, Hara T, Tanaka H, Kondo T, Betsuyaku S, Sawa S, Sakagami Y, Aimoto S, Kakimoto T**  
673 (2013) Differential Effects of the Peptides Stomagen, EPF1 and EPF2 on Activation of MAP  
674 Kinase MPK6 and the SPCH Protein Level. *Plant and Cell Physiology* **54**: 1253-1262

675 **Jones VA, Dolan L** (2017) MpWIP regulates air pore complex development in the liverwort  
676 *Marchantia polymorpha*. *Development*

677 **Jones VAS, Dolan L** (2012) The evolution of root hairs and rhizoids. *Annals of Botany* **110**: 205-212

678 **Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu JK, Torii KU** (2008)  
679 SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to *Arabidopsis*  
680 stomatal differentiation. *Plant Cell* **20**: 1775-1785

681 **Kondo T, Kajita R, Miyazaki A, Hokoyama M, Nakamura-Miura T, Mizuno S, Masuda Y, Irie K,**  
682 **Tanaka Y, Takada S, Kakimoto T, Sakagami Y** (2010) Stomatal Density is Controlled by a  
683 Mesophyll-Derived Signaling Molecule. *Plant and Cell Physiology* **51**: 1-8

684 **Lampard GR, Lukowitz W, Ellis BE, Bergmann DC** (2009) Novel and Expanded Roles for MAPK  
685 Signaling in Arabidopsis Stomatal Cell Fate Revealed by Cell Type-Specific Manipulations.  
686 *Plant Cell* **21**: 3506-3517

687 **Lampard GR, Macalister CA, Bergmann DC** (2008) Arabidopsis stomatal initiation is controlled by  
688 MAPK-mediated regulation of the bHLH SPEECHLESS. *Science* **322**: 1113-1116

689 **Lau OS, Davies KA, Chang J, Adrian J, Rowe MH, Ballenger CE, Bergmann DC** (2014) Direct roles of  
690 SPEECHLESS in the specification of stomatal self-renewing cells. *Science* **345**: 1605-1609

691 **Lee JS, Hnilova M, Maes M, Lin YCL, Putarjuna A, Han SK, Avila J, Torii KU** (2015) Competitive  
692 binding of antagonistic peptides fine-tunes stomatal patterning. *Nature* **522**: 435-+

693 **Lee JS, Kuroha T, Hnilova M, Khatayevich D, Kanaoka MM, McAbee JM, Sarikaya M, Tamerler C,  
694 Torii KU** (2012) Direct interaction of ligand-receptor pairs specifying stomatal patterning.  
695 *Genes & Development* **26**: 126-136

696 **Lehmann P, Or D** (2015) Effects of stomata clustering on leaf gas exchange. *New Phytologist* **207**:  
697 1015-1025

698 **Liu T, Ohashi-Ito K, Bergmann DC** (2009) Orthologs of Arabidopsis thaliana stomatal bHLH genes and  
699 regulation of stomatal development in grasses. *Development* **136**: 2265-2276

700 **Luo L, Zhou WQ, Liu P, Li CX, Hou SW** (2012) The development of stomata and other epidermal cells  
701 on the rice leaves. *Biologia Plantarum* **56**: 521-527

702 **MacAlister CA, Bergmann DC** (2011) Sequence and function of basic helix-loop-helix proteins  
703 required for stomatal development in Arabidopsis are deeply conserved in land plants.  
704 *Evolution & Development* **13**: 182-192

705 **MacAlister CA, Ohashi-Ito K, Bergmann DC** (2007) Transcription factor control of asymmetric cell  
706 divisions that establish the stomatal lineage. *Nature* **445**: 537-540

707 **McAdam SAM, Brodribb TJ** (2016) Linking Turgor with ABA Biosynthesis: Implications for Stomatal  
708 Responses to Vapor Pressure Deficit across Land Plants. *Plant Physiology* **171**: 2008-2016

709 **McElwain JC, Chaloner WG** (1995) Stomatal Density and Index of Fossil Plants Track Atmospheric  
710 Carbon Dioxide in the Palaeozoic. *Annals of Botany* **76**: 389-395

711 **Menand B, Yi KK, Jouannic S, Hoffmann L, Ryan E, Linstead P, Schaefer DG, Dolan L** (2007) An  
712 ancient mechanism controls the development of cells with a rooting function in land plants.  
713 *Science* **316**: 1477-1480

714 **Meng XZ, Chen X, Mang HG, Liu CL, Yu X, Gao XQ, Torii KU, He P, Shan LB** (2015) Differential  
715 Function of Arabidopsis SERK Family Receptor-like Kinases in Stomatal Patterning. *Current*  
716 *Biology* **25**: 2361-2372

717 **Merced A, Renzaglia K** (2014) Developmental changes in guard cell wall structure and pectin  
718 composition in the moss *Funaria*: implications for function and evolution of stomata. *Annals*  
719 *of Botany* **114**: 1001-1010

720 **Merced A, Renzaglia KS** (2013) Moss stomata in highly elaborated *Oedipodium* (*Oedipodiaceae*) and  
721 highly reduced *Ephemerum* (*Pottiaceae*) sporophytes are remarkably similar. *American*  
722 *Journal of Botany* **100**: 2318-2327

723 **Merced A, Renzaglia KS** (2016) Patterning of stomata in the moss *Funaria*: a simple way to space  
724 guard cells. *Annals of Botany* **117**: 985-994

725 **Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J** (2002) Arabidopsis OST1 protein kinase  
726 mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive  
727 oxygen species production. *Plant Cell* **14**: 3089-3099

728 **Ohashi-Ito K, Bergmann DC** (2006) Arabidopsis FAMA controls the final proliferation/differentiation  
729 switch during stomatal development. *Plant Cell* **18**: 2493-2505

730 **Olsen JL, Rouze P, Verhelst B, Lin YC, Bayer T, Collen J, Dattolo E, De Paoli E, Dittami S, Maumus F,  
731 Michel G, Kersting A, Lauritano C, Lohaus R, Topel M, Tonon T, Vanneste K, Amirebrahimi  
732 M, Brakel J, Bostrom C, Chovatia M, Grimwood J, Jenkins JW, Jueterbock A, Mraz A, Stam  
733 WT, Tice H, Bornberg-Bauer E, Green PJ, Pearson GA, Procaccini G, Duarte CM, Schmutz J,**

734 **Reusch TBH, Van de Peer Y** (2016) The genome of the seagrass *Zostera marina* reveals  
735 angiosperm adaptation to the sea. *Nature* **530**: 331-+

736 **Paton J, Pearce J** (1957) The occurrence, structure and functions of the stomata in British  
737 bryophytes *In*, Vol 3. British Bryological Society, pp 228-259

738 **Paton JA, Pearce JV** (1957) The occurrence, structure and functions of the stomata in British  
739 bryophytes. *Trans Brit Bryol Soc* **3**: 228-259

740 **Payne WW** (1979) Stomatal patterns in embryophytes - Their evolution, ontogeny and  
741 interpretation. *Taxon* **28**: 117-132

742 **Peterson KM, Rychel AL, Torii KU** (2010) Out of the Mouths of Plants: The Molecular Basis of the  
743 Evolution and Diversity of Stomatal Development. *Plant Cell* **22**: 296-306

744 **Pillitteri LJ, Bogenschutz NL, Torii KU** (2008) The bHLH protein, MUTE, controls differentiation of  
745 stomata and the hydathode pore in *Arabidopsis*. *Plant Cell Physiol* **49**: 934-943

746 **Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU** (2007) Termination of asymmetric cell division and  
747 differentiation of stomata. *Nature* **445**: 501-505

748 **Pressel S, Goral T, Duckett JG** (2014) Stomatal differentiation and abnormal stomata in hornworts.  
749 *Journal of Bryology* **36**: 87-103

750 **Qi X, Han S-KI, Dang JH, Garrick JM, Ito M, Hofstetter AK, Torii KU** (2017) Autocrine regulation of  
751 stomatal differentiation potential by EPF1 and ERECTA-LIKE1 ligand-receptor signaling. *eLife*  
752 **6**: e24102

753 **Qiu YL, Li L, Wang B, Chen Z, Knoop V, Groth-Malonek M, Dombrowska O, Lee J, Kent L, Rest J**  
754 (2006) The deepest divergences in land plants inferred from phylogenomic evidence. *Proc*  
755 *Natl Acad Sci USA* **103**

756 **Qiu YL, Li LB, Wang B, Chen ZD, Knoop V, Groth-Malonek M, Dombrowska O, Lee J, Kent L, Rest J,**  
757 **Estabrook GF, Hendry TA, Taylor DW, Testa CM, Ambros M, Crandall-Stotler B, Duff RJ,**  
758 **Stech M, Frey W, Quandt D, Davis CC** (2006) The deepest divergences in land plants inferred  
759 from phylogenomic evidence. *Proceedings of the National Academy of Sciences of the*  
760 *United States of America* **103**: 15511-15516

761 **Raissig MT, Abrash E, Bettadapur A, Vogel JP, Bergmann DC** (2016) Grasses use an alternatively  
762 wired bHLH transcription factor network to establish stomatal identity. *Proceedings of the*  
763 *National Academy of Sciences* **113**: 8326-8331

764 **Ran JH, Shen TT, Liu WJ, Wang XQ** (2013) Evolution of the bHLH Genes Involved in Stomatal  
765 Development: Implications for the Expansion of Developmental Complexity of Stomata in  
766 Land Plants. *Plos One* **8**: 11

767 **Raven JA** (2002) Selection pressures on stomatal evolution. *New Phytologist* **153**: 371-386

768 **Remy W, Gensel PG, Hass H** (1993) The Gametophyte Generation of Some Early Devonian Land  
769 Plants. *International Journal of Plant Sciences* **154**: 35-58

770 **Rudall PJ, Hilton J, Bateman RM** (2013) Several developmental and morphogenetic factors govern  
771 the evolution of stomatal patterning in land plants. *New Phytologist* **200**: 598-614

772 **Rudall PJ, Knowles EVW** (2013) Ultrastructure of stomatal development in early-divergent  
773 angiosperms reveals contrasting patterning and pre-patterning. *Annals of Botany* **112**: 1031-  
774 1043

775 **Ruhfel BR, Gitzendanner MA, Soltis PS, Soltis DE, Burleigh JG** (2014) From algae to angiosperms–  
776 inferring the phylogeny of green plants (Viridiplantae) from 360 plastid genomes. *BMC*  
777 *Evolutionary Biology* **14**: 1-27

778 **Ruszala EM, Beerling DJ, Franks PJ, Chater C, Casson SA, Gray JE, Hetherington AM** (2011) Land  
779 Plants Acquired Active Stomatal Control Early in Their Evolutionary History. *Current Biology*  
780 **21**: 1030-1035

781 **Rychel AL, Peterson KM, Torii KU** (2010) Plant twitter: ligands under 140 amino acids enforcing  
782 stomatal patterning. *Journal of Plant Research* **123**: 275-280

783 **Shaw AJ, Schmutz J, Devos N, Shu S, Carrell AA, Weston DJ** (2016) Chapter Five - The Sphagnum  
784 Genome Project: A New Model for Ecological and Evolutionary Genomics. *In* AR Stefan, ed,  
785 Advances in Botanical Research, Vol Volume 78. Academic Press, pp 167-187  
786 **Shimamura M** (2016) *Marchantia polymorpha*: Taxonomy, Phylogeny and Morphology of a Model  
787 System. *Plant Cell Physiol* **57**: 230-256  
788 **Shpak ED, McAbee JM, Pillitteri LJ, Torii KU** (2005) Stomatal patterning and differentiation by  
789 synergistic interactions of receptor kinases. *Science* **309**: 290-293  
790 **Simmons AR, Bergmann DC** (2016) Transcriptional control of cell fate in the stomatal lineage.  
791 *Current Opinion in Plant Biology* **29**: 1-8  
792 **Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Hara-Nishimura I** (2010) Stomagen  
793 positively regulates stomatal density in *Arabidopsis*. *Nature* **463**: 241-U130  
794 **Szovenyi P, Frangedakis E, Ricca M, Quandt D, Wicke S, Langdale JA** (2015) Establishment of  
795 *Anthoceros agrestis* as a model species for studying the biology of hornworts. *Bmc Plant*  
796 *Biology* **15**: 7  
797 **Takata N, Yokota K, Ohki S, Mori M, Taniguchi T, Kurita M** (2013) Evolutionary Relationship and  
798 Structural Characterization of the EPF/EPFL Gene Family. *PLoS ONE* **8**: e65183  
799 **Tam THY, Catarino B, Dolan L** (2015) Conserved regulatory mechanism controls the development of  
800 cells with rooting functions in land plants. *Proceedings of the National Academy of Sciences*  
801 *of the United States of America* **112**: E3959-E3968  
802 **Torii KU** (2015) Stomatal differentiation: the beginning and the end. *Curr Opin Plant Biol* **28**: 16-22  
803 **Trembath-Reichert E, Wilson JP, McGlynn SE, Fischer WW** (2015) Four hundred million years of  
804 silica biomineralization in land plants. *Proceedings of the National Academy of Sciences of*  
805 *the United States of America* **112**: 5449-5454  
806 **Villagarcia H, Morin A-C, Shpak ED, Khodakovskaya MV** (2012) Modification of tomato growth by  
807 expression of truncated ERECTA protein from *Arabidopsis thaliana*. *Journal of experimental*  
808 *botany* **63**: 6493-6504  
809 **Villarreal AJ, Renzaglia KS** (2006) Structure and development of *Nostoc* strands in *Leiosporoceros*  
810 *dussii* (Anthocerotophyta): a novel symbiosis in land plants. *Am J Bot* **93**: 693-705  
811 **Villarreal JC, Renzaglia KS** (2015) The hornworts: important advancements in early land plant  
812 evolution. *Journal of Bryology* **37**: 157-170  
813 **Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S** (2007) Stomatal Development and Patterning Are  
814 Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in *Arabidopsis*.  
815 *The Plant Cell* **19**: 63-73  
816 **Wickett NJ, Mirarab S, Nguyen N, Warnow T, Carpenter E, Matasci N, Ayyampalayam S, Barker MS,**  
817 **Burleigh JG, Gitzendanner MA, Ruhfel BR, Wafula E, Der JP, Graham SW, Mathews S,**  
818 **Melkonian M, Soltis DE, Soltis PS, Miles NW, Rothfels CJ, Pokorny L, Shaw AJ, DeGironimo**  
819 **L, Stevenson DW, Surek B, Villarreal JC, Roure B, Philippe H, dePamphilis CW, Chen T,**  
820 **Deyholos MK, Baucom RS, Kutchan TM, Augustin MM, Wang J, Zhang Y, Tian Z, Yan Z, Wu**  
821 **X, Sun X, Wong GK-S, Leebens-Mack J** (2014) Phylotranscriptomic analysis of the origin and  
822 early diversification of land plants. *Proceedings of the National Academy of Sciences* **111**:  
823 E4859-E4868  
824 **Xu ZZ, Jiang YL, Jia BR, Zhou GS** (2016) Elevated-CO<sub>2</sub> Response of Stomata and Its Dependence on  
825 Environmental Factors. *Frontiers in Plant Science* **7**: 15  
826 **Yang K-Z, Jiang M, Wang M, Xue S, Zhu L-L, Wang H-Z, Zou J-J, Lee E-K, Sack F, Le J** (2014)  
827 Phosphorylation of Serine 186 of bHLH Transcription Factor SPEECHLESS Promotes Stomatal  
828 Development in *Arabidopsis*. *Molecular Plant* **8**: 783-795  
829 **Yang M, Sack FD** (1995) The too many mouths and four lips mutations affect stomatal production in  
830 *Arabidopsis*. *Plant Cell* **7**: 2227-2239  
831 **Yang T, Liu X** (2015) Comparing photosynthetic characteristics of *Isoetes sinensis* Palmer under  
832 submerged and terrestrial conditions. *Scientific Reports* **5**: 17783  
833 **Zeiger E, Farquhar GD, Cowan IR** (1987) Stomatal function. Stanford University Press, Stanford, Calif.

834 **Zhao LM, Sack FD** (1999) Ultrastructure of stomatal development in *Arabidopsis* (Brassicaceae)  
835 leaves. *American Journal of Botany* **86**: 929-939  
836