Active stomatal control of *Marsilea crenata*, an amphibious fern, in response to CO₂ and exogenous application of ABA

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ABSTRACT: Angiosperms have active stomatal control in response to rising CO_2 and plant regulator abscisic acid (ABA). Whether ferns have similar response is controversial. To evaluate its stomatal response, we measured leaf photosynthetic gas exchange of *Marsilea crenata* (an amphibious fern), grown under full light and shaded condition, in response to variations in CO_2 concentration ([CO_2]) and exogenous application of ABA. The results showed that stomatal conductance (g_s) of *M. crenata* significantly decreased while photosaturated photosynthetic rate (A_{max}) increased as [CO_2] increased from 0 to 600 ppm, resulting in increments in water use efficiency (WUE). The reduction in g_s when [CO_2] was elevated from 400 to 800 ppm was more in leaves of full light-grown than those of shade-grown plants, however, the increment in A_{max} was similar. Leaves of *M. crenata* gradually closed stomata after 30 minutes of application of exogenous ABA, resulting in a 52.1 % reduction in g_s and a 40 % in A_{max}, hence a 25 % increase of WUE. A more than two-fold increment of ABA contents was also measured in the leaves after the ABA application. This study showed that stomata of *M. crenata* do respond to the increase of ambient [CO_2] from 0 to 600 ppm and to the ABA application, and the response to the elevated ambient [CO_2] is affected by growth conditions.

KEY WORDS: Active stomatal control, abscisic acid, CO₂, *Marsilea crenata*, water use efficiency.

INTRODUCTION

The apparatus of stomata, the valves regulating the gas exchange between plants and the atmosphere, is often considered as one of the most important innovation in terrestrial plants. The regulation of opening and closure of stomata helps plants balancing water loss from transpiration and CO_2 uptake for photosynthesis. Francis Darwin was the first to show that stomata on leaves responded to environmental stimuli (Darwin, 1898). After Darwin, many researchers have studied stomatal response and mechanisms of stomatal control under varying environmental conditions. Environmental factors such as vapor pressure deficit (VPD), light intensity, the concentrations of carbon dioxide ([CO_2]) and plant endogenous hormones were found to affect stomatal aperture (Tardieu and Davies, 1992; Maherali *et al.*, 2003; Bunce, 2004; Shimazaki *et al.,* 2007).

Two different processes are responsible for the stomatal closure, the hydro-passive and active processes (Haworth *et al.*, 2013). In the hydro-passive process, aperture of stomata changes with leaf water status. Guard cells lose turgor when the amount of water evaporating out of these cells is more than that moving in. When that happens, the guard cells are not able to maintain shape, and the aperture is closed. It has been shown that, in lycophytes, ferns, and conifers, VPD-induced stomatal closure is a hydro-passive response to reduced leaf turgor (McAdam and Brodribb, 2015). The active process is considered as a physiological control via active ion transport in guard cells, which is regulated by

plant signalling pathway and then induces the change of osmotic potential of guard cells. The response of stomata to [CO_2], abscisic acid (ABA), and light is cnsidered active process. McAdam *et al.* (2016) showed VPD-induced ABA synthesis indicating that an active, ABA-mediated component is involved in the VPD induced stomatal closure in angiosperms.

As CO_2 diffuses into the leaf through stomata for photosynthesis, concomitantly water fluxes out of the leaves by transpiration. Accordingly, stomatal behaviour is important in regulating CO_2 uptake and water loss. It has been found that elevated atmospheric [CO_2] increased rate of photosynthesis and light-use efficiency (Hikosaka *et al.*, 2003; Guo and Trotter, 2006; Tomimatsu *et al.*, 2014) while reduces stomatal conductance (g_s) and hence transpiration rate (Drake *et al.*, 1997; Centritto *et al.*, 2002). Consequently, photosynthetic water use efficiency (WUE) during photosynthesis is enhanced under elevated CO_2 condition. In addition, it has been shown that CO_2 sensitivity of stomata varied with growth condition of plants. For example, stomatal sensitivity to CO_2 in leaves of *Vicia faba* differed between plants grown in the growth chamber and those in the field (Talbott *et al.*, 1996). Field studies also revealed that leaves from upper canopy had greater stomatal sensitivity to CO_2 than those from middle or low canopy (Wullschleger *et al.*, 2002).

Some researchers reported that active control of stomatal closure is absent in early-diverging vascular plants, including lycophytes and ferns (Doi *et al.*, 2006; Doi and Shimazaki, 2008; Brodribb *et al.*, 2009;

Brodribb and McAdam, 2011). Doi and Shimazaki (2008) showed that unlike the stomata of angiosperms, the stomata of the fern, *Adiantum capillus-veneris*, did not respond to changes in $[CO_2]$ in the dark. Furthermore, Brodribb and McAdam (2011) proposed that the ancestral state for stomatal control of water balance acts as passive hydraulic valves. According to the hypothesis, the stomatal behavior in ferns and lycophytes is regulated by hydraulic status of guard cells instead of environmental cues. Therefore, stomata of ferns and lycophytes are incapable of actively sustaining homeostatic water use efficiency. The evolution of efficient water use in early seed plants provides them a competitive advantage over ferns and lycophytes in terrestrial environment which partially contributes to the decline of the dominance of ferns and lycophytes in ecosystems of the late Paleozoic (McAdam and Brodribb, 2012). Brodribb and McAdam (2013) reported that stomata of angiosperms opened in response to low $[CO_2]$ in the dark and closed at $[CO_2]$ higher than ambient condition in the light, whereas the lycophytes and ferns did not possess similar stomatal behavior. Hõrak *et al.*, (2017) reported that stomatal response to VPD is probably more than just a hydro-passive process in some ferns. A study on conifer species (*Metasequoia glyptostroboides*), which is phylogenetically midway between the fern and angiosperm clades, found its stomatal behavior is intermediate between the passively controlled ferns and ABA-dependent actively controlled angiosperms (McAdam and Brodribb, 2014). These results evoke a reconsideration of the evolution of stomatal behavior.

In contrast to the aforementioned studies by Doi and Shimazaki (2008), Ruszala *et al.* (2011) found that stomata of a lycophyte, *Selaginella unicinata*, did respond to variation of ambient $[CO_2]$ and to the drought hormone ABA. They reported that not only do stomata of this lycophyte respond to CO_2 and ABA in the same way as those of flowering plants, but also the mechanisms of ABA signal transduction pathway are highly similar between guard cells of *Selaginella* and *Arabidopsis*. Besides, a research on moss by Chater *et al.* (2011) provided evidence that stomata of sporophytes of two mosses (*Physcomitrella patens* and *Funaria hygrometrica*) did respond to ABA and $[CO_2]$. The core regulatory components involved in ABA signalling pathway of guard cells in flowering plants are also operational in the two mosses. These results indicated that not only lycophytes but also mosses can respond to the environmental and exogenous cues. The results also suggest that active regulation behaviour of stomata was originated at least as far back before the emergence of the ferns. To understand the evolution of stomatal control, surveys of the stomatal behaviour in ferns have been expanded.

With more examination on the stomatal response of ferns, more evidence challenged the evolutionary hypothesis proposed by Brodribb and McAdam (2011), which suggested that the active regulation of stomata was evolved after the seed plant emerged. After investigating diverse fern species grown under contrasting irradiance, Creese *et al.* (2014) reported that plants grown under high irradiance were more responsive to the variations of irradiance, VPD, and CO_2. Recently, Hõrak *et al.* (2017) also reported that stomata in three temperate fern species (*Athyrium filix-femina*, *Dryopteris carthusiana*, and *Dryopteris filixmas*) responded to the variations of ABA and CO_2 concentration. There is increasing evidence that active stomatal control occurs in ferns, and/or that the sensitivity of the response depends on growth conditions (Soni *et al.*, 2012; Creese *et al.*, 2014; Hõrak *et al.*, 2017). These results strongly suggested that the stomatal response of some ferns is hydro-passive, as described by Brodribb and McAdam (2011), but other fern species have active mechanisms for stomatal regulation. The debate over the origins of signaling pathways for both ABA and CO_2 continues.

Marsilea sp., an amphibious fern, grows in aquatic habitat and terrestrial environment experiencing larger variation in water availability as compared to most other fern species. In addition, *Marsilea* sp. grows in habitats with high light availability. Accordingly, its stomatal response might also differ from most other fern species. To test the hypothesis, we investigated the stomatal response of this fern to $[CO_2]$ and exogenous ABA and the consequences of the response. Specifically, we asked following questions. Do stomata of *Marsilea* sp. respond to changes in $[CO_2]$? Does the response vary between *Marsilea* sp. grown under different light regimes? Do stomata of the fern possess active regulating process in response to changes in environmental factors? To answer these questions, we measured photosynthetic gas exchange of leaves of *M. crenata*, a native fern in Taiwan, grown under full light and shading treatments, in response to changes in CO_2 concentration and to the drought hormone, ABA.

MATERIALS AND METHODS

Plant materials

Rhizomes of *M. crenata* with 2~4 nodes were planted in plastic pots (diameter of 7 inch) filled with a mixture of perlite: vermiculite: peat of 1: 1: 2 by volume. Plants were grown under natural light in a greenhouse of temperature controlled at 25°C. Following measurements were conducted on fully expanded leaves.

Gas exchange in response to $[CO_2]$

Photosynthetic gas exchange in response to changes in ambient $[CO_2]$ was measured by a portable photosynthetic system (LI-6400, LI-COR, USA) on

leaves of *M. crenata* grown under natural light. During the gas exchange measurement, the vapor pressure deficit between leaf and air (VPD) was controlled at 1.1~1.3 kPa, and the photosynthetic photon flux (PPF) at 900 μmol m^{-2} s^{-1}. The ambient CO_2 concentration ($[CO_2]$) was adjusted at 0, 50, 100, 150, 200, 300, 400, 600, 700, 800, 1000, and 1200 μmol mol^{-1}, respectively. Under each $[CO_2]$, the steady-state rates of photosaturated CO_2 assimilation rate (A_{max}), stomatal conductance (g_s), transpiration rate (E) and the intercellular CO_2 concentration (Ci) were recorded. Photosynthetic water use efficiency (WUE) was calculated as A_{max}/E.

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

To evaluate the effect of growth light regimes on stomatal response, leaf photosynthetic gas exchange of *M. crenata* grown under full light (n = 6) and shading treatment (n = 6), where light intensity was 30 % of full light, was compared. The average PPF at midday under full light condition in greenhouse was around 800 μmol m^{-2} s^{-1}. Photosynthetic gas exchange of the most recently, fully expanded leaves was measured. The VPD and PPF were controlled at 1.1~1.3 kPa and 900 μmol m^{-2} s^{-1}, respectively, and the $[CO_2]$ was adjusted at 400 μmol mol^{-1}. After the steady-state of gas exchange rates at $[CO_2]$ of 400 μmol mol^{-1} were measured, the ambient $[CO_2]$ was elevated to 800 μmol mol^{-1}, and the g_s, A_{max} and E at steady-state were recorded.

Gas exchange in response to exogenous ABA

Rhizomes (n = 7) of *M. crenata*, each with 1~2 nodes (bearing 4 leaflets in each node), were excised from the cultivated plants, transferred to flasks containing Murashige and Skoog (MS) medium and grown in a greenhouse for one week. Plants in flasks were then brought into the lab, and their gas exchange parameters were measured on one of the fully expanded leaflets. During gas exchange measurement, the VPD, light intensity, and $[CO_2]$ was controlled at 1.1~1.3 kPa, 900 μmol m^{-2} s^{-1} and 400 μmol mol^{-1}, respectively. When a steady-state value of g_s was measured, two leaflets were excised for the determination of ABA concentration (Pre-ABA treatment). After the two leaflets excised, ABA (final concentration of 10^{-4} M) or distilled water (as control) was added to the medium. Following the application of ABA or distilled water, the g_s was subsequently recorded every minute for consecutively 3 hours. Afterward, the remaining two leaflets were excised for the measurement of ABA content (Post-ABA treatment). Leaf endogenous ABA was extracted with 80 % methanol and quantified with ELISA using Phytodetek® monoclonal enzyme immunoassay ABA test kit (Agdia, USA), according to the method of Hurng *et al.* (1994).

Statistical analyses

All statistical tests were performed using the computer software Origin 9.1 (OriginLab Corporation, USA). Paired t-test was conducted for the comparisons of the gas exchange response and ABA content of same leaf before and after ABA treatment. The gas exchange parameters between leaves of full light-grown and shading treatment plants were compared with the Student's t-test. Significant levels are reported as $p < 0.05$.
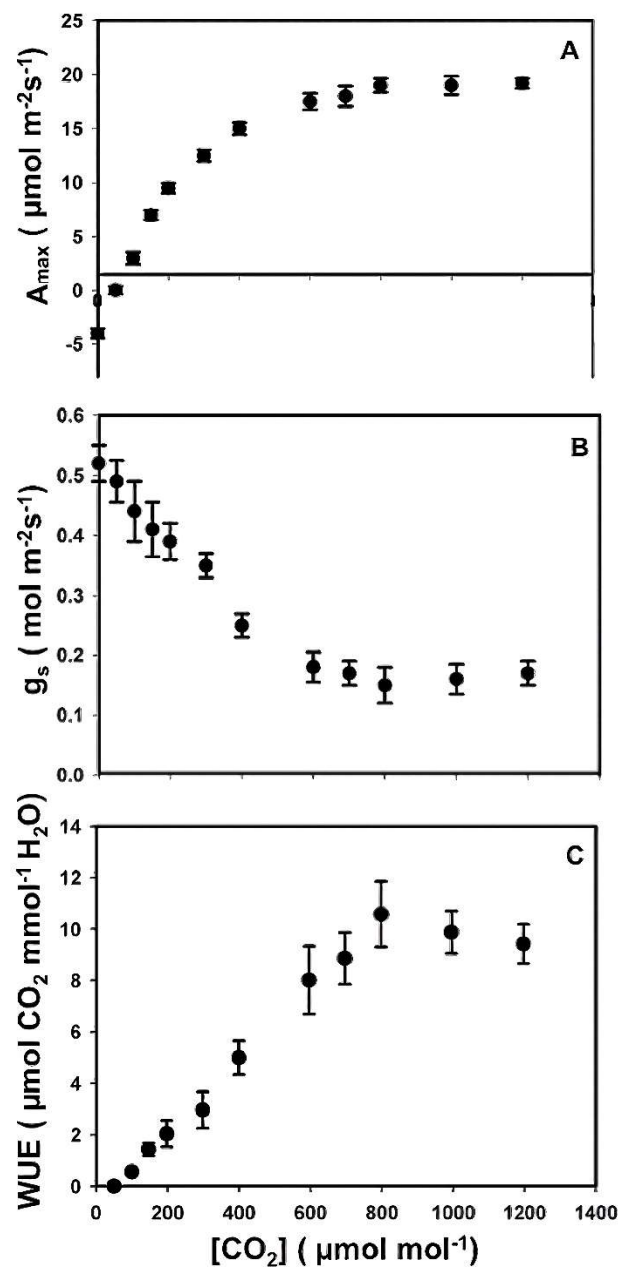

Fig. 1. The response of (A) photosaturated photosynthetic rate (A_{max}), (B) stomatal conductance (g_s), and (C) water use efficiency (WUE) in response to variations of ambient CO_2 concentration ($[CO_2]$) in leaves of *M. crenata*. (mean ± s.e., n = 6)

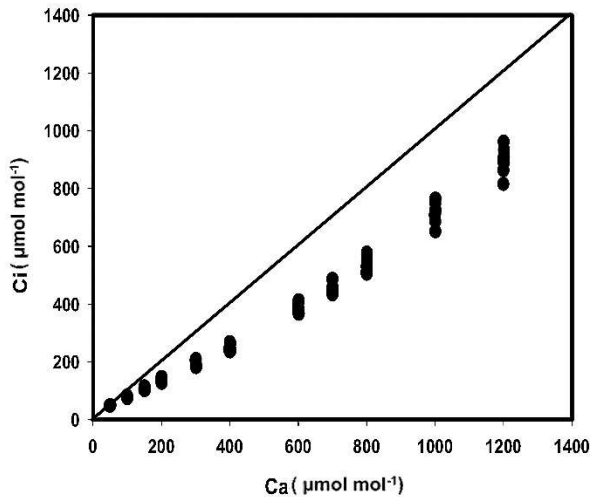**Fig. 2**. The response of intercellular CO_2 concentration (Ci) in response to variations of ambient CO_2 concentration (Ca) in leaves of *M. crenata*.

RESULTS

Leaf gas exchange in response to [CO₂]

The photosaturated photosynthetic rate (A_{max}) increased with the increment of ambient [CO_2] from 0 to 800 μmol mol⁻¹ (Fig. 1A). A saturation of A_{max} was obtained at the ambient [CO_2] of 800 μmol mol⁻¹. In contrast to the response of A_{max} to increasing [CO_2], the stomatal conductance (g_s) declined with the increase of ambient [CO_2] from 0 to 600 μmol mol⁻¹ (Fig. 1B). No further reduction in g_s was found when [CO_2] was elevated from 600 to 800 μmol mol⁻¹. Photosynthetic water use efficiency (WUE) also increased as [CO_2] increased from 0 to 800 μmol mol⁻¹ (Fig. 1C). In comparison to under the [CO_2] of 400 μmol mol⁻¹, the value of g_s decreased by 41.5 % while that of A_{max} and WUE increased by 26.7 % and 72.9 %, respectively, at CO_2 concentration of 800 μmol mol⁻¹.

The intercelluar [CO_2] (Ci) also increased with the increase of ambient [CO_2] (Fig. 2). However, the degree of increment declined as the ambient [CO_2] increased. Significantly higher A_{max} and g_s measured at [CO_2] of 400 μmol mol⁻¹ were found in leaves of plants grown in full light than in those of plants in shading treatment (Fig. 3). However, leaves of both treatments had similar WUE at 400 μmol mol⁻¹ of CO_2. Similar pattern of the response of A_{max} (and g_s) to elevated CO_2 from 400 to 800 μmol mol⁻¹ were found in leaves of full light and shading treatment plants. In leaves of plants of both treatments, A_{max} increased when [CO_2] increased from 400 to 800 μmol mol⁻¹. Similar degree of increment was found in leaves of plants in both treatments.

In contrast to A_{max}, leaves of plants grown under different light regimes showed different degree of reduction in g_s in response to elevated CO_2 from 400 to 800 μmol mol⁻¹ (Fig. 3B). Leaves of plants grown under

full light had a 56.8% reduction in g_s, while those of plants under shading had a 38.7% reduction (Fig. 3). As a result, at [CO_2] of 800 μmol mol⁻¹, leaves of full light grown plants had significantly higher WUE than those of shading treatment plants (Fig. 3C).
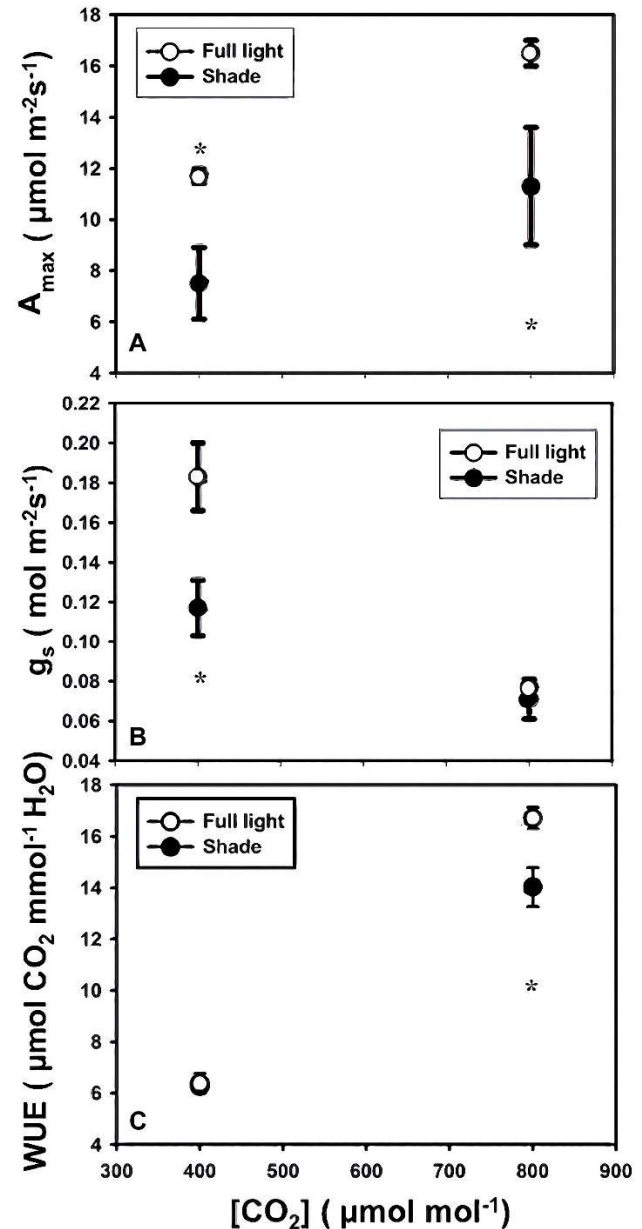

Fig. 3. The (A) photosaturated photosynthetic rate (A_{max}), (B) stomatal conductance (g_s), and (C) photosynthetic water use efficiency (WUE), measured at [CO_2] of 400 and 800 μmol mol⁻¹, in leaves of *M. crenata* grown under different light regimes. An asterisk indicates significant difference between light regimes.

Gas exchange and leaf ABA content in response to ABA application

The g_s and A_{max} remained unchanged in response to the application of ABA in 30 minutes, afterward g_s steadily declined in the following 100 minutes and finally
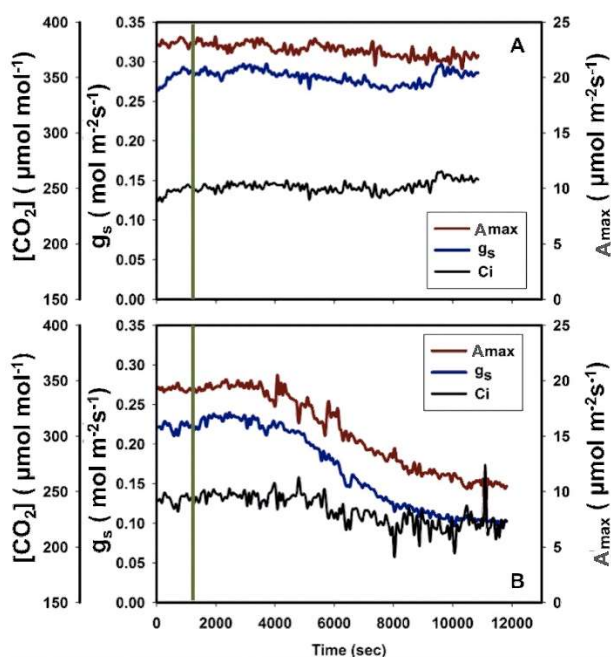
Fig. 4. A representative of time course of changes in photosaturated photosynthetic rate (A_{max}), stomatal conductance (g_s), and intercellular CO_2 concentration (Ci) of leaves of *M. crenata* after (A) double distilled water (ddH$_2$O) or (B) ABA application. The vertical line indicated the time of ddH$_2$O or ABA application.

reached a lowest value after around 120 minutes of ABA application (Fig. 4B). In contrast, A_{max} and g_s of leaves of control treatment remained fairly constant before and after water application (Fig. 4A).

Table 1 summaries the results of gas exchange measurement and ABA content of leaves before and after the ABA application. An average of 52.1 % and 40 % reduction in g_s and A_{max} was measured, respectively, after the application of exogenous ABA. In contrast to the reductions in g_s and A_{max}, WUE was increased after the application of ABA. The ABA content of leaves significantly increased from 2481 picomol g^{-1}FW (control) to 5538 picomol g^{-1}FW after the exogenous application of ABA.

DISCUSSION

In this study, we found that stomata of the amphibious fern, *M. crenata*, do respond to the elevations of ambient [CO_2] from 0 to 600 μmol mol^{-1} and to the exogenous application of ABA. Both responses result in significant increase in WUE of the leaves. These results indicate that *M. crenata* has active stomatal control in response to changes in environmental factors.

CO_2 response of stomata

In comparison to the stomatal response of the three clades of plants, ferns/lycophytes, gymnosperms, and angiosperms, examined by Brodribb *et al.* (2009), the

Table 1. A summary of results of photosaturated photosynthetic rate (A_{max}), stomatal conductance (g_s), the ratio of intercellular CO_2 to ambient CO_2 concentration (Ci/Ca), water use efficiency (WUE) and the ABA content of leaves of *M. crenata* before (pre-treatment) and after (post-treatment) ABA application. Values (mean ± s.e., n = 7) within the same row followed by different superscripts represent significant difference at *p* = 0.05.

Parameters	pre-treatment	post-treatment
A_{max} (μmol m^{-2}s^{-1})	20.01 ± 0.38 [a]	11.98 ± 0.78 [b]
g_s (mol m^{-2}s^{-1})	0.23 ± 0.02 [a]	0.11 ± 0.01 [b]
Ci/Ca	0.603 ± 0.020 [a]	0.530 ± 0.012 [b]
WUE	6.16 ± 0.37 [b]	7.68 ± 0.49 [a]
ABA content (picomol g^{-1})	2481 ± 231 [b]	5538 ± 681 [a]

stomatal response of *M. crenata* was more similar to that of angiosperms than to that of conifers or ferns/lycophytes. Less than 50 % of increment of g_s in conifers and ferns/lycophytes, and 70 % in angiosperms were measured when ambient [CO_2] was reduced from 400 μmol mol^{-1} to 100 μmol mol^{-1} (Brodribb *et al.*, 2009). While our measurement showed a 69.2 % increment of g_s in *M. crenata* in response to changes in [CO_2] from 400 μmol mol^{-1} to 100 μmol mol^{-1}. In response to increase in [CO_2] from 400 μmol mol^{-1} to 600 μmol mol^{-1}, angiosperms showed significant reduction in g_s and increased in WUE. There is no significant change in g_s and in WUE of leaves of conifers and ferns/lycophytes (Brodribb *et al.*, 2009). Similar patterns were found in the responses of A_{max} and WUE to variations in [CO_2] between *M. crenata* in this study (Fig. 1) and angiosperms reported by Brodribb *et al.* (2009). Creese *et al.* (2014) found species variation in stomatal sensitivity to an elevated CO_2 of 2000 μmol mol^{-1} in fern, in which high sensitivity was found in species from open habitat. Huang (2015) compared six fern species and found different degrees of their g_s in response to elevated [CO_2]. In this study we also found that stomata of *M. crenata*, an open habitat growing species, had high sensitivity to elevated [CO_2]. Leaf intercellular CO_2 concentration (Ci) increased with the increases of ambient CO_2 concentration (Ca) (Fig. 2). However, the increment of Ci declined as Ca increased resulting in gradual decreases in [Ci/Ca] (indicating increases of WUE). This result also suggests that *M. crenata* regulates the stomatal aperture in response to variations of [CO_2] resulting in increases in WUE. Accordingly, we reconfirm that the stomatal sensitivity to elevated [CO_2] does occur in this fern. It has been suggested that the regulating mechanism for the response might have evolved in common ancestors of land plants and been conserved in some ferns but lost in others during plant diversification (Franks and Britton-Harper, 2016).

Phenotypic plasticity was found in *M. crenata* grown under different light regimes (Fig. 3). Measured at [CO_2] of 400 μmol mol^{-1}, leaves of full light-grown plants had significantly higher A_{max} and corresponding g_s than those of shade-grown plants. However, the g_s of leaves of full light-grown and shade-grown plants showed different

degree of response to elevated [CO_2]. In consistent with the results of Creese *et al.* (2014) that ferns grown under high irradiance were more responsive to [CO_2], we also found that g_s of full light-grown *M. crenata* plants were more sensitive to increment of CO_2 concentration than those grown under shading treatment.

ABA responses

ABA is an important plant hormone that regulates plant growth and stomatal aperture, particularly when plants are under drought. During water stress, most photosynthetic organisms show increments of ABA content (Hartung, 2010). Not only the endogenous ABA but also the application of exogenous ABA led to stomatal closure in angiosperms and gymnosperms (Franks and Farquhar, 2001). Similar phenomena were reported in bryophytes, which synthesize ABA under stress and show stomatal closure in response to exogenous ABA (Hartung, 2010; Chater *et al.*, 2011; Ruszala *et al.*, 2011). In *M. quadrifolia,* it has been shown that adding ABA to the liquid culture medium, plants with leaves of submerged type produced leaves of morphological characteristics similar to the land leaves (Liu, 1984). The land-form characteristics induced by ABA included differentiation of stomata and trichomes and development of lateral roots (Liu, 1984), which are all related to water relation of the plants. The production of trichomes could reduce transpirational water loss (Wu and Kao, 2009), while the development of lateral roots can increase water uptake. Liu (1984) suggested that upon exposure to land conditions, reduction in water availability for *M. quadrifolia* causes an increase in the endogenous level of ABA, which in turn leads to the development of land leaf characteristics. In addition, the endogenous ABA level in leaves of *M. quadrifolia* increases, in parallel with the morphogenetic transition in leaves from submerged form to the aerial form, after being transferred from basal medium (osmotic potential of 131 mmol Kg^{-1}) to MS medium (osmotic potential of 194 mmol Kg^{-1}) (Lin and Yang, 1999). Thus, the increase in endogenous ABA level might be induced by a reduction in water potential of the MS medium. The aforementioned studies all indicated that ABA is involved in the drought response of *M. quadrifolia*. Other *Marsilea* species might also have similar response. In this study, we found that stomata on leaves of *M. crenata* did close in response to exogenous application of ABA. The degree of closure response in *M. crenata* was similar to that reported in angiosperms but different from that in some other ferns (Franks and Farquhar, 2001; Brodribb and McAdam, 2011; Hõrak *et al.*, 2017). Recently, Cai *et al.* (2017) demonstrated that two aquatic ferns, *Azolla filiculoides* and *Salvinia cucullata*, phylogenetically close to *M. crenata*, have ABA reception and membrane transporter gene families. They also found stomatal apertures of two other land ferns, *Polystichum proliferum* and *Nephrolepis exaltata*, did

close after ABA application. In comparison to angiosperms, *M. crenata* has similar degree of stomatal response to ABA as seen in this study. Furthermore, there is a larger than 2-fold increase in leaf ABA content following ABA application. Accordingly, we concluded that the stomata of *M. crenata* also have active control process.

Contrasting observations on stomatal response in ferns have been reported. Results of this study supported the statements that stomatal control occurs in ferns and the sensitivity of the response depends on growth conditions. Franks (2013) suggested that the relative importance of hydro-passive and active stomatal control in different plant species or lineages may vary as a result of a number factors. For example, the inhibition of ABA-induced stomatal closure by cytokinins and auxins has been reported in angiosperms (Das *et al.*, 1976; Snaith and Mansfield, 1982). The interactive effects of phytohormones might also affect stomatal response to ABA in ferns. In addition, most of the studies on stomatal response in ferns focused on C_3 lineages. Some ferns utilize CAM photosynthetic pathway (Keeley, 1983; Wong and Hew, 1976). Stomatal response of these fern lineages containing CAM awaits investigation.

In conclusion, we clearly demonstrate that stomata of the amphibious fern, *M. crenata*, do respond to CO_2 concentration and exogenous ABA application in this study. The degree of g_s response is comparable to those reported for angiosperms. The results indicate that *M. crenata* has active control of the stomatal aperture. The active stomata control can help *M. crenata* to optimize its water use efficiency which might confer its ability to live in terrestrial environment.

ACKNOWLEDGMENTS

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