Fern Stomatal Responses to ABA and CO₂ Depend on Species and Growth Conditions

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Changing atmospheric CO₂ levels, climate, and air humidity affect plant gas exchange that is controlled by stomata, small pores on plant leaves and stems formed by guard cells. Evolution has shaped the morphology and regulatory mechanisms governing stomatal movements to correspond to the needs of various land plant groups over the past 400 million years. Stomata close in response to the plant hormone abscisic acid (ABA), elevated CO₂ concentration, and reduced air humidity. Whether the active regulatory mechanisms that control stomatal closure in response to these stimuli are present already in mosses, the oldest plant group with stomata, or were acquired more recently in angiosperms remains controversial. It has been suggested that the stomata of the basal vascular plants, such as ferns and lycophytes, close solely hydropassively. On the other hand, active stomatal closure in response to ABA and CO₂ was found in several moss, lycophyte, and fern species. Here, we show that the stomata of two temperate fern species respond to ABA and CO₂ and that an active mechanism of stomatal regulation in response to reduced air humidity is present in some ferns. Importantly, fern stomatal responses depend on growth conditions.

Stomatal pores, formed by guard cells on plant leaves and stems, mediate CO₂ uptake for photosynthesis and water loss via transpiration. Adequate regulation of the stomatal aperture in response to changing environmental conditions is essential for thriving plant growth. In angiosperms, whose stomatal regulation has been studied the most, stomata close in response to abscisic acid (ABA), elevated CO₂ concentration, reduced air humidity, darkness, and air pollutants, whereas they open in response to light, increased air humidity, and decreased CO₂ concentration.

In recent years, the evolution of plant stomatal signaling pathways has become a subject of intensive study and passionate debate. The central role of ABA in angiosperm stomatal responses has been known for a long time (Cutler et al., 2010). More recently, experiments assessing stomatal responses to ABA have been conducted with mosses and basal vascular plants such as ferns and lycophytes. Lack of stomatal closure in response to ABA treatment in several fern and lycophyte species led to the hypothesis that these plant groups use only hydropassive mechanisms for stomatal regulation (Brodribb and McAdam, 2011). Moreover, high ABA levels induced in response to drought did not inhibit the opening of fern and lycophyte stomata upon rehydration, suggesting that endogenous ABA did not control stomatal responses in these plant species (McAdam and Brodribb, 2012).

On the other hand, stomata in the epidermal strips of the lycophyte Selaginella uncinata and in the sporophytes of the mosses Physcomitrella patens and Funaria hygrometrica closed in response to ABA in a concentration-dependent manner (Chater et al., 2011; Ruszala et al., 2011), indicating a conserved role for ABA in the stomatal responses of plants. Recently, dose-dependent ABA-induced stomatal closure also was shown to be present in the ferns Polystichum proliferum and Nephrolepis exaltata (Cai et al., 2017). Several genes encoding proteins involved in ABA signal transduction are expressed in the stomata-bearing sporophyte of the moss P. patens (O’Donoghue et al., 2013) and in the epidermal fraction of the fern P. proliferum (Cai et al., 2017). Furthermore, the P. patens and Selaginella moellendorffii homologs of OPEN STOMATA1 (OST1), a SnRK-type kinase that participates in ABA-induced stomatal closure via phosphorylation of the central guard cell anion channel SLOW ANION CHANNEL1 (SLAC1) in Arabidopsis (Arabidopsis thaliana; Geiger et al., 2009; Lee et al., 2009, Vahisalu et al., 2010), complemented the ABA insensitivity of stomatal closure in the Arabidopsis ost1 mutant (Chater et al., 2011; Ruszala et al., 2011). The P. patens deletion mutant that lacked OST1 had impaired ABA-induced stomatal closure (Chater et al., 2011), and
Plant stomata open or close in response to subambient or above-ambient CO₂ concentration, respectively. This is the basic mechanism through which the supply of photosynthetic CO₂ is regulated. The question of stomatal responsiveness to CO₂ in plant groups older than angiosperms remains controversial. Gas-exchange analysis of several fern and lycophyte species indicated that the stomata of these plants open in response to subambient CO₂ concentrations but do not close at above-ambient CO₂ levels (Brodribb et al., 2009; Brodribb and McAdam, 2013). Similarly, stomata in the mosses P. patens and F. hygrometrica had strong CO₂ responses in the range of 0 to 400 ppm CO₂ but did not close markedly at above-ambient CO₂ levels (Chater et al., 2011). However, stomata of the lycophyte S. uncinata responded to both subambient and above-ambient CO₂ concentrations (Ruszala et al., 2011), as did the stomata of the fern P. scolopendrium (Mansfield and Willmer, 1969). Recently, several fern species were shown to close their stomata in response to CO₂ elevation from the ambient 400 to 800 ppm, similar to the angiosperm Arabidopsis (Franks and Britton-Harper, 2016). It was hypothesized that different growth conditions and species-specific behaviors may account for the varied results obtained in the analysis of stomatal responses of mosses, ferns, and lycophytes (Roelfsema and Hedrich, 2016). For example, in some fern species, stomata closed in response to CO₂ elevation from 0 to 400 ppm, in others they did not respond or even opened, whereas in some species the response depended on growth light intensity (Creese et al., 2014). Thus, a consensus on whether there is a universal mechanism that governs stomatal responses to CO₂ and ABA in all land plants has not been reached to date, and further experiments assessing the effect of growth conditions and species are required. Importantly, the above-mentioned studies paid little attention to the fact that fern stomata are morphologically different from those of angiosperms: they lack subsidiary cells, and there is little mechanical interaction between guard cells and epidermal cells during guard cell swelling (i.e. diminished mechanical advantage of epidermal cells over guard cells; Franks and Farquhar, 2007). Large kidney-shaped stomata of ferns that grow in the forest understorey characterized by deep shade and more humid air have been suggested to have slow dynamics of stomatal movements (Hetherington and Woodward, 2003). These differences in guard cell morphological and mechanical properties undoubtedly translate into differences in stomatal functioning.

Here, we provide evidence of growth condition- and species-dependent stomatal responsiveness to ABA and CO₂ in three temperate fern species. The results indicate that, while the stomata of some ferns can respond to ABA and CO₂, the kinetics and, thus, the underlying mechanisms of stomatal regulation differ, at least partly, between ferns and angiosperms. Furthermore, the stomatal response to decreased air humidity is slowed down at low CO₂ concentration in Athyrium flīx-femīna, indicating that fern stomatal responses are not purely hydropassive. Thus, species and growth conditions affect fern stomatal responsiveness and should be taken into account when drawing major conclusions about the evolution of the mechanisms that control stomatal regulation in ferns.

RESULTS AND DISCUSSION

The Stomatal Response to ABA Is Species Specific and Depends on Growth Conditions in Ferns

As the presence of active stomatal responses in ferns has remained controversial to date, we studied whether their stomatal responses to ABA and CO₂ could be influenced by growth conditions. Three temperate fern species (A. flīx-femīna, Dryopteris carthusiana, and Dryopteris flīx-mas) were collected from nature and transferred to the laboratory, where they were grown in two different growth conditions: in a growth cabinet characterized by constant relative air humidity of 70% both day and night (from here on referred to as the growth cabinet) or in a growth room with lower and variable relative air humidity (RH: ~30%–40% during the day and ~40%–65% at night; from here on referred to as the growth room). The light regime was 16/8 h and 12/12 h light/dark in the growth room and the growth cabinet, respectively. After the ferns had developed new leaves indoors, gas-exchange analysis was carried out with a custom-built device that enabled parallel recording of stomatal conductance in four plants (see “Materials and Methods”); one measurement cuvette is shown in Fig. 1A). Similar experiments were carried out with the angiosperm Arabidopsis (Columbia-0) grown in identical conditions and using another custom-built device that enabled parallel recording of stomatal conductance in eight plants (see “Materials and Methods”). Stomatal responses of the ferns and Arabidopsis to 10 μM ABA sprayed on leaves or changes in CO₂ concentration were measured.

The stomatal conductance of the angiosperm Arabidopsis decreased strongly in response to 10 μM ABA application in plants grown both in the growth cabinet as well as in the growth room (Fig. 1B), pointing to prominent ABA responsiveness of Arabidopsis stomata irrespective of growth conditions. However, the growth
conditions affected stomatal responsiveness in ferns. When grown in the growth cabinet at 70% RH, the stomatal conductance of *A. filix-femina* and *D. filix-mas* decreased in response to spraying with ABA, whereas *D. carthusiana* did not respond to ABA treatment (Fig. 1C). None of the tested fern species responded to ABA when grown in the growth room at lower RH (Fig. 1C). Thus, fern species differ in their ability to close stomata in response to ABA, and stomatal responsiveness is affected by growth conditions.

Air humidity has been shown to affect stomatal morphology and responsiveness in various angiosperm species. High relative air humidity during growth has been associated with longer and more open stomata (Torre et al., 2003; Nejad and van Meeteren, 2005; Fanourakis et al., 2011; Aliniaeifard et al., 2014), lower levels of ABA (Zeevaart, 1974; Nejad and van Meeteren, 2007; Okamoto et al., 2009; Aliniaeifard et al., 2014), and decreased stomatal responsiveness to ABA (Fospíšilová, 1996; Fanourakis et al., 2013; Pantin et al., 2013b; Arve et al., 2014). Exposure to low RH can enhance stomatal ABA responsiveness in young leaves that are constantly in a microenvironment with high RH (Pantin et al., 2013b). This indicates that reduced RH is required to prime stomatal ABA responses, potentially via increased ABA levels, as ABA application can restore stomatal ABA responsiveness (Pantin et al., 2013b) and results in normal stomatal development (Fanourakis et al., 2011) in plants grown at high RH. However, in these reports, the RH that led to reduced ABA sensitivity was very high: 90% or more. In our experiment, lower growth RH (30%–65%) led to the loss of stomatal ABA responsiveness in two ferns compared with growing them at 70% RH (Fig. 1C). This could be explained by increased ABA levels induced by low air humidity, as observed in other species (Bauer et al., 2013; McAdam and Brodribb, 2015), which could potentially lead to partial ABA-induced closure of the stomata of ferns grown in the growth room and no further effect of ABA treatment on stomatal conductance. If the ABA response is already activated in ferns grown at lower RH, these plants would be expected to have lower stomatal conductance. However, as the magnitude of ABA-induced stomatal closure was modest even in the ferns grown at 70% RH, such a difference in stomatal conductance could remain undetected. It has been shown that the ABA levels of ferns do not increase in response to short-term low-humidity treatment (McAdam and Brodribb, 2015), but ABA levels in ferns subjected to long-term growth at low RH remain to be characterized.

The guard cell ABA response appears to be present in some fern species and not in others (Fig. 1C; Brodribb and McAdam, 2011; Cai et al., 2017), suggesting that the ABA responsiveness may have been lost in some lineages of ferns. In addition to the direct effect on stomata, ABA reduces leaf hydraulic conductance in angiosperms (Shatil-Cohen et al., 2011; Pantin et al., 2013a). Thus, the ABA-induced decrease in stomatal conductance in *A. filix-femina* and *D. filix-mas* grown at 70% RH could have occurred due to changes in leaf hydraulics and not due to the direct effect of ABA on guard cells. Different procedures for ABA application could explain the varying ABA responsiveness of ferns and lycophytes found in experiments. It is possible that ABA applied via the transpiration stream is incapable of inducing stomatal

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**Figure 1.** Stomatal responses of ferns and Arabidopsis grown in two different conditions to foliar ABA spraying (10 μM). A, *D. filix-mas* in the gas-exchange measurement chamber. B, Mean ± st stomatal conductance before and 47 min after treatment with ABA in Arabidopsis grown in the growth room and in the growth cabinet (n = 7). C, Mean ± st stomatal conductance before and 47 min after treatment with ABA in the ferns *A. filix-femina*, *D. filix-mas*, and *D. carthusiana* grown in different conditions (n = 4 or 7 for *A. filix-femina*, n = 4 or 7 for *D. filix-mas*, and n = 5 or 4 for *D. carthusiana*, grown in the growth room or the growth cabinet, respectively). Statistically significant differences are denoted with asterisks (repeated-measures ANOVA with Tukey’s posthoc test).

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Conditions in ferns and lycophytes, whereas ABA applied to epidermal peels or by leaf spraying enters guard cells directly and, thus, is more efficient in promoting stomatal closure (Fig. 1C; Brodribb and McAdam, 2011; Ruszala et al., 2011; Cai et al., 2017).

Although the stomata of two fern species closed in response to ABA, the extent of the closure was small compared with the angiosperm Arabidopsis (Fig. 1, B and C). This indicates that, while several ferns are capable of ABA perception and response, the kinetics and underlying molecular mechanisms of this response could be different in ferns and in angiosperms. The components of the core stomatal ABA signaling pathway have been shown to be present in mosses, lycophytes, ferns, and angiosperms (Hanada et al., 2011; Cai et al., 2017), indicating an early origin of ABA signal transduction. Nevertheless, it is possible that not all of these proteins have strictly stomata-related functions in ferns, as was shown recently for a homolog of the OST1 kinase that participates in sex determination in a fern (McAdam et al., 2016). However, other kinases besides OST1 can activate anion channels toward stomatal closure, and although OST1 is the most important among them in Arabidopsis (Kollist et al., 2014), ABA-induced signaling pathways do not have to be identical in ferns and angiosperms. Thus, it is possible that the classical stomatal ABA-response pathway functions only partially in ferns and is not capable of inducing stomatal closure in response to ABA treatment in all fern species. A wider study of stomatal ABA responsiveness across the evolutionary tree of ferns would be required to understand how guard cell ABA responsiveness has evolved. For example, an analysis of different fern classes showed that, whereas stomatal opening in response to blue light is present in lycophytes and most classes of ferns, it has been lost in the fern group Polypodiopsida (Doi et al., 2015). Thus, it is conceivable that the stomatal ABA responsiveness has been lost in some branches of the evolutionary tree of ferns, and systematic analysis of stomatal responses in different taxa is required to bring further insight to this question.

### Fast Stomatal Opening

Fast stomatal opening in response to a reduction of CO\textsubscript{2} concentration from 400 to 100 ppm occurred in all ferns irrespective of growth conditions (Fig. 2, C and D; Supplemental Tables S2 and S3). Elevation of CO\textsubscript{2} from 100 ppm back to 400 ppm and then further to 800 ppm caused stomatal closure in nearly all tested fern species and conditions (Fig. 2, C and D; Supplemental Tables S2 and S3). There was one exception: *D. flexuosa* grown in the growth room did not respond significantly to above-ambient levels of CO\textsubscript{2}, whereas its stomatal opening in response to a change from 400 to 100 ppm and closure induced by CO\textsubscript{2} elevation from 100 to 400 ppm was one of the largest (Fig. 2, C and D; Supplemental Tables S2 and S3). Previously, high RH has been shown to enhance the response to CO\textsubscript{2} elevation in *Vicia faba* (Talbott et al., 2003). In accordance with this, stomatal responses to CO\textsubscript{2} elevation were reduced at least partially in ferns grown at lower RH. In addition to *D. flexuosa*, the low RH of the growth room also affected CO\textsubscript{2} responsiveness in *A. flexifolia*, whose final stomatal conductance achieved after the transition from 100 to 400 ppm was significantly higher than the pretreatment value at 400 ppm, indicating a slowed closure response (Fig. 2D; Supplemental Table S2). Thus, as in experiments with ABA, growth in the growth room with lower RH tended to reduce the stomatal sensitivity in *D. flexuosa* and *A. flexifolia*. As stomatal responses to CO\textsubscript{2} are tightly coupled with ABA responses (Xue et al., 2011; Merilo et al., 2013; Chatter et al., 2015), the analogous dampening of responses to ABA and elevated CO\textsubscript{2} found in *A. flexifolia* and *D. flexuosa* when grown at low RH suggests that the underlying mechanisms responsible for the effect of growth conditions could be similar and potentially related to ABA levels.

All the fern species studied here had the ability to open stomata due to CO\textsubscript{2} withdrawal and close stomata due to CO\textsubscript{2} enrichment, both in the subambient and above-ambient range of CO\textsubscript{2} levels. However, their stomatal opening in response to low CO\textsubscript{2} was markedly faster than stomatal closure in response to elevated CO\textsubscript{2} (Fig. 2C). Thus, the previously documented presence and absence of stomatal responses to subambient and above-ambient CO\textsubscript{2} concentration, respectively (Brodribb et al., 2009; Brodribb and McAdam, 2013), could be explained by the relatively slow response to elevated CO\textsubscript{2} in ferns. The closure kinetics of the elevated CO\textsubscript{2} response was clearly different between the studied ferns and the angiosperm Arabidopsis; the latter showed a fast drop in stomatal conductance in response to the transition from 400 to 800 ppm compared with the steady decrease in ferns, whereas, in ferns, the opening under subambient CO\textsubscript{2} levels was faster than in Arabidopsis (Fig. 2, A and C). A similar difference in the kinetics of high CO\textsubscript{2}-induced stomatal closure in ferns and Arabidopsis has been found before (Franks and Britton-Harper, 2016). These differences in the kinetics of CO\textsubscript{2} responses between ferns and angiosperms may result from their different stomatal morphology and related mechanical properties. Ferns have lower stomatal conductance and a diminished mechanical...
advantage of epidermal cells over guard cells (Franks and Farquhar, 2007) that could result in faster opening of their stomata. However, ferns also show reduced rates of change in stomatal aperture compared with angiosperms due to less lateral movement of guard cells and reduced shuttling of osmotica between guard and epidermal cells, which could explain their slower closure responses (Franks and Farquhar, 2007).

Interestingly, the pretreatment stomatal conductance of Arabidopsis plants grown at lower humidity in the growth room was higher than that in the plants grown in the growth cabinet in experiments with both ABA
and CO₂ (Figs. 1B and 2, A and B; Students t test, P < 0.05). This seems counterintuitive, as previous studies have shown higher stomatal conductance or wider stomatal aperture at higher RH in several angiosperm species (Torre et al., 2003; Nejad and van Meeteren, 2005; Fanourakis et al., 2011; Aliniaeifard et al., 2014). However, here, the measurements of stomatal behavior were carried out at ~70% RH, which was higher than the growth room RH of ~40%. Thus, it is possible that, in Arabidopsis, stomatal opening occurred when plants were transferred from the growth room to the measurement chamber. This fast opening response was not present in any of the tested fern species, where the stomatal conductance of plants grown in the growth room was similar to that in the growth cabinet (Fig. 1B and 2, C and D; two-way ANOVA with Tukey’s posthoc test where appropriate; Supplemental Tables S4 and S5). These data suggest a greater flexibility and faster stomatal responsiveness to changes in vapor pressure deficit (VPD) in angiosperms. This could be expected, as the responses to changes in VPD are linked with the biosynthesis and presence of ABA (Bauer et al., 2013; McAdam and Brodribb, 2015) as well as ABA signaling (Merilo et al., 2013), and stomatal ABA responses in angiosperms are faster and stronger than in ferns (Brodribb and McAdam, 2011; Fig. 1, B and C).

Dampening of the Response to High VPD at Low CO₂ Concentration Suggests the Presence of Active Stomatal Regulation in Ferns

The stomatal responses of ferns have been proposed to be regulated solely hydropassively by leaf water status (Brodribb and McAdam, 2011). If fern stomata indeed act as purely passive valves, it would be expected that the stomatal response to increased VPD would be faster in plants with higher stomatal conductance, as this would result in higher transpiration, greater loss of water, and more negative leaf water potential. To test this, we analyzed stomatal responsiveness to reduction in RH corresponding to higher VPD in either low (50 ppm) or ambient (400 ppm) CO₂ concentration in ferns grown in the growth room and the growth cabinet. As the stomata of A. filix-femina responded to ABA and those of D. carthusiana did not, we chose these two species for the experiments. As expected, the stomatal conductance was higher in low CO₂ concentration in both species, albeit the difference was greater for A. filix-femina (Fig. 3A; Supplemental Fig. S1A).

Stomata of A. filix-femina closed in response to a rapid increase in VPD from 0.7 to 1 kPa to 2 to 2.2 kPa in both low and ambient CO₂ concentrations (Fig. 3). However, the response to high VPD was significantly slower in low than in ambient CO₂ (Fig. 3C). This was true for ferns grown both in the growth room as well as in the growth cabinet, indicating the robustness of the response. This finding is not in accordance with the model of passive VPD-dependent stomatal regulation in ferns, since stomatal conductance was roughly 2 times higher at low CO₂ and this should have caused a faster hydropassive response to VPD. This experiment indicates that there is more than just a passive mechanism that regulates the stomatal response to a change in VPD in this fern species. As ABA and the components of its signaling pathway mediate stomatal responses to changes in VPD in angiosperms (Bauer et al., 2013; Merilo et al., 2013; McAdam and Brodribb, 2015) and A. filix-femina is a fern with ABA-responsive stomata (Fig. 1C), ABA is an obvious candidate as a mediator of stomatal response to high VPD in A. filix-femina. Stomatal

![Figure 3. Stomatal response of A. filix-femina to reduced air humidity at different CO₂ concentrations. A, Time-resolved stomatal conductance of A. filix-femina. At time point 0 min, VPD was increased from 0.7 to 1 kPa to 2 to 2.2 kPa. Mean ± se is shown (n = 7 or 8 for the growth room or the growth cabinet, respectively). B, Stomatal conductance in relative units, calculated based on the data presented in A. C, Stomatal half-response times, calculated based on the data presented in A. Different letters denote statistically significant differences (two-way ANOVA with Tukey’s posthoc test; the effect of CO₂ concentration was statistically significant).](image-url)
closure in response to increased VPD appeared to be delayed in low CO₂ concentration also in *D. carthusiana* (Supplemental Fig. S1) grown in both the growth room and the growth cabinet, but there was no statistically significant difference between stomatal half-response times to increased VPD at low and ambient CO₂ concentrations. Thus, it is possible that, in *D. carthusiana*, hydropassive stomatal control is more important in VPD responses.

Interestingly, despite the low stomatal conductance of ferns, their net CO₂ assimilation rate ranged between 4.1 and 4.5 μmol m⁻² s⁻¹ compared with 5.5 μmol m⁻² s⁻¹ in Arabidopsis. This resulted in twice as high instantaneous water use efficiency of ferns in comparison with Arabidopsis. As ferns naturally grow in the shaded conditions of the forest understory, where CO₂ concentration is higher than in upper layers (Bazzaz and Williams, 1991), it is possible that the low stomatal conductance of ferns is an adaptive trait associated with growth habitat. Adaptation to growth in shade and increased CO₂ concentration also could explain the strong stomatal sensitivity to CO₂ withdrawal in ferns.

**CONCLUSION**

The results presented here indicate that, while the stomatal responses of some ferns may be hydropassive, as described before (Brodbibb and McAdam, 2011), other fern species have active mechanisms for stomatal regulation as well. Large-scale studies of stomatal responsiveness in ferns from different families and evolutionary age would be necessary to gain further insight into the evolution of passive and active stomatal control mechanisms in ferns. Moreover, as shown here, growth conditions can have strong effects on stomatal responsiveness in ferns; for example, responses to ABA and CO₂ were weakened in ferns grown at RH of 30% to 40% compared with 70% RH, while fast stomatal responses were maintained in the angiosperm Arabidopsis grown in both conditions. Whether contrasting patterns of stomatal ABA sensitivity detected for ferns in this study are accompanied by differences in ABA synthesis in variable conditions remains to be studied in the future.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

The ferns *Althyrion flisx-femina*, *Dryopteris carthusiana*, and *Dryopteris flisx-nas* were used in the experiments. Specimens of *D. flisx-nas* were collected from wild gardens in Kooraste and Näräpää in Põlvamaa, southern Estonia, in August 2016. Specimens of *D. carthusiana* were collected from a clear-cut forest area in Kooraste, and specimens of *A. flisx-femina* were collected from a forest in Näräpää between August and October 2016. All the ferns were transported to the laboratory, transplanted into a 4:2:3 peat-vermiculite-water mixture, and grown either in a growth room at 16/8 h light/dark, low RH (30%-40%) during the day and 40%-65% at night), and 100 to 150 μmol m⁻² s⁻¹ light or in a growth cabinet (MAC1600; Sneders Scientific) at 12/12 h light/dark, 70% RH, and 100 μmol m⁻² s⁻¹ light. These growth conditions are referred to in this article as the growth room and the growth cabinet, respectively. Only leaves developed indoors in the respective conditions were used for gas-exchange measurements. Arabidopsis (*Arabidopsis thaliana*) Columbia-0 plants used for comparison were grown in identical conditions and were 23 to 27 d old during the experiments.

**Gas-Exchange Experiments**

The stomatal conductance of ferns was recorded with a thermostated four-chamber, custom-built, flow-through gas-exchange device. The main body of the system consists of four thermostated gas-exchange cuvettes formed by two glass cylinders (i.d. 10.6 cm, height 13.6 cm) and a thermostated water jacket between them. The cuvettes are placed on a stand composed of two well-fitted glass plates that form a bottom for the gas-exchange cuvette. One of these plates contains perforations for the plant stem and is removable. The other glass plate contains gas input and output ports, a temperature sensor, and a fan to guarantee high turbulence and uniform gas mixing. The fan ensured that the boundary layer conductance was high (11–12 cm s⁻¹) and, further, that heat exchange between leaves and the chamber air was very good, reducing the effect of transpiration on leaf temperature. Modeling gum was used to ensure an air-tight separation of plant shoots within the cuvette from roots in the soil. The chambers are hermetically sealed and operate under slight overpressure of a few millibars to avoid uncontrolled intake of ambient air. Air flow rate through the chamber was 2.5 L min⁻¹. Ambient air passing through a large buffer volume of 25 L was used. The air temperature inside the chambers was measured by thermistors with negative temperature coefficient thermistors (model -001; RTI Electronics) and was between 24°C and 25°C. Leaf temperature was determined from leaf energy balance based on absorbed light and transpiration. All tubing and connections were made of Teflon and stainless steel. For illumination, four 50-W halogen lamps provided photosynthetic photon flux density of 500 μmol m⁻² s⁻¹ to each cuvette. Concentrations of CO₂ and water vapor in the reference channel (i.e., air entering the measuring cuvette) and measurement channel (air coming out from the cuvette) were measured with an infrared gas analyzer (Li7000; Li-Cor), and stomatal conductance and net assimilation rate were calculated with a custom-written program as described by Kollist et al. (2007).

The stomatal conductance of the plants was measured at ~70% RH, 500 μmol m⁻² s⁻¹ light, and 24°C to 25°C air temperature. When stomatal conductance had stabilized, plants were treated with ABA; change in CO₂ concentration, or change in VPD. For ABA treatment, cuvette covers were removed, 10 μM ABA with 0.012% Silwet L-77 (Duchefa) and 0.05% ethanol was sprayed on leaves, cuvette covers were returned, and measurement of stomatal conductance continued. In experiments with CO₂, the CO₂ concentration in the chamber was reduced from 400 to 100 ppm for 2 h, thereafter returned to 400 ppm for 2 h, and then elevated to 800 ppm for 2 h. In experiments with high VPD, stomatal conductance of the plants was allowed to stabilize in the measurement cuvette with either 30 or 400 ppm CO₂. Thereafter, the air humidity in the chamber was lowered from ~70% to ~30% to 40%, yielding a change in VPD from 0.7 to 1 kPa to 2 to 2.2 kPa.

The stomatal conductance of Arabidopsis was recorded with an eight-chamber, custom-built, temperature-controlled gas-exchange device analogous to the one described before (Kollist et al., 2007). The stomatal conductance of plants was measured at ~70% RH, 150 μmol m⁻² s⁻¹ light, and 24°C to 25°C air temperature. When stomatal conductance had stabilized, plants were treated similarly to ferns with either ABA spraying or changes in CO₂ concentration.

**Data Analysis and Statistics**

In the ABA and CO₂ experiments, stomatal conductance values before and after application of these stimuli were compared by repeated-measures ANOVA with Tukey’s posthoc test. In experiments with reduced air humidity, stomatal half-response times were calculated by scaling the whole 42-min stomatal response to a range from 0% to 100% and by calculating the time when 50% stomatal closure was achieved. Half-response times were compared by two-way ANOVA with Tukey’s posthoc test. All statistical analyses were carried out with Statistica (StatSoft). All effects were considered significant at P < 0.05.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Stomatal response of *D. carthusiana* to reduced air humidity in different CO₂ concentrations.

**Supplemental Table S1.** Repeated-measures ANOVA with Tukey’s HSD posthoc test for Figure 2B.

**Supplemental Table S2.** Repeated-measures ANOVA with Tukey’s HSD posthoc test for Figure 2D, plants grown in the growth room.
Growth Conditions Affect Fern Stomatal Responses

Supplemental Table S3: Repeated-measures ANOVA with Tukey’s HSD posthoc test for Figure 2D, plants grown in the growth cabinet.

Supplemental Table S4: Two-way ANOVA for pretreatment stomatal conductance in Figure 1C.

Supplemental Table S5: Two-way ANOVA with Tukey’s HSD posthoc test for pretreatment stomatal conductance in Figure 2D.

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