Atavistic Stomatal Responses to Blue Light in Marsileaceae[OPEN]

Anna S. Westbrook,1 and Scott A.M. McAdam1,2,3

Purdue Center for Plant Biology, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

ORCID IDs: 0000-0002-0742-3781 (A.S.W); 0000-0002-9625-6750 (S.A.M.M).

Stomata respond to changes in light environment through multiple mechanisms that jointly regulate the tradeoff between carbon assimilation and water loss. The stomatal response to blue light is highly sensitive, rapid, and not driven by photosynthesis. It is present in most vascular plant groups but is believed to have been lost in the ancestor of leptosporangiate ferns. Schizaeales and Salviniales are the only leptosporangiate orders that have not been tested for stomatal responses to a low fluence of blue light. We report that these stomatal responses are absent in *Lygodium japonicum* (Schizaeales). In contrast, we observed stomatal responses to a low fluence of blue light in *Regnellidium diphyllum* and *Marsilea minuta* (Marsileaceae, Salviniales). In *R. diphyllum*, blue light triggered stomatal oscillations. The oscillations were more sensitive to atmospheric carbon dioxide concentration than to humidity, suggesting that the blue light responses of Marsileaceae stomata differ from those of angiosperms. Our findings suggest that Marsileaceae have physiologically diverged from other leptosporangiate ferns, achieving unusually high photosynthetic capacities through amphibious lifestyles and numerous anatomical convergences with angiosperms. Blue light stomatal responses may have contributed to this divergence by enabling high rates of leaf gas exchange in Marsileaceae.

The evolution of homoiohydric land plants required a mechanism to balance rates of carbon acquisition and water loss. This requirement drove the evolution of stomata, the turgor-operated valves on leaves that open and close in response to numerous environmental signals, including leaf water status, photosynthetic rate, carbon dioxide concentration, and light availability (Darwin, 1898). Mobile stomata enable high photosynthetic capacities by providing a means of optimizing rates of gas exchange under variable conditions and conserving water during drought (Sussmilch et al., 2019; Brodribb et al., 2020). Stomatal responses to light are key components of this regulatory system that take two forms: a photosynthetically active radiation (PAR) response, often referred to as the red light or photosynthesis-dependent response, and an independent blue light response (Zeiger, 1983). Unlike the PAR response, which requires light intensities sufficient to induce increases in photosynthesis, the blue light response can occur under a very low fluence of blue light (fluence too low to increase the photosynthetic rate) in the presence of a high-intensity light of a different wavelength (Ogawa et al., 1978; Shimazaki et al., 2007). In these cases, blue light acts as a signal while PAR is the energy source. In a few species, stomata only open when the blue light and photosynthesis-dependent responses are simultaneously activated (Doi et al., 2015).

The PAR stomatal response can be triggered by signals originating from the mesophyll and guard cells. In angiosperms, the mesophyll-based component appears to be associated with photosynthesis and carbon assimilation (Morison, 1987; Hanstein et al., 2001; Roelfsema et al., 2002). This overlap between light and CO2 responses might explain stomatal responses to CO2 in the dark (Roelfsema et al., 2002; Messinger et al., 2006) and in albino leaf patches (Roelfsema et al., 2006). The signaling pathway linking mesophyll photosynthesis to stomatal function remains unclear (Lawson and Matthews, 2020), although there is some evidence that the signal is aqueous (Lee and Bowling, 1992; Fujita et al., 2013) rather than gaseous (Mott and Peak, 2013; Mott et al., 2014). It is also unclear whether this function is conserved across vascular plants. The angiosperm PAR response might reflect a combination of mesophyll signals and largely unknown pathways involving guard cell photosynthesis and/or metabolism (Olsen et al., 2002; Messinger et al., 2006; Lawson et al., 2008; Zhu et al., 2020), although the mesophyll component is often dominant (Roelfsema et al., 2002; Mott et al., 2008; Fujita et al., 2013). In seed-free plants, the mesophyll-based response to light is

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1These authors contributed equally to the article.  
2Author for contact: smcadam@purdue.edu.  
3Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Scott A.M. McAdam (scmadam@purdue.edu).  
S.A.M.M. designed the research; A.S.W. and S.A.M.M. performed the research and analyzed data; and A.S.W. wrote the manuscript with revisions from S.A.M.M.  
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apparently absent, and the PAR stomatal response is entirely dependent on guard cell photosynthesis and/or metabolism (Doi and Shimazaki, 2008; McAdam and Brodribb, 2012). The lack of a mesophyll-based response may explain why nonangiosperm stomata appear to be insensitive to low CO2 in the dark (Doi and Shimazaki, 2008; Brodribb and McAdam, 2013) and close slowly (Franks and Britton-Harper, 2016), if they close at all (Brodribb et al., 2009; Brodribb and McAdam, 2013), in response to very high CO2 concentrations in the light.

The blue light stomatal response appears to be ancestral in vascular plants (Doi et al., 2015) and primarily contained within the guard cells (Shimazaki et al., 2007; Suetsugu et al., 2014). Although there is also evidence of a stomatal response to blue light with origins outside of the guard cells, the mechanism underlying this secondary response remains unclear (Ballard et al., 2019). In all lineages, the primary pathway occurring within the guard cells is initiated by blue light perception by phototropins and culminates in guard cell H+\text{-}ATPase activation, which hyperpolarizes the plasma membrane and consequently increases guard cell turgor (Kinoshi et al., 2001; Doi et al., 2015; Li et al., 2015b; Falhof et al., 2016; Yamauchi et al., 2016). The intermediate steps of this pathway are increasingly well resolved in Arabidopsis (Arabidopsis thaliana). Upon blue light perception, phototropin molecules autophosphorylate and then recognize and phosphorylate BLUE LIGHT SIGNALING1 (BLUS1; Inoue et al., 2008; Takemiya et al., 2013). BLUS1 appears to phosphorylate a third kinase, BLUE LIGHT-DEPENDENT H+\text{-}ATPASE PHOSPHORYLATION (BHP), which (despite its name) does not interact directly with the H+\text{-}ATPase (Hayashi et al., 2017). The remaining uncharacterized steps likely involve TYPE 1 PROTEIN PHOSPHATASE (PP1), which is a known positive regulator of H+\text{-}ATPase (Takemiya et al., 2006; Hayashi et al., 2017).

The intermediate components of this blue light-signaling pathway in Arabidopsis are not universal. Both BLUS1 and BHP evolved after the divergence of angiosperms (Sussmilch et al., 2019; Harris et al., 2020), so their functions must be performed by unidentified proteins in earlier-diverging lineages (Doi et al., 2015). There are similar phylogenetic differences in secondary stomatal signaling pathways involving blue light. For instance, blue light can facilitate stomatal opening through the inhibition of slow (S)-type anion efflux channels in angiosperms (Marten et al., 2007). This process is dependent on kinases called CONVERGENCE OF BLUE LIGHT AND CO2 (CBC1) and CBC2, which are also implicated in the inhibition of S-type anion efflux channels in response to CO2 deficit (Hiyama et al., 2017; Sussmilch et al., 2019). CBC1 and CBC2 therefore represent an opportunity for coordination between the blue light and PAR pathways. This opportunity may not be available to early-diverging lineages: CBC1 and CBC2 probably evolved in an ancestor to modern seed plants and may not have a functional analog in earlier-diverging lineages (Sussmilch et al., 2019).

Differences between light receptors may also help explain differences in stomatal responses to light across major groups of vascular plants. The phototropin and phytochrome phylogenies show convergent duplication patterns across clades (Li et al., 2015b). For instance, a single phototropin copy that originated in an ancestor to the Viridiplantae underwent independent duplications in the seed plants, leptosporangiate ferns, and lycophytes (and several duplications in the bryophytes), which repeatedly resulted in the specialization of one copy for blue light reception under high-light conditions and the other for low-light conditions (Li et al., 2015b). Phototropins and phytochromes may also have coevolved: these two classes of photoreceptors show similar duplication patterns and may achieve physical or functional coordination through subfunctionalizations among orthologs (Li et al., 2015a; Li and Mathews, 2016). At the same time, some groups of land plants use photoreceptors without functional analogs in angiosperms. Neochrome is a phototropin-phytochrome chimera, first observed in leptosporangiate fern lineages, that helps optimize light reception in the low-light environments that most fern species have occupied since the evolution of angiosperm canopies (Kawai et al., 2003; Kangae et al., 2006). This unique photoreceptor was acquired by horizontal gene transfer from hornworts and has been secondarily transferred between fern lineages (Li et al., 2014).

The most dramatic phylogenetic difference in stomatal light responses is an absence of the blue light response in certain groups. Unlike the PAR stomatal response, which is functionally conserved across vascular land plants despite mechanistic differences between lineages (Doi and Shimazaki, 2008; McAdam and Brodribb, 2012; Doi et al., 2015), the stomatal response to a low fluence of blue light has been lost in the most derived fern lineages (Fig. 1). Previous studies have reported that the response is present in eusporangiate fern groups but apparently lost in the leptosporangiate ferns, or Polypodidae (Doi et al., 2015). These studies have reported an absence of stomatal responses to a low fluence of blue light in eight leptosporangiate fern species: Osmunda japonica (Osmundales), Dicranopteris linearis (Gleicheniales), Alsophila mertensiana (Cyatheales), Adiantum capillus-veneris (Polypodiales), Pteris cretica (Polypodiales), Asplenium scolopendrium (Polypodiales), Thelypteris acuminata (Polypodiales), and Lepisorus thunbergianus (Polypodiales; Doi et al., 2006, 2015). Those eight species represent four of the seven orders of leptosporangiate ferns, one of which lacks stomata (Smith et al., 2006). We sought to test for stomatal responses to blue light in the remaining two orders, the Schizaeales and Salviniales.

Here we report the first observation of stomatal responses to blue light within the leptosporangiate ferns. These responses occurred within the Marsileaceae, a small family of semiaquatic ferns in the order Salviniales. The Marsileaceae are an unusual group exhibiting several convergences with angiosperms, including...
heterospory (Schneider and Pryer, 2002), vessel ele-
ments (Schneider and Carlquist, 2000), high rates of
gas exchange (Wu and Kao, 2011; Deans et al., 2019),
and superficially angiosperm-like stomatal kinetics
(Deans et al., 2019). Marsileaceae also exhibit several
nonstomatal responses to blue light (Lin and Yang,
1999; Kao and Lin, 2010). Considered in this context,
our finding suggests that blue light responses, includ-
ing stomatal responses, helped enable the extensive
physiological and ecological divergence of Marsilea-
cean from other leptosporangiate ferns. We also report
preliminary evidence suggesting that the signaling
networks integrating blue light stomatal responses with
other influences on stomatal aperture differ between
Marsileaceae and angiosperms. These results are con-
sistent with a hypothesis of a secondary evolution of
stomatal responses to blue light in Marsileaceae, which
may have been driven by a selective pressure for high
photosynthetic capacities and rapid responses to envi-
ronmental change.

RESULTS
We examined Lygodium japonicum (Schizaeales),
Marsilea minuta (Salviniales), and Regnellidium diphy-
lum (Salviniales) for the presence of stomatal re-
sponses to blue light by tracking stomatal conductance
during a transition from 600 μmol m⁻² s⁻¹ red light to
600 μmol m⁻² s⁻¹ red light + 5 μmol m⁻² s⁻¹ blue
light. In L. japonicum, we observed no change in sto-
matal conductance or photosynthetic rate when a low
fluence of blue light was superimposed onto a red light
background (Fig. 2A). In contrast, both M. minuta and
R. diphyllum showed increases in stomatal conductance
and photosynthetic rate (Fig. 2, B and C).

In R. diphyllum, the magnitude of the increase in
stomatal conductance after the imposition of blue light
was dependent on the intensity of the blue light im-
posed, up to the wavelength composition of 300 μmol
m⁻² s⁻¹ blue light + 300 μmol m⁻² s⁻¹ red light (Fig. 3).
Increases in photosynthetic rate sometimes accompa-
nied these increases in stomatal conductance, but the

Figure 1. Stomatal responses to blue light in ferns and outgroups. Phylogeny (simplified from PPG I, 2016) showing previous reports of the presence (black) and absence (red) of stomatal responses to blue light (Doi et al., 2015, 2006), with new data presented in this study shown in bold text. Hymenophyllales lack stomata (Smith et al., 2006).

Figure 2. Stomatal responses to a low fluence of blue light are absent in Schizaeales and present in Salviniales. Photosynthetic rate (green) and stomatal conductance (blue) were measured in leaves of L. japonicum (Schizaeales; A), M. minuta (Salviniales; B), and R. diphyllum (Salviniales; C), with transitions between light envi-
ronments indicated by vertical lines and red (RL) and blue (BL) light intensities given between lines.
photosynthetic changes were proportionally smaller and not dose dependent (Fig. 3). The imposition of blue light on a red-light background also triggered stomatal oscillations in this species. The oscillations occurred under low intensities of blue light (5–40 μmol m⁻² s⁻¹) but not under high intensities of blue light (100–300 μmol m⁻² s⁻¹; Fig. 3). Imposing 40 μmol m⁻² s⁻¹ blue light triggered several clear oscillations when atmospheric CO₂ was held at 400 μmol mol⁻¹ CO₂ but did not trigger oscillations at 800 μmol mol⁻¹ CO₂ (Fig. 4). The high-CO₂ environment also muted the increase in stomatal conductance due to blue light. In contrast, vapor pressure deficit (VPD) appeared to have relatively little effect on either the magnitude of the blue light response or the emergence of oscillations under blue light (Fig. 5). In the average response to 40 μmol m⁻² s⁻¹ blue light imposed at low VPD (1–1.3 kPa), the initial oscillation amplitude was 0.043 mol m⁻² s⁻¹ and the initial period was 24 min. At high VPD (2–2.4 kPa), the initial amplitude was 0.027 mol m⁻² s⁻¹ and the initial period was 20.5 min.

We also investigated the relationships between stomatal conductance (gₛ), photosynthetic rate (A), intercellular CO₂ concentration (Cᵢ), and intrinsic water-use efficiency (iWUE; A/gₛ) during the stomatal oscillations associated with the imposition of blue light in R. diphyllum. We confirmed that the higher stomatal conductances associated with the imposition of a low fluence of blue light were not matched by proportional increases in photosynthetic rate, resulting in increased Cᵢ values and reduced iWUE values (Fig. 6). The troughs in Cᵢ and the peaks in iWUE corresponded to the steady-state values of Cᵢ and iWUE observed under the same intensities of exclusively red light. In other words, blue light was associated with higher Cᵢ and lower water use efficiency throughout the oscillatory cycles.

Finally, we compared rates of stomatal closure upon transition to darkness. These rates depended on the initial light environment. The removal of a mixture of blue and red wavelengths caused stomata in Marsileaceae species to close at initially rapid rates, which could be matched to exponential models with time constants of 2.28 (M. minuta) and 3.24 min (R. diphyllum; Fig. 7). In contrast, exposure to darkness from exclusively red wavelengths caused stomata to close along very slow exponential (M. minuta; 12.00 min) or linear (R. diphyllum) trajectories.

DISCUSSION

Blue Light Responses in Marsileaceae

The stomata of L. japonicum, a representative of the order Schizaeales, did not respond to a low fluence of
blue light imposed on a red light background. This finding is consistent with results from all leptosporangiate ferns previously studied (Doi et al., 2006, 2015). Unexpectedly, we did discover a stomatal response to low fluence of blue light on a red light background in two species from the Marsileaceae (Salviniales), *M. minuta* and *R. diphyllum* (Fig. 2). This finding represents the first report of stomatal responses to a low fluence of blue light in a group of leptosporangiate ferns (Fig. 1).

The order Salviniales contains two families: Marsileaceae (genera *Marsilea*, *Regnellidium*, and *Pilularia*) and Salviniaceae (genera *Salvinia* and *Azolla*). As both representatives examined here belong to the family Marsileaceae, we do not know whether stomatal responses to blue light are present throughout the order Salviniales or restricted to the Marsileaceae. Given that the exclusively aquatic *Salvinia* spp. have only vestigial stomata and *Azolla* spp. have highly modified, doughnut-shaped stomata formed from a single guard cell (van Cotthem, 1970; Busby and Gunning, 1984; de la Sota and Cassa de Pazos, 1990), we suspect that these responses are only found in Marsileaceae. The Salviniaceae are an ancient order with an extensive fossil record but largely unknown origins that may date back to the late Jurassic (Yamada and Kato, 2002; Collinson et al., 2013; Hermsen, 2019). Future investigations into early fossil members of this order might help clarify whether common ancestors of extant Marsileaceae and Salviniaceae produced leaves with stomata, achieved high rates of photosynthesis, and/or experienced water shortages, any of which may indicate the evolutionary origin of these stomatal responses in this clade.

Two evolutionary scenarios can account for the presence of stomatal responses to low fluence of blue light in Marsileaceae and their absence in other leptosporangiate groups (Fig. 1): either these responses have been repeatedly lost (at least four times) in leptosporangiate ferns or they were lost once in the common ancestor of leptosporangiate ferns and regained in Marsileaceae. The ease with which a group could regain this function would depend on the nature of the lesion.

Figure 4. Stomatal responses of *R. diphyllum* to blue light (BL) transitions at ambient and high atmospheric CO2. Mean (± se; *n* = 3–4) photosynthetic rate (green) and stomatal conductance (blue) at 400 (A) and 800 μmol mol−1 CO2 (B) in leaves acclimated to 600 μmol m−2 s−1 red light (RL) then exposed to 40 μmol m−2 s−1 BL and 560 μmol m−2 s−1 RL (vertical line).

Figure 5. Stomatal responses of *R. diphyllum* to the imposition of blue light (BL) by VPD. Mean (± se; *n* = 3) photosynthetic rate (green) and stomatal conductance (blue) at a low VPD (1–1.3 kPa; A) and a high VPD (2–2.4 kPa; B). Leaves were acclimated to 600 μmol m−2 s−1 red light (RL) then exposed to 40 μmol m−2 s−1 BL and 560 μmol m−2 s−1 RL (vertical line).
to the blue light stomatal signaling pathway, which will be difficult to determine until all components of the pathway have been identified in both angiosperms and ferns. This effort is complicated by evidence that some components of the blue light stomatal signaling pathway differ across major groups of land plants (Doi et al., 2015; Sussmilch et al., 2019; Harris et al., 2020). Furthermore, it seems likely that the signaling pathway responsible for stomatal responses to blue light in Marsileaceae differs from the pathway found in the ancestor to all ferns (and/or the ancestor to all vascular plants; Doi et al., 2015), especially given that most, if not all, components of the canonical stomatal response to blue light also serve unrelated functions or are closely related to genes that do so (Inoue et al., 2008; Takemiya et al., 2013; Hayashi et al., 2017; Sussmilch et al., 2019).

Blue Light-Induced Stomatal Oscillations in Regnellidium

We found that a low fluence of blue light superimposed onto higher intensities of red light triggered stomatal oscillations in *R. diphyllum*. Stomatal oscillations are worth studying because they have direct fitness consequences and may provide insight into stomatal regulation (Cowan, 1972; Farquhar and Cowan, 1974). While oscillations are common in *R. diphyllum* and not exclusive to any light environment, they are unusually large under blue light and frequently disappeared upon the removal of the blue light. This effect is the opposite of the behavior previously observed in angiosperms, where blue light inhibited stomatal oscillations in the monocots *Musa acuminata* (Zait et al., 2017) and *Tradescantia pallida* (Ballard et al., 2019).

**Figure 6.** Effects of blue light (BL)-induced stomatal oscillations on photosynthetic efficiency in *R. diphyllum*. Photosynthetic rate (green) and stomatal conductance (blue; A), Ci (B), and iWUE (C) are shown across a single series of light transitions, indicated by vertical lines. BL and red light (RL) intensities are given between lines.

**Figure 7.** Rates of stomatal closure in response to darkness in *M. minuta* and *R. diphyllum*. Mean (± se; n = 3) relative stomatal conductance across the transition from exclusively red light (RL; red lines) or combined red and blue light (BL; 40 μmol m⁻² s⁻¹ blue + 560 μmol m⁻² s⁻¹ RL; purple lines) to darkness (indicated by the vertical line) in *M. minuta* (A) and *R. diphyllum* (B). Mean (± se) rates of stomatal conductance at t = 10 were 0.060 ± 0.014 mol m⁻² s⁻¹ (*M. minuta*, RL), 0.171 ± 0.057 mol m⁻² s⁻¹ (*M. minuta*, RL + BL), 0.124 ± 0.024 mol m⁻² s⁻¹ (*R. diphyllum*, RL), and 0.247 ± 0.053 mol m⁻² s⁻¹ (*R. diphyllum*, RL + BL).
This angiosperm stomatal behavior has been explained by attributing oscillations under red light to a hydraulic mismatch between transpiration rate and leaf water supply (Zait et al., 2017; Ballard et al., 2019). A hydraulic mismatch can cause leaf turgor and stomatal conductance to oscillate in opposite phases (Barps, 1971; Farquhar and Cowan, 1974), particularly if stomatal responses to leaf water status are delayed because they depend on metabolic regulation. This hydraulic explanation accounts for most known stomatal oscillations, although other causes are possible (Yang et al., 2003). Blue light may inhibit hydraulic oscillations through two distinct mechanisms, one occurring in the guard cells and another based in the surrounding tissue (Ballard et al., 2019). The guard cell mechanism is probably the archetypal blue light response, which may increase the osmotic load in guard cells enough to overcome hydraulic triggers that would otherwise cause stomatal closure. The other mechanism remains largely uncharacterized, but there is some indication that it involves changes in the bundle sheath to increase the hydraulic supply to the guard cells (Zait et al., 2017). If so, this pathway would presumably not occur in ferns, because the bundle sheath is an angiosperm synapomorphy (Lee goodwill, 2008).

In *R. diphyllum*, blue light oscillations were much more common under a low fluence (5–40 μmol m\(^{-2}\) s\(^{-1}\)) than under a high fluence (100–300 μmol m\(^{-2}\) s\(^{-1}\)) of blue light at a constant total light intensity (Fig. 3). The absence of oscillations under high fluences of 100 and 300 μmol m\(^{-2}\) s\(^{-1}\) blue light suggests that, as in angiosperms (Zait et al., 2017; Ballard et al., 2019), a high intensity of blue light can generate sufficient osmotic pressure to maintain high guard cell turgor and stomatal aperture even in the presence of other closing signals, such as reduced water availability or even reduced photosynthetic rate. However, the intensity threshold beyond which the blue light signal dominates appears to be higher in Marsileaceae than in angiosperms previously studied (Ballard et al., 2019).

The strong opening response and lack of oscillation under 50% blue light (Fig. 3) suggest that oscillations are not a necessary consequence of changes to leaf water status in *R. diphyllum*. That stomata opened normally and oscillated in response to a low fluence of blue light at high VPD (Fig. 5) further suggests that leaf water status may not effectively explain the presence of oscillations when they do occur. In contrast, a high concentration of atmospheric CO\(_2\) muted the stomatal blue light response and prevented stomatal oscillation in response to a low fluence of blue light (Fig. 4). These data suggest that the oscillations in *R. diphyllum* triggered by a low fluence of blue light may be largely, if not exclusively, due to a photosynthetic feedback signal.

A better understanding of fern stomatal responses to PAR and CO\(_2\) would facilitate the investigation of possible photosynthetic feedback influencing stomatal conductance. For instance, we sometimes observed rapid stomatal closure during oscillations under blue light (Figs. 2 and 6). Marsileaceae stomata also close quickly upon the removal of combined blue and red light, but they close slowly upon the removal of exclusively red light (Fig. 7). The closure speeds observed following the transition from red light to darkness are more reminiscent of other leptosporangiate ferns (Xiong et al., 2018; Kübarsepp et al., 2020), most of which lack blue light stomatal responses (Doi et al., 2015). Taken together, these findings suggest that oscillations in *R. diphyllum* involve an unknown signal that actively inhibits the blue light response instead of merely driving closure through an independent pathway. Studies of stomatal regulation in angiosperms have revealed that feedback between light and CO\(_2\) signaling (Hiyama et al., 2017; Ando and Kinoshita, 2018) are important components of a complex regulatory system (Zhang et al., 2018; Sussmilch et al., 2019). The oscillations observed in *R. diphyllum* under blue light may reflect similar interactions in this species of fern.

**Ecological Significance of Blue Light Responses**

The evolution of stomatal responses to light can be attributed to the same fundamental driver that underlies all active stomatal regulation: land plants need to balance carbon acquisition against water loss (Raven, 2002; Brodribb et al., 2020). As the optimal approach to this tradeoff can vary over short timescales, many evolutionarily successful strategies emphasize rapid responses to environmental changes (Lawson and Blatt, 2014). One well-established explanation for blue light stomatal responses extends this paradigm, focusing on the fact that carbon availability limits photosynthesis during the lag period between the photosynthetic response to increased light and the stomatal opening response (Drake et al., 2013). In angiosperms, stomatal opening in the presence of blue light is much faster than stomatal opening under purely red light (Sharkey and Ogawa, 1987; Shimazaki et al., 2007). For this reason, blue light responses hasten stomatal opening at dawn, a period in which low intensities of blue light quickly become available (Zeiger et al., 1981). Rapid stomatal responses may also help angiosperms simultaneously achieve high time-averaged photosynthetic rates and efficient water use in variable light environments (Kirschbaum and Pearcy, 1988; Shimazaki et al., 2007).

The explanation involving variable light availability is interesting in the context of fern evolution because ferns underwent a major adaptive radiation in the shady environments underneath angiosperm canopies (Schneider et al., 2004; Schuettpelz and Pryer, 2009). Although the presence of the blue light stomatal response (Doi et al., 2015) and even the phototropin duplication unique to ferns (Li et al., 2015b) are ancestral to the lineage, which emerged prior to the angiosperm explosion (Pryer et al., 2004), responses to light acquired a new ecological significance for ferns after the
rise of angiosperms. Competitive and mutualistic relationships with angiosperms both tend to entail light limitation (Schneider et al., 2004; Schuettpelz and Pryer, 2009). Both for this evolutionary reason and because they lack various physiological adaptations found in angiosperms (Brodribb et al., 2005, 2007; Feild and Brodribb, 2013), ferns typically have low photosynthetic capacities relative to angiosperms (Brodribb et al., 2005; Tosens et al., 2016). Consequently, the fitness consequences of light limitation and the best possible physiological responses may differ between ferns and angiosperms. Light limitation is thought to explain the adaptive value of numerous fern innovations, such as neochrome, the phytochrome-phototropin chimera (Kawai et al., 2003; Kanegae et al., 2006) found in the Polypodiales, Gleicheniales, and Cyatheales (Li et al., 2014). Neochrome facilitates adaptation to shady environments through increased light sensitivity, chloroplast movement, and phototropism (Li and Mathews, 2016). The acquisition of neochrome is not a causal explanation for the absence of blue light stomatal responses in most leptosporangiate ferns. Neochrome is not associated with stomatal responses (Li and Mathews, 2016) and has not been observed in the Schizaeales and Osmundales, orders in which stomatal responses to a low fluence of blue light also have not been observed (Fig. 1; Li et al., 2014; Doi et al., 2015). Nonetheless, the presence of both these unusual light-related features in most leptosporangiate ferns suggests a broader evolutionary trend in which efficiency has become less important than maximizing assimilation in low-light environments.

The Marsileaceae have a unique evolutionary history differentiating them from other ferns. The aquatic and semiaquatic habitats of these species (Pryer, 1999) may occur under less dense canopies than those under which most leptosporangiate ferns have radiated (Schneider et al., 2004; Schuettpelz and Pryer, 2009). The Marsileaceae are also unusual in that they have achieved some of the highest rates of gas exchange recorded in ferns (Wu and Kao, 2011; Deans et al., 2019), magnifying the costs associated with delayed stomatal responses to changing light environments (Vico et al., 2011; McAusland et al., 2016). Efficient stomatal responses to variation in light availability could therefore be more important in the Marsileaceae than in other ferns, which generally do not achieve high water-use efficiency in variable environments (McAdam and Brodribb, 2012).

Support for the idea of increased efficiency in Marsileaceae emerges from data on closure speeds. Marsileaceae representatives *M. quadrifolia* and *M. hirsuta* close stomata rapidly in response to darkness (McAdam and Brodribb, 2012; Kübarsepp et al., 2020). In this study, we found that *M. minuta* and *R. diphyllum* both achieved rapid stomatal closure if (and only if) the stomatal response to blue light had previously been activated (Fig. 7). This result suggests that stomatal responses to blue light help Marsileaceae make use of transient periods of light availability by shortening the period of low water-use efficiency that occurs at the end of those periods. This adaptation might be especially useful in the partially terrestrial lineages that account for a large number and increasing proportion of Marsileaceae species (Nagalingum et al., 2007).

The finding that Marsileaceae exhibit stomatal responses to a low fluence of blue light, unlike all leptosporangiate ferns previously studied (Doi et al., 2015), also provides strong support for the idea that these unique ferns have undergone positive selection for increased stomatal opening. Stomatal responses to blue light in Marsileaceae, as in eusporangiate ferns and other groups of vascular plants (Shimazaki et al., 2007; Doi et al., 2015), are highly sensitive to low intensities of blue light provided a higher-intensity background of PAR is also available. Alongside the finding that stomatal conductance increases with the amount of blue light provided, even with total light intensity held constant (Fig. 2), this result indicates that blue light opens stomata wider than the apertures achievable by the PAR stomatal response. As in angiosperms, this capacity may support photosynthesis under low-light conditions (Zeiger, 1983). Under higher-light conditions, the blue light response might contribute to the high stomatal conductances and photosynthetic rates observed in Marsileaceae (Wu and Kao, 2011; Deans et al., 2019).

CONCLUSION

Our observations support a view in which most leptosporangiate ferns have adopted an evolutionarily successful strategy tailored for shady forest understories (Schneider et al., 2004; Schuettpelz and Pryer, 2009). Lacking some of the hydraulic innovations (Brodribb et al., 2005, 2007; Feild and Brodribb, 2013) and stomatal control mechanisms (Sussmilch et al., 2019; Brodribb et al., 2020) found in seed plants, ferns have lower photosynthetic capacities (Brodribb et al., 2005; Tosens et al., 2016). Many ferns also have ready access to water (Mehltreter et al., 2010). Taken together, these facts suggest that the optimal strategy for fern stomatal regulation usually promotes photosynthesis whenever water is available. Stomatal responses to blue light, which promote closure as well as opening, could be unnecessary or even counterproductive. However, these responses may have provided adaptive benefits for the Marsileaceae, which have followed an evolutionary trajectory different from that of other leptosporangiate ferns (Pryer, 1999, 2004). The high photosynthetic capacities of most Marsileaceae (Wu and Kao, 2011; Deans et al., 2019) are facilitated by numerous short-term and long-term responses to environmental conditions (Johnson, 1986). Our study adds blue light stomatal responses to this list of adaptations known to maximize rates of leaf gas exchange in Marsileaceae. The absence of these responses in all leptosporangiate ferns previously studied and the differences in these responses between Marsileaceae and angiosperms jointly suggests that stomatal blue light...
responses may have been lost in an ancestor of leptosporangiate ferns and secondarily evolved in Marsileaceae. Our results highlight the potential evolvability of stomatal light response pathways and indicate that differences in these pathways may emerge at high taxonomic resolution.

MATERIALS AND METHODS

**Legiodium japonicum**, *Marsilea minuta*, and *Regnellidium diphylum* were grown under controlled conditions at day/night temperatures of 23°C/18°C under a natural photoperiod in the glasshouses at Purdue University. All plants were grown in standing water in IN Miami topsoil, watered daily, and given monthly applications of complete liquid fertilizer.

A LI-6800 Portable Photosynthesis System was used for gas exchange measurements. To test for stomatal responses to low fluence of blue light, we followed the protocol of Doi et al. (2015). Conditions in the cuvette were maintained for the duration of the experiment with air temperature set at 23°C to 24°C, CO₂ concentration at 365 (Marsileaceae) or 400 μmol mol⁻¹ (Legiodium). VPD at 1.08 to 1.54 kPa, flow rate at 500 μmol s⁻¹, and fan speed 10,000 rpm. Plants were acclimated to darkness before exposure to 600 μmol quanta m⁻² s⁻¹ of red light. After stomata opened and gas exchange rates stabilized, an additional 5 μmol quanta m⁻² s⁻¹ of blue light was added to the red light background. Both wavelengths were turned off after 10 min. In Marsileaceae species, we waited for gas exchange rates to stabilize, eliminated the blue light, and waited for stability again before exposing leaves to darkness.

To compare the effects of different intensities of blue light, we set conditions in the cuvette to 23°C to 28°C, 400 μmol mol⁻¹ CO₂, 1.6 to 2.3 kPa VPD, 500 μmol s⁻¹ flow, and 10,000 rpm fan speed. To compare the effects of different concentrations of CO₂ on blue light responses, we set conditions in the cuvette to 23°C, 1.0 to 1.3 kPa VPD, 500 μmol s⁻¹ flow, and 10,000 rpm fan speed. To compare the effects of different VPD values on blue light responses, we set conditions in the cuvette to 23°C, 400 μmol mol⁻¹ CO₂, 500 μmol s⁻¹ flow, and 10,000 rpm fan speed. To analyze the effect of initial light environment on rates of stomatal closure in darkness, we set conditions in the cuvette to 22°C to 24°C, 0.8 to 1.5 kPa VPD, 500 μmol s⁻¹ flow, and 10,000 rpm fan speed, and the light environment to 500 to 600 μmol m⁻² s⁻¹ at either 100% red light or 90% red light with a maximum of 40 μmol m⁻² s⁻¹ blue light. We conducted a minimum of three biological replicates for each of these experiments.

All leaf gas exchange data were automatically logged every 30 s and corrected for leaf area in the cuvette by taking measurements from photographs in ImageJ (version 1.52; Schneider et al., 2012). Gaps in the traces indicate the removal of transitional spikes and artifacts due to analyzer matching. The initial oscillation amplitude was calculated as half the change in gₑ between the first peak following the imposition of blue light and the subsequent trough. The initial oscillation period was the time elapsed between the first two peaks following the imposition of blue light. iWUE was calculated as the quotient of photosynthetic rate and stomatal conductance to water vapor. Rates of stomatal closure in darkness were analyzed by normalizing each trace to gₑΔ = 1 at t = 0, averaging within each species by treatment combination, and fitting exponential (following Vico et al., 2011) or linear models in R (version 4.0.2) to each averaged sequence, excluding oscillations at the beginning or end when necessary.

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