Research Article

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Research Area: Ecophysiology and Sustainability (A secondary area is a Signaling and Response)
Stomatal blue light response is present in early vascular plants

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One Sentence Summary: We surveyed stomatal blue light responses in a wide lineage of plants and found that the responses are present in all these plants except the fern species of Polypodiopsida.
Footnotes:

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Abstract

Light is a major environmental factor required for stomatal opening. Blue light (BL) induces stomatal opening in higher plants as a signal under the photosynthetic active radiation. The stomatal BL response is not present in the fern species of Polypodiopsida. The acquisition of a stomatal BL response might provide competitive advantages in both the uptake of CO$_2$ and prevention of water loss with the ability to rapidly open and close stomata. We surveyed the stomatal opening in response to strong red light (RL) and weak BL under the RL with gas exchange technique in a diverse selection of plant species from euphyllophytes, including spermatophytes and monilophytes, to lycophytes. We showed the presence of RL-induced stomatal opening in most of these species and found that the BL responses operated in all the euphyllophytes except Polypodiopsida. We also confirmed that the stomatal opening in lycophytes, the early vascular plants, is driven by plasma membrane H$^+$-ATPase and K$^+$ accumulation in guard cells, which is the same mechanism operating stomata of angiosperms. These results suggest that the early vascular plants respond to both RL and BL and actively regulate stomatal aperture. We also found three plant species, which absolutely requires blue light for both stomatal opening and photosynthetic CO$_2$ fixation, including a gymnosperm *Cycas revoluta*, and the ferns *Equisetum hyemale* and *Psilotum nudum*. 
Introduction

Stomata regulate gas exchange between plants and the atmosphere (Zeiger 1983; Assmann, 1993; Roelfsema and Hedrich, 2005; Shimazaki et al., 2007; Kim et al., 2010). Acquisition of stomata was a key step in the evolution of terrestrial plants by allowing uptake of CO₂ from the atmosphere and accelerating the provision of nutrients via the transpiration stream within the plant (Hetherington and Woodward, 2003; McAdam and Brodribb, 2013). Stomatal aperture is regulated by changes in the turgor of guard cells, which are induced by environmental factors and endogenous phytohormones. Light is a major factor in the promotion of stomatal opening and the opening is mediated via two distinct light-regulated pathways that are known as photosynthesis- and blue light (BL)-dependent responses under photosynthetic active radiation (PAR) (Vavasseur and Raghavendra, 2005; Shimazaki et al., 2007; Lawson et al., 2014).

The photosynthesis-dependent stomatal opening is induced by a continuous high intensity of light and the action spectrum for the stomatal opening resembles that of photosynthetic pigments in leaves (Willmer and Fricker, 1996). Both mesophyll and guard cells possess photosynthetically active chloroplasts and their photosynthesis has been suggested to contribute to stomatal opening in leaves. The decrease in the concentration of intercellular CO₂ (Ci) caused by photosynthetic CO₂ fixation or some unidentified mediators and metabolites from mesophyll cells are supposed to elicit stomatal opening, although the exact nature of the events is unclear (Wong et al. 1979; Vavasseur and Raghavendra 2005; Roelfsema et al., 2006; Mott et al., 2008; Lawson et al. 2014).

BL-dependent stomatal opening requires strong intensity of PAR as a background: weak BL solely scarcely elicits stomatal opening, but the same intensity of BL induces the fast and large stomatal opening in the presence of strong red light (RL) (Ogawa et al., 1978, Shimazaki et al., 2007). Since such stomatal opening requires BL under the RL or PAR, we call the opening reaction as BL-dependent response of stomata. BL-dependent stomatal response takes place and proceeds under the natural environments because the sunlight contains both BL and RL, and facilitates photosynthetic CO₂ fixation (Assmann 1988, Takemiya et al., 2013). In this stomatal response, BL and PAR (BL, RL, and other wavelength of light) seems to act as a signal...
and an energy source, respectively.

The BL-dependent stomatal opening is initiated by the absorption of BL by phototropins (phot1, phot2), the plant specific blue light receptors, in guard cells, followed by activation of the plasma membrane H⁺-ATPase (Kinoshita and Shimazaki, 1999). Two newly identified proteins, protein phosphatase 1 and BLUS1 (blue light signaling 1), mediate the signaling between phototropins and H⁺-ATPase (Takemiya et al., 2006; Takemiya et al., 2013a; Takemiya et al., 2013b). The activated H⁺-ATPase evokes a plasma membrane hyperpolarization, which drives K⁺ uptake through the voltage-gated, inward-rectifying K⁺ channels (Assmann, 1993; Shimazaki et al., 2007; Kim et al., 2010; Kollist et al., 2014). The accumulation of K⁺ causes water uptake and increases turgor pressure of guard cells, and finally results in stomatal opening. The BL-dependent opening is enhanced by RL and BL at low intensity is effective in the presence of RL (Ogawa et al., 1978; Iino et al., 1985; Shimazaki et al., 2007; Suetsugu et al., 2014). These stomatal responses by RL and BL are commonly observed in a number of seed plants so far examined.

Fine control of stomatal aperture to various environmental factors has been characterized in many angiosperms. Although morphological and mechanical diversity of stomata is widely documented, little is known about the functional diversity (Willmer and Fricker, 1996; Hetherington and Woodward, 2003). Our previous study indicated that BL-dependent stomatal response is absent in the major fern species of Polypodiopsida, including Adiantum capillus-veneris, Pteris cretica, Asplenium scolopendrium, and Nephrolepis auriculata, but the stomata of these species open by PAR including RL (Doi et al., 2006). When the epidermal peels isolated from A. capillus-veneris are treated with photosynthetic electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1dimethylurea (DCMU) (Doi and Shimazaki, 2008), the response is completely inhibited, but the responses in the seed plants of Vicia faba and Commelina communis are relatively insensitive to DCMU (Schwartz and Zeiger, 1984). These findings suggest that there is functional diversity in light-dependent stomatal response in different lineages of land plants. In accord with this notion, the different sensitivities of stomatal response to abscisic acid (ABA) and CO₂ have been reported among the plant species of angiosperm, gymnosperm, ferns, and lycophytes (Mansfield and Willmer, 1969; Brodribb and McAdam, 2011), although the exact responsiveness to ABA and CO₂ is still debated (Chater et al., 2011; Ruszala et al., 2011; Chater et al.,
To address the origin and distribution of stomatal light responses, we investigated the presence of a stomatal response using a gas exchange method and various lineages of vascular plants, including euphyllophytes and lycophytes. Unexpectedly, all plant lineages except Polypodiopsida in monilophytes exhibited a stomatal response to blue light in the presence of RL, suggesting that the response was present in the early evolutionary stage of vascular plants. We also report the stomatal opening in response to RL in these plant species.
Results

Typical blue light responses of stomata in angiosperms and ferns

To distinguish the stomatal response specific to BL from that of RL, we measured stomatal conductance by gas exchange technique using a dual beam protocol (Ogawa et al., 1978; Assmann et al., 1985; Iino et al., 1985; Shimazaki et al., 1986). The method allows for discrimination of the photosynthesis- and BL-dependent stomatal opening. All the plants tested were kept in darkness for 12 h before measurements. When the dark-adapted A. thaliana leaf was irradiated with a high intensity of RL at 600 µmol m⁻² s⁻¹, a gradual increase in stomatal conductance occurred and reached maximum within 30 min with an immediate photosynthetic CO₂ uptake at maximum within 10 min (Fig. 1A). Such CO₂ uptake by RL is often found in Arabidopsis and is due to the significant stomatal opening in the dark, which allows sufficient provision of CO₂ for photosynthesis without further opening (Lascève et al., 1997; Caird et al., 2007). When a low intensity of BL at 5 µmol m⁻² s⁻¹ was superimposed on RL, stomatal conductance increased rapidly and reached a peak, followed by a fast decline after turning off the BL. No increase in the conductance occurred when RL at 5 µmol m⁻² s⁻¹ was superimposed on the RL instead of BL (not shown). We call this response BL-dependent stomatal opening, but this response occurs under the natural environment because the sunlight consists of both RL and BL.

In recent phylogenetic analyses, the vascular plants are divided into euphyllophytes and lycophytes (Pryer et al., 2001; Smith et al., 2006). The euphyllophytes consist of spermatophytes and monilophytes; the monilophytes comprise four fern species: Polypodiopsida, Equisetopsida, Marattiopsida, and Psilotopsida (Fig. 2). Polypodiopsida, which diversified after the advent of angiosperms as modern fern species (Schneider et al., 2004), is the largest fern lineage and consists of seven major groups: Osmundales, Hymenophyllales, Gleicheniales, Schizaeales, Salviniales, Cyatheales, and Polypodiales (Smith et al., 2006; Wolf et al., 2011). Hymenophyllales ferns, which reside in the water, do not have stomata. We have reported that five fern species within Schizaeales and Polypodiales lack the stomatal responses to BL (Doi et al., 2006). To clarify whether the absence of stomatal BL response is a common feature among these species, we investigated stomatal responses in four lineages, including Osmundales, Gleicheniales, Cyatheales, and...
Polypodiales. Light responses of stomata and photosynthetic CO₂ uptake in *Osmunda japonica* (Osmundales), *Dicranopteris linearis* (Gleicheniales), *Alsophila mertensiana* (Cyatheales), and *Lepisorus thunbergianus* (Polypodiales). Light responses of stomata and photosynthetic CO₂ uptake in *Osmunda japonica* (Osmundales), *Dicranopteris linearis* (Gleicheniales), *Alsophila mertensiana* (Cyatheales), and *Lepisorus thunbergianus* (Polypodiales).
(Polypodiales) were presented (Fig. 1 B-F). When the strong RL (600 µmol m$^{-2}$ s$^{-1}$) that saturates photosynthesis was applied to the upper surface of the leaf, photosynthetic CO$_2$ uptake increased rapidly to the maximum value. Stomatal conductance showed almost the same time course as that of the CO$_2$ uptake in response to RL, and reached the maximum about 20-60 min after the onset of RL. The stomatal responses to RL in Polypodiopsida ferns were faster than that of *Arabidopsis* in general (Fig.1) as has been reported previously (Doi et al., 2006). Prolonged exposure to RL caused a slight and gradual decrease in the conductance in *D. linearis, A. mertensiana, T. acuminata* and *L. thunbergianus*. A weak BL (5 µmol m$^{-2}$ s$^{-1}$) superimposed on RL did not increase stomatal conductance in these Polypodiopsida ferns. The results indicate that all these stomata examined here open by RL, while they do not exhibit BL-dependent stomatal opening. The lack of BL-dependent stomatal response was confirmed in five evolutionary diverse fern species of Polypodiopsida (Doi et al., 2006).

Stomatal response to light in gymnosperms

Given the lack of BL response of stomata in Polypodiopsida ferns, it is interesting to know whether other plant lineages, including gymnosperms and...
lycophytes, show the response (Fig. 2). Gymnosperms comprise four clades, including Cycadopsida, Ginkgoopsida, Coniferopsida, and Gnetopsida. Figure 3 shows typical light responses of stomatal conductance and photosynthesis in a representative species of each clade: *Zamia furfuracea* (Cycadopsida), *Cycas revoluta* (Cycadopsida), *Ginkgo biloba* (Ginkgoopsida), *Chamaecyparis obtusa* (Coniferopsida), and *Gnetum* spp. (Gnetopsida).
Irradiation of leaves with RL at 600 µmol m$^{-2}$ s$^{-1}$ increased stomatal conductance in these species except for *C. revoluta*. When weak BL at 5 µmol m$^{-2}$ s$^{-1}$ was applied to the leaf superimposed on the RL, stomatal conductance and the CO$_2$ uptake increased in all these species. Stomata of *C. revoluta* did not respond to RL and only a slight CO$_2$ uptake occurred, but a large increase in conductance was elicited by BL with a simultaneous large CO$_2$ uptake (Fig. 3B, Fig. 1SA). From these results we conclude that the gymnosperms possess BL response of stomata. Furthermore, a sole irradiation of *C. revoluta* leaf with weak BL did not cause an increase in stomatal conductance but a subsequent RL induced large stomatal opening and CO$_2$ uptake (Fig. 1SB), suggesting that RL is required for BL-dependent stomatal opening in this plant species.

Stomatal responses in fern species other than Polypodiopsida

We hypothesized that BL response of stomata was acquired in plants after the evolution of seed plants because both gymnosperms and angiosperms possess the response but the extant major fern Polypodiopsida does not. We therefore investigated whether the stomata of fern species that evolved earlier than Polypodiopsida were able to open stomata in response to BL.

Unexpectedly, as shown in Figure 4, three fern classes of Equisetopsida, Marattiopsida, and Psilotopsida (Fig. 2) showed stomatal opening in responses to BL except Polypodiopsida (Fig.1). When these fern species were irradiated with strong RL, stomata of *Angiopteris lygodiifolia* (Marattiopsida) and *Botrychium tennum* (Psilotopsida) opened and photosynthetic CO$_2$ uptake proceeded, but stomata of *Equisetum hyemale* (Equisetopsida) and *Psilotum nudum* (Psilotopsida) did not open and no CO$_2$ uptake occurred. Interestingly, stomata of *P. nudum* and *E. hyemale* showed a large stomatal opening by BL and photosynthetic CO$_2$ uptake occurred in parallel with an increase in stomatal conductance. The results indicated that the ferns of Equisetopsida, Marattiopsida, and Psilotopsida have BL response of stomata, and the BL responses of Equisetopsida and Psilotopsida are essential for photosynthetic CO$_2$ fixation in these plant species by eliminating the barrier against CO$_2$ entry into the leaf.

Stomatal responses in lycophytes

As described above, stomatal BL response operates in fern species older
than Polypodiopsida, and this observation prompted us to investigate the responses in lycophytes, which are the most primitive extant vascular plants that appeared about 420 million years ago. We used *Selaginella moellendorfii* and *Selaginella uncinata*, a sister group to the ferns and the seed plants, as a representative of lycophytes (Fig. 2). *Selaginella* species have fronds that consist of microphylls and stems and their stomata are in line with the central part of the microphyll (Soni et al., 2012). Stomatal conductance and photosynthetic CO$_2$ uptake were increased by strong RL (Fig. 5). After conductance reached a steady state, weak BL on the RL further increased both stomatal conductance and the CO$_2$ uptake in *S. moellendorfii* and *S. uncinata* (Fig. 5A, C). Since a short pulse of blue light is able to elicit stomatal opening in Arabidopsis and other seed plants (Assmann et al., 1985; Iino et al., 1985; Shimazaki et al., 2007), we

![Figure 4. Stomatal conductance (blue line) and photosynthetic CO2 uptake (red line) in response to light in leaves of *Equisetum hyemale* (A), *Angiopteris lygodiiifolia* (B), *Botrychium ternatum* (C), and *Psilotum nudum* (D). Plants were treated with light as shown in Fig. 1.](image-url)
tested the effect of a pulse on the microphylls. A pulse of BL superimposed on RL also
induced fast stomatal opening in these plants (Fig. 5B, D) and stomata in the plants
closed more slowly after the pulse than those in Arabidopsis. From these results, we
conclude that BL-dependent stomatal response operates in lycophytes although the
detailed mechanism has yet to be clarified.

H+-ATPase and K+ accumulation in guard cells are responsible for stomatal
opening in lycophytes

We have shown that BL-dependent stomatal response operates in vascular
plants except Polypodiopsida ferns. It is unclear whether the stomatal responses in
these diverse lineages of plants have the same mechanisms as those in angiosperms,
Arabidopsis. To test this, we investigated the involvement of H\(^+-\)ATPase and K\(^+\) accumulation in the opening response using the earliest evolved vascular plants of S. uncinata. Recent investigation reported light-induced stomatal opening in this same species (Ruszala et al., 2011) with concomitant K\(^+\) accumulation in guard cells. Furthermore, fusicoccin, an activator of the plasma membrane H\(^+-\)ATPase, induced stomatal opening, as has been reported in several plant species of angiosperms (Shimazaki et al., 2007). We performed essentially the same experimental work. When the detached microphylls from S. uncinata were irradiated by both RL (600 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) and BL (5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) for 2 hours, stomata opened (Supplemental Fig. S2A) and accumulated a substantial amount of K\(^+\) in guard cells (Supplemental Fig. S2B). When fusicoccin was administered to the epidermal peels from this plant under the dark, stomata opened but the apertures of stomata were not as large as those under the light (Fig. S2A). The results suggest that the H\(^+-\)ATPase drives K\(^+\) uptake in guard cells and the accumulated K\(^+\) is responsible for stomatal opening, at least partially, in S. uncinata. Quantitative relationships among stomatal aperture, H\(^+-\)ATPase activity, and the accumulation of K\(^+\) in guard cells could not be obtained because of technical difficulty.
**Discussion**

BL response of stomata is present in early vascular plants

Stomatal responses to light have been characterized in angiosperms such as *Arabidopsis thaliana*, *Commelina communis*, and *Vicia faba* (Zeiger, 1983; Assmann, 1993; Willmer and Fricker, 1996; Roelfsema et al., 2006; Shimazaki et al., 2007). In these plants, stomatal opening is mediated by two light signaling cascades, BL-dependent and photosynthesis-induced responses (Shimazaki et al., 2007; Kollist et al., 2014; Lawson et al., 2014). Both responses are observed in angiosperms, but no comprehensive analysis had yet been done on the evolution of stomatal response across phylogenetically divergent species of vascular plants. We have shown that the stomata of Polypodiopsida ferns lack the BL-dependent stomatal response in intact plants, and that RL-induced stomatal opening is mediated by guard cell chloroplasts using the isolated epidermal peels from the fern (Doi et al., 2006; Doi and Shimazaki, 2008). We therefore suspected that the stomatal responses to light have diverged across species of vascular plants. To examine functional diversity of stomata, we investigated stomatal responses to strong RL (photosynthesis-induced) and weak BL superimposed on RL in three large lineages of the vascular plants, including gymnosperms, ferns and lycophytes.

We confirmed the lack of stomatal BL response in four major lineages of modern ferns Polypodiopsida (Schneider et al., 2004). Stomata in all Polypodiopsida tested in this study did not open in response to BL in the presence of RL, although RL induced stomatal opening (Fig. 1, 2). We also found that most of the plant species in euphyllophytes and lycophytes (Fig. 2) showed stomatal opening in response to RL except two species of fern, *Equisetum hyemale* and *Psilotum nudum* (Fig. 4), and gymnosperms of *Cycas revoluta* (Fig.3). However, all these vascular plant species, including fern species except Polypodiopsida, exhibited the typical BL response of stomata: weak BL required strong RL as a background to induce stomatal opening. We note here that plant species of *Equisetum hyemale*, *Psilotum nudum*, *Cycas revoluta* (Fig. 1SB), *Selaginella uncinata*, and *Selaginella moellendorfii* did not respond to weak BL in the absence of RL. From these results, we concluded that the common ancestor of vascular plants of euphyllophytes and lycophytes emerged about 420 million years ago and acquired BL-dependent stomatal response although the associated molecular
mechanisms in these plants have yet to be determined. The modern ferns of Polypodiopsida likely lost the ability to respond to BL when they evolved under the canopy of higher plants or grew in environments that reduced sensitivity to blue light (Schneider et al., 2004). Instead, it appears that Polypodiopsida acquired a chimera of the red/far-red light receptor phytochrome and phototropin (NEOCHROME), and showed extremely high sensitivity to white light in chloroplast accumulation and phototropic bending, which might enhance fitness of these plants when growing under a canopy (Kawai et al., 2003; Suetsugu et al., 2005).

Recent studies indicated that the stomatal responses to CO₂ and abscisic acid are absent in Polypodiopsida and lycophytes, and the stomatal aperture of these plants is regulated by passive control of guard cell turgor by leaf water status (McAdam and Brodribb, 2013), differing from angiosperms (Doi and Shimazaki, 2008; Brodribb and McAdam, 2011; McAdam and Brodribb, 2012). By contrast, other groups reported that the stomata of mosses and lycophytes are sensitive to both CO₂ and abscisic acid (Chater et al., 2011; Ruszala et al., 2011), and that even the stomata in sporophytes of moss *Physcomitrella patens*, the most basal land plant group, exhibited light and H⁺-ATPase-driven stomatal opening (Chater et al., 2011). Our studies on the light-induced stomatal opening from euphylllophytes to lycophytes indicated that stomatal movement is regulated by both photosynthesis- and BL-dependent ion transport. Although it was not determined whether phototropins function as BL receptors in this plant species, it is clear that the BL-dependent stomatal opening is present in the initial stage of land plant evolution.

**Molecular mechanisms of Selaginella BL response of stomata**

The signaling pathway regulating the BL-dependent stomatal response is known to contain blue light receptor phototropins, a protein kinase BLUS1 (blue light signaling1), a type1 protein phosphatase, the plasma membrane H⁺-ATPase, and inward-rectifying K⁺ channels in *Arabidopsis* (Shimazaki et al., 2007; Zhao et al., 2012; Takemiya et al., 2013a; Kollist et al., 2014). Although all of the older land plants except Polypodiopsida show blue light-dependent stomatal response, the signaling components responsible for these processes are largely unknown among the diverse species. DNA sequence
databases of *Selaginella moellendorffii* indicate the presence of homologs of phototropins and those of H'\(^+\)-ATPase (Banks et al., 2011) but homologs of BLUS1 and a K\(^+\)\(_{\text{in}}\) channels have not been identified in the lycophytes (Gomez-Porras et al., 2012). However, both *S. moellendorffii* and *S. uncinata* exhibited clear blue light-specific stomatal opening (Fig. 5). This agrees with previous observations that a specific activator of the H'\(^+\)-ATPase, fusicoccin, promotes stomatal opening in epidermal peels from *S. uncinata* under dark with concomitant accumulation of K\(^+\) in the guard cells (Ruszala et al., 2011). These results suggest that the H'\(^+\)-ATPase drives K\(^+\) uptake in guard cells and the K\(^+\) functions as osmotica in stomatal opening in a manner consistent with angiosperms, although *Selaginella* does not have homologs of KAT1, KAT2 and AKT1. BK-like channels found in *Selaginella* might compensate for the absence of Shaker-like K\(^+\)\(_{\text{in}}\) channels during K\(^+\) uptake in guard cells (Gomez-Porras et al., 2012).

We note here that the primitive non-vascular land plant of *Physcomitrella patens* opens stomata in response to fusicoccin (Chater et al., 2011) and possesses four genes coding K\(^+\)\(_{\text{in}}\)-like channels (Gomez-Porras et al., 2012).

Light (RL plus BL) enhances stomatal opening in *Selaginella* epidermis, and the stomatal aperture was larger under light than in the presence of fusicoccin under dark. This is opposite to the response of stomata in the epidermis of angiosperms; in this latter case, the stomatal aperture exposed to fusicoccin was larger than stomata exposed to light in Arabidopsis and other higher plants (Shimazaki et al., 1993; Shimazaki et al., 2007; Takemiya et al., 2013a). These observations may suggest differences in the mechanism of stomatal opening between angiosperms and lycophytes. It is possible that stomatal opening is enhanced by inhibition of anion channels by BL in lycophytes as has been suggested in Arabidopsis and *Vicia faba* (Marten et al., 2007) and this response is greater in lycophytes than angiosperms. Inhibition of anion channels would not occur with the application of fusicoccin.

**The role of blue light response of stomata**

We found that the stomata in some species of ferns and gymnosperms (Fig. 3 and 4) did not respond to RL, but responded to the superimposed weak BL with simultaneous CO\(_2\) uptake. These plant species must experience a drastic reduction of intercellular CO\(_2\) concentration (Ci) under strong RL because of photosynthetic CO\(_2\) fixation in the leaf and closed stomata. These observations indicate that the decreased Ci with RL is
not sufficient for stomatal opening in these plant species and further implicates the functional roles of BL-dependent stomatal opening. The BL response of stomata depends on $Ci$ in the leaf and therefore one role of this response is to meet the demand of CO$_2$ for photosynthesis by opening stomata (Assmann, 1988). This notion is supported by a recent study showing that a mutant variant lacking a stomatal BL response was less sensitive to decreased CO$_2$ than wild-type plants (Takemiya et al., 2013a). The other proposed role is that the BL response can indirectly monitor the magnitude of photosynthetic CO$_2$ fixation through the absorption of PAR in guard cell chloroplasts (Suetsugu et al., 2014). The responses described above also suggest the presence of fine control of stomatal aperture in ferns and gymnosperms through light quality and intensity.
Material and method

Plants and growth conditions

Zamia furfuracea, Dicranopteris linearis, Angiopteris lygodiiifolia, Botrychium ternatum, Equisetum hyemale, Psilotum nudum, Selaginella moellendorfii, and Selaginella uncinata were purchased from local nurseries. Chamaecyparis obtusa was kindly provided by Dr. Eiji Gotoh (Faculty of Agriculture, Kyushu University). Lepisorus thunbergianus, Thelypteris acuminata, Cycas revoluta, and Ginkgo biloba were transplanted from Hakozaki campus. Osmunda japonica, was transplanted from Karatsu in the northern part of Kyushu islands. Chamaecyparis obtusa was grown outside. Lepisorus thunbergianus, Thelypteris acuminata, Dicranopteris linearis, Botrychium ternatum and Psilotum nudum were grown in a growth room under a white fluorescent lamp (50 µmol m^{-2} s^{-1}) on a 14/10 h light/dark cycle at 24 °C. Selaginella moellendorfii and Selaginella uncinata were grown in a growth room maintained at more than 90% relative humidity under a white fluorescent lamp (20 µmol m^{-2} s^{-1}) on a 14/10 h light/dark cycle at 24 °C. Zamia furfuracea, Osmunda japonica, Angiopteris lygodiiifolia and Equisetum hyemale were grown in the greenhouse under natural light conditions.

Gas exchange measurements

Measurement of gas exchange of intact plants was carried out using an open path gas exchange system (LI-6400, LI-COR Inc., Lincoln, NE, USA) equipped with a normal chamber (LI-COR Inc.). Plants were kept in the dark overnight before the measurements in order to induce stomatal closing. The leaves and/or fronds were clamped with a gas-tight normal chamber. The leaf temperature was maintained at 24 °C. Measurements were conducted under a constant CO2 concentration of 365 µl l^{-1} and a relative humidity of 50-60 %. Red and blue light were provided by a light emitting diode (ISL-150×150; CCS Inc.). Data were recorded at 20 s intervals and processed with a KaleidaGraph™ (Synergy Software, PA). Photon fluence rates were determined with a LI-2500 light meter equipped with an LI190SA quantum sensor (LI-COR Inc.).

Determination of stomatal apertures

Selaginella plants were kept in the dark overnight before measurements. Lateral
microphylls were obtained from the plants with tweezers under a safety light. These microphylls were immersed in a solution containing 10 mM MES, 30 mM KCl, and 0.1 mM CaCl₂, pH 6.05 in petri dishes and kept in the dark for 2 hours. The microphylls was irradiated by red light together with blue light for 2 hours. FC (10 μM) was added to the solution in which the microphylls were immersed they were incubated in the dark for 2 hours. Micrographs of over 50 stomata were obtained using a Nikon fluorescence microscope equipped with a CCD camera. The stomatal aperture was defined as the width to length ratio of the stomatal pores.

Cytochemical detection of K⁺ in guard cells

Lateral microphylls were obtained and immersed in the same solution as described above. After 2 hours incubation under light, accumulation of K⁺ by guard cells was quantified by staining with sodium hexanitrocobaltate (III) as described previously (Green et al., 1990; Willmer and Fricker, 1996).
Figure 1. Stomatal conductance (blue line) and photosynthetic CO₂ uptake in response to light in leaves of *Arabidopsis thaliana* (A) and Polypodiopsida ferns (B-F). The plant leaves were irradiated with continuous red light (RL) at 600 µmol m⁻² s⁻¹ and blue light (BL) at 5 µmol m⁻² s⁻¹ at the position of the upward red- and blue-arrows, respectively. BL was superimposed on the RL. Downward arrows of red and blue indicate the termination of RL and BL, respectively. The experiments for stomatal conductance in each plant species were done at least three times (typically five times), and typical data were presented.

Figure 2. Phylogenetic tree showing relationships between major groups of extant vascular plants, based on previously published phylogenetic studies (Pryer et al., 2001; Wikström and Kenrick, 2001; Schneider et al., 2004; Smith et al., 2006).

Figure 3. Stomatal conductance (blue line) and photosynthetic CO₂ uptake (red line) in response to light in leaves of *Zamia furfuracea* (A), *Cycas revoluta* (B), *Ginkgo biloba* (C), *Chamaecyparis obtuse* (D), *Gnetum spp.* (E). Plants were treated with light as shown in Fig. 1.

Figure 4. Stomatal conductance (blue line) and photosynthetic CO₂ uptake (red line) in response to light in leaves of *Equisetum hyemale* (A), *Angiopteris lygodilfolia* (B), *Botrychium ternatum* (C), and *Psilotum nudum* (D). Plants were treated with light as shown in Fig. 1.

Figure 5. Stomatal conductance (blue line) and photosynthetic CO₂ uptake (red line) in response to light in leaves of *Selaginella moellendorffii* (A, B) and *Selaginella uncinata* (C, D). Plants were treated with light as shown in Fig. 1. Upward arrows of blue in panels B and D indicate the application of BL pulse at 150 µmol m⁻² s⁻¹.

Supplemental Figure S1. Stomatal conductance (blue line) and photosynthetic CO₂ uptake (red line) in response to light in leaves of *Cycas revoluta*. The plant leaves were irradiated with red light (RL) at 600 µmol m⁻² s⁻¹ and blue light (BL) at 5 µmol m⁻² s⁻¹ at
the position of the upward red- and blue-arrows, respectively. Downward arrows of red
and blue indicate the termination of RL and BL, respectively. Note that the irradiation
order of RL and BL is reversed in panels A and B.

Supplemental figure S2. (A) Stomata in lateral microphylls of *Selaginella* plants open in
response to light and fusicoccin. Microphylls were irradiated by red light (600 µmol m$^{-2}$
s$^{-1}$) and blue light (5 µmol m$^{-2}$ s$^{-1}$) for 2h, or treated with 10µM FC for 2h in the dark.
Stomatal apertures of 100 stomata were determined by microscopic examination in
each treatment. The data are means of three independent experiments with standard
errors. Asterisk indicates significant differences between the two treatments ($P<0.05$ by
Student's *t* test). (B) K$^+$ accumulation in guard cells. Cytochemical detection of K$^+$ in
guard cells was carried out as described in Materials and Methods.


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