Evolutionary Conservation of ABA Signaling for Stomatal Closure in Ferns

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Summary

ABA-driven stomatal regulation reportedly evolved after the divergence of ferns, during the early evolution of seed plants approximately 360 Mya. This hypothesis is based on the observation that the stomata of certain fern species are unresponsive to ABA, but exhibit passive hydraulic control. However, ABA-induced stomatal closure was detected in some mosses and lycophytes. Here, we observed that a number of ABA signaling and membrane transporter protein families diversified over the evolutionary history of land plants. The aquatic ferns *Azolla filiculoides* and *Salvinia cucullata* have representatives of 23 families of proteins orthologous to those of *Arabidopsis thaliana* and all other land plant species studied. Phylogenetic analysis of the key ABA signaling proteins indicates an evolutionarily conserved stomatal response to ABA. Moreover, comparative transcriptomic analysis has identified a suite of ABA responsive genes that differentially expressed in a terrestrial fern species, *Polystichum proliferum*. These genes encode proteins associated with ABA biosynthesis, transport, reception, transcription, signaling, and ion and sugar transport, which fit the general ABA signaling pathway constructed from *Arabidopsis thaliana* and *Hordeum vulgare*. The retention of these key ABA-responsive genes could have had a profound effect on the adaptation of ferns to dry conditions. Furthermore, stomatal assays have shown the primary evidence for ABA-induced closure of stomata in two terrestrial fern species *P. proliferum* and *Nephrolepis exaltata*. In summary, we report new molecular and physiological evidence for the presence of active stomatal control in ferns.

Keywords: comparative genomics, gene expression, ion transporters, stomatal evolution, stomatal closure

One sentence summary

New evidence for ABA-induced stomatal closure in fern and known evidence in earlier diverging lineages does not support the hypothesis that stomatal responsiveness to ABA evolved first in seed plants.

List of author contributions

Z.H.C. conceived research plans and designed the experiments; S.C. and G.C. performed the experiments; S.C., G.C., Y.W., Y.H., Q.Y., D.B.M., G.J., D.X. and Z.H.C. analyzed the data; Z.H.C. and M.R.B. wrote the article with contributions of all the authors.

Introduction

Fossil plants have provided insights into the broader physiological and ecological adaptations that enable land plants to reduce water loss via specialized morphological structures (Edwards et al., 1998; Raven, 2002). Early land plants were exposed to high levels of UV radiation, elevated temperatures and extremely dry soils. Thus, adaptations to regulate gas exchange via active control mechanisms would have helped avoid catastrophic dehydration and allow early land plants to survive long enough to reproduce (Ruszala et al., 2011). Stomata were a key innovation in the earliest phases of the colonization of land by terrestrial plants, an event that fundamentally altered the global landscape and climate. Stomata have been observed in Silurian fossils of sporophytes and gametophytes of early land plants (Raven, 2002). The presence of structurally defined stomata in the epidermis of above-ground organs of sporophytes appears to be the ancestral condition for terrestrial plants (Qiu et al., 1998; Raven, 2002). That these structures appear more or less unchanged for over 400 million years (Edwards et al., 1998; Beerling and Franks, 2009) suggests that they are an essential adaptation to terrestrial plant life. Land plants acquired stomata to regulate gas exchange by opening and closing of the stomatal pore (Hetherington and Woodward, 2003; Berry et al., 2010; Chater et al., 2011). While liverworts lack stomata, mosses and most other land plants have a typical stomatal pore surrounded by at least a pair of guard cells (Franks and Farquhar, 2007; Chen et al., 2017).

The response of stomatal guard cells to hydraulic and non-hydraulic signaling supports a fundamental role of abscisic acid (ABA) in plant signaling in a changing environment (Brodribb and McAdam, 2011; Pantin et al., 2013). ABA induces stomatal closure in many seed plant species (Willmer and Fricker, 1996; Wolf et al., 2006; Chen et al., 2012b; Wang et al., 2013; Chen et al., 2016). ABA-induced stomatal closure has also been reported in a lycophyte, *Selaginella uncinata* (Ruszala et al., 2011) and the mosses:

86 *Physcomitrella patens* and *Funaria hygrometrica*, but not in all species of mosses and
87 lycophytes (Brodribb and McAdam, 2011; Merced and Renzaglia, 2014; Lind et al., 2015).
88 However, the role of ABA in stomatal regulation in the other major clades of land plants is
89 still under debate. A major shift in the stomatal control process between lycophytes and ferns
90 versus seed plants (gymnosperms and angiosperms) has been proposed; fern and lycophyte
91 guard cells lack responsiveness to endogenous ABA, which evolved subsequently in seed
92 plants (Brodribb and McAdam, 2011; McAdam and Brodribb, 2012a,b; McAdam et al.
93 2016). The ecological significance of this change is that seed plants may have a greater
94 capacity than lycophytes and ferns to actively control water loss during drought (McElwain,
95 2011). However, molecular evidence supporting this shift is still lacking (Chater et al., 2011;
96 Ruszala et al., 2011; Chater et al., 2013; Lind et al., 2015; Chen et al., 2017).

97 Investigations of ABA signaling and membrane transport to date have focused largely
98 on angiosperms (Blatt, 2000). ABA metabolism and transport, ABA perception and signal
99 transduction, and ABA signal response and modulation are parts of the ABA signaling
100 pathway in plants (Cutler et al., 2010; Hauser et al., 2011). ABA metabolism includes genes
101 encoding zeaxanthin epoxidases (ZEPs) (Marin et al., 1996), 9-cis-epoxycarotenoid
102 dioxygenase (NCEDs) (North et al., 2007), abscisic aldehyde oxidases (AAOs) (Seo et al.,
103 2000) that are crucial for ABA biosynthesis, and *CYP707As* encoding ABA 8'-hydroxylases
104 in the ABA catabolic pathway (Saito et al., 2004). ABA transport is mediated by the ATP
105 binding cassette transporters ABCG25 and ABCG40 (Kang et al., 2010; Kuromori et al.,
106 2010) and nitrate transporter NRT1.2 (Kanno et al., 2012). Cytosolic ABA perception consists
107 of Pyrabactin resistance (PYR)/ PYR Like (PYL)/regulatory component of ABA receptor
108 (RCAR) (Ma et al., 2009; Park et al., 2009), protein phosphatase 2Cs (PP2Cs) (Schweighofer,
109 2004) and snf1-related protein kinase 2 (SnRK2) (Umezawa et al., 2009). RCAR–PP2C
110 complex formation leads to inhibition of PP2C activity, thereby allowing activation of

111 SnRK2s. Two G protein-coupled receptors-Type G proteins GTG1 and GTG2 were identified
112 as plasma membrane ABA receptors (Pandey et al., 2009). ABA-induced reactive oxygen
113 species (ROS) and nitric oxide (NO) production down-regulates the activity of the PP2C
114 phosphatases and activates Ca^{2+}-permeable channels and anion channels (Grabov and Blatt,
115 1998; Hamilton, et al., 2000; Köhler and Blatt 2002; Garcia-Mata et al., 2003; Wang et al.,
116 2013; Chen et al., 2016). Interactions with the H_2S gasotransmitter have also been implicated
117 to overlap with ABA regulation of guard cell ion transport (Papanatsiou et al., 2015). During
118 ABA-induced stomatal closure, elevated cytosolic Ca^{2+} activates Ca^{2+}-dependent protein
119 kinases (CDPKs) that activate Ca^{2+} channel (Harmon et al., 2000) and directly phosphorylate
120 PP2Cs and targets like slow anion channels (SLAC1/SLAHs) (Vahisalu et al., 2008; Geiger et
121 al., 2009, 2010). While the ABA pathways are firmly resolved in angiosperms, a
122 comprehensive comparative study in ferns, sister group to the seed plants, is needed to
123 address the conflicting results (Brodribb and McAdam, 2011; Hanada et al., 2011; Ruszala et
124 al., 2011).

125 The evolutionary timing of the acquisition of active stomatal control has emerged as a
126 key question because of its importance for understanding the influence on the rise of global
127 vegetation. In this study we addressed fundamental questions regarding stomatal regulation in
128 ferns, a group that to date has been understudied, so as to better evaluate the evolution of this
129 key adaptation across land plants. Was active stomatal regulation retained in ferns, following
130 its origin in early land plants? We utilized genomic, transcriptomic and physiological tools to
131 test the hypothesis that ABA-induced stomatal closure governed by membrane transporters
132 and ABA signaling components is ancestral in ferns. Our results suggest that genes of ABA
133 reception and signaling components are found in all stomata-bearing terrestrial plants
134 including ferns. Our molecular and physiological evidence indicates that active stomatal
135 control is present not only in seed plants but also in early-diverged extant vascular plants and

136 ferns. These findings discount previous claims (McAdam et al., 2016) that ferns lack the
137 necessary signaling components for active, ABA-mediated stomatal closure.

138

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Results

Genomic evidence of an essential set of ABA signaling genes and proteins in ferns

To identify potential orthologues of 63 ABA reception and membrane transport genes known to be involved in stomatal regulation in *A. thaliana*, we performed bioinformatics analyses of predicted stomatal ABA receptor and membrane transport genes in 23 gene families across 36 species (including chlorophytic and streptophytic green algae, read algae and plants; Figures 1 and 2). There was an overall increase in the number of these genes and gene families from algae to seed plants (Figure 1). To avoid potential bias when using a single selection criterion for the comparative analysis of candidate genes, we tested a range of selection criteria (e.g. E-value<10^{-10} and E-value<10^{-5} with or without query coverage>50%) that all give comparable results (data not shown). Using a simple selection criterion of E-value<10^{-5}, we found that the water fern species *Azolla filiculoides* and *Salvinia cucullata* have orthologues of all 23 gene families (Figure 2). Among those 23 gene families, we found orthologues of 109 and 71 putative stomatal guard cell genes and 364 and 233 genes in the 23 families of *A. filiculoides* and *S. cucullata,* respectively (Figure 2A and 2B; Tables S1, S2, S3 and S4). The corresponding numbers are 336 and 138 genes in all 23 tested families for *A. thaliana* and a charophyte alga, *Klebsormidium flaccidum*, respectively (Figure 2A; Tables S1 and S2). Even with the strict selection criteria (E-value<10^{-10} and query coverage>50%), *A. filiculoides* and *S. cucullata* have at least one putative guard cell gene in 19 and 17 of the 23 ABA reception and membrane transport gene families, respectively (Figure 2B; Tables S3 and S4). The number of genes for water ferns were similar to those of the other non-seed plants such as the lycophyte *S. moellendorffii* and moss *P. patens*, but much higher than those for the representative algal species (Figures 1 and 2).

We next used the predicted protein sequences of RCAR11s, PP2Cs, SnRK2s, SLAC1s, and vacuolar H^+-pyrophosphatase 1 (AVP1s) in 36 species for phylogenetic

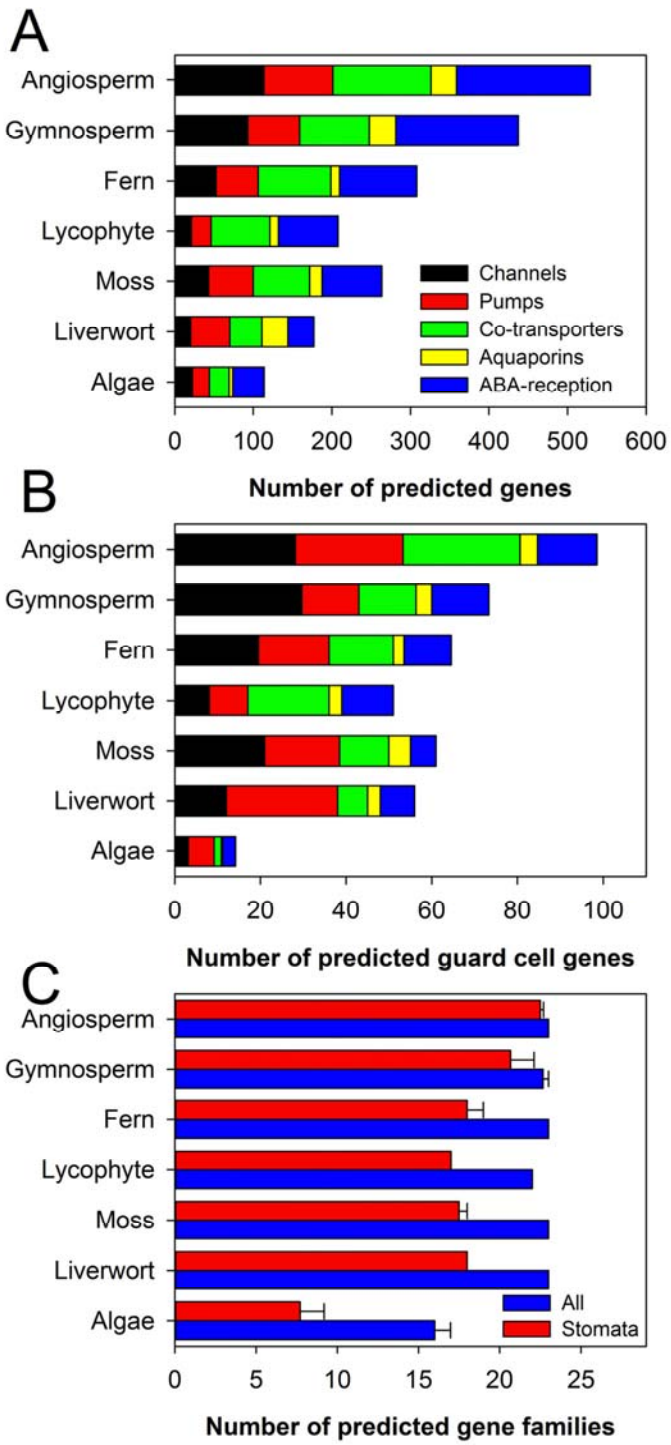

Figure 1. Number of predicted membrane transporters and ABA reception complex proteins and families in different taxa.

analysis. Core ABA signaling has been evolutionarily present in land plants including ferns (Figures 2 and 3). All the PP2C and SnRK2s protein families have been identified across all tested land plant and algal species, however, RCARs are not found in the seven algae

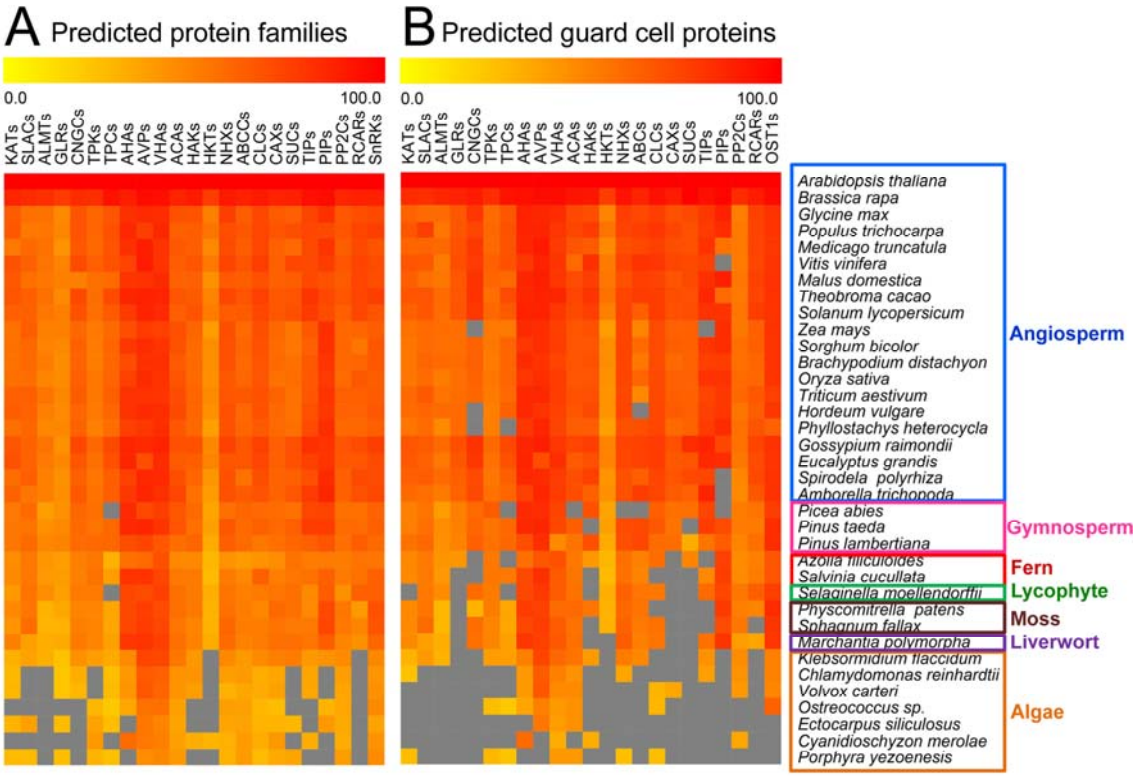

Figure 2. Similarity heat map for the evolution of membrane transporters and ABA reception complex proteins in different species.

examined (Figures 2 and 3). Importantly, it appears that the two key ion transporters SLAC1 and AVP1 evolved in concert with green plant evolution (Figure 3D and 3E) as plants adapted to land.

Fern retains ABA responsive genes for active stomatal control

The water fern species examined may not respond to ABA due to their unusual guard cell and pore development (Busby and Gunning, 1984). We therefore used two terrestrial fern species *Polystichum proliferum* and *Nephrolepis exaltata* (both are more typical of ferns in terms of morphology and habitat) in the following experiments. Well-established ABA responsive genes and signaling pathway in *A. thaliana* (Table S5) were used as a control for the ABA-induced gene expression in epidermal layers of *P. proliferum.* The results showed that ferns share core ABA metabolism, reception, signaling and membrane transporter genes (Figures 4 and 5) with *A. thaliana, H. vulgare, O. sativa, P. patens* and *K. flaccidum* (Table S5). In

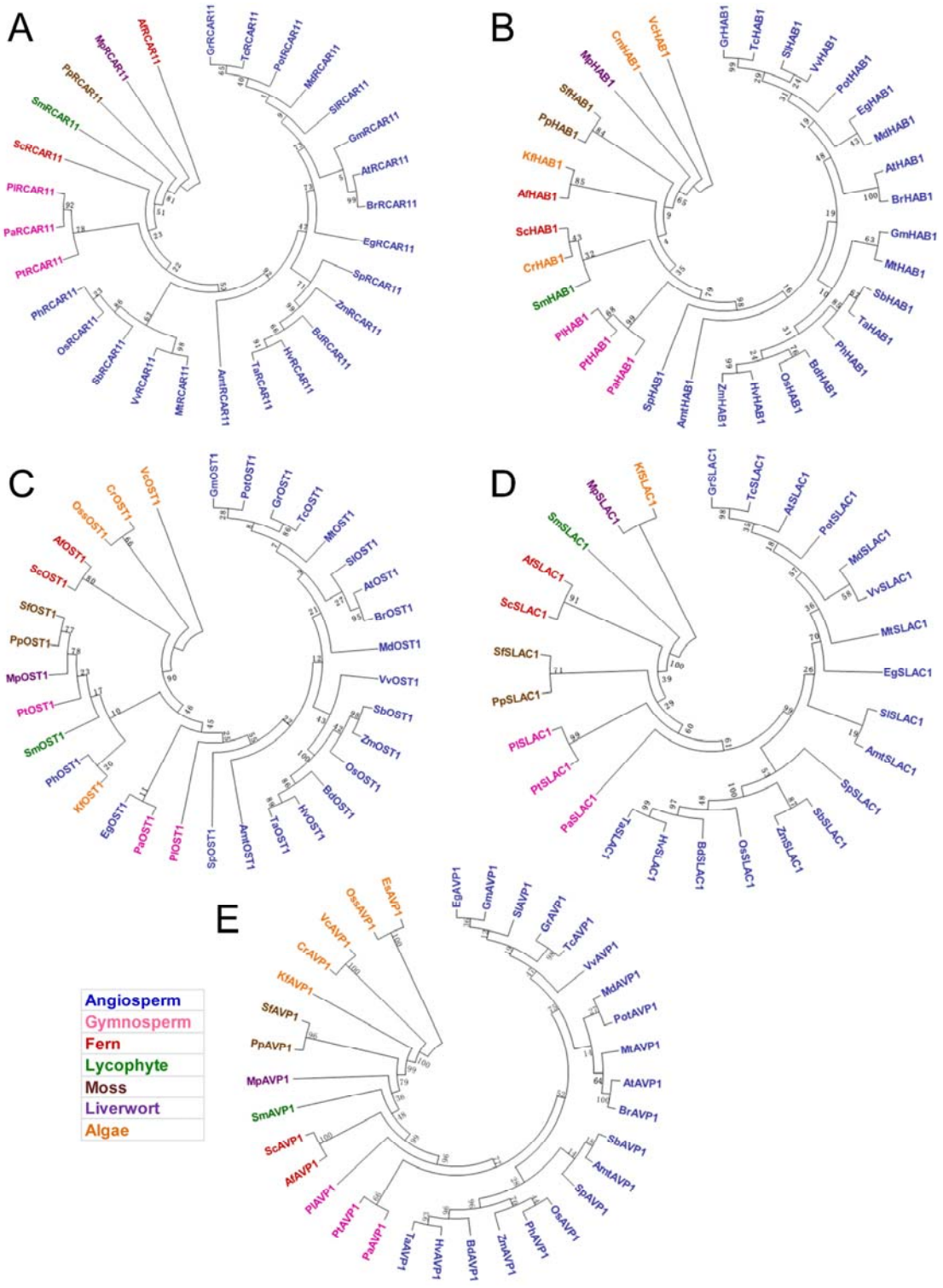

Figure 3. Phylogenetic trees of key ABA signaling proteins in species of plants and algae.

quantitative Real Time PCR (qPCR) experiments, all 40 selected key genes (Table S6) were successfully amplified from the ABA-treated lower epidermis of *P. proliferum* and many orthologous genes in *A. thaliana* guard cells were significantly up-regulated or down-

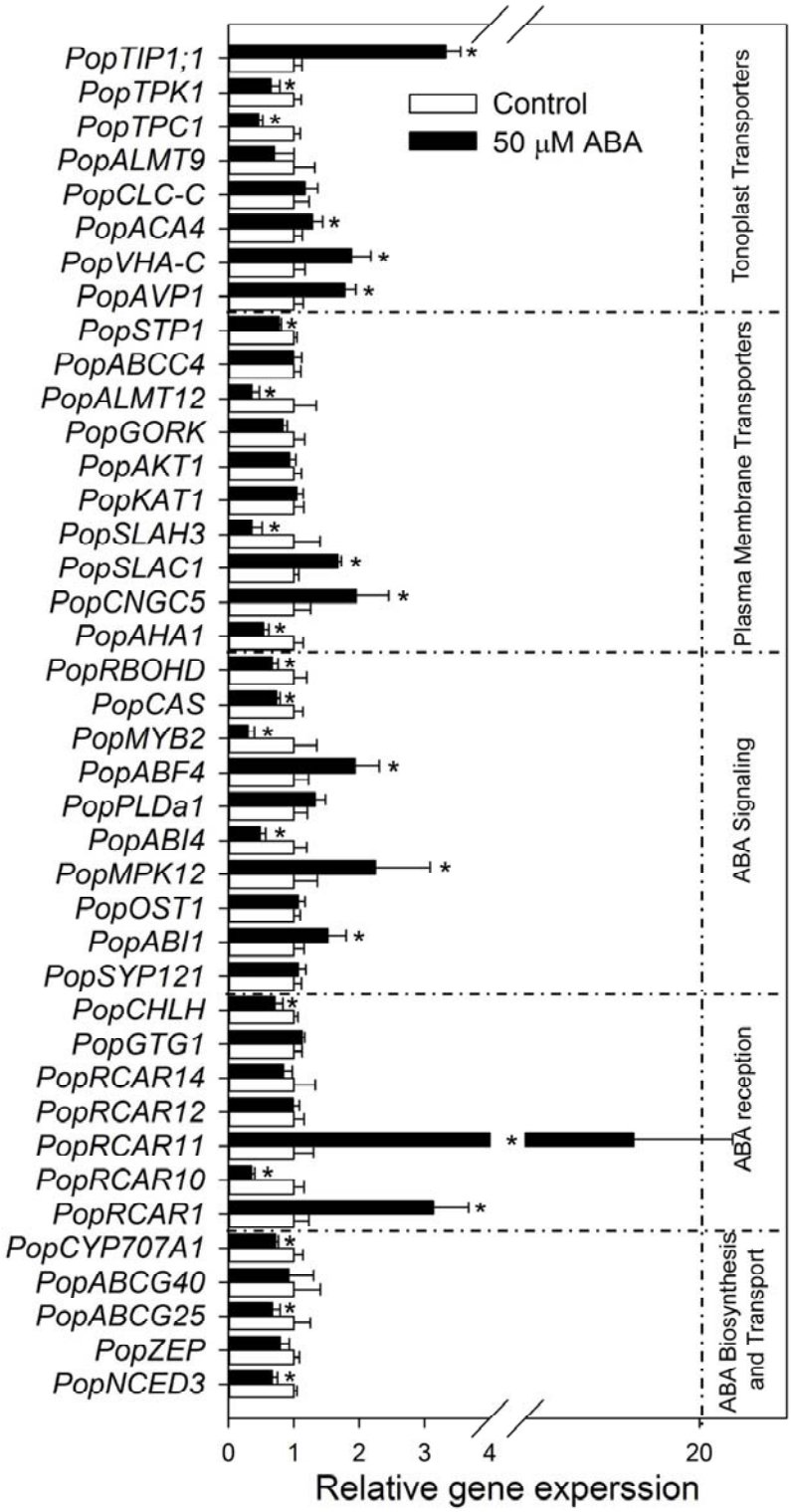

Figure 4. Relative expression of ABA signaling genes in epidermal peels of *Polystichum proliferum*.

regulated in *P. proliferum* compared to the control (Figure 4). We detected at least one gene family member in *P. proliferum* representing each core node of the ABA signaling pathways (Figures 4 and 5).

186 *PopNCED3* and *PopZEP*, two key genes of ABA biosynthesis, were significantly
187 down-regulated when ABA was applied to *P. proliferum*. The expression levels of *P.*
188 *proliferum* orthologues *PopABCG25* and *PopABCG40*, which transport ABA, were slightly
189 reduced by ABA treatment. The expression of ABA catabolic gene, *PopCYP707A1* was
190 significantly reduced upon ABA treatment (Figure 4). ABA treatment resulted in up-
191 regulation of ABA receptor genes *PopRCAR1* and *PopRCAR11* up to 18-fold while the
192 expression of *PopRCAR10* was significantly reduced. The orthologue of the plasma
193 membrane ABA receptor gene *PopGTG1* showed no response to ABA. Moreover, the
194 orthologue of *ABA insensitive 1* (*PopABI1*) was significantly upregulated and *open stomata 1*
195 (*PopOST1*) was little affected. Surprisingly, the transcripts of *mitogen-activated protein*
196 *kinase 12* (Jammes et al., 2009) *PopMPK12* in *P. proliferum* was doubled by ABA treatment.
197 Among other signaling components for ABA response, transcription factors *ABA insensitive 4*
198 (*PopABI4*) and *myb domain protein 2* (*PopMYB2*) were down-regulated while ABRE binding
199 factor 4 (*PopABF4*) was significantly up-regulated by ABA. The Calcium sensor (Han et al.,
200 2003) *PopCAS* and the ROS homeostasis gene *PopRBOHD* were both down-regulated in
201 ABA-treated epidermis of *P. proliferum* (Figure 4).

202 Most of the transporter genes were differentially regulated in leaf epidermis of *P.*
203 *proliferum* after ABA treatment. Expressed orthologues of *A. thaliana* guard cell *S-type anion*
204 *channel homolog 3* (*PopSLAH3*), *PopALMT12*, Ca^{2+} *activated vacuolar* K^{+} *channel*
205 (*PopTPK1*), and *slow vacuolar* Ca^{2+} *channel* (*PopTPC1*) were significantly down-regulated
206 whilst *PopSLAC1* and *cyclic nucleotide gated channel 5* (*PopCNGC5*) was up-regulated upon
207 ABA treatment. Importantly, the plasma membrane H^{+}*-ATPase 1* (*PopAHA1*) and *sucrose/*H^{+}
208 *cotransporter 1* (*PopSTP1*) were significantly down-regulated, but the expression of *vacuolar*
209 H^{+}*-pyrophosphatase 1* (*PopAVP1*) and *vacuolar* H^{+}*-ATPase C* (*PopVHA-C*) was up-
210 regulated by ABA. Furthermore, a key ABA responsive water channel encoding *tonoplast*

intrinsic protein 1;1 (*TIP1;1*) gene was up-regulated by more than 300% (Figure 4). These observations indicate a set of signaling pathways that regulate gene expression in ways similar to that of angiosperms. However, the fact that these genes may be regulated similarly does not necessarily mean that the responsiveness of guard cells to ABA is the same in ferns.

Comparative transcriptomic analysis reveals conserved genes for active stomatal regulation across land plant lineages

To identify genes potentially involved in stomatal regulation along the green plant tree of life, we first interrogated a collection of microarray and RNA-sequencing (RNA-seq) datasets from the angiosperms *A. thaliana* (Leonhardt et al., 2004; Wang et al., 2011) and *O. sativa* (Lenka et al., 2011), the moss *P. patens* (Stevenson et al., 2016) and the green algae *K. flaccidum* (Holzinger et al., 2014). We also conducted qPCR on the fern *P. proliferum* (Figure 4) and RNA-seq on the angiosperm *H. vulgare* (Figure 5). The combined results further supported our hypothesis that the ABA signaling pathway is conserved throughout the examined plants despite only a limited number of genes tested with qPCR in *P. proliferum* (Figures 4 and 5; Table S6). The ABA- or drought-induced transcriptomic data showed that there are a few thousand genes up-regulated or down-regulated and that these genes vary among species. However, key genes are conserved across the studied species (Table S5).

The presence of a gene in an epidermal preparation does not mean that it is expressed in guard cells or that it participates in guard cell ABA signaling. However, our recent work on comparative analysis of RNA-sequencing of epidermal peels and guard cell protoplasts has shown similar transcriptome profiles in *A. thaliania* (Zhao et al. unpublished). Here we isolated RNA from lower leaf epidermal peels of *P. proliferum* and *H. vulgare*. The use of *H. vulgare* genotypes has yielded a total of 6,676 differentially expressed genes (DEGs) from epidermal peels (Table S5). The data provided clear transcriptome profiles of stomatal-specific transcripts. RNA-seq on whole leaves is not likely to have good representation of

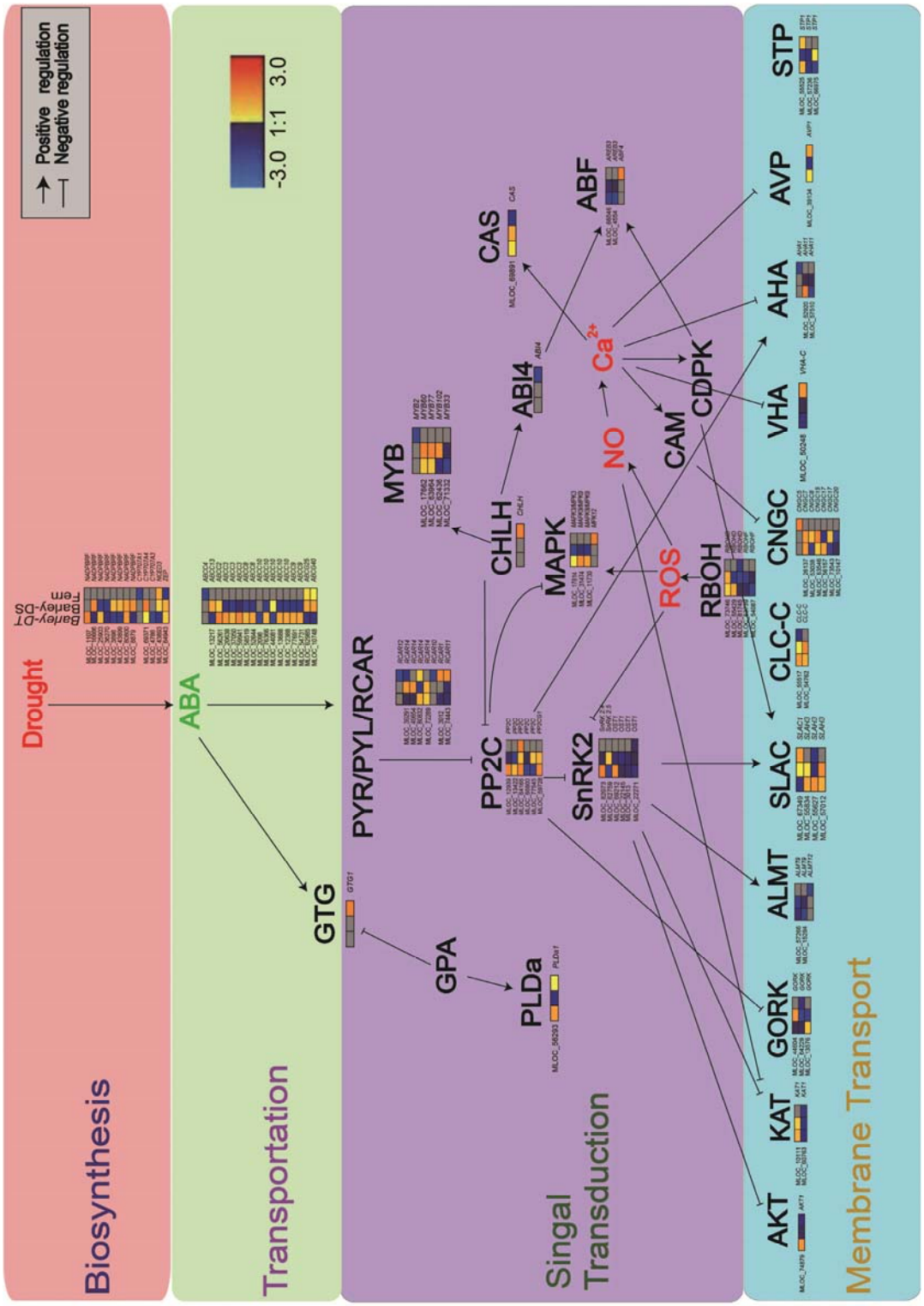

Figure 5. Conserved ABA signaling pathway in epidermis of *Hordeum vulgare* and *Polystichum proliferum*.

guard cell DEGs due to "masking" effects from the low ratio (1/100) of guard cells to all other leaf cells (Zhao et al. unpublished). Epidermal peels reduce the ratio of guard cells to other cells ratio to ~1/5, which significantly increases the detection of guard cell transporter

239 transcripts that are usually in low abundance (Chen et al., 2016). In our RNA-seq
240 experiments on *H. vulgare* epidermal peels, 579 ABA signaling, 55 ABA biosynthesis and
241 transport, 87 ABA reception components, and 117 membrane transport DEGs were detected
242 (Figure 5). Based on the RNA-seq of *H. vulgare* leaf epidermal peels, we were able to
243 construct an ABA signaling pathway containing most of the key ABA-responsive genes
244 identified in drought-tolerant and drought-sensitive *H. vulgare* genotypes and in *P. proliferum*
245 (Figure 5). Interestingly, qPCR data collected from leaf epidermal peels of *P. proliferum* were
246 able to fit into most of the important nodes of the ABA signaling pathways (Figure 5).
247 Therefore, these data provided transcriptomic evidence that stomata in *P. proliferum* may
248 respond to ABA.

249 **ABA induces stomatal closure in two fern species**

250 Despite the genomic and transcriptomic findings on the conservation of ABA-responsive
251 genes and signaling pathways, this evidence alone does not prove that the stomata of ferns
252 respond to ABA. Therefore, we explored the physiological impact of ABA on stomatal
253 closure in two fern species. We used a well-established methodology adapted from stomatal
254 assay and electrophysiology (Blatt et al., 1990; Chater et al., 2011; Chen et al., 2012a), which
255 was different from the previously published work in ferns (Brodribb and McAdam, 2011;
256 McAdam and Brodribb, 2012a,b). In epidermal peels of the two fern species *P. proliferum*
257 and *N. exaltata,* most of the stomata were open during the day in a growth chamber (Figures
258 6-8). Stomata of *P. proliferum* and *N. exaltata* showed only a slight decrease in the measuring
259 buffer for a period of 120 min under the microscopic light at 100 μmol m^{-2} s^{-1}
260 photosynthetically active radiation (PAR) (Figure 6A and 6B). Stomatal sensitivity was also
261 tested with 10 mM $CaCl_2$ (commonly used to trigger Ca^{2+}-induced stomatal closure), which
262 led to enhanced stomatal closure (Figure 6A and 6B). Most importantly, stomatal aperture
263 was significantly decreased ($P<0.01$) by 30.8% and 44.9% at 60 min and 120 min,

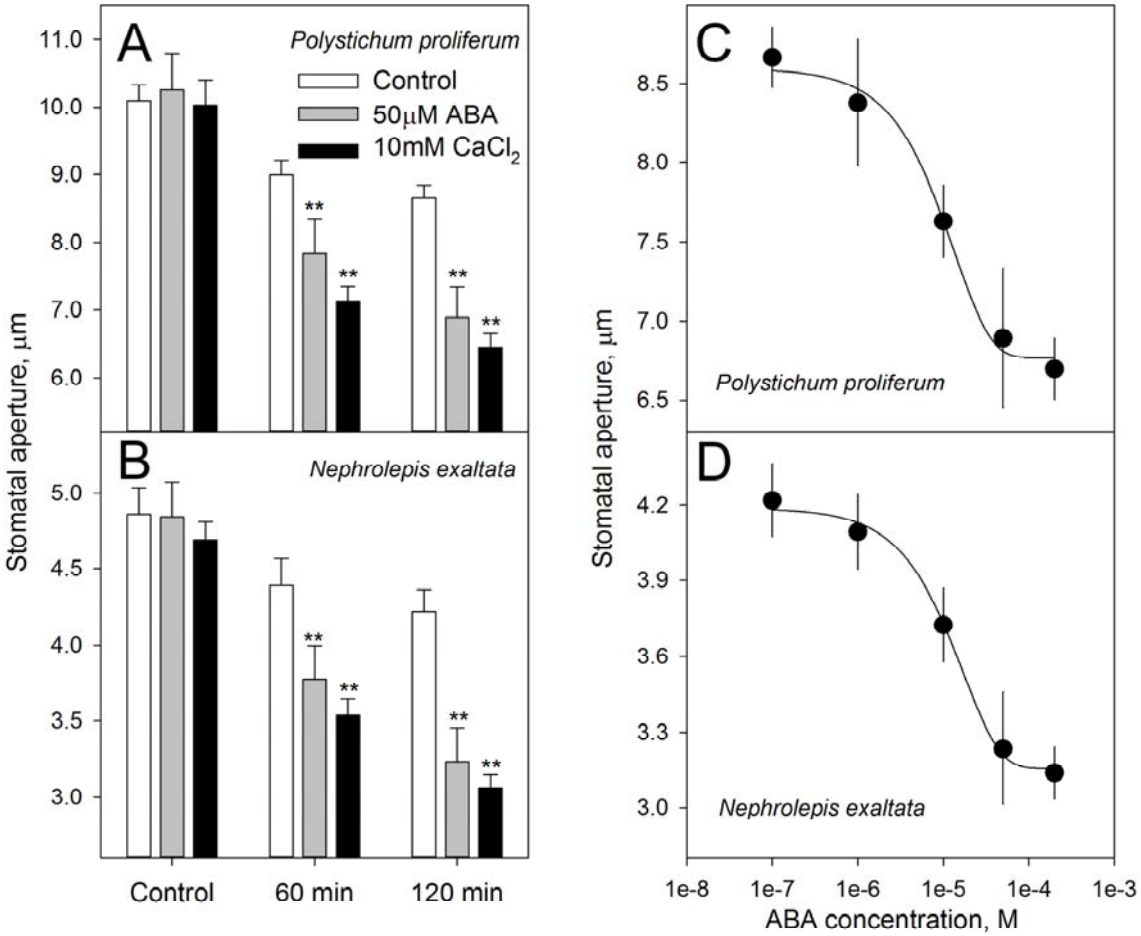

Figure 6. Stomatal responsiveness to ABA and $CaCl_2$ in ferns.

respectively, in 50 μM ABA treatment in *P. proliferum* (Figure 6A) and did so in a dose-dependent manner (Figure 6C and 6D) in both fern species. Moreover, ABA-induced stomatal closure was consistent in epidermal peels of both *P. proliferum* and *N. exaltata* (Figure 7).

Vacuoles are critical for controlling guard cell volume, which regulates the opening and closure of stomata (Blatt, 2000; Franks et al., 2001; Shope et al., 2003; Chen et al., 2012b). We tested whether stomatal closure is related to vacuole changes (Gao et al., 2005; Meckel et al., 2007) of guard cells in *P. proliferum* and *N. exaltata*. ROS staining using confocal microscopy showed that large vacuoles are found in guard cells of open stomata of *P. proliferum* and *N. exaltata* (Figure 8A). Following ABA treatment, vacuoles shrank

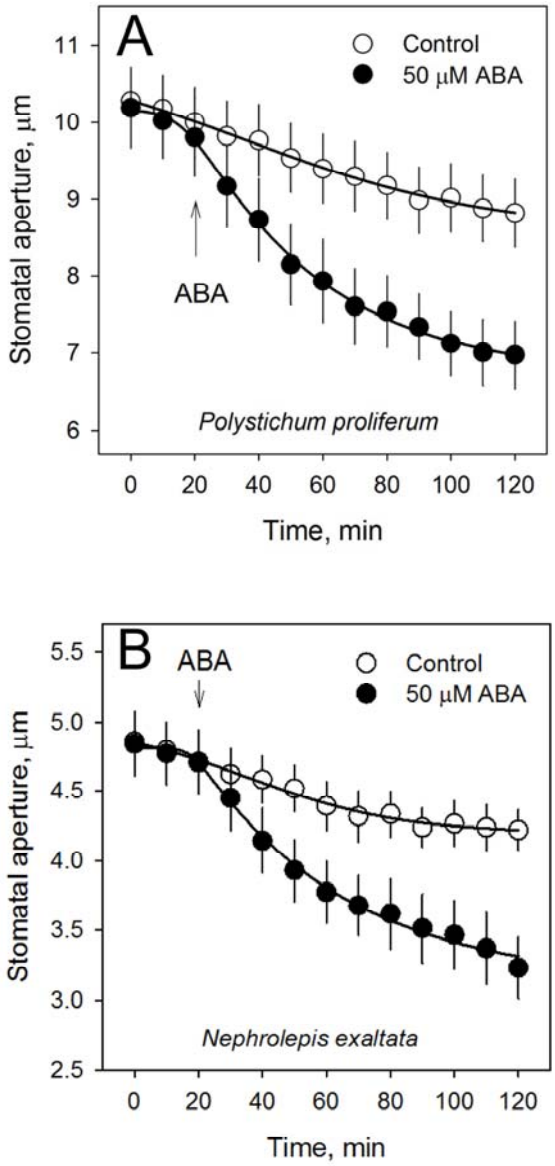

Figure 7. ABA-induced stomatal closure in ferns.

274 significantly in guard cells of half-open and closed stomata (Figure 8B and 8C). Interestingly,
275 the estimated total volume of vacuoles of each stomata was significantly correlated with the
276 stomatal aperture ($P<0.0001$) in both fern species (Figure 8D).

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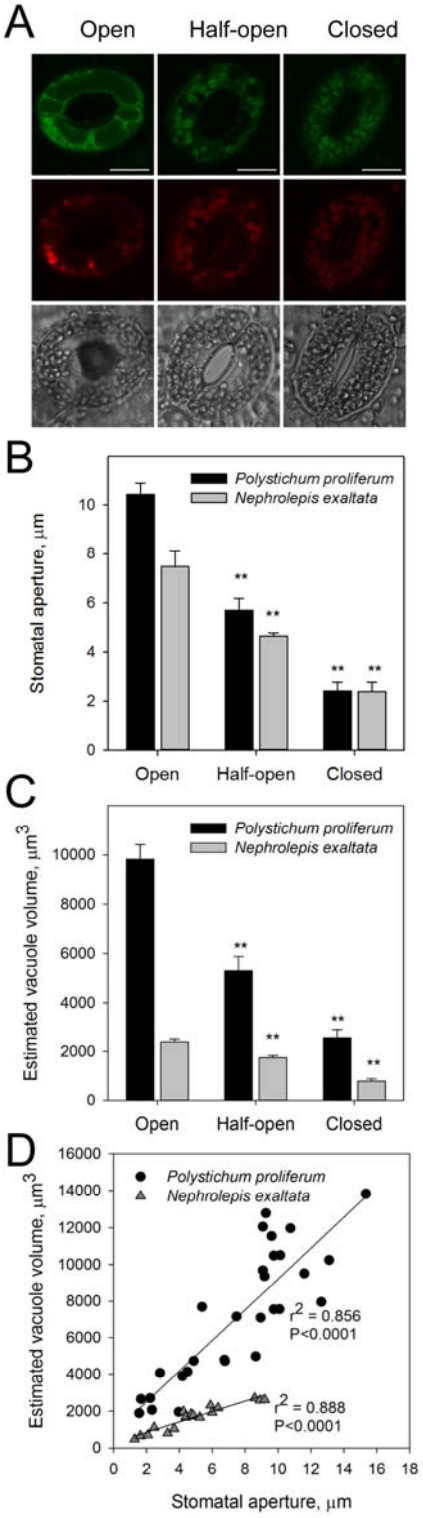

Figure 8. ABA-induced stomatal guard cell volume changes in ferns.

Discussion

Evolutionary genomic analysis reveals the genetics of active stomatal regulation in ferns

281 Molecular biological and genomic analyses provide evidence that a core ABA signaling
282 pathway (Hauser et al., 2011) consisting of PYR/PYL/RCAR, PP2Cs and OST1-like kinases
283 and their target genes were established during the transition from an aquatic to a terrestrial
284 environment over 400 million years ago during the origin of land plants (Munemasa et al.,
285 2015). Nonetheless, the origin of the active response of stomata to endogenous and
286 environmental cues is still unclear. Stomata of the moss *P. patens* and in the lycophyte *S.*
287 *uncinata* respond to environmental signals in a manner similar to those observed in seed
288 plants. The orthologues of the key ABA signaling components in drought response are
289 involved in stomatal control in early land plants (Raven, 2002; Chater et al., 2011; Ruszala et
290 al., 2011; Chater et al., 2013). ABA responsive *PpLEA-1::GUS* lines of *P. patens* revealed a
291 pattern of localized expression around the stomatal ring of sporophytes, which further
292 strengthens previous observations of the ABA response of stomata in mosses (Garner and
293 Paolillo, 1973; Chater et al., 2011; Stevenson et al., 2016). The stomatal response of the *A.*
294 *thaliana ost1* mutant to ABA is rescued by substitution with the *P. patens* orthologue
295 *Pp*OST1. Also, the targeted knockout of the *Ppost1-1* exhibits a significantly attenuated ABA
296 response of stomata. It indicates important features for guard cell ABA signaling during land
297 plant evolution (Cuming et al., 2007; Lind et al., 2015). ABA-related genes from *A. thaliana*
298 generate 11 orthologous clusters of ABA-related genes from *A. thaliana*, *Arabidopsis lyrata*,
299 *Populus trichocarpa*, *O. sativa*, *S. moellendorffii*, and *P. patens*. Phylogenetic analyses
300 indicated that the common ancestor of these six species possessed most of the key protein
301 functions of ABA-related genes, suggesting that the expansion of the gene families related to
302 ABA signaling pathways may have contributed to the sophisticated stress tolerance
303 mechanisms of seed plants (Hanada et al., 2011).

304 Here, we filled a crucial gap in our understanding of ABA signaling by including
305 genomic and transcriptomic evidence for ABA signaling of ferns. The essential orthologues

306 to *A. thaliana* core ABA reception components (RCARs, OST1s, and PP2Cs) and guard cell
307 membrane transporters (e.g. SLAC1/SLAHs, AHAs, TPKs, ALMTs, AVPs) are found in the
308 two water fern (Figures 2 and 3) and a land fern species (Figure 4). These highly conserved
309 ABA signaling components reinforce the pivotal role that stomata have played in the
310 evolution of terrestrial plants (Raven, 2002; O'Donoghue et al., 2013). Although the functions
311 of those key genes in ferns need to be determined in future experiments, our results suggest
312 that ferns are equipped with a set of essential ABA signaling genes necessary for ABA-
313 induced stomatal closure. These findings discount previous claims based on a more limited
314 analysis centered on a single kinase pathway leading to control of a subset of anion channels
315 (McAdam et al., 2016).

316 **Stomatal membrane transport and ABA signaling genes are conserved in a terrestrial**
317 **fern**

318 Comparative transcriptomic analysis among *P. proliferum, H. vulgare, O. sativa, A. thaliana*,
319 *P. patens*, and *K. flaccidum* (Table S5; Figure 5) has generated some important insights into
320 the ABA responsiveness in fern stomata. Our results for ABA-induced gene expression in *P.*
321 *proliferum* are consistent with those of the model plant *A. thaliana* (Leonhardt et al., 2004;
322 Wang et al., 2011), *O. sativa* (Lenka et al., 2011), *H. vulgare* (Figure 5), and *P. patens*
323 (Stevenson et al., 2016). Further evidence for establishing the evolutionary timeline for the
324 appearance of functional ABA signaling gene expression in guard cells could be helpful to
325 refine evolutionary models (Munemasa et al., 2015).

326 The stomatal response to ABA in land plants may have been recruited from a pre-
327 existing transport and signaling network in their common ancestor and ferns appear to have
328 these stomatal regulatory features. During drought stress, *A. thaliana* AtNCEDs and AtZEPs
329 (Xiong and Zhu, 2003) promote the biosynthesis of ABA, which is then transported to guard
330 cells via AtABCG25 and AtABCG40 (Kang et al., 2010; Kuromori et al., 2010). At the same

time, ABA catabolic gene At*CYP707As* (Okamoto et al., 2006) is significantly reduced. For membrane transport, ABA inhibits AtAHA1 activity by reducing the phosphorylation of AtAHAs (Merlot et al., 2007) and AtKATs (Sato et al., 2009). ABA also triggers Ca^{2+} influx and Ca^{2+} release from internal stores to the cytosol that leads to elevated cytosolic Ca^{2+}, which, in turn, suppresses AtKAT1 activity while promoting that of AtSLAC1 and depolarizing the membrane (Grabov and Blatt, 1998; Hamilton et al., 2000; Chen et al., 2010; Chen et al., 2012b). Membrane depolarization activates AtGORK resulting in K^+ efflux from guard cells (Hosy et al., 2003). AtSLAC1 is involved in anion efflux guard cells (Vahisalu et al., 2008) and is directly activated by OST1, which is involved in core ABA signaling (Geiger 2009). AtALMT12 is a plasma membrane malate-induced, R-type anion channel in guard cells controlling stomatal response (Imes et al., 2013). The tonoplast chloride channel C AtCLC-C and AtALMT9 have roles in the anion fluxes that control stomatal movements (Jossier et al., 2010; De Angeli et al., 2013). The tonoplast AtTPK1 (Gobert et al., 2007) regulate stomatal closure via K^+ release from vacuoles. Also, ABA-induced Cytosolic pH change has a significant role for stomatal closing (Blatt and Armstrong, 1993; Grabov and Blatt, 1997, 1998; Chen et al., 2012b) and AtAVP1 regulated NO_3^- transport and cytosolic pH (Rea and Poole, 1993; Zancani et al., 2007). These genes along with others were found to be differentially expressed in the fern *P. proliferum* in the control and ABA treatment (Figure 4). In addition, recruitment of the regulatory genes controlling root function evolved after stomata, providing plants with improved capacities for water and nutrient uptake from soils (Menand et al., 2007). The parallel acquisition of those two adaptive features allowed early land plants to access water and nutrients more readily.

Ferns have both passive and active stomatal regulation

The fern clade shows a remarkable diversity with 11,961 species, outnumbering the number of species of extant gymnosperms (Pryer and Schuettpelz, 2009; McElwain, 2011;

356 Schuettpelz and Schneider, 2016) and only angiosperms have more species (>350,000;
357 http://www.theplantlist.org/browse/A/). Passive control of stomata via regulation of xylem
358 water supply presumably played a role in the success of the fern clade (Brodribb and
359 McAdam, 2011; McAdam and Brodribb, 2012a,b). The evolution of stomata and xylem are
360 interconnected. Stomata primarily control transpirational water loss while the xylem
361 modulates water supply. Their coordinated functions have to be fine-tuned in response to
362 drought. A species with greater coordination of stomatal and xylem functions would have an
363 advantage especially when comparing ferns to seed plants (Sperry, 2004; McElwain, 2011).
364 However, "leaky" stomata in ferns have generally been considered a significant
365 ecophysiological limitation (Testo and Watkins, 2012).

366 We cannot rule out the possibility that the stomata of some ferns do not respond to
367 ABA, given the huge diversity of fern species and their habitats (Creese et al., 2014) and the
368 fact that only few species were examined in this study. However, active control of stomatal
369 opening and closure may be ancestral for land plants with stomata, as evidenced by stomatal
370 ABA sensitivity in the moss *P. patens* and the lycophyte *S. uncinata* (Chater et al., 2011;
371 Ruszala et al., 2011). Further, addition of ABA caused dose-dependent stomatal closure and
372 inhibition of stomatal opening in response to light in *S. uncinata* (Ruszala et al., 2011). The
373 overall ABA dose-dependent stomatal closure in *P. proliferum* and *N. exaltata* (Figure 6C and
374 6D) was similar to that in *S. uncinata*. However, there is a notable difference in their stomatal
375 sensitivity to ABA. ABA-induced significant stomatal closure in *S. uncinata* was observed in
376 1 μM ABA treatment (Ruszala et al., 2011), which was not found in ferns at this
377 concentration (Figure 6). Many taxa do not have ABA-induced stomatal closure, including
378 some mosses and lycophytes (Brodribb and McAdam, 2011). Stomatal ABA sensitivity is
379 present in lineages of vascular plants that diverged prior to ferns as well as in lineages that
380 diverged after ferns. Will it be possible to have an evolutionary scenario in which stomatal

ABA sensitivity is present in ferns?

Here, we showed that stomatal closure can be measured directly in excised epidermis in the presence of ABA (Figures 6 and 7) in two terrestrial fern species *P. proliferum* and *N. exaltata*. We employed similar stomatal assay methods to those used in analyses of *A. thaliana*, *Vicia faba*, and *H. vulgare* (Chen et al., 2010; Chen et al., 2012a; Liu et al., 2014). Our results are consistent with recent reports that stomatal response of ferns to CO_2, vapor pressure deficit, and light is neither lost nor controlled only by hydraulic (i.e. passive) regulation of turgor pressure (Creese et al., 2014; Doi et al., 2015; Franks and Britton-Harper, 2016). A crucial point to consider is that the methodologies differ between this study and that by McAdam and Brodribb (2012a,b), which introduced ABA to the transpiration stream to measure responses to endogenous ABA concentrations. The addition of exogenous ABA to epidermal peels and spraying of ABA used in this study could be largely responsible for the different results. Also, there is an overlapping fern species *N. exaltata* in our study and those of Brodribb and McAdam (2011). It appears that the sensitivity of *N. exaltata* responding to ABA under these two experimental conditions was very different and was not directly comparable. By contrast, the range of physiological and molecular techniques used in the present study is more comparable to those used in moss and lycophyte species by Chater et al (2011) and Ruszall et al (2011). Clearly, it will be of interest to see whether, as a group, the fern might fall between two or more subsets with different stomatal characteristics.

Is SnRK2s regulation of SLACs the only mechanism for stomatal closure?

OST1/SnRK2s have important roles in stomatal regulation via the activation of SLAC/SLAH anion channels in *A. thaliana* (Geiger et al., 2010), as well as the species of moss (Chater et al., 2011) and lycophte examined (Ruszala et al., 2011; Lind et al., 2015). However, McAdam et al (2016) recently reported that GAIA1, a homolog of OST1 in the water fern *Ceratopteris richardii*, regulates ABA signaling for gametophyte sex determination, rather than stomatal

406 regulation. Fern and lycophyte SnRK2s were found to be unable to activate native
407 endogenous SLACs. Therefore, these authors concluded that the ABA-signaling pathway
408 through SnRK2-mediated SLAC activation for stomatal closure may not appear to be
409 operating in the fern *C. richardii*. Instead, they suggested that connection between ABA and
410 stomatal control via the specific activation of SLAC/SLAH anions channels is a more recent
411 innovation that did not evolve until after the divergence of ferns and seed plants (McAdam et
412 al., 2016).

413 We call into question the conclusions of McAdam et al (2016). Not only can we
414 demonstrate ABA-induced stomatal closures in two fern species, but recent advances point to
415 alterative pathways for ABA-induced stomatal closure. These findings imply that the focus
416 on the OST1/SnRK2-SLAC1 pathway might not be universal in all land plants. Indeed, the
417 suggestion is hardly surprising; in *A. thaliana*, multiple calcium-dependent kinases CPK3,
418 CPK5, CPK6, CPK21, and CPK23 (Brandt et al., 2012, 2015; Geiger et al., 2010; Scherzer et
419 al., 2012) and our recent data on CPKs and CRKs (Pornsiriwong et al. unpublished) are
420 thought to modulate ABA-activated guard cell anion channels. Orthologous genes (e.g.
421 OST1, CPKs, CRKs) can be found in the genome and transcriptome of *C. richardii*
422 (Marchant et al. unpublished). Therefore, these data raise the question of whether CrSnRK2
423 in *C. richardii* is the only kinase essential for ABA-induced stomatal closure in ferns. We
424 note, too, that McAdam et al (2016) offer no stomatal aperture assays for *C. richardii*.
425 Clearly, if alternative pathways for ABA signaling exist, then the lack of anion channel
426 activation by CrSnRK2 cannot prove an ABA insensitivity of stomata of *C. richardii*. Further
427 experiments should focus on the identification of CPKs and CRKs in *C. richardii* and their
428 interaction with SLAC/SLAH anion channels in combination with stomatal physiological
429 measurements in order to confirm whether there is a general ABA insensitivity of stomata in
430 *C. richardii*. In conclusion, despite the advantages of passive hydraulic control for fern

stomata, the molecular mechanisms of ABA-induced stomatal closure may exist in certain fern species.

Materials and Methods

Plant materials and growth conditions

Drought-tolerant (X5) and -sensitive (X54) Tibetan annual wild barley (*H. vulgare* spp. *spontaneum*) genotypes were used in this study. Seeds were sown in 4-L pots with potting mixture and five healthy and uniform seedlings were maintained per pot. Plants were grown in a greenhouse at a 22±2°C and 60% relative humidity (RH) during the day, and 20±2°C and 60% RH at night, in 12h/12h light/dark cycle. The average PAR was 400 μmol m^{-2} s^{-1}. All plants were well-watered before drought treatment, which was commenced five weeks after sowing. Fully-expanded leaves were sampled when the water holding capacity decreased to 10% (v/v). Abaxial epidermal strips were peeled using a pair of fine forceps and frozen immediately in liquid nitrogen.

Small sporophytes of the ferns *P. proliferum* and *N. exaltata* were purchased from a local plant nursery (Bunnings, Penrith, Australia) and grown in a growth chamber. Plants were grown under 12h/12h day/night, 20±1°C, 100 μmol m^{-2} s^{-1} PAR, and 60% RH. Plants were irrigated weekly with half strength Hoagland's solution. Plants were grown for at least four weeks before stomatal assay and qPCR. Treatment was applied by spraying 50 μM ABA to the upper and lower epidermis of fern leaves for 1 h before epidermal peeling. We used fine forceps to peel abaxial epidermal strips from young fully expanded leaves.

Evolutionary bioinformatics analysis

All 63 query *A. thaliana* guard cell genes and 23 gene families are from Chen et al (2017). Genome sequence data were downloaded from the National Center for Biotechnology

455 Information (NCBI) (http://www.ncbi.nlm.nih.gov) and Ensembl Plants
456 (http://plants.ensembl.org/index.html). The genome sequences of *A. filiculoides* and *S.*
457 *cucullata* are from Assistant Professor Fay-Wei Li (Personal Communications). Genesis
458 software was used to estimate the similarity among protein sequences and families. Candidate
459 protein sequences were selected by BLASTP researches which satisfied the criteria of E-
460 value and query coverage at different levels. Amino acid sequences were translated from
461 nucleotide sequences using BioEdit software and the phylogenies were constructed with
462 MEGA 6 using the Maximum Likelihood (ML) method of evolution (Tamura et al., 2013).
463 Values from 1,000 bootstrap replicates are shown at the nodes.

464 **RNA-Sequencing**

465 Total RNA was extracted from barley epidermal peels with the miRNeasy mini kit (QIAGEN,
466 Germany) following the manufacturer's instructions. RNA quantity and quality check, library
467 construction and sequencing were performed as described in (Dai et al., 2014; Wang et al.,
468 2016). The Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA)
469 was used to construct the library following the manufacturer's instructions. Total RNA
470 samples were purified by oligo-dT beads and poly (A)-containing mRNA were then
471 fragmented. The cDNA was generated by First/Second Strand Master Mix and Super Script II
472 (Invitrogen, Carlsbad, CA, USA) reverse transcription and adapters were ligated onto both
473 ends to generate end-repaired DNA followed by amplification and enrichment. After
474 purification, Ampure XP Beads were added to the PCR products, and size-selected for
475 sequencing using Agilent 2100. The raw data obtained from Illumina HiSeq 2500 were
476 cleaned by trimming and removing empty reads, low quality bases (Q<30 and length<50 bp)
477 and adaptor sequences at the 3' end. We used *BWA* to map clean reads to the genome
478 reference of barley (http://plants.ensembl.org/) and *Bowtie* for gene reference (Langmead et
479 al., 2009). The mapped reads assembling, abundance estimation and differentially expressed

480 genes (DEGs) were done using Cuffdiff of Cufflinks v2.1.1 (Roberts et al., 2011).

481 **Quantitative RT-PCR**

482 Quantitative PCR was carried out following the protocol of Chen et al. (2016) with some
483 modification. RNA of epidermal peels of *P. proliferum* was extracted using Trizol reagent
484 (Life Technologies, Australia) following the manufacturer's procedure, and the residual
485 genomic DNA was removed with amplification grade DNase I (Ambion, Australia). First-
486 strand cDNA was synthesized with the SensiFAST™ Kit (Bioline, Australia). Transcript
487 levels of the target genes were determined by the SensiFAST^{TM} SYBR No-ROX Kit (Bioline,
488 Australia) with *Ceratopteris richardii* specific primers (Table S6) for *P. proliferum* genes
489 using a Rotor-Gene® Q6000 (QIAGEN, Germany). The primers design was based on the
490 amplification of transcripts encoding conserved protein domains between *C. richardii* and *A.*
491 *thaliana*. qPCR conditions were consisted: 1.) polymerase activation at 95°C for 2 min; 2.) 40
492 cycles of 5 s denaturation at 95°C, 10 s annealing at 63°C, and 15 s extension at 72°C; 3.)
493 SYBR green signal data were acquired at the end. *Beta tubulin* (*PopTUB*) was used as the
494 reference for normalization of relative gene expression. Final values were averaged from
495 three independent biological replicates.

496 **Stomatal aperture assay**

497 Stomatal aperture assays were carried out using similar methods as described earlier (Chen et
498 al., 2010; Eisenach et al., 2012; Chen et al., 2016). Peels from the lower epidermis of
499 *Polystichum proliferum and Nephrolepis exaltata* plants were placed in glass bottom petri
500 dishes and were pre-treated for 10 min in a measuring buffer [10 mM KCl and 5 mM 2-(N-
501 morpholino)propanesulfonic acid (MES) at pH 6.1 with $Ca(OH)_2$] under 100 μmol m^{-2} s^{-1}
502 PAR light. The samples were imaged in the measuring buffer for 20 min as the control under
503 a Nikon microscope attached with a Nikon NIS-F1 CCD camera and a Nikon DS-U3

504 controller (Nikon, Tokyo, Japan). Treatments were applied as ABA (0.1, 1, 10, 50, and 200
505 μM) or $CaCl_2$ (10 mM) and measured for another 100 min. Control experiments were also
506 performed by measuring stomatal aperture in the measuring buffer for 120 min. All the
507 stomatal aperture measurements were conducted under constant microscopy light of 100
508 μmol m^{-2} s^{-1} PAR to avoid dark-induced stomatal closure. Images were taken every 5 min
509 and stomatal apertures were measured and analyzed with Image J software (NIH, USA). For
510 every data point, there were 30-40 stomata measured and the experiments were independently
511 repeated 5 times.

512 **Confocal microscopy**

513 ROS production in the cytoplasm of guard cells was determined using the fluorescent
514 indicator 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Life Technologies,
515 Australia). The styryl dye (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl)
516 Hexatrienyl) Pyridinium Dibromide) (FM4-64) has been reported to selectively stain
517 tonoplast and endomembrane (Life Technologies, Australia). Epidermal peels were pre-
518 treated with the opening buffer for 2 h before loading 20 μM H2DCFDA for 20 min in the
519 dark, followed by a 5-min flush in the measuring buffer to remove excess dye. The strips
520 were subsequently incubated in the measuring buffer containing 50 μM ABA for 120 min
521 with a 10-min sampling interval under confocal microscopes (Zeiss and Leica, Germany).
522 The fluorescence images were collected with excitation at 488 nm, and emission at 505-525
523 nm for H2DCFDA and emission at 610-640 nm for FM4-64. Vacuole volume of both guard
524 cells was estimated using the formula: $V=4/3\times S\times r$, where S is the area of the vacuole and r is
525 the radius of the guard cells. Guard cell volume was also calculated as a control to the
526 vacuolar volume using the Henry III software (University of Glasgow, UK).

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Figure legends

Figure 1. Number of predicted membrane transporters and ABA reception complex proteins and families in different taxa. (A) The number of predicted transporters and ABA reception families (A) and guard cell genes (B) are grouped into seven taxa**.** Genesis software was used to estimate the similarity among protein sequences (A) and family (B). Candidate protein sequences were selected by BLASTP searches that satisfied the criteria of E-value<10^{-10} and query coverage>50% for guard cell proteins and E-value<10^{-5} for all proteins. (C) The number of gene families in different taxa. Data are mean ± SE for those with error bars.

Figure 2. Similarity heat map for the evolution of membrane transporters and ABA reception complex proteins in different species. Genesis software was used to estimate the similarity among protein family (A) and sequences (B). Candidate protein sequences were selected by BLASTP searches that satisfied the criteria of E-value<10^{-5} only (A) and E-value<10^{-10} and query coverage>50% (B). Colored squares indicate protein sequence similarity from zero (yellow) to 100% (red). Clades are indicated by different colors on the right: angiosperm (blue), gymnosperm (pink), fern (red), lycophyte (green), moss (brown), liverwort (purple), algae (orange). Gray squares indicate that no proteins were found that satisfied the selection criteria. KATs represent the AKTs/KATs/GORKs proteins. Abbreviations: AKT, Arabidopsis inwardly-rectifying K^+ channel; KAT, guard cell inwardly-rectifying K^+ channel; GORK, guard cell outwardly-rectifying K^+ channel; HAK, high-affinity K^+ transporter; TPK, tonoplast K^+ channel; ALMT, aluminum-activated malate transporter; SLAC, slow anion channel; GLR, glutamate receptor-like Ca^{2+} channel; CNGC, cyclic nucleotide gated channel; TPC, two-pore channel; ACA, autoinhibited Ca^{2+}-ATPase; AHA, Arabidopsis plasma membrane H^+-ATPase; AVP, Arabidopsis vacuolar H^+-pyrophosphatase; VHA, vacuolar H^+-

560 ATPase; CLC, chloride channel; NHX, Na^+/H^+ antiporter; ABCC, ATP-binding cassette C
561 transporter; HKT, high-affinity K^+/Na^+ transporter; CAX, cation proton exchanger; SUC,
562 sucrose transporter; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein;
563 ABI, Protein Phosphatase 2C; RCAR, regulatory component of ABA receptor; SnRK2,
564 SNF1-related protein kinase 2.
565
566 **Figure 3.** Phylogenetic trees of key ABA signaling proteins in species of plants and algae.
567 The regulatory component of ABA receptor 11 (RCAR11) (A), ABA insensitive 1 (ABI1)
568 (B), open stomatal 1 (OST1) (C), slow anion channel 1 (SLAC1) (D), and vacuolar H^+-
569 pyrophosphatase 1 (AVP1) (E) were estimated. The maximum-likelihood method was used to
570 construct the trees and evolutionary distances were computed in MEGA 6. Bootstrap values
571 are shown next to each branch of the trees. Clades are indicated by different colors:
572 angiosperm (blue), gymnosperm (pink), fern (red), lycophyte (green), moss (brown),
573 liverwort (purple), algae (orange). Abbreviations of species: Af, *Azolla filiculoides*, Amt,
574 *Amborella trichopoda*, At, *Arabidopsis thaliana*, Bd, *Brachypodium distachyon*, Br, *Brassica*
575 *rapa*, Cm, *Cyanidioschyzon merolae*, Cr, *Chlamydomonas reinhardtii*, Eg, *Eucalyptus*
576 *grandis*, Es, *Ectocarpus siliculosus*, Gm, *Glycine max*, Gr, *Gossypium raimondii*, Hv,
577 *Hordeum vulgare*, Kf, *Klebsormidium flaccidum*, Md, *Malus domestica*, Mp, *Marchantia*
578 *polymorpha*, Mt, *Medicago truncatula*, Os, *Oryza sativa*, Oss, *Ostreococcus sp.*, Pa, *Picea*
579 *abies*, Ph, *Phyllostachys heterocycla*, Pl, *Pinus lambertiana*, Pot, *Populus trichocarpa*, Pp,
580 *Physcomitrella patens*, Pt, *Pinus taeda*, Py, *Porphyra yezoenesis*, Sb, *Sorghum bicolor*, Sc,
581 *Salvinia cucullata*, Sf, *Sphagnum fallax*, Sl, *Solanum lycopersicum*, Sm, *Selaginella*
582 *moellendorffii*, Sp, *Spirodela polyrhiza*, Ta, *Triticum aestivum*, Th, *Theobroma cacao*, Vc,
583 *Volvox carteri*, *Vv, Vitis vinifera*, Zm, *Zea mays*.
584

585

586 **Figure 4.** Relative expression of ABA signaling genes in epidermal peels of *Polystichum*
587 *proliferum*. Forty genes involved in the ABA signaling pathway were classified into five
588 groups by function. Relative expression levels were calculated and normalized. Data are
589 mean ± SE. Significant up- or down-regulation is indicated with an asterisk at $P<0.05$.

590

591 **Figure 5.** Conserved ABA signaling pathway in epidermis of *Hordeum vulgare* and
592 *Polystichum proliferum*. The signaling network is formed by four main functional categories:
593 ABA biosynthesis (pink), ABA transportation (green), signal transduction (purple) and the
594 down-stream ion channels and transporters (light blue). Heatmap of transcriptome of *H.*
595 *vulgare* epidermis in drought stress (left and middle) and qPCR of *P. proliferum* epidermis in
596 ABA treatment (right) are shown. Abbreviations: ABCC, ATP-binding cassette C transporter;
597 ABCG, ATP-binding cassette G; ABF, ABA responsive elements-binding factor; ABI4, ABA
598 insensitive 4; AHA, arabidopsis plasma membrane H^+-ATPase; AKT1, serine/threonine
599 kinase 1; ALMT, aluminum-activated malate transporter; AREB, AREB-like protein; AVP,
600 arabidopsis vacuolar H^+-pyrophosphatase; CAS, calcium sensing receptor; CHLH,
601 protoporphyrin IX magnesium chelatase, subunit H; CLC-C, chloride channel C; CNGC,
602 cyclic nucleotide gated channel; CYP707A, cytochromeP450 family 707 superfamily A;
603 GORK, gated outwardly-rectifying K^+ channel; GTG, GPCR-type g protein 1; KAT, guard
604 cell inwardly-rectifying K^+ channel; MAPK, mitogen activated kinase-like protein; MYB,
605 MYB domain protein; NADPBRF, NAD(P)-binding Rossmann-fold superfamily protein;
606 NCED3, 9-cis-epoxycarotenoid dioxygenase 6; OST1, open stomata 1; PLDa1,
607 phospholipase D alpha 1; PP2C, Protein Phosphatase 2C; RBOH, respiratory burst oxidase
608 homolog protein; RCAR, regulatory component of ABA receptor; SLAC, slow anion
609 channel; STP1, sugar transporter 1; VHA, vacuolar H^+-ATPase; ZEP, zeaxanthin epoxidase.

Figure 6. Stomatal responsiveness to ABA and $CaCl_2$ in ferns. Stomatal aperture of *Polystichum proliferum* (A) and *Nephrolepis exaltata* (B) in the control, 50 μM ABA and 10 mM $CaCl_2$ treatments at 0, 60 and 120 min. (B) ABA dose-dependent (0.1, 1, 10, 50 and 200 μM ABA) stomatal closure in *Polystichum proliferum* (C) and *Nephrolepis exaltata* (D). Data are mean±SE (n= 30-40 stomata from 5 biological replicates). ** indicates significant difference at $P<0.01$ level.

Figure 7. ABA-induced stomatal closure in ferns. Stomatal aperture of *Polystichum proliferum* (A) and *Nephrolepis exaltata* (B) in the control (0-20min) and 50 μM ABA (20-120 min) treatment. Blank (control) stomatal aperture measurements were also conducted for 120 min in both species. Data are mean ± SE (n= 30-40 stomata from 5 biological replicates).

Figure 8. ABA-induced stomatal guard cell volume changes in ferns. (A) Representative confocal images of open, half-open and closed stomata in *Polystichum proliferum*. Green and red fluorescence indicate the ROS and endomembrane stain, respectively. Bars = 20 μm. Stomatal aperture (B) and estimated vacuole volume (C) of open, half-open and close stomata in *Polystichum proliferum* and *Nephrolepis exaltata*. Data are mean±SE (n= 6-10 stomata from 3 biological replicates). (D) Correlation between stomatal aperture and estimated vacuole volume. Data are plotted from all the measured stomata in confocal imaging.

631 **Supplementary Materials**

632 **Table S1.** Number of predicted membrane transporters and ABA reception complex proteins
633 in 36 plant and algal species with E-value<10^{-5}.

634 **Table S2.** Similarity analysis for the evolution of predicted membrane transporters and ABA
635 reception complex proteins in 36 plant and algal species with E-value<10^{-5}.

636 **Table S3.** Number of predicted proteins of guard cell transporters and ABA reception
637 complex in 36 plant and algal species with E-value<10^{-10} and query coverage>50%.

638 **Table S4.** Similarity analysis for the evolution of predicted guard cell transporters and ABA
639 reception complex proteins in 36 plant and algal species with E-value<10^{-10} and query
640 coverage>50%.

641 **Table S5.** Comparative transcriptomic analysis of plant and algal species to ABA, drought or
642 desiccation stress. Data are from publically available datasets and the *Hordeum vulgare*
643 transcriptome from this study.

644 **Table S6.** Primers for quantitative RT-PCR of ABA signaling genes in the fern *Polystichum*
645 *proliferum*.

646

647

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