Evolution of chloroplast retrograde signaling facilitates green plant adaptation to land

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Published online February 25, 2019. This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1812092116/-/DCSupplemental.

Chloroplast retrograde signaling networks are vital for chloroplast biogenesis, operation, and signaling, including excess light and drought stress signaling. To date, retrograde signaling has been considered in the context of land plant adaptation, but not regarding the origin and evolution of signaling cascades linking chloroplast function to stomatal regulation. We show that key elements of the chloroplast retrograde signaling process, the nucleotide phosphatase (SAL1) and 3-phosphoadenosine-5-phosphate (PAP) metabolism, evolved in streptophyte algae—the algal ancestors of land plants. We discover an early evolution of SAL1-PAP chloroplast retrograde signaling in stomatal regulation based on conserved gene and protein structure, function, and enzyme activity and transit peptides of SAL1s in species including flowering plants, the fern *Ceratopteris richardii*, and the moss *Physcomitrella patens*. Moreover, we demonstrate that PAP regulates stomatal closure via secondary messengers and ion transport in guard cells of these diverse lineages. The origin of stomata facilitated gas exchange in the earliest land plants. Our findings suggest that the conquest of land by plants was enabled by rapid response to drought stress through the deployment of an ancestral SAL1-PAP signaling pathway, intersecting with the core abscisic acid signaling in stomatal guard cells.

**Author contributions:** D.E.S., B.J.P., and Z.-H.C. designed research; C.Z., Y.W., K.X.C., and D.B.M. contributed equally to this work. C.Z., Y.W., K.X.C., D.B.M., G.C., P.S.S., D.E.S., B.J.P., and Z.-H.C. analyzed data; and P.J.F., J.H.L.-M., P.S.S., D.E.S., B.J.P., and Z.-H.C. wrote the paper.

**Significance**

The projected increase in drought severity and duration worldwide poses a significant threat to the health of terrestrial ecosystems. We reveal that unique genetic features of desiccation sensing and protection in streptophyte algae not only distinguish them from chlorophyte algae, but also represent a crucial evolutionary step that may have facilitated colonization and subsequent diversification of terrestrial habitats. We demonstrate the evolutionary significance of a molecular mechanism underlying how plants sense drought stress via the coordination of chloroplast retrograde signaling to trigger the closure of stomata, protecting vital photosynthetic tissue. Our findings constitute a significant step forward in understanding the evolution of plant drought tolerance, contributing to the diversification of terrestrial plant communities through past global climate transitions.

Based on fossil evidence (1, 2), green plants (Viridiplantae) colonized land at least 450 million years ago (Mya), with molecular estimates of ~500 Mya (3). This transition to land represents one of the most important steps in the evolution of life on Earth, ultimately leading to the formation of current terrestrial ecosystems. Movement of plants to land significantly altered atmospheric oxygen concentration, facilitating the evolution of terrestrial animals (4, 5). However, crucial steps in green plant terrestrialization have not been fully understood (6–8). Viridiplantae comprise two clades: Streptophyta, which contain streptophyte algae and land plants (embryophytes) (SI Appendix, Fig. S1), and Chlorophyta, which include all the remaining green algae (9). Although there are many extant land-dwelling chlorophyte algae, a group of freshwater streptophyte algae represents the lineage that is sister to embryophytes. Environmental stress tolerance features of streptophyte algae may have facilitated their transition to land (6, 9).

Drought and excess light are inescapable threats to land plants due to the risk of desiccation, photooxidation and associated heat, and UV damage (9). Drought and excess light are key inducers of chloroplast-nucleus communication, which activate acclimatory cellular responses (6, 8, 10). The stress hormone abscisic acid (ABA), the synthesis of which is initiated in chloroplasts, is critical for the cellular response to drought and excess light. Streptophyte algae may possess all of the required features for ABA responsiveness and chloroplast-to-nucleus retrograde signaling (11, 12). The 3-phosphoadenosine-5-phosphate (PAP) signaling network is critical for the cellular response to drought and excess light. PAP regulates stomatal closure via secondary messengers and ion transport in guard cells of these diverse lineages. The origin of stomata facilitated gas exchange in the earliest land plants. Our findings suggest that the conquest of land by plants was enabled by rapid response to drought stress through the deployment of an ancestral SAL1-PAP signaling pathway, intersecting with the core abscisic acid signaling in stomatal guard cells.
retrograde signal has been demonstrated to function in ABA-regulated processes in drought stress and stomatal closure in the flowering plant Arabidopsis thaliana (13). Presumably, the functions and interrelationships of ABA and PAP signaling are present in both streptophyte algae and land plants (6). We hypothesize that chloroplast retrograde and ABA signaling played crucial roles in the process of terrestrialization, enabling ancestral land plants to sense and respond to fluctuating environmental conditions.

The evolution of stomata more than 400 Mya was a key innovation that enabled land plants to regulate gas exchange and hydration and to endure drought as they colonized land (14). As the gateways for terrestrial carbon and water fluxes, stomata have strongly influenced global water and carbon cycles over geological time and respond rapidly to drought (14–16). The ABA signaling network is likely to have evolved before the land plants (17–20), stomata, and stomatal guard cells. Drought-induced ABA production increases guard cell cytosolic Ca\(^{2+}\), hydrogen peroxide (H\(_2\)O\(_2\)), and nitric oxide (NO) to inhibit the K\(^{+}\) channel and to activate the Ca\(^{2+}\)/H\(_2\)O\(_2\) and K\(^{+}\) channels, thereby triggering stomatal closure in A. thaliana (18, 19, 21). In contrast, the mechanisms by which chloroplasts sense drought and contribute to the signal cascade that ultimately triggers stomatal closure during drought are only beginning to be unraveled and have not yet been fully considered in an evolutionary context (6, 22).

Chloroplast retrograde signaling involves multiple signaling pathways necessary to coordinate chloroplast function and plant cell responses to environmental stimuli and to alter plant physiological responses (10). One such chloroplast retrograde signaling pathway involves the drought- and high light-induced phosphoanodinose PAP, a by-product of the reaction of sulfotransferase enzymes (SOTs) including tyrosyl protein sulfotransferases (TPSs) (10, 23). The turnover of PAP in chloroplasts is primarily mediated by the nucleotide phosphatase SAL1/FRY1 (24, 25). Inactivation of SAL1 by transient silencing or loss-of-function led to improved drought tolerance in wheat (Triticum aestivum) and A. thaliana, respectively (26, 27). In A. thaliana, accumulation of PAP mediates large changes of the transcriptome during excess light and drought treatments, enhancing drought tolerance and stimulating stomatal closure with similar kinetics to ABA (13, 28, 29), but whether or not this occurs in other plants is unknown. These findings raise questions about the timing of the origin of retrograde PAP signaling and the evolution of its critical role in stress response and guard cell function.

Here, we address a critical knowledge gap with comparative genetic evidence of key features of drought sensing and protection mechanisms in streptophyte algae, including the sister group to land plants, Zyggnematophyceae. We also trace the evolutionary conservation of SAL1 function throughout the streptophytes and demonstrate how the chloroplast retrograde signal PAP interacts with the ABA-signaling pathway to drive stomatal closure across a phylogenetically broad suite of land plants.

**Results**

**Streptophyte Algae and the Movement of Plants to Land.** Using two methods, we conducted a comparative genetic similarity analysis (15, 30) of predicted sequences from 61 protein families from 31 species representing the major clades (31, 32) of land plants, streptophyte algae, and chlorophyte algae (Fig. 1 and SI Appendix, Tables S1–S3). These 61 protein families (Fig. 1 and SI Appendix, Tables S1–S3) were selected based on their key roles in chloroplast retrograde signaling of SAL1-PAP and sulfur metabolism (33), reactive oxygen species (ROS) and NO signaling (34), photoreceptors (35), Ca\(^{2+}\)-dependent protein kinases (36), and membrane transport and ABA signaling (15, 30).

The overall similarity in these conserved protein families among species of land plants and streptophyte algae is clear (Fig. L4). Not surprisingly, across these protein families, the two streptophyte algae Klebsormidium flaccidum (K. nitens) and Chara braunii showed higher similarity to embryophytes than to the chlorophyte algae (Fig. 1B). Significantly, many components that subsequently form the key guard-cell-signaling pathways were present in streptophyte algae predating the origin of land plants and the evolutionary emergence of stomata (SI Appendix, Fig. S1).

**Molecular Analyses Reveal a Broadly Conserved SAL1-PAP Pathway.** In the sulfur metabolism pathway in which PAP is produced and degraded, TPS and its related SOT19–SOT21 were evolutionarily conserved in all land plants and streptophyte algae, while most cytosolic SOTs (SOT1–SOT18) were not conserved in representative species of streptophyte algae, liverworts, mosses, lycophytes, and ferns (Fig. L4 and SI Appendix, Tables S2 and S3). Amino acid alignment and Logo analysis of the protein domains showed conserved sequence similarity for SAL1s and TPSTs, but not for SOT15s (a representative cytosolic SOT) in some species (SI Appendix, Fig. S2 A–C). Analysis using the 1000 Plant Transcriptome (1KP; www.onekp.com) database (37) showed that the presence and structure of SAL1s and TPSTs are highly conserved in sequences from streptophytes and chlorophytes (Fig. 2A and SI Appendix, Figs. S2 D and E and Table S4). Moreover, using the PlantOrDB (38), SAL proteins were identified in 35 land plants and 6 chlorophyte algae (SI Appendix, Fig. S3A). Gene structure comparison using the PIECE2 (39) software showed conservation of protein domain and exon and intron gene structure in SAL1s and TPSTs from angiosperms to chlorophytes, but these features are not conserved in SOT15s (SI Appendix, Figs. S2F and S3 and Table S4).

We found predicted chloroplast transit peptides (cTPs), important for guiding and translocating the SAL1s into chloroplasts, in all tested land plants. Strong cTP predictions were recovered from analyses of ZcSAL1 and CoSAL1 sampled from Zygnema circinatum (Zygmatophyta) and Coleochaeta orbicularis (Coleochaetales), respectively, which are closely related to land plants. Interestingly, K. flaccidum (Klebsormidiales) was found to have SAL1 homologs (KISAL1/KNsAL1) with putative mitochondrial transit peptide (mTP) as predicted by TargetP and a putative cTP recovered using ChloroP, albeit with different sequence characteristics relative to land plant SAL1 TPs (Fig. 2B and SI Appendix, Figs. S4 and Table S5).

We also experimentally validated the evolutionary conservation of SAL1 catalytic activity against PAP via the cloning and functional analysis of CrSAL1 from the model fern species Ceratopteris richardii and PpSAL1 from the model moss species Physcomitrella patens, respectively. CrSAL1 and PpSAL1 proteins showed PAP catalytic activity, but CrSAL1 activity was lower than PpSAL1 (Fig. 2 C and D). However, both proteins still exhibited enzymatic properties characteristic of AtSAL1 (28). Addition of Mg\(^{2+}\) as a cofactor or reduced DTT (DTTred) as a reductant increased the activity of CrSAL1 and PpSAL1, while known SAL1 inhibitors in A. thaliana, Li\(^{+}\) and oxidized DTT (DTTox) (13, 24), decreased their activity, with stronger inhibition observed in CrSAL1 compared with PpSAL1 (Fig. 2 C and D).

**Evolutionarily Conserved PAP-Induced Stomatal Closure, H\(_2\)O\(_2\) and NO Signaling, and Ion Transport Are Key Features in Land Plants.** We investigated whether the physiological responses to PAP signaling are indeed evolutionarily conserved. Addition of 100 μM PAP resulted in significant closure of stomata in all tested plant species except Marchantia polymorpha, which has pores but no stomata (Fig. 3A and SI Appendix, Fig. S1). We then explored a possible evolutionary link between PAP retrograde signaling and H\(_2\)O\(_2\) and NO, which are vital for abiotic and biotic stress response in most living organisms (40, 41). Treatment with 100 μM PAP led to significant increases in the H\(_2\)O\(_2\) and NO levels in guard cells of two angiosperms (A. thaliana and Hordeum vulgare), a fern (Nephrolepis exaltata), and a moss (Sphagnum fallax) (Fig. 3 B and C).
We further studied the extent of the conserved evolution of PAP action on guard cell ion transport using microelectrode ion flux measurement (MIFE). Across two angiosperms (*A. thaliana* and *H. vulgare*), a fern (*N. exaltata*), and a moss (*S. fallax*), net uptake of K\(^+\) and Cl\(^-\) of guard cells in the control was reversed to net efflux by 100 μM PAP treatment (Fig. 3D and E). Meanwhile, the net K\(^+\) and Cl\(^-\) efflux was accompanied by an average of a 4.3-fold increase of net Ca\(^{2+}\) influx into guard cells of the four species (Fig. 3F). K\(^+\) and Cl\(^-\) flux across the guard cell plasma membrane are regulated by the outwardly rectifying K\(^+\) channel (GORK), inwardly rectifying K\(^+\) channels (KATs), and slow anion channels (SLACs) (19). In situ PCR on guard cells of *A. thaliana* wild-type plants showed that GORK and SLAC1 are significantly up-regulated while KAT2 is significantly down-regulated by 100 μM PAP (*SI Appendix*, Fig. S5A). When expressing these genes in *Xenopus laevis* oocytes, none of the KAT1, KAT2, and SLAC1 channels were directly affected by PAP (*SI Appendix*, Fig. S5B). In *A. thaliana*, I\(_{Ca^{2+}}\) and I\(_{anion}\) in wild-type guard cells showed typical characteristics of anion and Ca\(^{2+}\) channels, and little change was observed over 15 min in the control (*SI Appendix*, Fig. S5C and D). Addition of 100 μM PAP for 15 min led to a significant increase of I\(_{Ca^{2+}}\) and I\(_{anion}\) in guard cells (Fig. 3G).

**Discussion**

The streptophyte algae comprise a grade of lineages that share varying degrees of coancestry with land plants, including the land plant sister group Zygnematophyceae (32, 42). Transcriptomes of streptophyte algae have revealed that algal ancestors of land plants evolved stress-signaling pathways before colonization of terrestrial habitats by land plants (6, 22). Phytohormone-mediated...
Signaling has been detected in streptophyte algae (12), which possessed a complex regulatory network that utilized many transcription factor families similar to those of land plants (43, 44). Thus, it might be expected that the sensing and signaling networks that land plants possess for acclimation and adaptation to the variable and harsh terrestrial environments existed in their algal ancestors (8). Importantly, our data show that SAL1s of the streptophyte algae are very similar to those of land plants. Our findings provide insights into the evolution of a suite of key protein families in the sister groups of land plants (Figs. 1 and 2 and SI Appendix, Fig. S1), with broad implications for understanding the evolution of plant abiotic stress responses and terrestrial plant life.

Here, SAL1 enzymatic capacity for PAP degradation and TPs for organellar targeting of SAL1 in C. richardii and P. patens (Fig. 2) suggest an evolutionarily conserved PAP-mediated signaling in seed-free land plant species. Approximately 95% of plastid proteins are nuclear-encoded, and their precursors contain N-terminal extension TPs (45), which direct the protein precursors into plastids through a conserved posttranslational mechanism (46). The identification of a putative TP in SAL1 of K. flaccidum and cTPs in SAL1s of C. orbicularis and Z. circumcarinatum demonstrates a possible stepwise evolution of TPs for plastidal targeting within the streptophyte algae (Fig. 2B and SI Appendix, Figs. S1 and S4 and Table S5). The acquisition of cTPs in SAL1s provides streptophyte algae with a fundamental functional advantage in gaining a chloroplastic oxidative stress sensor that potentially links the retrograde signaling SAL1-PAP pathway to plant tolerance to desiccation, which these algae frequently experience (6, 8, 22, 46). In this study, we have focused on the evolution of PAP signaling in relation to stomatal closure. This raises the question as to what other functions of SAL1-PAP signaling are conserved. In this context, it is worth noting that streptophyte algae lack guard cells and that PAP signaling has different roles in different cell types in angiosperms, such as having opposing effects on Mg2+ and Li+ ions (46).
ROS accumulation in guard cells and bundle sheaths (13, 24). It would be of interest for future studies to investigate specific functions for PAP and SAL1 in a streptophyte alga and assess how the roles of PAP evolved and specialized during the evolution of multicellularity.

With regard to PAP synthesis, SOTs display significant differences and overlap in their substrate specificities and diversity (23, 47). TPST is exceptional in its size and structure, and the growth of multicellularity.

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Materials and Methods

Evolutionary Bioinformatics. Comparative genetic similarity analysis of protein families and source of the genome dataset are described in refs. 15 and 30. The 1KP database (37) was used to retrieve the mRNA sequences of SAL1s, SOT71s, and PTP5s, and the Interactive Tree of Life resource (https://itol.embl.de) was used to annotate phylogenetic trees. The protein secondary structure of CYPs and mTPs was predicted using Inet (www.compbio.dundee.ac.uk/pred/ legacy/inet) and the Ipred 4 server (50).

Plant Materials and Growth. Plant species were selected to represent divergent lineages across plant land phylogeny (51). The growth conditions of plants are described in refs. 21, 30, and 52.

Stomatal Bioassay. Stomatal aperture and air-pore aperture assays were carried out on epidermal peels (21, 30). The H2O2 and NO production in Stomatal Bioassay. plants are described in refs. 21, 30, and 52.

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Molecular Biology. Cloning of CsSAL1 of C. richardii and of PpSAL1 of P. patens, purification of SAL1 proteins, and AMP produced from degradation of PAP was conducted according to refs. 24 and 28.

Electrophysiology. Net K+, Ca2+, and Cl− flux was measured using the non-invasive MIFE technique (52). Electrophysiological recordings of anion and Ca2+ channel currents in guard cells of ColLer and osf2-2 using double-barreled microelectrodes are described in ref. 21. See SI Appendix for full details of the materials and methods.

ACKNOWLEDGMENTS. We thank Dr. A. Sali, Dr. Y. Wang, Dr. S. Cai, Dr. B. Xu, Dr. X. Wang, Dr. Y. Huang, A. Athman, L. Westmoreland, and M. Mak for their technical support; Dr. F.-W. Li for the femL genome data; and Dr. G. Jin and Dr. F. Xu for bioinformatics. This project is supported by the Australian Research Council (Grants DE1401011143, CE140100008, DP150104007); National Natural Science Foundation of China (Grants 31571578, 31620103912); University of Florida Genetics Institute; and UK Biotechnology and Biological Sciences Research Council.

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