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Report

Regulatory Mechanism Controlling Stomatal Behavior Conserved across 400 Million Years of Land Plant Evolution

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Summary

Stomatal pores evolved more than 410 million years ago [1, 2] and allowed vascular plants to regulate transpirational water loss during the uptake of CO_2 for photosynthesis [3]. Here, we show that stomata on the sporophytes of the moss *Physcomitrella patens* [2] respond to environmental signals in a similar way to those of flowering plants [4] and that a homolog of a key signaling component in the vascular plant drought hormone abscisic acid (ABA) response [5] is involved in stomatal control in mosses. Cross-species complementation experiments reveal that the stomatal ABA response of a flowering plant (*Arabidopsis thaliana*) mutant, lacking the ABA-regulatory protein kinase OPEN STOMATA 1 (OST1) [6], is rescued by substitution with the moss *P. patens* homolog, *PpOST1-1*, which evolved more than 400 million years earlier. We further demonstrate through the targeted knockout of the *PpOST1-1* gene in *P. patens* that its role in guard cell closure is conserved, with stomata of mutant mosses exhibiting a significantly attenuated ABA response. Our analyses indicate that core regulatory components involved in guard cell ABA signaling of flowering plants are operational in mosses and likely originated in the last common ancestor of these lineages more than 400 million years ago [7], prior to the evolution of ferns [8, 9].

Results and Discussion

Moss Stomata Respond to Environmental and Exogenous Cues

Mosses are the most basal land plant group with stomata (Figure 1A), and the moss *Physcomitrella patens* provides a powerful model for investigation of the evolution of early land plants because of the available genomic resources [10, 11]. However, the physiology and molecular genetics of moss stomata [10] are poorly understood. We therefore undertook experiments characterizing stomatal behavior in *P. patens* and a further moss species, also within the Funariaceae (*Funaria hygrometrica*), in response to environmental and exogenous cues. Stomata of the Funariaceae are formed from single dikaryotic guard cells which are located in a ring around the base of the diploid sporophyte structure [12, 13] (see Figure S1 available online). Our results indicate that the stomata of expanding green sporophytes of both moss species close in response to exogenous abscisic acid (ABA) and CO_2 and open in response to a diurnal light rhythm (Figure 1B; Figure S2).

We investigated the stomatal ABA and CO_2 responses in further detail by focusing on *P. patens*. In these experiments, stomatal apertures of *P. patens* decreased as the ABA dose increased from 0 μM to 100 μM, with significant concentrations at 50 and 100 μM ABA relative to 0 μM ABA (Figure 2A). This nonlinear ABA dose-response relationship is similar to that reported for stomata on the sporophytes of the hornwort *Anthoceros punctatus* [14] (Figure 1A) and vascular plants [4]. Parallel aperture closure responses were observed as the atmospheric CO_2 concentration increased from 0 ppm to 1000 ppm (Figure 2B), with the strongest response between 0 and 400 ppm.

To investigate whether the biochemical mechanisms driving the observed opening and closing of stomatal pores in mosses are similar to those of vascular plants, we undertook a further experiment employing the fungal toxin fusicoccin. In the stomata of vascular land plants, fusicoccin artificially activates the H^+-ATPase pump that initiates turgor-driven stomatal opening. Downregulation of this proton pump is critical for ABA-induced stomatal closure in flowering plants [15]. We found that treating sporophytes of *P. patens* and *F. hygrometrica* with 10 μM fusicoccin induced significant stomatal opening (Figure 1B). A similar response has been observed for the stomata on the sporophytes of *A. punctatus* exposed to 15 μM fusicoccin [14]. These results for nonvascular land plants suggest that the biochemical mechanism of turgor-driven stomatal aperture responses is conserved across plant groups and diverse sporophyte structures. Together with our physiological data (Figure 1; Figure 2), this evidence challenges the suggestion that active stomatal responses to CO_2 and ABA only evolved after the evolutionary appearance of ferns 365 million years ago [8, 9].

Sporophyte ABA-Regulated Gene Expression Is Localized to the Stomatal Region

ABA signaling is known to mediate environmental stress responses in the gametophyte of bryophytes [14, 16–19], but the functional role of ABA in the stomata-bearing sporophytes of mosses is unknown. Here we report evidence for ABA-regulated gene expression in *P. patens* sporophytes from the analysis of transgenic lines in which a *P. patens* ABA-responsive promoter was translationally fused to the *GUS* reporter gene [19]. These *PpLEA-1::GUS* lines allowed detection of ABA responsiveness via GUS histochemical staining and revealed a pattern of localized expression confined to the basal region of the sporophyte around the stomatal ring (Figure 3). Taken together with our measurement of ABA-mediated reductions in moss stomatal apertures (Figure 1B; Figure 2A), these results implicate the involvement of ABA signaling in moss stomata, a view further strengthened by previous observations on the stomata of *F. hygrometrica* [20] and *A. punctatus* [14].

ABA Signaling Genes Upregulated in Sporophytes

The genome sequence of *P. patens* contains potential homologs of several vascular plant ABA-signaling components,

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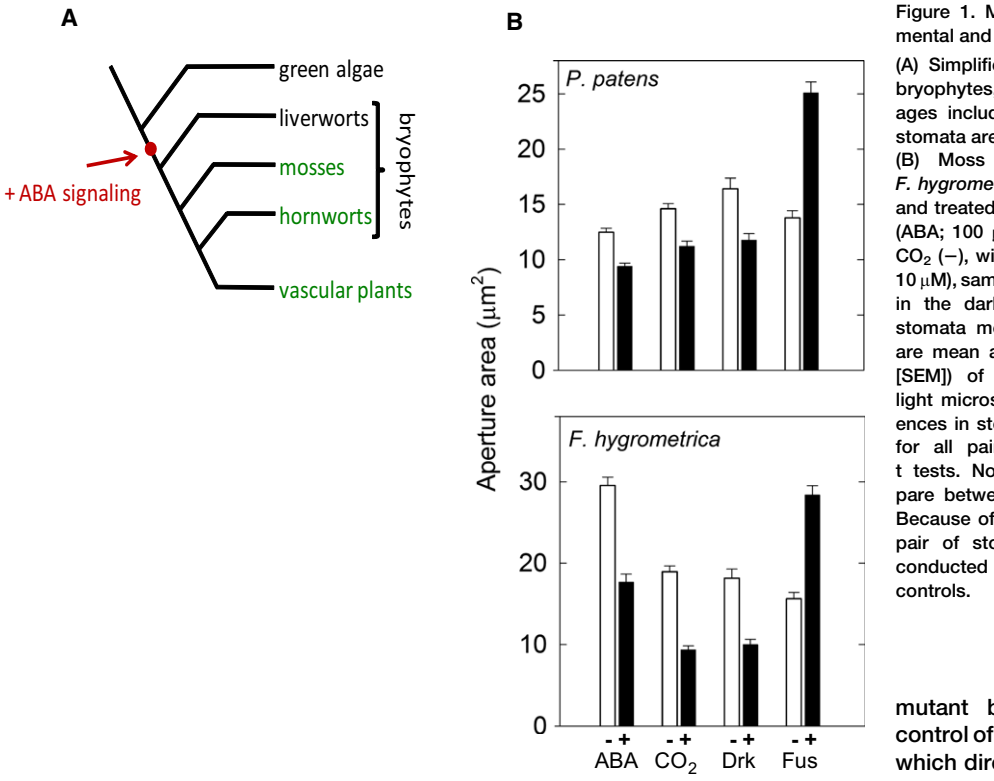

Figure 1. Moss Stomata Respond to Environmental and Exogenous Cues

(A) Simplified land plant phylogeny illustrating bryophytes, which comprise three separate lineages including the mosses [9]. Lineages with stomata are listed in green.

(B) Moss sporophytes of *P. patens* and *F. hygrometrica* were maintained in CO_2-free air and treated with (+) or without (−) abscisic acid (ABA; 100 μM), with (+) or without (−) ambient CO_2 (−), with (+) or without (−) fusicoccin (Fus; 10 μM), sampled predawn and stomata measured in the dark (+Drk), or sampled midday and stomata measured in the light (−Drk). Values are mean area (± standard error of the mean [SEM]) of stomatal apertures, measured by light microscopy. Significant ($p < 0.001$) differences in stomatal aperture area were identified for all pairwise comparisons with Student's t tests. Note that it is not possible to compare between the four treatment experiments. Because of the large number of samples, each pair of stomatal treatment experiments was conducted on different days, with appropriate controls.

and transcriptomic analyses have confirmed their expression in gametophytes [11, 21] (Figure S3). Our *PpLEA-1::GUS* results additionally indicate ABA responsiveness in the sporophyte life-cycle stage (Figure 3). We therefore investigated whether components of the core guard cell ABA-signaling network recently identified to regulate stomatal behavior in the flowering plant *Arabidopsis thaliana* [4, 5, 22, 23] are expressed in *P. patens* sporophytes (Figure 4A). We isolated RNA from expanding green sporophytes and performed RT-PCR using appropriate gene-specific primers for *P. patens* homologs of the *PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR* (*PYL/RCAR*) ABA receptor [22, 23], the *ABA INSENSITIVE 1* (*ABI1*) protein phosphatase 2C, and the *OPEN STOMATA 1* (*OST1*) sucrose nonfermenting 1-related protein kinase 2 gene families [6]. These experiments indicated that at least one gene family member representing each step in the core ABA-signaling pathway, and additionally an H^+-ATPase gene homolog [15], is expressed in sporophytes (Figure 4A; Figure S3).

Conservation of OST1 Function across 400 Million Years of Land Plant Evolution

In *A. thaliana*, OST1 is a key ABA-inducible protein kinase within the network regulating ABA-mediated reductions in stomatal aperture [5]. *A. thaliana* mutants lacking OST1 kinase activity have severely impaired stomatal closure in response to ABA [6]. The *P. patens* genome contains four putative *OST1* genes, one of which (designated here *PpOST1-1*) is transcriptionally upregulated in response to ABA and drought [16] (Figure S3). To determine whether OST1 function is conserved across land plant evolution, we performed a cross-species complementation experiment with *PpOST1-1*. We expressed *PpOST1-1* in *A. thaliana ost1* mutant backgrounds [24] under the control of the *A. thaliana OST1* promoter, which directs gene expression to guard cells [6, 25], and verified the resulting transformants by RT-PCR (Figure 4). Subsequent stomatal measurements indicated that the moss *PpOST1-1* rescued the ABA-induced stomatal closure response in three independently transformed *Arabidopsis ost1* mutant lines [6, 25], with significant reductions in stomatal aperture observed following exposure to 20 μM ABA (Figure 4).

To confirm the function of *OST1*-related genes in *P. patens*, we constructed a moss deletion mutant lacking the *PpOST1-1* gene by homologous recombination and verified this gene knockout by RT-PCR (Figure 5) and Southern blotting (Figure S4). Observations of the stomata on the resulting *P. patens* sporophytes indicated that the *Ppost1-1* knockouts had significantly attenuated stomatal responses to ABA (Figure 5). The remaining ABA responsiveness is likely attributable to the three remaining *PpOST1* gene homologs. Together, the complementation and knockout results confirm that the moss *OST1-1* gene can substitute for the loss of OST1 function in a flowering plant. This key finding indicates that its molecular and guard cell-directed function has been conserved since the divergence of vascular plants and mosses. In *Arabidopsis*, OST1 activates the S-type anion channel SLAC1, triggering depolarization of the guard cell membrane, a critical step in stomatal closure [4]. It will be interesting to determine whether PpOST1 phosphorylates an orthologous ion channel in *P. patens*.

Conclusions

Our integrated physiological and molecular genetic analyses contribute to building a more complete view of early land plant evolution that complements paleobotanical discoveries [1–3, 7]. We show that extant moss sporophytes share components of the same core molecular genetic "toolkit" for coping with drought as vascular plants, even though the two lineages diverged more than 400 million years ago [7]. This suggests

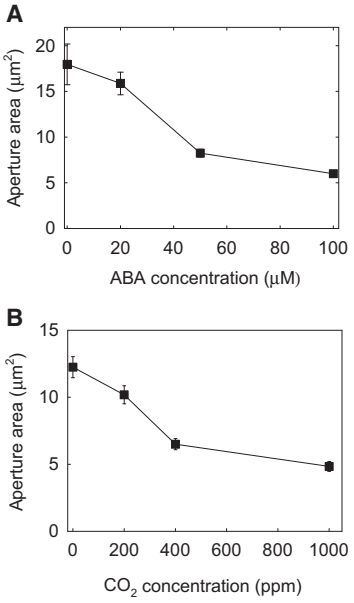

Figure 2. Moss Stomata Respond to ABA and Atmospheric CO_2 Concentration

Changes in aperture area of stomata of *P. patens* sporophytes in response to increasing dosage of ABA (A) and atmospheric CO_2 concentration (B). Values are mean (±SEM) of stomatal apertures, measured with light microscopy. In (A), significant (analysis of variance [ANOVA], $p < 0.001$) reductions in stomatal aperture area were detected at 50 and 100 μM ABA relative to 0 μM ABA. In (B), significant (ANOVA, $p < 0.001$) reductions in stomatal aperture area were detected at 400 ppm and 100 ppm CO_2 concentrations relative to 0 ppm CO_2.

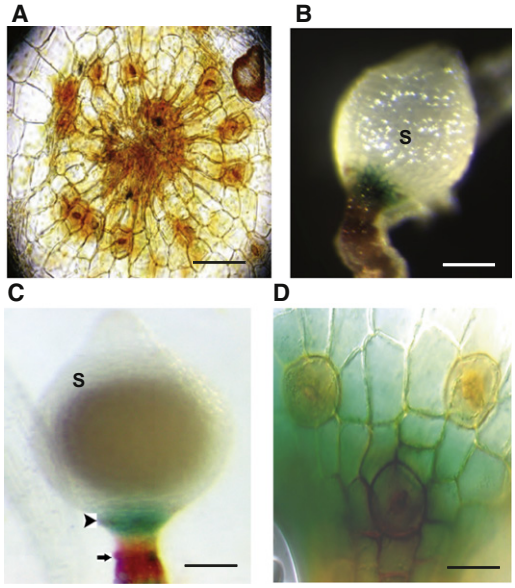

Figure 3. Expression of an ABA Reporter Gene Is Localized around Moss Stomata

(A) Surface view of excised *P. patens* sporophyte showing ring of stomata located around the proximal end.
(B and C) Expanding (B) and expanded (C) *P. patens* sporophytes (S) of a *PpLEA-1::GUS*-expressing line assayed for GUS activity, revealed by blue staining. Darker spots in the blue ring (arrowhead in C) are the stomata. The foot of the sporophyte (light brown tissue below blue) and gametophyte (reddish tissue indicated by arrow) show no blue coloration.
(D) Close-up of GUS staining at base of apophysis near three stomata.
Scale bars represent 50 μm (A), 150 μm (B and C), and 20 μm (D).

that the stomatal ABA response of higher plants was recruited and redeployed from a preexisting gene network present in the last common ancestor of these two plant groups. Recruitment of the regulatory genes controlling rooting function in the sporophyte from the gametophyte stage also occurred early in land plant evolutionary history [26], providing plants with improved access to soil water and nutrients [3]. Our experimental evidence indicates that the parallel acquisition of a regulatory pathway for controlling stomata apertures probably represents a further crucial step in allowing plants to achieve permanent hydration (i.e., homoiohydry) [2] prior to the evolution of club mosses and ferns [9]. We suggest that

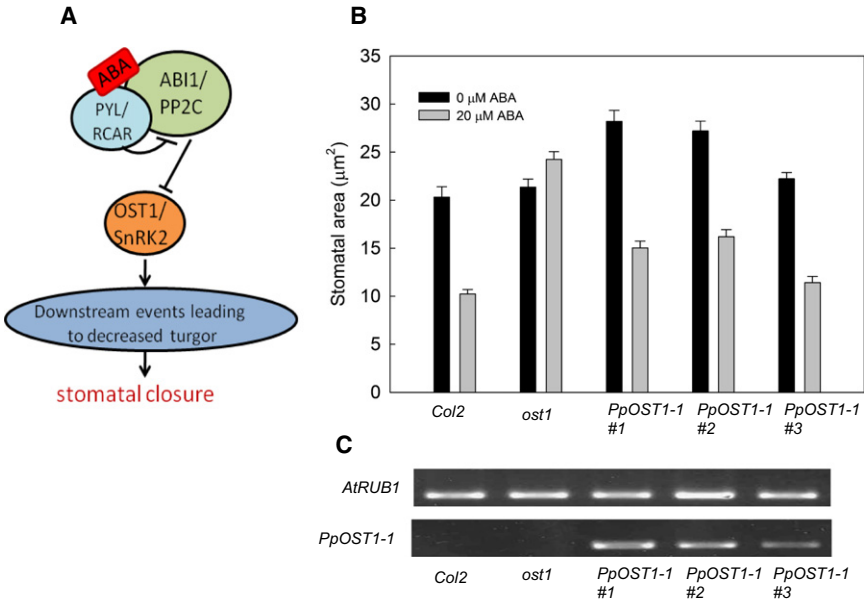

Figure 4. Cross-species Complementation of *A. thaliana ost1* Mutant with *PpOST1-1* Restores Stomatal Sensitivity to ABA

(A) Schematic of core guard cell ABA-signaling genes in the flowering plant *A. thaliana*, illustrating the key role of the OST1 regulatory kinase [22]. PYR/PLY or RCAR family proteins are believed to be ABA receptors [22, 23]. ABA binding promotes their interaction with members of the ABA INSENSITIVE 1 (ABI1) family of protein phosphatase 2C negative regulators of ABA signaling. This interferes with ABI1 binding and inhibition of the OPEN STOMATA 1 (OST1) family of SnRK2 protein kinases, activity of which is required for ABA-induced stomatal closure.
(B) Mean (±SEM) stomatal aperture measurements of *A. thaliana* lines treated with or without 20 μM ABA for 2 hr in CO_2-free air. Stomatal pore areas were significantly different after ABA treatment for each genotype (ANOVA, $p < 0.001$), except for the nontransformed *ost1* mutant.
(C) RT-PCR analysis of *PpOST1-1* expression in wild-type *Col2*, the *ost1* mutant, and three lines of *ost1* transformed with *pOST::PpOST1-1*. *AtRUB1* was used as a positive control.

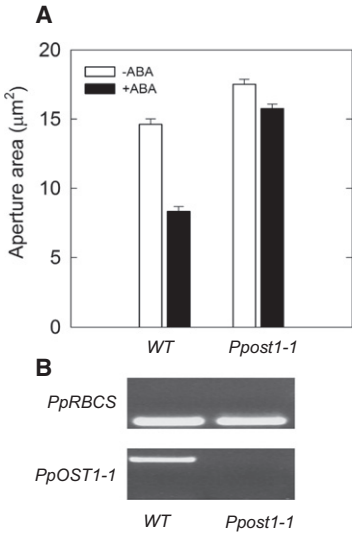

Figure 5. Deletion of *PpOST1-1* from the *P. patens* Genome Causes Attenuated Stomatal Closure Response to ABA

(A) Mean stomatal aperture measurements of *P. patens* wild-type (WT) and *Ppost1-1* deletion mutant sporophytes treated with or without 100 μM ABA for 2 hr in CO_2-free air (±SEM). Stomatal pore areas of ABA-treated *Ppost1-1* deletion mutants were significantly larger than ABA-treated wild-type stomata (ANOVA, $p < 0.001$; 95% confidence limits for wild-type: 5.5–7.0 μm^2; *Ppost1-1*: 1.0–2.5 μm^2).

(B) RT-PCR verification of *P. patens Ppost1-1* knockout mutant. *Ppost1-1* and wild-type cDNA were amplified with primers specific to *PpOST1-1* or positive control *PpRBCS*.

this may represent a key factor in facilitating the earliest phase of the sporophyte-driven colonization of the continents [3] that fundamentally changed the ecology and climate of the planet [27].

Supplemental Information

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.04.032.

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