Rapid response of leaf photosynthesis in two fern species *Pteridium aquilinum* and *Thelypteris dentata* to changes in CO$_2$ measured by tunable diode laser absorption spectroscopy

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**Abstract** We investigated stomatal conductance ($g_s$) and mesophyll conductance ($g_m$) in response to atmospheric CO$_2$ concentration [CO$_2$] in two primitive land plants, the fern species *Pteridium aquilinum* and *Thelypteris dentata*, using the concurrent measurement of leaf gas exchange and carbon isotope discrimination. [CO$_2$] was initially decreased from 400 to 200 μmol mol$^{-1}$, and then increased to 200 to 700 μmol mol$^{-1}$, and finally decreased to 700 to 400 μmol mol$^{-1}$. Analysis by tunable diode laser absorption spectroscopy (TDLAS) revealed a rapid and continuous response in $g_m$ within a few minutes. In most cases, both ferns showed rapid and significant responses of $g_m$ to changes in [CO$_2$]. The largest changes (quote % decrease) were obtained when [CO$_2$] was decreased from 400 to 200 μmol mol$^{-1}$. This is in contrast to angiosperms where an increase in $g_m$ is commonly observed at low [CO$_2$]. Similarly, fern species observed little or no response of $g_s$ to changes in [CO$_2$] whereas, a concomitant decline of $g_m$ and $g_s$ with [CO$_2$] is often reported in angiosperms. Together, these results suggest that regulation of $g_m$ to [CO$_2$] may differ between angiosperms and ferns.

**Keywords** CO$_2$ response · Ferns · Mesophyll conductance · Pteridophytes · Photosynthesis

**Introduction**

Atmospheric CO$_2$ is a substrate for leaf photosynthesis in land plants, and thus CO$_2$ availability at the carboxylation site is one of the most important limiting factors for leaf photosynthesis. In the process of leaf photosynthesis in C$_3$ land plants, CO$_2$ diffuses from the atmosphere through stomata, intercellular air spaces, and the leaf mesophyll to the site of carboxylation in the chloroplasts. CO$_2$ concentration in the chloroplast is lower than that in the atmosphere because of significant resistance to CO$_2$ diffusion through this diffusional pathway, i.e., limitations in CO$_2$ diffusion strongly reduce leaf photosynthesis. There are two major CO$_2$ diffusional limitations; CO$_2$ conductance though stomata, $g_s$, and that from substomatal cavities to the chloroplast, termed $g_m$.

Atmospheric CO$_2$ levels have changed substantially over the evolutionary history of land plants. It is estimated that atmospheric CO$_2$ levels were approximately 10 times higher than the present when land plants started to evolve 360–480 million years ago (Royer et al. 2004). Ferns are a major component of the fossil flora, and although they are primitive, non-seed plants, they are closely related to seed plants (Pryer et al. 2001). Atmospheric CO$_2$ levels fell abruptly during the Cretaceous period (Kuypers et al. 1999), which coincides with a major diversification in the fern group (Pryer et al. 2004). On the other hand,
angiosperms, which are currently the dominant group of seed plants, emerged during a period when atmospheric CO2 level was only two- to three-fold higher than the present (Haworth et al. 2011). This implies that ferns and angiosperms evolved under different selection pressures, which may have resulted in different mechanisms of CO2 diffusion between these two plant groups (Carriquí et al. 2015). Changes in the mechanisms of CO2 diffusion in plant evolutionary history have been suggested because stomatal frequency in fossil plants, which strongly affects CO2 diffusional limitations in extant ferns with extant angiosperms (Wright et al. 2005). In ferns, both have much lower photosynthetic capacity than angiosperms in the fossil record. Although the difference in cannot be determined through similar anatomical imprints in the fossil record. Although the difference in g_m is possibly still partially reflected in extant plants of angiosperms and ferns. Leaf mesophyll anatomy affecting g_m, including chloroplast surface area facing the intercellular airspaces and cell wall thickness, could have changed from ferns to angiosperms (Carriquí et al. 2015), which may be affected by the decrease in atmospheric CO2 level. A comparison of CO2 diffusional limitations in extant ferns with extant angiosperms could provide crucial information to estimate how photosynthesis traits have evolved in land plants. If atmospheric CO2 levels can influence selection pressure, phylogenetically distant fern groups may also vary in internal morphology and g_m.

In the present atmospheric CO2 conditions, fern species have much lower photosynthetic capacity than angiosperms (Wright et al. 2005). In ferns, both g_s and g_m are lower than in angiosperms. A lower g_m is suggested to be the major mechanism underlying the lower photosynthetic capacity of fern species (Carriquí et al. 2015). However, there are only three published determinations of g_m of fern species to the best of our knowledge (Carriquí et al. 2015; Gago et al. 2013; Volkova et al. 2009). Anatomical and physiological mechanisms underlying the low g_m of fern species still remain to be confirmed.

The response of g_s to atmospheric CO2 concentration [CO2] is different between angiosperms and ferns. Extensive studies on angiosperms have shown that g_s typically increases with a decrease in [CO2] (e.g., Brodribb et al. 2009; Messinger et al. 2006). However, three ferns, Osmunda regalis, Blechnum gibbum and Nephrolepis extata, showed small responses to changes in [CO2] (Gago et al. 2013). The averaged g_s for six ferns and lycophytes showed no response to an increase in [CO2] above ambient, while they showed a slight increase with a decrease in [CO2] (Brodribb et al. 2009). Studies for the response of g_m to [CO2] are limited compared with g_s in angiosperms, and to the best of our knowledge, there is only one published study on the response of g_m to [CO2] in fern species (Gago et al. 2013). For angiosperms, there is conflicting evidence as to how g_m responds to [CO2]. Some studies reported insignificant effects of [CO2] on g_m (Harley et al. 1992; Tazoe et al. 2009), whereas other studies reported a decline in g_m at high [CO2] (Bunce 2010; Douthe et al. 2011; Hassiotou et al. 2009; Loreto et al. 1992, Tazoe et al. 2011), or showed curved responses to changes in [CO2] (Flexas et al. 2007; Vrábl et al. 2009). Gago et al. (2013) obtained a curved response in g_m with changes in [CO2] for three fern species. However, the observed decline in g_m at low [CO2] in angiosperms and ferns (sub-stomatal CO2 concentration, C_i < 50 µmol mol⁻¹) may be an artifact related to partially photorespired CO2 (Tholen et al. 2012). Furthermore, the chlorophyll fluorescence technique used can lead to errors in the estimation of g_m in conditions of changing [CO2] (Gilbert et al. 2011). Because of these potential artifacts and errors, it is necessary to confirm previous studies on the response of g_m to [CO2] in angiosperms and ferns through the use of complimentary methods. We chose specifically to look at ferns because of the limited information published on CO2 responses and to determine if like stomatal responses to CO2, ferns also differed in g_m responses compared with angiosperms.

The purposes of this study were to determine: (1) the photosynthetic traits of ferns, including g_m and g_s at the present [CO2] (400 µmol mol⁻¹) for comparison against published values for ferns and angiosperms, and (2) the rapid and continuous response of g_m, g_s and photosynthetic rate of ferns to changing [CO2]. For these purposes, we developed a custom-designed gas exchange system using a concurrent measurement of gas exchange and carbon isotope ratio using tunable diode laser absorption spectroscopy (TDLAS), to quantify the rapid, continuous responses in g_m in fern species in response to changes in [CO2] with a time resolution of a few minutes (Tazoe et al. 2009, 2011). O2 gas was used at a level of 2 % for gas exchange measurements in order to minimize the effect of photorespiration on carbon isotope measurements. To the best of our knowledge, this is the first study to examine continuous responses in g_m in fern species in response to changes in [CO2]. We also determined leaf anatomical traits using light micrographs and calculated photosynthetic parameters using the light–response curve and A/C_i curve, to compare the photosynthetic traits of ferns with those of angiosperms reported previously.

We selected two fern species from order Polypodiales, Pteridium aquilinum and Thelypteris dentata. From recent phylogenetic studies, Polypodiales is the most modern order among the seven fern orders in Polypodiopsida (Smith et al. 2006). The estimated divergence time of Pteridium (Dennstaedithaceae family) and Thelypteris (Thelypteridaceae family, Eupolipods II) is ~90 and ~65 million years, respectively (Pryer et al. 2004), when
atmospheric CO$_2$ levels decreased with time from ~2,000 to ~500 ppm (Bice and Norris 2002). *P. aquilinum* was possibly distributed worldwide in the Oligocene (Der et al. 2009) when the atmospheric CO$_2$ levels had decreased (~400 ppm; Zhang et al. 2013). *P. aquilinum* and *T. dentata* grow in open sites and show higher photosynthetic rates than those of other ferns that grow in shady sites. High photosynthetic rate assures high accuracy in the estimation of $g_m$ using the carbon isotope method.

**Materials and methods**

**Plants materials**

*Pteridium aquilinum* (L.) Kuhn (Fig. 1a) and *T. dentata* (Forssk.) E. P. St. John (Fig. 1b) were used. *P. aquilinum* is a deciduous fern that grows in open habitats and is distributed widely in temperate zones in the Northern hemisphere. *T. dentata* is an evergreen fern that grows in open habitats in tropical or subtropical zones, and which has recently expanded into southern coastal areas in Japan (Murakami et al. 2007). Rhizomes of *P. aquilinum* and *T. dentata* were purchased commercially (Takayama Engei, Kyoto, Japan) and collected around the greenhouse at Kyoto Institute of Technology (Ukyo-ku, Kyoto, Japan), respectively. Five rhizomes of each species were planted in 3-liter pots filled with mixed soil (peat moss:humus:sand = 3:3:1 volume ratio) in a 50 % shaded glasshouse. Five plants of both species were used for light–response curve, $A/C_i$ curve, and anatomical analysis. Three or four of the five plants were used for CO$_2$ response measurements. Average daytime photosynthetic photon flux density (PPFD) in the glasshouse was 221 ± 7 µmol m$^{-2}$ s$^{-1}$. Plants were watered every 2 days, fertilized with a 1/2,000 solution of Hyponex 2009) when the atmospheric CO$_2$ levels had decreased with time from ~2,000 ppm (Zhang et al. 2013). Average temperature and relative humidity in the glasshouse from frond emergence to the experiments were 20.7 ± 0.1 °C and 74.5 ± 0.3 %, respectively.

**Estimation of $g_m$**

Gas exchange and carbon isotope discrimination were measured concurrently using a custom-designed system constructed at the National Institute for Agro-Environmental Sciences (Fig. 2), which was based on previous studies (Nelson et al. 2008; Tuzson et al. 2008; Wada et al. 2011). A custom-made gas exchange system was connected to a CO$_2$ isotope analyzer, a tunable diode laser absorption spectroscope (QC-TILDAS-ISO, Aerodyne Research Inc., Billerica, MA, USA) for the sequential measurement of CO$_2$ isotopologues. A custom leaf chamber with a diameter of 12 cm with temperature and humidity controlled by a thermo chiller (SMC, HRS018-AF-10) and a bubbler, respectively, was connected to the CO$_2$/H$_2$O analyzer (LI-7000, LI-COR) for the gas exchange measurements. Leaf area was measured using scanned images of the cut leaves immediately after the measurement, using ImageJ software (http://imagej.nih.gov/ij/). We measured the leaf boundary layer conductance in accordance with the leaf area by obtaining a calibration curve using saturated filter papers with different areas. The fan placed inside the chamber mixed the air in the chamber completely. A thermocouple placed inside the chamber was connected to the gas exchange analyzer in order to record leaf temperature. A red and blue light emitting diode (LED) light source (red: blue 8:1, LEDRB-630DL, Opto Code Corp., Tokyo, Japan) was set onto the chamber, with PPFD of 500 µmol m$^{-2}$ s$^{-1}$ at the leaf surface. The flow rate was set at 500 ml min$^{-1}$, and leaf temperature at 25–28 °C. Vapor pressure deficit (VPD) was set at <1.5 kPa. N$_2$ and O$_2$ gas were mixed using mass controllers (SEC-E40, HORIBA Ltd., Kyoto, Japan) to generate 2 % O$_2$. To determine photosynthetic responses to changes in atmospheric CO$_2$ concentration [CO$_2$], a fully expanded mature leaf was clamped into the chamber, and the ambient CO$_2$ concentration ($C_a$) was kept at 400 µmol mol$^{-1}$ for 40–60 min. After that, $C_a$ was first reduced to 200 µmol mol$^{-1}$ for 80 min, and then increased to 700 µmol mol$^{-1}$ for 80 min. Finally, $C_a$ was decreased to 400 µmol mol$^{-1}$. We varied $C_a$ from 200 to 700 µmol mol$^{-1}$ because leaf photosynthesis of ferns was CO$_2$-limited in this range of $C_a$ in $A/C_i$ curve analysis. Measurements were performed at 30 s intervals, calibrated every 30 min using two standard gas cylinders, 200 and 700 µmol mol$^{-1}$, during the measurement (Fig. 3). Stability of TDLAS was tested using standard CO$_2$ gas at a CO$_2$ concentration of 400 µmol mol$^{-1}$ for 2 h before and after the measurements. Analysis with Allan variance showed that

Fig. 1 Whole plant images of a *Pteridium aquilinum* and b *Thelypteris dentata. 83 × 55 mm (300 × 300 DPI)
deviation of $\delta^{13}C$ for 30 min was $<0.03\%$. The $\delta^{13}C$ of the gas was stabilized completely within 10 min. Observed carbon isotope discrimination during photosynthesis ($\delta_o$) was calculated using the following equation (Evans et al. 1986),

$$
\delta_o = \frac{1000\xi (\delta^{13}C_a - \delta^{13}C_{ref})}{1000 + \delta^{13}C_a - \xi (\delta^{13}C_a - \delta^{13}C_{ref})}
$$

(1)

where $\delta^{13}C_a$ and $\delta^{13}C_{ref}$ are the carbon isotope composition in the leaf chamber and in reference air. $\xi = C_{ref}/(C_{ref} - C_a)$, where $C_a$ and $C_{ref}$ are the CO$_2$ concentration in the leaf chamber and in reference air. $\xi$ was kept at $<9$ during measurements in order to ensure high precision and accuracy for $g_m$ estimation (Pons et al. 2009). Mesophyll conductance was calculated using the equations reported by Evans and von Caemmerer (2013) assuming no photorespiration:

$$g_m = \frac{1+t}{1-t} \left( b - a_i - \frac{eR_d}{(A + R_d)} \right) \frac{A}{C_a}/(\delta_i - \delta_o - \delta_e)
$$

(2)

$$t = (1 + a')E/2g_{ac}'$$

where $a'$ is a combined fractionation factor through the boundary layer and stomata,

$$a' = \frac{a_b (C_a - C_i) + a (C_i - C_{c})}{(C_a - C_i)}
$$

(3)

where $a_b$ (2.9 ‰) and $a$ (4.4 ‰) are the fractionation through CO$_2$ diffusion in the boundary layer and air, respectively (Evans et al. 1986). $C_s$ and $C_i$ are the CO$_2$ concentration at the leaf surface and in the leaf intercellular air space, respectively. $E$ is the transpiration rate, and $g_{ac}$ is total conductance to CO$_2$ diffusion. $b$ (30 ‰) is the fractionation associated with Rubisco carboxylation (Roeske and O'Leary 1984), $a_i$ (1.8 ‰) is the fractionation factor for dissolution and diffusion through water (O’Leary 1981), and $R_d$ is day respiration. The parameter $\varepsilon$, which is associated with day respiration, was calculated as $\varepsilon = \delta^{13}C_{tank} - \delta^{13}C_{atmosphere}$ assuming no fractionation by day respiration (Evans and von Caemmerer 2013; Tazoe et al. 2009). $\delta^{13}C_{tank}$ was from $-34$ to $-36$ ‰, and $\delta^{13}C_{atmosphere}$ was assumed to be $-8$ ‰. $\delta_i$ is fractionation when $C_i = C_c$ without respiratory fractionation:

$$\delta_i = \frac{1}{1-t} a' + \frac{1}{1-t} \left( (1+t)b - a' \right) \frac{C_i}{C_a}
$$

(4)
δ_e is fractionation with respiration, which was calculated as:

\[ \delta_e = \frac{1 + t}{1 - t} \left( \frac{eR_d}{(A + R_d)C_a} \right) (C_i - \Gamma^*) \]  

(5)

\( \Gamma^* \) is the CO₂ compensation point in the absence of \( R_d \), which was estimated following the procedure reported by Laisk et al. (1984). We used \( C^* \), the apparent CO₂ compensation point provided by Laisk et al. (1984), as a proxy of \( \Gamma^* \) (Douthe et al. 2011). \( C^* \) was 65.1 ± 3.9 and 65.1 ± 4.4 μmol mol⁻¹ in \( P. \ aquilinum \) and \( T. \ dentata \), respectively, and \( R_d \) was 3.8 ± 0.2 and 3.4 ± 0.2 μmol m⁻² s⁻¹, respectively.

**Estimation of photosynthetic parameters**

Leaf photosynthetic parameters were estimated from the light–response curve model (Ogren and Evans 1993) and the A/C\(_i\) curve fitting method (A/C\(_i\) Curve Fitting 10.0.xls, http://landflux.org/Tools.php, Ethier and Livingston 2004; Ethier et al. 2006) using a photosynthesis system (Li-6400, LI-COR). Leaf temperature and vapor pressure deficit (VPD) were set at 25 °C and 1.5 kPa, respectively. \( C_a \) was 400 μmol mol⁻¹ for the light–response curve analysis. PPFD was decreased stepwise from 500 to 0 μmol m⁻² s⁻¹. Thereafter, PPFD was returned to 400 μmol m⁻² s⁻¹ and then increased stepwise to 1,500 μmol m⁻² s⁻¹. Light-saturated photosynthesis rate (\( A_{sat} \)), curvature factor, quantum use efficiency, dark respiration rate, light compensation point, and \( g_s \) were obtained from light–response curves. For the A/C\(_i\) curve analysis, PPFD was set at 1,000 and 500 μmol m⁻² s⁻¹ for \( P. \ aquilinum \) and \( T. \ dentata \), respectively, because our previous experiment obtained saturated PPFD of 1,000 and 500 μmol m⁻² s⁻¹ for \( P. \ aquilinum \) and \( T. \ dentata \), respectively. \( C_a \) was decreased stepwise from 400 to 50 μmol mol⁻¹, then returned to 400 μmol mol⁻¹, and increased stepwise to 2,000 μmol mol⁻¹. Maximum carboxylation rate (\( V_{cmax} \)) and electron transport rate (\( J \)) were calculated from the A/C\(_i\) curve, assuming constant \( g_m \) during the changes in C\(_i\).

**Analysis of leaf morphological traits**

Leaf mass per area was calculated as leaf dry weight divided by leaf area. Leaf mesophyll anatomy was determined using light and transmission electron micrographs. Leaf sections of 2 × 3 mm were fixed in 5 % glutaraldehyde and 1 % osmium tetroxide, and were embedded in Spurr’s resin (Low Viscosity Resin kit, TAAB, Aldermaston, UK). Transverse Sects. (800 nm thick) were stained with 1 % toluidine blue solution. Anatomical characteristics were determined from digitized images of micrographs.
taken at ×400 magnification (BX51-33, OLYMPUS, Tokyo, Japan). The surface area of mesophyll cells and chloroplasts exposed to intercellular air spaces per unit leaf area (Smes and Sc) were estimated for transverse sections as described by Hanba et al. (2002). Transverse Sects. (70 nm thick) were stained with 2 % uranyl acetate and Reynold’s lead citrate. Thickness of cell walls covered with chloroplasts (cell wall thickness), and chloroplast thickness and width were measured from ×6,000 and ×2,500 magnification images, respectively, from micrographs taken by a transmission electron microscope (JEM-1220, JOEL, Tokyo, Japan), analyzed using ImageJ software (http://imagej.nih.gov/ij/).

Statistical analysis

Differences in mean values between species were tested using an unpaired t test to analyze photosynthetic parameters and leaf morphological traits. The effect of [CO2] on leaf gas exchange was analyzed using an unpaired t test. These statistical analyses were conducted using EZR version 1.24 (Kanda 2013; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html).

Results

Photosynthesis rate (A) was light saturated at a PPFD of 500 μmol m⁻² s⁻¹ for both P. aquilinum and T. dentata (Fig. 4a). For T. dentata, A tended to decrease when PPFD exceeded 1,000 μmol m⁻² s⁻¹. There were no significant differences in Amax, curvature factor, quantum use efficiency, respiration rate, or light compensation point between the two species (Table 1). When PPFD decreased from 500 to 0 μmol m⁻² s⁻¹, gs in P. aquilinum tended to decrease but that of T. dentata was almost constant (Fig. 4b), with a significant increase in intercellular CO₂ concentration (Ci) in both species (P < 0.05; Fig. 4c). The gs of P. aquilinum was compared with that of T. dentata and showed no significant difference at a PPFD of 2,000 μmol m⁻² s⁻¹. Vmax and J calculated using A/Ci curves were also not significantly different between the two species (Fig. 5a; Table 1). When Ci was decreased from 300 to 50 μmol mol⁻¹, the gs of both species significantly increased (P < 0.05; Fig. 5b). With Ci of 400 μmol mol⁻¹ to 1,800 μmol mol⁻¹, gs values remained almost constant in both species.

Transverse sections of the fronds of P. aquilinum (Fig. 6a, c) and T. dentata (Fig. 6b, d) showed that both ferns had loosely packed mesophyll cells and lacked distinct palisade tissue. Both ferns also had chloroplasts in the upper and lower epidermal cells. The width and thickness of chloroplasts in T. dentata were significantly larger than those of P. aquilinum (Table 2). The chloroplasts had large spaces between them and, as a result, 59 and 44 % of Smes were not covered with chloroplasts for P. aquilinum and T. dentata, respectively. The Smes and internal air spaces of P. aquilinum were significantly larger than those of T. dentata (Table 2). Other traits including Sc, LMA, leaf thickness, cell wall thickness, and chloroplast width/thickness were not significantly different between the two species.

When atmospheric CO₂ concentration [CO₂] was decreased from 400 to 200 μmol mol⁻¹, gs of both ferns increased slightly with time (Fig. 7a), with a 3.7 mmol m⁻² s⁻¹ increase in P. aquilinum and a 5.4 mmol m⁻² s⁻¹ increase...
in *T. dentata* on average (Table 3). In contrast, \(g_m\) of both ferns decreased rapidly (Fig. 7d), with a 8.6 mmol m\(^{-2}\) s\(^{-1}\) decrease in *P. aquilinum* and a 2.1 mmol m\(^{-2}\) s\(^{-1}\) decrease in *T. dentata* (Table 3).

### Table 1 Photosynthetic parameters obtained from the light-response and \(A/C_i\) curves of the ferns *Pteridium aquilinum* and *Thelypteris dentata*

| Parameters                                | *P. aquilinum*       | *T. dentata*       | \(P\) |
|-------------------------------------------|----------------------|--------------------|------|
| \(A_{sat}\) (μmol m\(^{-2}\) s\(^{-1}\)) | 7.54 ± 0.93          | 6.24 ± 0.90        | n.s. |
| Curvature factor                          | 0.87 ± 0.02          | 0.83 ± 0.06        | n.s. |
| Quantum use efficiency (μmol CO\(_2\) μmol photon\(^{-1}\)) | 0.074 ± 0.003        | 0.061 ± 0.006      | n.s. |
| Dark respiration rate (μmol m\(^{-2}\) s\(^{-1}\)) | 0.35 ± 0.06          | 0.46 ± 0.10        | n.s. |
| Light compensation point (μmol m\(^{-2}\) s\(^{-1}\)) | 3.98 ± 0.66          | 7.47 ± 1.89        | n.s. |
| \(g_s\) (mmol m\(^{-2}\) s\(^{-1}\)) at PPFD of 2,000 μmol m\(^{-2}\) s\(^{-1}\) | 115.0 ± 22.1         | 163.6 ± 24.1       | n.s. |
| \(V_{max}\) (μmol m\(^{-2}\) s\(^{-1}\)) | 40.4 ± 3.7           | 35.6 ± 5.4         | n.s. |
| \(J\) (μmol m\(^{-2}\) s\(^{-1}\)) | 72.0 ± 6.8           | 58.8 ± 8.1         | n.s. |

\(V_{max}\) and \(J\) were obtained from \(A/C_i\) curves in accordance with Ethier and Livingston (2004). Values are mean ± SE from five different plants \((n = 5)\). Statistical analysis was done using \(t\) test.

\(A_{sat}\), curvature factor, quantum use efficiency, dark respiration rate and light compensation point were calculated from light–response curves in accordance with Ogren and Evans (1993). *n.s.* not significant.

![Fig. 5](image1.png)

**Fig. 5** Response of a photosynthesis rate (\(A\)) and b stomatal conductance \((g_s)\) to intercellular CO\(_2\) concentration \((C_i)\) in *Pteridium aquilinum* (filled circles) and *Thelypteris dentata* (open circles). Ambient CO\(_2\) concentration was first decreased from 400 to 50 μmol mol\(^{-1}\), then returned to 400 μmol mol\(^{-1}\) and increased to 2000 μmol mol\(^{-1}\). Data points are means with bars for standard errors \((n = 5)\).

![Fig. 6](image2.png)

**Fig. 6** Light micrograph of transverse leaf sections of a *Pteridium aquilinum* and b *Thelypteris dentata* at 400× magnification. Transmission electron microscope images of c *Pteridium aquilinum* and d *Thelypteris dentata* at 6,000× magnification. 83 × 117 mm (300 × 300 DPI)
was 47 and 65.1 μmol mol⁻¹ in P. aquilinum and T. dentata, respectively (Fig. 7m).

When [CO₂] was increased from 200 to 700 μmol mol⁻¹, gₛ did not change in either species (Fig. 7b; Table 3). gₘ of P. aquilinum decreased by 1.1 mmol m⁻² s⁻¹, whereas it increased by 4.9 mmol m⁻² s⁻¹ in T. dentata (Table 3; Fig. 7e). A and Cₛ increased rapidly, by 8 and 6.8 μmol m⁻² s⁻¹ and 405.5 and 414.9 μmol mol⁻¹ for P. aquilinum and T. dentata, respectively (Fig. 7 h, k), and the respective increases in Cₑ were 175 and 254.5 μmol mol⁻¹ (Fig. 7n).

When [CO₂] was decreased from 700 to 400 μmol mol⁻¹, gₛ of both species increased slightly with time (Fig. 7c), with a 8.3 mmol m⁻² s⁻¹ increase in P. aquilinum and a 0.9 mmol m⁻² s⁻¹ increase in T. dentata on average (Table 3). The gₘ of P. aquilinum increased by 6.5 mmol m⁻² s⁻¹, whereas that of T. dentata decreased by 6.6 mmol m⁻² s⁻¹ on average (Table 3; Fig. 7f). A and Cₛ decreased rapidly, by 4.0 and 3.2 and 255.4 and 266.6 μmol mol⁻¹ for P. aquilinum and T. dentata, respectively (Fig. 7i, l), and the respective decreases in Cₑ were 115.1 and 213.2 μmol mol⁻¹ (Fig. 7o).

### Discussion

**Steady-state leaf photosynthetic traits and morphology in ferns**

There was no significant difference in photosynthesis traits between P. aquilinum and T. dentata obtained from the analysis of light–response curves and A/Cₛ curves (Table 1). P. aquilinum and T. dentata are Polypodiales, which is the most modern among the fern orders (Smith et al. 2006). Photosynthesis traits were compared with those reported in previous studies of fern species in Polypodiales. Aₛₐₜ, dark respiration rate, light compensation point, Vₘₐₓ, and J were within the range of those reported in previous studies, although the growth conditions differed (Carriquí et al. 2015; Gago et al. 2013; Sessa and Givnish 2014). At present, there is little evidence that photosynthetic traits are different between phylogenetically distant fern groups; two basal ferns Equisetum telmateia (Equisetales) and O. regalis (Osmundales) had photosynthetic traits within the range of those in Polypodiales (Carriquí et al. 2015; Gago et al. 2013). However, photosynthesis data for basal ferns are so scarce that further systematic studies are needed for phylogenetic consideration. The present result confirmed that the photosynthetic capacity of ferns is generally lower than angiosperms; ferns had lower Aₛₐₜ, dark respiration rate, gₛ, Vₘₐₓ, and J than angiosperms (Carriquí et al. 2015). The values reported in this study are in the range of those reported in Carriquí et al. (2015) for fern species.

Although photosynthesis traits are similar between P. aquilinum and T. dentata, internal air spaces and Sₘₑˢₛ were larger in P. aquilinum than in T. dentata, because of its loosely packed mesophyll cells. The smaller size of chloroplasts in P. aquilinum (Table 2) offset the effect of higher Sₘₑˢₛ, which involves similar Sₑ between P. aquilinum and T. dentata. This similar Sₑ may relate to the similar photosynthetic traits between species in the present study. Compared with angiosperms, where the lowest Sₑ so far reported was 5.0 m² m⁻² in a tree species Acer rufinerve grown in shade (Hanba et al. 2001), the Sₑ measurements of fern species here were among the lowest values, with an Sₑ of 3.9 ± 0.6 m² m⁻² in P. aquilinum and 5.0 ± 0.4 m² m⁻² in T. dentata. Terashima et al. (2006) reported that in seed plants, the average Sₑ was 15.01 m² m⁻² in annuals, 12.05 m² m⁻² in deciduous trees, and 14.45 m² m⁻² in evergreen trees. Carriquí et al. (2015) reported that the average Sₑ for seven angiosperms and ferns was 10.3 and 7.6 m² m⁻², respectively. These previous studies, together with our results, indicate that the Sₑ of ferns is lower than the Sₑ of most angiosperms. Small Sₑ measurements in fern species are related to small mesophyll thickness with large intercellular airspaces, and may also be partly affected by the size of chloroplasts (Table 2). Sₑ is one of the most significant factors affecting gₘₑ, where high Sₑ allows plants to increase diffusion of CO₂ into chloroplasts. Angiosperms had much lower atmospheric CO₂ levels than ferns at their emergence period, and this may have been crucial for angiosperms to increase diffusional surface for CO₂ to achieve high photosynthetic rates.

Cell wall thickness was 0.23 ± 0.02 μm in P. aquilinum (Table 2), which is similar to the cell wall thickness of 0.194 μm in P. aquilinum reported by Carriquí et al. (2015). The cell wall thickness of P. aquilinum and T. dentata

| Parameters                      | P. aquilinum | T. dentata | P      |
|--------------------------------|--------------|------------|--------|
| Sₑ (m⁴ m⁻²)                    | 3.9 ± 0.6    | 5.0 ± 0.4  | n.s.   |
| Sₑₑ (m³ m⁻²)                   | 11.0 ± 0.6   | 9.0 ± 0.3  | 0.01   |
| Leaf mass per area (g m⁻²)     | 31.7 ± 3.1   | 39.7 ± 4.0 | n.s.   |
| Leaf thickness (μm)            | 163.4 ± 9.1  | 149.9 ± 9.3| n.s.   |
| Internal air space (%)         | 39.6 ± 5.5   | 20.8 ± 2.8 | 0.02   |
| Cell wall thickness (μm)       | 0.23 ± 0.02  | 0.30 ± 0.03| n.s.   |
| Chloroplast width (μm)         | 4.51 ± 0.23  | 6.65 ± 0.26| <0.01  |
| Chloroplast thickness (μm)     | 2.43 ± 0.21  | 3.97 ± 0.07| <0.01  |
| Chloroplast width/thickness    | 1.94 ± 0.15  | 1.68 ± 0.04| n.s.   |

Values are mean ± SE from five different plants (n = 5). Statistical analysis was done using t test.

n.s. not significant
Fig. 7 Changes in stomatal conductance ($g_s$), mesophyll conductance ($g_m$), photosynthesis rate ($A$), intercellular CO$_2$ concentration ($C_i$) and chloroplast CO$_2$ concentration ($C_c$) in response to atmospheric CO$_2$ concentration [CO$_2$] for Pteridium aquilinum (filled circles) and Thelypteris dentata (open circles). [CO$_2$] was first decreased from 400 to 200 μmol mol$^{-1}$ (left panels), then increased from 200 to 700 μmol mol$^{-1}$ (middle panels), and finally decreased from 700 to 400 μmol mol$^{-1}$ (right panels). Data points were averaged for two data (1 min) from 3 or 4 different plants with bars for standard errors ($n$ = 3 or 4). 167 × 196 mm (300 × 300 DPI)
phylogenic group (from 0.194 to 0.687 μm in ferns, Carriquí et al. 2015). As previously described, S_c values in P. aquilinum and T. dentata (3.9 ± 0.6 and 5.0 ± 0.4 m^2 m^-2) were lower than those of most seed plants including annuals (15.0 m^2 m^-2), deciduous broadleaved trees (12.1 m^2 m^-2), and evergreen trees (14.5 m^2 m^-2) (Terashima et al. 2006). When anatomical traits in the present study were compared with those of an angiosperm, Lysimachia minoricensis that had similar LMA (31.4 g m^-2) to our study (31.7 and 39.7 g m^-2), the cell wall thickness was similar (0.213 μm and 0.28 μm), but S_c was much smaller in the present study (3.9 and 5.0 m^2 m^-2) than L. minoricensis (8.9 m^2 m^-2; Carriquí et al. 2015). Therefore, the low g_m of fern species may be at least partly affected by low S_c; a significant positive correlation was obtained between g_m and S_c for angiosperms (Terashima et al. 2006, 2011). The concentration and activities of membrane proteins that transport CO₂ into mesophyll cells, such as aquaporins (Hanba et al. 2004; Kawase et al. 2013; Terashima and Ono 2002), could also account for the low g_m of ferns.

**Steady-state g_m of fern species compared with angiosperms at the present [CO₂]**

One of the goals of our study was to determine steady-state g_m values of ferns and compare them with the published values for angiosperms at the present [CO₂] (400 μmol mol^-1). For the estimation of g_m, carbon isotope, chlorophyll fluorescence, and A/C_i curve-fitting methods have all been used previously (Pons et al. 2009). There are only three studies that reported the g_m of ferns. Gago et al. (2013) showed that the g_m of O. regalis, B. gibbum and N. exaltata were between 30 and 73 mmol m^-2 s^-1 using a chlorophyll fluorescence method, and between 30 and 112 mmol m^-2 s^-1 using an A/C_i curve-fitting method. Volkova et al. (2009) showed that g_m of Dicksonia antarctica grown in the shade and at high irradiance was 115 ± 35 and 155 ± 64 mmol m^-2 s^-1, respectively, estimated from A/C_i curve fitting. Carriquí et al. (2015) reported that g_m varies from 26 to 253 mmol m^-2 s^-1 for seven fern species using a chlorophyll fluorescence method. We used a different method from previous studies reporting the g_m of ferns (Carriquí et al. 2015; Gago et al. 2013; Volkova et al. 2009). The g_m values in the present study (35.6 to 52.5 mmol m^-2 s^-1) were among the lowest values reported from these three previous studies. Furthermore, the g_m values of P. aquilinum and T. dentata in the present study were lower than those of typical seed plants, including angiosperms and gymnosperms (Flexas et al. 2008, 2012).

The cause of the low g_m in fern species remains to be clarified, but some anatomical traits have been suggested to play a role (Gago et al. 2013; Carriquí et al. 2015). As previously described, S_c values in P. aquilinum and T. dentata (3.9 ± 0.6 and 5.0 ± 0.4 m^2 m^-2) were lower than those of most seed plants including annuals (15.0 m^2 m^-2), deciduous broadleaved trees (12.1 m^2 m^-2), and evergreen trees (14.5 m^2 m^-2) (Terashima et al. 2006). When anatomical traits in the present study were compared with those of an angiosperm, Lysimachia minoricensis that had similar LMA (31.4 g m^-2) to our study (31.7 and 39.7 g m^-2), the cell wall thickness was similar (0.213 μm and 0.28 μm), but S_c was much smaller in the present study (3.9 and 5.0 m^2 m^-2) than L. minoricensis (8.9 m^2 m^-2; Carriquí et al. 2015). Therefore, the low g_m of fern species may be at least partly affected by low S_c; a significant positive correlation was obtained between g_m and S_c for angiosperms (Terashima et al. 2006, 2011). The concentration and activities of membrane proteins that transport CO₂ into mesophyll cells, such as aquaporins (Hanba et al. 2004; Kawase et al. 2013; Terashima and Ono 2002), could also account for the low g_m of ferns.
Dynamic response of \( g_s \) and \( g_m \) in response to changes in [\( \text{CO}_2 \)]

The slight increase in \( g_s \) (<10.4 %) followed a decrease in \( C_a \) from 400 to 200 \( \mu \text{mol mol}^{-1} \) (Table 3; Fig. 7). This was consistent with Brodribb et al. (2009), who reported that six ferns/lycopods showed small increases in \( g_s \) when \( C_a \) decreased from 380 to 100 \( \mu \text{mol mol}^{-1} \). In a study on \( B. \ gibbum \) and \( O. \ regalis \), Gago et al. (2013) reported a slight decrease in \( g_s \) when \( C_a \) decreased from 400 to 50 \( \mu \text{mol mol}^{-1} \), and a similar result was obtained in our study (Fig. 5b). No change was observed in \( g_s \) after an increase in \( C_a \) (from 200 to 700 \( \mu \text{mol mol}^{-1} \)). This result supports Brodribb et al. (2009), who reported no significant changes in \( g_s \) when \( C_a \) was increased from 380 to 600 \( \mu \text{mol mol}^{-1} \) for three Polypodiales ferns. The insensitivity of stomata to the increase in \( C_a \) contrasts with the response seen in angiosperms, which showed a significant decrease (Brodribb et al. 2009). Although the physiological control of stomatal response to [\( \text{CO}_2 \)] in angiosperms remains poorly understood, Brodribb et al. (2009) hypothesized that a signaling pathway between mesophyll and guard cells in response to [\( \text{CO}_2 \)] may be present in angiosperms but not in ferns. However, the slight increase in \( g_s \) in ferns following decreased \( C_a \) is similar (but less distinct) to the response in angiosperms, suggesting that the mechanisms involved in \( \text{CO}_2 \) sensing in angiosperms may be partly functional in ferns.

In contrast to the increase in \( g_s \), the \( g_m \) of the two fern species in the present study decreased quickly when \( C_a \) was decreased from 400 to 200 \( \mu \text{mol mol}^{-1} \) (Fig. 7d). Decreased \( g_m \) at low \( C_i \) (<200 \( \mu \text{mol mol}^{-1} \)) has been reported for some angiosperms (Flexas et al. 2007; Žáril et al. 2009) and for three ferns (Gago et al. 2013) using chlorophyll fluorescence or isotope methods. However, previous studies pointed out that the decrease in \( g_m \) at low \( C_i \) might be because of artifacts caused by respiration and photorespiration, when measurements were performed at atmospheric \( \text{O}_2 \) level (20 %, Gago et al. 2013; Tholen et al. 2012). As far as we know, three previous studies have been conducted to estimate the \( g_m \) response to [\( \text{CO}_2 \)] using 2 % or 1 % \( \text{O}_2 \) to minimize the effect of photorespiration (Mizokami et al. 2015; Tazoe et al. 2009, 2011). Tazoe et al. (2009, 2011) reported that in \( \text{Nicotiana plumbaginifolia} \), both \( g_m \) and \( g_s \) decreased with \( C_i \) on the application of abscisic acid (ABA), but they increased in the absence of ABA, which suggested that \( g_s \) has an effect on \( g_m \) and its response to [\( \text{CO}_2 \)] (Tazoe and Santrucek 2015). In our study, however, the rapid decrease in \( g_m \) after decreasing \( C_i \) is in contrast to the gradual and slight increase in \( g_s \) for two fern species (Fig. 7a, d). The effect of photorespiration was minimized, because we measured gas exchange at 2 % \( \text{O}_2 \). The present result suggested that the decrease in \( g_m \) at 200 \( \mu \text{mol mol}^{-1} \) of \( C_s \) in the two ferns may be independent of \( g_s \).

The \( g_m \) did not decrease from 200 to 700 \( \mu \text{mol mol}^{-1} \) with [\( \text{CO}_2 \)] for \( T. \ dentata \) and \( P. \ aquilinum \) (Fig. 7e; Table 3), which is in contrast to the results that reported significant decreases in \( g_m \) from 200 to 1,000 \( \mu \text{mol mol}^{-1} \) of [\( \text{CO}_2 \)] for three angiosperms (Tazoe et al. 2011). Although a trend where \( g_m \) declines at high [\( \text{CO}_2 \)] is frequently reported in angiosperms (Bunce 2010; Douthe et al. 2011; Flexas et al. 2007; Mizokami et al. 2015; Žáril et al. 2009) and also in ferns (Gago et al. 2013), the dependence of \( g_m \) on \( \text{CO}_2 \) varied between studies and species. \( g_m \) was highest at >200 \( \mu \text{mol mol}^{-1} \) of \( C_i \) in \( O. \ regalis \), whereas \( N. \ exaltata \) showed highest \( g_m \) at \( C_i \) of 400 \( \mu \text{mol mol}^{-1} \) (Gago et al. 2013). These results suggest that plant species have “optimum” \( g_m \) at different \( \text{CO}_2 \) levels, which might reflect atmospheric \( \text{CO}_2 \) levels during their evolution. \( P. \ aquilinum \) showed its highest \( g_m \) at [\( \text{CO}_2 \)] of 400 \( \mu \text{mol mol}^{-1} \) (Table 3), with this species distributed worldwide in the Oligocene (Der et al. 2009) when the \( \text{CO}_2 \) level had decreased near to the present level (~400 ppm; Zhang et al. 2013). \( T. \ dentata \) showed its highest \( g_m \) at [\( \text{CO}_2 \)] of 700 \( \mu \text{mol mol}^{-1} \) (Table 3), and the estimated divergence time of \( T. \ dentata \) is ~65 million years (Pryer et al. 2004) when \( \text{CO}_2 \) levels were high (~1,000 ppm; Bice and Norris 2002).

The physiological mechanisms for \( g_m \) responses to changes in [\( \text{CO}_2 \)] have not been identified for angiosperms or ferns. Mizokami et al. (2015) suggested that the decrease in \( g_m \) in response to the increase in [\( \text{CO}_2 \)] may be mediated by inactivation of the plasma membrane intrinsic proteins via carbonic anhydrase activity. However, an increased \( g_m \) in response to increased [\( \text{CO}_2 \)] in the present study (Fig. 7e) suggested that, in \( T. \ dentata \), other mechanisms may be involved. Terashima et al. (2011) suggested that porosity and tortuosity of the cell wall, or diffusion of HCO3− could be affected by \( \text{pH} \) via changes in [\( \text{CO}_2 \)], and thus affect \( \text{CO}_2 \) diffusion through cell walls. Irrespective of the mechanisms for \( g_m \), \( g_m \) imposes major photosynthetic limitations in ferns (Carriqué et al. 2015). This is a major reason why the \( g_m \) response of ferns to [\( \text{CO}_2 \)] has a large effect on the response of photosynthesis (\( A \) to [\( \text{CO}_2 \)] (Fig. 7 g–l)). A decrease in \( g_m \) in response to low [\( \text{CO}_2 \)] is clearly not advantageous for photosynthesis in the present low atmospheric \( \text{CO}_2 \) environment because low \( g_m \) (Fig. 7d) causes low \( C_i \) (Fig. 7 m) and thus a diminished rate of photosynthesis (Fig. 7). The regulation mechanism of \( g_m \) with changes in [\( \text{CO}_2 \)] may have developed with plant evolution in response to historical changes in atmospheric \( \text{CO}_2 \) levels and \( g_s \).
Conclusion

In a steady-state with the present level of CO₂ in the environment (400 μmol mol⁻¹), two ferns P. aquilinum and T. dentata had lower mesophyll conductance (gₘ) than the angiosperms measured so far, which may be partly imposed by the small Sₑ observed. The dynamic response of gₛ to changes in [CO₂] confirmed previous studies that reported slight increases in gₛ after changes in [CO₂] from the present level to below the present level (e.g., 200 μmol mol⁻¹) for fern species, in which the sensitivity of gₛ to the decrease in [CO₂] was much lower than in angiosperms. Although a causal mechanism for the CO₂ response of gₘ still remains to be clarified, the dynamic response in gₘ showed a rapid and significant decrease to decreased [CO₂] (from 400 to 200 μmol mol⁻¹), which was in contrast to the response of angiosperms. These dynamic CO₂ responses in gₛ and gₘ in P. aquilinum and T. dentata suggest that fern species have not evolved efficient regulatory mechanisms to cope with the low CO₂ levels. Future studies of the CO₂ response of gₘ and gₛ in ferns, and studies of gₛ in other primitive plants, such as mosses, will be helpful to further elucidate how photosynthetic traits have evolved in the history of land plants.

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