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# Stomatal blue light response is present in *Marsilea crenata*, an amphibious fern

**Tai-Chung WU[1], Bai-Ling LIN[2], Wen-Yuan KAO[1,*]**

*1. Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei, Taiwan.*
*2. Genomics Research Center, Academia Sinica, Taipei, Taiwan.*
*[*]Corresponding author's email: wykao@ntu.edu.tw*

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ABSTRACT: The active and passive regulation of stomatal response to environmental stimulation has been well studied in angiosperms but rarely in ferns. Active stomatal control of *Marsilea crenat*a, an amphibious fern, in response to $CO_2$ concentration and exogenous application of abscisic acid was reported in a previous study. The objectives of the current study were to investigate whether *M. crenata* has stomatal blue light (BL) response and to evaluate the sensitivity of the response. Leaf stomatal conductance ($g_s$) of the fern grown under two light regimes and the apertures of stomata on isolated epidermal strips irradiated with photosynthetically saturated red light (RL) or with RL plus BL were measured. Stomatal response of two angiosperms and two other ferns (*Adiantum capillus-veneris* and *Nephrolepis auriculata*) were also measured. Application of 50 μmol $m^{-2}$ $s^{-1}$ BL caused a significant increase in $g_s$ of *M. crenata* and *Sambucus chinensis* (an angiosperm) leaves irradiated with photosynthetically saturated RL of 900 μmol $m^{-2}$ $s^{-1}$, indicating the presence of stomatal BL-specific response. The response was not detected in the two other ferns. In comparison to sole RL irradiated epidermal strips, a 43 % increment of the ratio of stomatal width/length was measured in *M. crenata* epidermal strips irradiated with RL plus BL. *M. crenata* grown under shading lost the stomatal BL-specific response. In conclusion, this is the first report of the presence of stomatal BL-specific response in a fern of Polypodiopsida. However, growth conditions have strong effects on the sensitivity of the specific response of the fern.

KEY WORDS: Fern, *Marsilea crenata*, Polypodiopsida, stomatal blue light-specific response.

## INTRODUCTION

Stomata first occurred in the fossil recorded around 400 million years ago in the late Silurian-early Devonian Period (Edwards *et. al.*, 1992). This structure is one of the critical evolutionary features contributing to the successful colonization of land by plants. A stoma is a pore which is surrounded by a pair of guard cells. Turgor changes in the guard cells determine the area of stomatal pore through which gaseous diffusion occurs. When guard cells are turgid and inflated, the pore opens, and the pore closes as the cells are deflated. This process allows plants to uptake $CO_2$ for photosynthesis while reduce water loss through transpiration (for a review see Buckley, 2005).

Light-induced stomatal responses were first reported by Darwin (1898). Stomata open in response to red light and blue light. Distinct mechanisms underlie the stomatal opening response to different wavelengths of light (Shimazaki *et al.*, 2007). Red light absorbed by chloroplasts of guard cells and mesophyll cells provides energy for carbon fixation. Due to the limitation of stomata to $CO_2$ diffusion, the process of carbon fixation results in reduction of the intercellular $CO_2$ concentration (Ci). The decrease of Ci activates the transport mechanism, induces membrane hyperpolarization hence results in stomatal opening. The induction of stomatal opening by the reduction of Ci is not specifically caused by the chloroplast absorption of red light. Blue light also has the same effect after being absorbed by chloroplasts of mesophylls and guard cells. However, it has been shown that red light induces stomatal opening even under constant Ci (Messinger *et al*., 2006; Lawson *et al*., 2008), which suggests that a Ci-independent mechanism is also involved in red light-induced stomatal opening. Recent study by Ando and Kinoshita (2018) found that red light-induced photosynthesis dependent phosphorylation of plasma membrane H+-ATPase in guard cells promotes stomatal opening in whole leaves. In addition to providing energy, blue light also acts as a signal inducing stomatal opening. In guard cells, blue light perceived by the photoreceptors, phototropins (phot1 and phot2), initiates a signal transduction cascade and ultimately activates the $H^+$-ATPase on plasma membrane (Kinoshita and Shimazaki, 1999; Briggs and Christie, 2002). The simultaneous acidification of apoplast induces the hyperpolarization of plasma membrane and drives $K^+$ uptake through voltage-gated $K^+$ channel, which results in stomatal opening. This way of stomatal opening is induced specifically by blue light and is independent of photosynthesis, therefore, is called stomatal blue light-specific response. In parallel to the activation of membrane ion transport, guard cell starch degradation is activated through the phot1/phot2-dependent signaling pathway, which accelerates stomatal opening in *Arabidopsis* (Horrer *et al*., 2016). It is thus suggested that guard cells integrate blue-light induced proton pumping with starch degradation to regulate stomatal opening (Horrer *et al*., 2016). The stomatal blue light-specific response is usually investigated with a dual-beam protocol in which a strong red light is used to saturate photosynthetically

mediated opening of stomata and a second beam of blue light is subsequently applied (Iino *et al*., 1985). Any additional opening produced by the supplemented blue light can be attributed to the blue light-specific response (Iino *et al.*, 1985; Frechilla *et al.*, 2004).

It has been proposed that the stomatal blue light-specific response is important for stimulating stomatal opening in the early morning. The response facilitates photosynthetic carbon gain early in the diel period, when the irradiance spectrum is enriched in blue wavelengths (Zeiger, 1984). The rapid stomatal opening could also be important for plants, particularly for those in the understory (Chazdon, 1988; Chazdon and Pearcy, 1991), to efficiently utilize the short duration of sun flecks for photosynthesis (Iino *et al.*, 1985).

The stomatal blue light-specific response has been studied mainly in angiosperms but few in ferns. For example, the stomatal blue light-specific response was not found in *Adiantum capillus-veneris* (a fern of Leptosporangiopsida), although it possesses both functional phototropin and plasma membrane $H^+$-ATPase (Doi *et al*., 2006). After surveying a variety of plant species of euphyllophytes, including spermatophytes, monilophytes, and lycophytes, Doi *et al*. (2015) found that the blue light-specific response operated in lycophytes and all euphyllophytes except Polypodiopsida. They also confirmed that lycophytes and angiosperms share the same regulation mechanism of blue light-specific stomatal response. These results suggest that the ancestor of lycophytes and euphyllophytes might have acquired blue light-specific stomatal response while modern ferns of Polypodiopsida, which evolved under canopy of angiosperms (Schneider *et al.*, 2004), likely lost the ability.

*Marsilea*, an amphibious fern, diverging earlier than most ferns of Polypodiopsida, exhibits many features similar to angiosperms (Kao and Lin, 2010; Schneider and Carlquist, 2000). Wu (2020) investigated stomatal response to environmental factors and found that stomata of *M. crenata* do respond to elevated $CO_2$ and the application of ABA, which is similar to the response of stomata of angiosperms but differs from that of most ferns of Polypodiopsida (Wu *et al*., 2020). In this study, we asked do stomata of *M. crenata* also have blue light-specific response that is found in most angiosperms? To answer the question, we compared stomatal conductance ($g_s$) of *M. crenata* leaves irradiated with photosynthetically saturated red light and subsequently with red light plus blue light pulse. In addition, we examined stomata on isolated epidermal strips of *M. crenata* leaves irradiated with red light only or with red light plus blue light. The sensitivity of stomatal response to blue light in *M. crenata* plants grown under different light regimes was also investigated.

## MATERIALS AND METHODS

**Stomatal conductance in response to a blue light pulse**

In addition to *Marsilea crenata*, an angiosperm (*Sambucus chinensis*) and two Polypodiopsida ferns (*Adiantum capillus-veneris* and *Nephrolepis auriculata*) were investigated for the reference. *Sambucus chinensis* has been shown to have stomatal blue light-specific response (Huang, 2015), whereas *A. capillis-veneris* and *N. auriculate* have no such response (Doi *et al.*, 2006). Studied plants were grown in a greenhouse receiving natural light and with temperature controlled at 25 °C. We conducted photosynthetic gas exchange measurements on the most recently fully expanded leaves or leaflets using a portable photosynthetic system (LI-6400, LI-COR, USA) equipped with a dual-beam (red and blue) light source (LI-6400-40, LI-COR, USA). For the gas exchange measurement, a leaf (or leaflet) was enclosed in a cuvette, with the vapor pressure deficit between leaf and air (VPD) controlled at 1.1~1.3 kPa and leaf temperature at 25 °C, and illuminated with a photosynthetically saturated red light (RL) of 900 μmol $m^{-2}$ $s^{-1}$. After reaching steady-state, the photosynthetic rate ($A_n$), stomatal conductance ($g_s$) and intercellular $CO_2$ concentration (Ci) of the leaf were continuously recorded every minute for at least 20 minutes. Thereafter, the leaf received a blue light (BL) pulse of 50 μmol $m^{-2}$ $s^{-1}$ in addition to the RL, subsequently the aforementioned gas exchange parameters of the leaf were recorded for 60 minutes. After 60 minutes, the BL was turned off and those parameters were recorded for another 60 minutes. The steady-state values of photosynthetic parameters, $A_n$, $g_s$ and Ci ($n = 4 \sim 6$ for each species), recorded before, during, and after the BL pulse were first analyzed with a One-Way Analysis of Variance (One-Way ANOVA) and then tested with Tukey test. Significant differences are reported as $p < 0.05$.

**Response of stomata on isolated epidermal strip**

Because the isolated stomata of *Rhoeo discolor* were reported to show response to light (Dayanandan and Kaufman, 1975), this plant was also included in this study for reference. The epidermis of 5 different plants (2 angiosperms and 3 ferns, including *R. discolor*, *S. chinensis*, *A. capillus-veneris*, *M. crenata*, *N. auriculata*) was isolated for observation of stomatal aperture. The epidermal strips were prepared by scrubbing off mesophylls with a razor blade (Mott *et al*., 2014) or were directly peeled off from leaves (Shen *et al.*, 2015). The isolated epidermal strips ($n = 4 \sim 6$ for each species) were floated in Petri dishes containing 10 ml stomatal opening buffer (50 mM KCl, 10 mM MES/KOH, pH 6.15) (McAinsh *et al*., 1991). After being kept in dark for 2 hours, the epidermal strips were irradiated with RL of 750 μmol $m^{-2}$ $s^{-1}$ or RL and 12 % blue light (RL+BL) (a

total of 750 μmol $m^{-2}$ $s^{-1}$ ) for 2 hours. Combinations of LED of different spectra, color glass filters (Andover Corporation, USA) and pieces of color cellophane were used to simulate the proportion of blue light of sun and of light source used in photosynthetic gas exchange measurement. The light intensity was adjusted to around 750 μmol $m^{-2}$ $s^{-1}$ measured with a quantum sensor (LI-190, LI-COR) and the resulting spectra of light source were measured with a spectroradiometer (HR2000, Ocean Optics). The epidermal strips were transferred to slides and observed under a light microscope (DME, Leica). The stomatal response was quantified by measuring stomatal width and length. Fifty to one hundred stomata were calculated from each isolated epidermal strip, and four to six strips were prepared for each plant species. The ratio of stomatal width to length between stomata on isolated epidermal strips in darkness, irradiated with RL and RL+BL were analyzed first with One-Way ANOVA and then tested with Tukey test.

**Stomatal sensitivity of *M. crenata* grown under different light regimes**

*M. crenata* were planted in 7-inch pots and grown under two different light regimes (full light and shading, n = 6 for each regime) in the greenhouse. Plants in shading treatment received 30 % of full light intensity. After two weeks of treatments, plants with new fully opened leaves were brought back to the laboratory for the photosynthetic gas exchange measurement. Leaf gas exchange was measured by a portable photosynthetic system, LI-6400 (LI-COR, USA), equipped with a dual-beam (red and blue) light source. Light-saturated photosynthetic rate ($A_n$), stomatal conductance ($g_s$) and intercellular $CO_2$ concentration (Ci) at steady-state were measured under two different light conditions, RL (sole red light of 900 μmol $m^{-2}$ $s^{-1}$) and RL+BL (50 μmol $m^{-2}$ $s^{-1}$ of blue light superimposed on the 900 μmol $m^{-2}$ $s^{-1}$ of red light). The steady-state values of $A_n$, $g_s$ and Ci measured between RL and RL+BL conditions were compared using Student's t-test. Significant differences are reported as $p < 0.05$.

## RESULTS

**Stomatal conductance in response to a blue light pulse**

Representative responses of the photosynthetic rate ($A_n$) and stomatal conductance ($g_s$) of leaves of *S. chinensis*, *M. crenata* and *N. auriculata* irradiated with RL or with RL and BL pulse were shown in Fig. 1. After the gas exchange rate of the leaf achieved steady-state under photosynthetically saturated RL, application of additional BL caused an obvious increase in $g_s$ of *S. chinensis* (an angiosperm, Fig. 1A) and *M. crenata* (Fig. 1B), and a small increase in $A_n$ in *S. chinensis* (Fig. 1A) and *M. crenata* (Fig. 1B). After the BL was turned off, $g_s$ of *S. chinensis* and *M. crenata* declined dramatically.

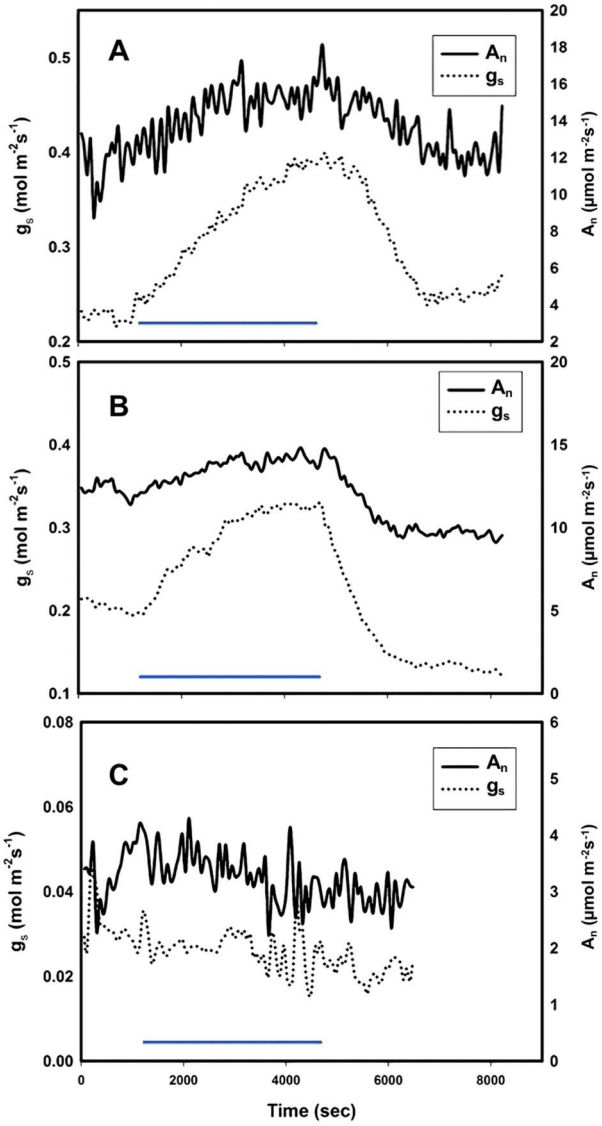


**Fig. 1**. The time course of changes in stomatal conductance ($g_s$) and photosynthetic rate ($A_n$) of leaves of (**A**) *Sambucus chinensis*, (**B**) *Marsilea crenata, and* (**C**) *Nephrolepis auriculata*. The blue bar indicates the time period when blue light (50 μmol $m^{-2}$ $s^{-1}$) was superimposed on the photosynthetically saturated red light.

In contrast, no significant changes in $g_s$ and $A_n$ in response to the supplement of BL were found in the leaf of *N. auriculata* (Fig. 1C).

Table 1 summarizes the means of the parameters obtained from the gas exchange measurements (n = 4~6 measurements for each species). Under illumination with photosynthetically saturated RL of 900 μmol $m^{-2}$ $s^{-1}$, an addition of 50 μmol $m^{-2}$ $s^{-1}$ BL caused a significant increase in $g_s$ and Ci of *S. chinensis* and *M. crenata* leaves but not in those of the ferns *A. capillus-veneris* and *N. auriculata*. The result indicates the presence of stomatal blue light-specific response in leaves of *S. chinensis* and *M. crenata* but not in those of *A. capillus-veneris* and *N. auriculata* under our experimental conditions.

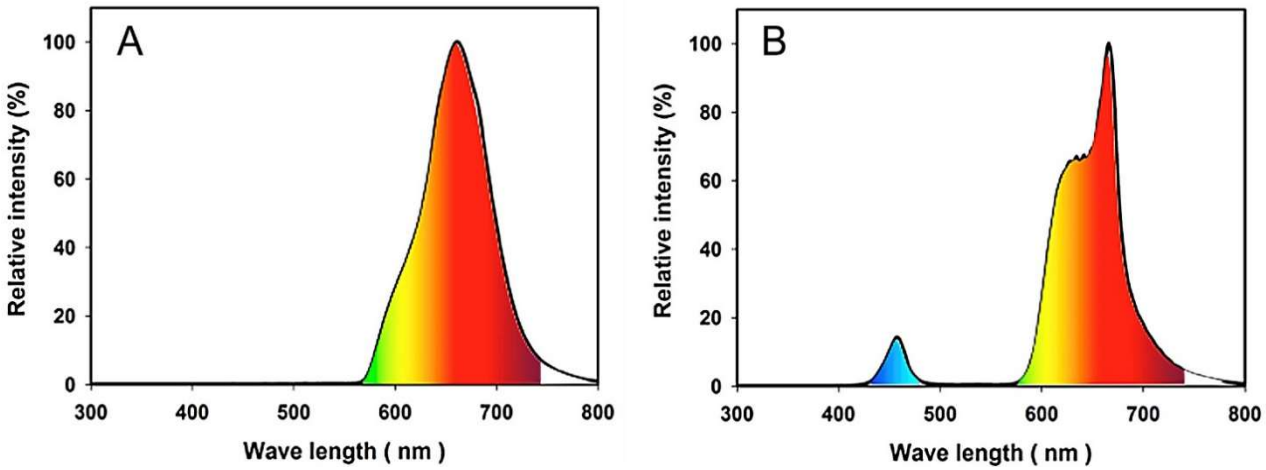


**Fig. 2** The spectrum of (**A**) red light and (**B**) red + blue light source used in examining the stomata on isolated epidermal strips.

**Table 1** The steady-state photosynthetic rate ($A_n$, μmol $m^{-2}$ $s^{-1}$ ), stomatal conductance ($g_s$, mmol $m^{-2}$ $s^{-1}$ ), and intercellular $CO_2$ concentration (Ci, μmol $mol^{-1}$ ) of leaves of *Sambucus chinensis*, *Marsilea crenata, Adiantum capillus-veneris,* and *Nephrolepis auriculata* irradiated with sole photosyntherically saturated red light (Red (before)), with red light and 50 μmol $m^{-2}$ $s^{-1}$ blue light (Red + Blue), and with red light only (after turning off the blue light) (Red (after)). Values (mean ± s.e., n = 4 ~ 6) within the same row followed by different superscripts represent significant difference at p = 0.05.

| Species/ Parameters | Red (before) | Red + Blue | Red (after) |
|---|---|---|---|
| *Sambucus chinensis* | | | |
| $A_n$ | 13.1 ± 2.1 $^{ab}$ | 15.2 ± 1.9 $^{a}$ | 11.9 ± 1.9 $^{b}$ |
| $g_s$ | 0.238 ± 0.082 $^{c}$ | 0.384 ± 0.095 $^{a}$ | 0.257 ± 0.075 $^{b}$ |
| Ci | 262.1 ± 10.2 $^{b}$ | 281.3 ± 9.7 $^{a}$ | 270.9 ±10.9 $^{b}$ |
| *Marsilea crenata* | | | |
| $A_n$ | 12.3 ± 1.2 $^{ab}$ | 14.2 ± 2.0 $^{a}$ | 10.1 ± 1.9 $^{b}$ |
| $g_s$ | 0.214 ± 0.028 $^{b}$ | 0.338 ± 0.033 $^{a}$ | 0.121 ± 0.019 $^{c}$ |
| Ci | 254.2 ± 6.2 $^{b}$ | 282.8 ±7.9 $^{a}$ | 243.1 ± 5.6 $^{c}$ |
| *Adiantum capillus-veneris* | | | |
| $A_n$ | 2.3 ± 0.1 $^{ab}$ | 2.4 ± 0.2 $^{a}$ | 1.6 ± 0.1 $^{b}$ |
| $g_s$ | 0.032 ± 0.008 $^{a}$ | 0.031 ± 0.007 $^{a}$ | 0.031 ± 0.010 $^{a}$ |
| Ci | 254.2 ± 11.2 $^{a}$ | 260.5 ± 13.9 $^{a}$ | 263.6 ± 11.8 $^{a}$ |
| *Nephrolepis auriculata* | | | |
| $A_n$ | 2.8 ± 0.3 $^{a}$ | 2.6 ± 0.4 $^{a}$ | 3.1 ± 0.5 $^{a}$ |
| $g_s$ | 0.029 ± 0.007 $^{a}$ | 0.023 ± 0.007 $^{a}$ | 0.020 ± 0.008 $^{a}$ |
| Ci | 262.0 ± 11.3 $^{a}$ | 258.0 ± 12.2 $^{a}$ | 250.0 ± 15.9 $^{a}$ |

**Table 2** The photosynthetic rate ($A_n$, μmol $m^{-2}$ $s^{-1}$), stomatal conductance ($g_s$, mol $m^{-2}$ $s^{-1}$), and intercellular $CO_2$ concentration (Ci, μmol $mol^{-1}$) of leaves irradiated with sole photosynthetically saturated red light (900 μmol $m^{-2}$ $s^{-1}$) (Red) or with red light (900 μmol $m^{-2}$ $s^{-1}$) plus blue light (50 μmol $m^{-2}$ $s^{-1}$) (Red + Blue) of full-light and shade (30 % of full light) grown *Marsilea crenata*. Values (mean ± s.e., n = 4 ~ 6) within the same light regime followed by different superscripts represent significant difference at p = 0.05 (means ± s.e., n=6).

| Light/ | Full light | | Shade | |
|---|---|---|---|---|
| Parameters | Red | Red + Blue | Red | Red + Blue |
| $A_n$ | 8.2 ± 1.0 $^{B}$ | 11.2 ± 0.3 $^{A}$ | 6.2 ± 0.9 $^{a}$ | 7.5 ± 0.8 $^{a}$ |
| $g_s$ | 0.100 ± 0.010 $^{B}$ | 0.168 ± 0.008 $^{A}$ | 0.107 ± 0.014 $^{a}$ | 0.117 ± 0.008 $^{a}$ |
| Ci | 260.3 ± 5.8 $^{B}$ | 281.3 ± 2.9 $^{A}$ | 295.7 ± 2.3 $^{a}$ | 287.7 ± 5.2 $^{a}$ |

## Response of stomata on isolated epidermal strip

Fig. 2 shows the spectra of the light sources, RL and RL + BL, irradiating on the isolated epidermal strips. The wavelength of the RL source was between 600-750 nm. In addition to the RL, a BL of wavelength between 400-500 nm was detected in RL + BL irradiation condition.

After being transferred from darkness to the RL irradiated condition (Fig. 2A) for two hours, no significant changes in the ratio of stomatal width to length were measured in the two angiosperms and the three ferns (Fig. 3, Fig. 4). Compared to those on epidermal strips irradiated with sole RL, stomata on the epidermal strips of two angiosperms had higher stomatal width/length ratio (37 % and 27 % increments for *R. discolor* and *S. chinensis*, respectively) after the supplement of BL. Similar to the stomatal response of the two angiosperms, stomata on the epidermal strips of *M. crenata* irradiated with RL+BL had significantly higher width/length ratio than those on the epidermal strips in darkness or irradiated with RL only (Fig. 4), a 43 % increment of the ratio was measured. No significant difference was found in the ratio of stomatal width/length among darkness, RL and RL+BL irradiated epidermal strips of *A. capillus-veneris* and *N. auriculata*. (Fig. 3, Fig. 4).

## Stomatal sensitivity of *M. crenata* grown under different light regimes

Table 2 illustrates that the effect of BL on $A_n$, $g_s$ and Ci differs between leaves of full light grown and shade grown plants of *M. crenata*. An addition of 50 μmol $m^{-2}$ $s^{-1}$ BL to RL significantly increases $A_n$, $g_s$ and Ci of leaves of full light grown *M. crenata*. In contrast, no significant differences were found in $A_n$, $g_s$, and Ci between RL irradiated leaves and RL+BL irradiated leaves of the shade grown *M. crenata*.

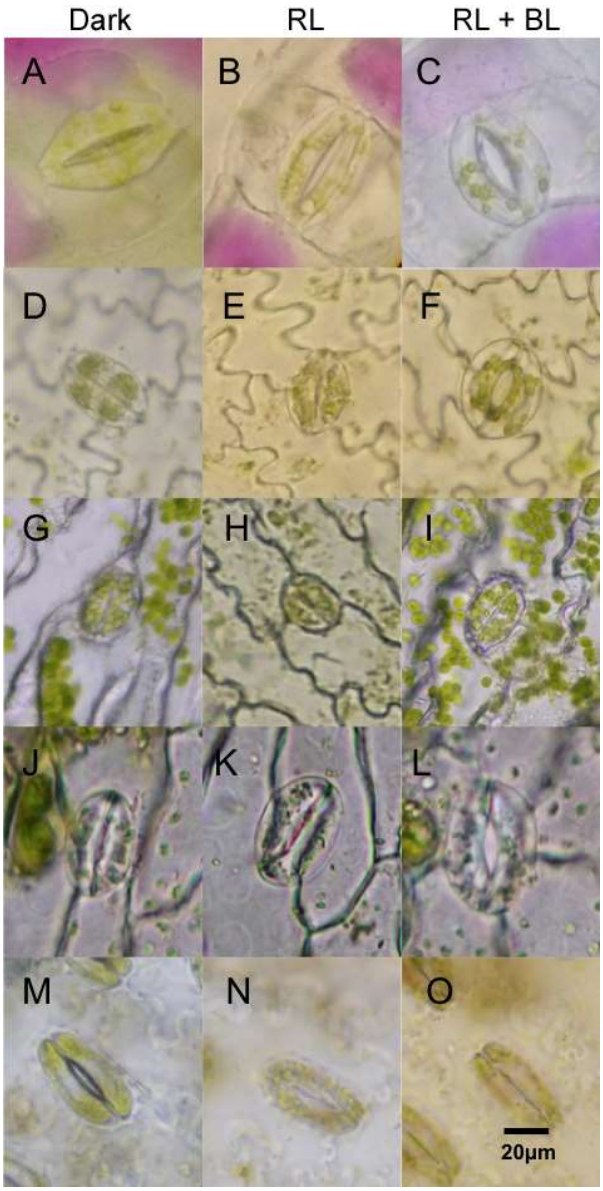


**Fig. 3** Micrographs of stomata on epidermal strips, in darkness (**A, D. G. J, M**), illuminated by red light (RL of 750 μmol $m^{-2}$ $s^{-1}$) (**B, E, H, K, N**), or red light with 12% blue light (RL + BL ≈ 750 μmol $m^{-2}$ $s^{-1}$) (C, F, I, L, O), of two angiosperms, *Rhoeo discolor* (**A, B, C**) and *Sambucus chinensis* (**D, E, F**), and three fern species, *Adiantum capillus-veneris* (**G. H, I**), *Marsilea crenata* (**J, K, L**), and *Nephrolepis auriculata* (**M, N, O**).

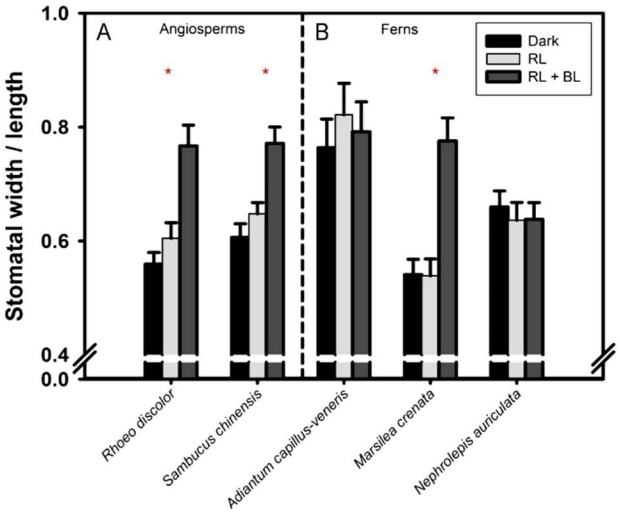


**Fig. 4.** The ratio of width to length of stomata on isolated epidermal strips of (**A**) two angiosperms and (**B**) three ferns. The isolated epidermal strips were kept in darkness (Dark), irradiated with red light (RL) or irradiated with red light plus blue light (RL + BL) for two hours. The asterisk indicates significant difference in the ratio (mean ± s.e., n = 4 ~ 6) between stomata on isolated epidermal strips irradiated with red light (RL) and red light plus blue light (RL + BL).

## DISCUSSION

Blue light has been found more effective than red light in inducing stomatal opening in many angiosperms (Lee *et al.,* 2007; Shimazaki *et al.*, 2007; Wang *et al.*, 2011). Similar to the stomata of angiosperms, the stomata of *M. crenata* were not fully opened under the irradiation of saturated RL while a supplement of BL caused stomata to open further and resulted in increasing $g_s$ (Fig. 1). This result clearly demonstrates the presence of stomatal blue light specific-response in the amphibious fern *M. crenata*.

Recently, Harris *et al*. (2020) provided evidence showing that many of the genes that pattern and operate stomata in modern tracheophytes were already present in the common ancestor of land plants. Accordingly, they suggested that simple stomata of modern bryophytes are a result of reductive evolution. Their results also support the hypothesis that the embryophyte common ancestor already possessed actively controlled stomata (Harris *et al*., 2020). This might explain why Marsileaceae, diversified in parallel with the rise of angiosperms (Schneider *et al.*, 2004), has stomatal behavior and other features similar to modern angiosperms (this study; Wu, 2020). *Marsilea crenata* has the highest photosynthetic capacity among the three studied ferns, and its photosynthetic rate is similar to that of the angiosperm *S. chinensis* (Fig. 1). Thus, the retaining of blue-light specific stomatal response would allow *M. crenata* to optimize photosynthetic performance. In addition to the possession of stomatal blue light specific-response, *Marsilea* has other angiosperm-like features. For example, *Marsilea* has advanced xylem system with vessel element (for a review, see Gifford and Foster, 1988), which would increase the efficiency of water uptake and transport. Cox (2018) suggested that the reductive evolution may not be unique to guard cells but may also have occurred in other elements of bryophyte, such as in the evolution of the vascular system. *Marsilea* also show diurnal leaf phototropism, which might increase photosynthetic performance (Kao and Lin, 2010). The possession of these angiosperm-like features might increase ecological breadth of *Marsilea.*

The results that stomata on isolated epidermis of the

studied plants did not open after irradiated with sole RL (Fig. 3) while a relatively high $g_s$ and a reduction in Ci were measured on intact leaves illuminated with RL (Fig. 1) imply that the mesophyll of these plants plays key influence on stomatal response to RL. Ando and Kinoshita (2018) suggested that mesophyll photosynthesis is required for RL-induced phosphorylation of plasma membrane $H^+$-ATPase, which leads to the accumulation of $K^+$ in guard cell, and hence induces stomatal opening.

Similar to the study by Frechilla *et al.* (2004), we also found that the stomatal blue light-specific response in *M. crenata* is affected by growth condition. *Marsilea crenata* lost the stomatal blue light-specific response after grown under shading environment (Table 2). The blue light response of stomata involved both the photosynthesis-dependent and photosynthesis-independent components (Talbott and Zeiger, 1998). The growth conditions might affect the relative contribution of these two components of blue light response of stomata (Wang *et al.*, 2011). Compared to *M. crenata* plants grown under full light, plants grown in shading condition had a lower photosynthetic capacity ($A_n$) but similar $g_s$ which resulted in a higher Ci (Table 2). Under a high Ci condition, $K^+_{in}$ channel is inactivated and osmotica, like sucrose and malate, are decreased in guard cells (Talbott and Zeiger, 1998; Shimazaki *et al.*, 2007). Therefore, it is possible that with a reduction in osmotica in the guard cells the stomata of shade-acclimated *M. crenata* could not open further after the BL illumination (Table 2).

In contrast to the stomata of *M. crenata* that showed blue light-specific response, the stomata of *A. capillus-veneris and N. auriculata* (Figs. 1, 3) and some other ferns of Polypodiopsida (Doi *et al*., 2006; Doi *et al*., 2015) did not have such a response. The absence of BL responses observed in these ferns could be associated with a few factors. For example, in addition to the results of reductive evolution (Horris *et al*., 2020), the condition in which the plants were grown (this study; Wu, 2020; Hõrak *et al*., 2017) and the time range that the analysis was performed (Franks and Britton-Harper, 2016; Lima *et al.*, 2019; Hõrak *et al*., 2017) might affect the blue-light specific response. These factors should be taken into account when evaluating stomatal response of ferns.

In conclusion, we found that stomata of *M. crenata*, an amphibious fern of Polypodiopsida, do respond to blue light application. Accordingly, this is the first report of the presence of stomatal blue light-specific response in a fern of Polypodiopsida. We also confirmed that this stomatal response of *M. crenata* is not induced by mesophyll chloroplasts, but relies on blue light signals. However, the growth condition has significant effect on this specific response. *Marsilea crenata* plants grown under shade condition have lost the specific response. Stomatal blue light-specific response could fulfill the high demand of $CO_2$ in photosynthesis in full light grown *M. crenata* plants.

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