Metabolism-mediated mechanisms underpin the differential stomatal speediness regulation among ferns and angiosperms

Silvio Cândido-Sobrinho[1], Valéria Lima[1], Francisco Freire[1], Leonardo de Souza[2], Jorge Gago[3], Alisdair R. Fernie[4], and Danilo Daloso[1]

[1]Universidade Federal do Ceara
[2]Max-Planck-Institut fur molekulare Pflanzenphysiologie
[3]Universitat de les Illes Balears
[4]Max-Planck Institute for Molecular Plant Physiology

February 22, 2024

Abstract

Recent results suggest that metabolism-mediated stomatal closure mechanisms are important to regulate differentially the stomatal speediness between ferns and angiosperms. However, evidence directly linking mesophyll metabolism and the slower stomatal conductance (gs) in ferns is missing. Here we investigated the effect of exogenous application of abscisic acid (ABA), sucrose and mannitol on gs kinetics and carried out a metabolic fingerprinting analysis of ferns and angiosperms leaves harvested throughout a diel course. Ferns stomata did not respond to ABA in the time period analysed. No differences in the relative decrease in gs was observed between ferns and the angiosperm following provision of sucrose or mannitol. However, ferns have slower gs responses to these compounds than angiosperms. Metabolomics analysis highlights that ferns have higher accumulation of secondary rather than primary metabolites throughout the diel course, with the opposite being observed in angiosperms. Our results indicate that metabolism-mediated stomatal closure mechanism is conserved among ferns and angiosperms and that the slower stomatal closure in ferns is associated to a reduced capacity to respond to mesophyll-derived sucrose and to a higher carbon allocation toward secondary metabolism, which likely modulates both photosynthesis-stomatal movements and growth-stress tolerance trade-offs.

Introduction

Stomata are an epidermal structure composed of a pore surrounded by a pair of guard cells and, in certain cases, by subsidiary cells (Lima*et al.* 2018). Fossil records coupled to recent genomics studies suggest that stomata exists since at least 400 million years ago, being found from Bryophytes to Tracheophytes, with a particular loss in Liverworts during Bryophytes evolution (Harris *et al.* 2020). The appearance of stomata greatly contributed for plant adaptation to the terrestrial environment, which is due to the fact that stomatal opening allows the exchange of H_2O and CO_2between the leaf and the environment (Qu *et al.* 2017; Medeiros*et al.* 2019). More importantly, the active regulation of stomatal movements and the faster stomatal responses found in angiosperms are important characteristics that contribute to explain the success of this plant group in dominating both natural and agricultural ecosystems, when compared to plants of the basal lineage (Brodribb *et al.* 2019). Furthermore, evidence suggests that slower stomata can limit the photosynthetic rate (A) by up to 10% (McAusland *et al.*2016) and faster stomatal responses can improve plant growth, yield, water use efficiency (WUE) and drought tolerance (Papanatsiou *et al.* 2019; Qu *et al.* 2020). Thus, understanding the mechanisms that modulate stomatal speediness is important to comprehend the dynamic of natural ecosystems and to breed plants towards photosynthesis and WUE improvement (Lawson & Vialet-Chabrand 2018).

Stomata respond to a wide range of endogenous and environmental cues, including changes in CO_2 concentration, light quantity and quality, vapour pressure deficit, and phytohormones (Gago *et al.* 2020). Additionally, it has been shown that exogenous application of sugars and genetic alteration of enzymes associated with sucrose metabolism substantially alter stomatal conductance (g_s) (Antunes *et al.* 2012; Kelly *et al.* 2013; Lugassi *et al.* 2015; Li *et al.* 2016; Daloso*et al.* 2016b; Antunes *et al.* 2017; Medeiros *et al.*2018; Kelly *et al.* 2019; Flütsch *et al.* 2020b; Freire*et al.* 2021). These studies strengthen the idea that mesophyll-derived metabolites have a great influence on the regulation of stomatal movement (Mott 2009; Fujita *et al.* 2019). Notably, reduced sugar import into guard cells compromisesg_s, A and plant growth (Antunes *et al.* 2017) and substantially reduces stomatal speediness (Flütsch*et al.* 2020a). On the other hand, exogenous application of high concentrations of sucrose induce stomatal closure in tomato (Kelly*et al.* 2013), Arabidopsis (Medeiros *et al.* 2018) and plants of the basal lineage (Kottapalli *et al.* 2018). Thus, sucrose seems to be an important metabolic signal that coordinates the$A$ -g_s trade-off (Flütsch & Santelia 2021). However, despite recent advances in our understanding of the regulation of stomatal speediness in model angiosperm species (Lawson & Vialet-Chabrand 2018), it remains unclear whether these mechanisms are also present in plants of the basal lineage and, if so, whether they contribute to explain the slower stomatal responses found in ferns, when compared to angiosperms.

Ferns and angiosperms share a common genetic machinery that controls both stomatal formation and stomatal responses to light, CO_2 and abscisic acid (ABA) (Sussmilch *et al.*2017, 2019; Harris *et al.* 2020). However, whether ferns are able to respond to endogenous ABA is extensively debated. The ABA-responsiveness of ferns has been supported by studies in which the magnitude of ABA-induced stomatal closure is very low (Ruszala *et al.* 2011; Hõrak *et al.* 2017) and/or the ABA concentration used is too high (e.g. 100 μM) (Plackett *et al.* 2021). On the other side, evidences suggest that hydraulic and osmotic mechanisms independent of ABA are the major drivers of stomatal closure in ferns (Cardoso *et al.* 2019; Cardoso & McAdam 2019). Indeed, it was recently shown that ferns and lycophyte species lack ABA-responsiveness but an osmotic-mediated stomatal closure mechanism is likely found in these species (Gong *et al.* 2021). This hypothesis is further supported by previous results suggesting that sucrose-induced stomatal closure is a conserved mechanism throughout land plant evolution (Kottapalli *et al.* 2018). However, the last two works were performed using extremely high concentrations of sucrose (200 mM) and sorbitol (800 mM). It is unclear therefore whether these results have any physiological relevance (McAdam *et al.* 2021), especially in ferns that have low both photosynthetic rate (Tosens *et al.* 2016; Gago *et al.* 2019) and capacity to produce ABA (Cardoso & McAdam 2019). It is clear therefore that further studies using relevant physiological concentration of ABA and osmotic compounds are needed to better understand the evolution of stomatal movements regulation.

Despite the controversies regarding the ABA-responsiveness of ferns, it is known that these plants are able to respond to blue light and changes in vapour pressure deficit (VPD) and CO_2 concentration (reviewed in McAdam and Sussmilch 2021). However, ferns in general have lower g_s values and their stomatal responses are much slower than those observed in angiosperms (Franks & Britton-Harper 2016; Gago *et al.* 2019). In this vein, we have recently shown that the faster high CO_2-induced stomatal closure found in angiosperms compared to ferns is positively correlated with leaf sucrose content (Lima *et al.* 2019). Our previous results further suggest that ferns have higher investment of the daily CO_2 assimilated to the synthesis of metabolites related to secondary as opposed to primary metabolism, when compared to angiosperms (Lima et al. 2019). Considering that increased content of flavonols, a class of secondary metabolites, leads to lower stomatal aperture and slower stomatal closure responses (Watkins *et al.*2014, 2017), we hypothesize that the lower $g_s$values found in ferns is due to a higher investment to the synthesis of secondary rather than primary metabolites throughout the diel course, as compared to angiosperms. Furthermore, given that sucrose is negatively correlated with g_s (Gago *et al.* 2016) and is likely involved in the regulation of stomatal movement throughout land plant evolution (Kottapalli *et al.* 2018; Lima *et al.*2019), we further hypothesize that sucrose-mediated stomatal closure mechanisms are important to regulate both the magnitude of stomatal opening throughout the diel course and the differential stomatal closure speediness between ferns and angiosperms. To test these hypotheses, we investigated the effect of exogenous application of sucrose ong_s kinetics in two ferns and a representative angiosperm species and carried out a liquid chromatography mass spectrometry

(LC-MS)-based metabolic fingerprinting analysis, that mostly detect secondary metabolites (Perez de Souza *et al.* 2021), in leaves from two ferns and two angiosperms species harvested throughout the diel course.

Material and Methods

Plant material and growth conditions

We studied two ferns *Microsorum scolopendria* (Burman) Copel. and*Phlebodium aureum* (L.) J. Sm. and two angiosperm species*Vigna unguiculata* (L.) Walp. and *Nicotiana tabacum* L. Both ferns were obtained at the sporophyte stage from commercial suppliers and the species was confirmed by a taxonomist (professor Dr. Alexandre Salino, Federal University of Minas Gerais, Belo Horizonte, Brazil). *V. unguiculata* and *N. tabacum* were germinated in petri dishes and the seedlings were transferred to a hydroponic system containing Hoagland´s solution (Hoagland & Arnon 1950), as previously described (Lima *et al.* 2019). The plants were kept under greenhouse condition with 12 hours natural photoperiod, maximum photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1}, average ambient temperature of 30 ± 4 °C and relative humidity 62 ± 2 °C.

Stomatal closure kinetic analysis

Stomatal closure kinetics were assessed as described previously (Ceciliato *et al.* 2019). Leaves were detached and the petiole tip immediately submerged in deionized water in a petri dish. After that, a second oblique cut was made in the petiole and transferred to a 2 mL microcentrifuge tube with deionized water. Gas exchange analysis was then initiated by using an infrared gas exchange analyser (IRGA) equipped with a 6 cm^2 leaf chamber (Li-6400XT, LI-COR Biosciences, Inc. Lincoln, NE, USA). The gas exchange was recorded every 10 seconds for 40 minutes under 1000 μmol photons $m^{-2}s^{-1}$. After g_s stabilization, different metabolites were separately added to the tube to reach the desired final concentration as follows: ABA (5 μM), sucrose (25 mM) and mannitol (25 mM). ABA is a well-known phytohormone that induces stomatal closure at this concentration (Cai *et al.* 2017), which was then used to confirm whether the stomatal kinetic approach is feasible for the species used here. The sucrose concentration was selected based in the fact that sucrose transport into guard cells saturate at 25 mM (Outlaw 1995). Given that stomatal closure can also occur through an osmotic mechanism (reviewed in Lima et al. 2018), we thus included mannitol treatment to investigate the osmotic effect on stomatal closure kinetics. After the addition of ABA, sucrose or mannitol to the tube containing the detached leaf, gas exchange was recorded for further 40 minutes. In order to avoid any circadian rhythm effect, all stomatal kinetic analyses were carried out at the morning period of the day, in which a higher rate of g_s is observed in these species (Lima *et al.* 2019).

Ultra-high-performance liquid chromatography high-resolution mass spectrometry analysis

We have previously carried out a gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling analysis in leaf disks from ferns (*M. scolopendria* and *P. aureum*) and angiosperms (*V. unguiculata* and *N. tabacum*) species harvested throughout the day (05:00 h, 08:00 h, 14:00 h and 17:00 h) (Lima *et al.* 2019). This GC-MS platform is mostly related to the analysis of primary metabolites (Lisec *et al.* 2006). Here, we extended the metabolic characterization of ferns and angiosperms by carrying out an ultra-high-performance liquid chromatography high-resolution mass spectrometry (LC-MS) analysis, which mostly detect secondary metabolites (Perez de Souza *et al.* 2021), using samples from the same experiment carried out previously (Lima *et al.* 2019). Metabolites extraction and the LC-MS analysis were carried out exactly as described earlier (Tohge & Fernie 2010). The mass spectral data was first analysed using a metabolic fingerprinting approach followed by the identification of metabolites through the use of MS-DIAL software (Tsugawa *et al.* 2015), XCMS (Smith *et al.* 2006) and the MetaboanalystR package on R (Pang *et al.* 2020).

Gathering previously published gas exchange and GC-MS data

In order to have a better understanding of the metabolic differences between ferns and angiosperms and how this influences the diel course of leaf gas exchange, we collected previous gas exchange and GC-MS data carried out in the same species studied here (Lima *et al.* 2019). The GC-MS data collected refer to primary metabolites detected in the same leaf samples used for LC-MS analysis. Polar metabolites were extracted

according to a well-established GC-MS metabolite profiling protocol and identified using TagFinder, exactly as described previously (Lisec *et al.* 2006; Luedemann *et al.* 2008). The gas exchange was determined in leaves of angiosperms and ferns throughout the day (05:00 h, 08:00 h, 14:00 h and 17:00 h) using IRGA, as described above. We further collected data from stomatal closure kinetics induced by high CO_2 or dark conditions (Lima *et al.* 2019) to compare the speed of stomatal closure induced by ABA, sucrose and mannitol with those observed under these environmental cues.

Integrating gas exchange and GC-MS data throughout the diel course

Angiosperms have higher absolute values of A andg_s (Franks & Britton-Harper 2016; Tosens*et al.* 2016; Gago *et al.* 2019; Lima *et al.* 2019). Thus, to investigate the dynamic of gas exchange and the accumulation of primary metabolites throughout the diel course, both gas exchange and GC-MS data were subjected to a maximum-minimum transformation (0-to-1 range) according to the equation below:

$f(x) = \frac{xi - \min(x)}{\max(x) - min(x)}$, where xi is an observation of a variable (metabolites or gas exchange parameters).

This equation was applied to each variable, transforming them to a 0-1 scale throughout the diel course (05:00 h, 08:00 h, 14:00 h and 17:00 h). The lowest average value of each variable was set to zero (0) and the highest to one (1). The values in between were then proportionally normalized between the maximum and the minimum observed throughout the diel course (Deans *et al.* 2019). Variables with similar trends throughout the diel course were clustered by Dynamic Time Warping (DTW) analysis, using the *dtwclust* package in R (Sardá-Espinosa 2019).

Statistical analysis

Regression analyses were performed using models which best fit the observed data considering the highest R^2. Derivatives of the regression equations were calculated to assess relativeg_s change after exogenous application of different compounds and maximum velocity (V_{max}) of g_s responses. The $g_s$rate of change was plotted *versus* time and undergone clustering classification for time-series comparison analysis using *dtwclust*package in R (Sardá-Espinosa 2019). The metabolomics data were analysed by orthogonal partial least squares discriminant analysis (orthoPLS-DA) and principal component analysis (PCA) using FactoMineR package (Lê*et al.* 2008). The heat maps and the hierarchical clustering analysis (HCA) were carried out using the MetaboanalystR package on R (Pang *et al.* 2020; R Core Team 2020).

Results

The relative changes in A and g_s are similar among ferns and angiosperms over the diel course

Given that stomatal movements, photosynthesis and plant metabolism all follow a circadian rhythm (Hotta 2021), we first investigated whether the the circadian rhythm of A and g s is similar between ferns and angiosperms in relative terms and which primary metabolites are clustered with these parameters throughout the diel course in both plant groups. Twelve clusters were generated according to the pattern of accumulation/degradation over the diel course by Dynamic Time Warping (DTW) analysis (Figure S1). Interestingly, both A andg_s from all species were clustered together (see Cluster 11 at Figure S1). Certain sugars, organic acids and secondary metabolites were also found in this cluster. This fact notwithstanding, only glycine is common between ferns and angiosperms (Table S1). The list of metabolites found in each cluster is presented in Table S1. Angiosperms have much higher absolute values of both A andg_s, but the relative changes in these parameters are identical between ferns and angiosperms throughout the diel course (Figures 1a-d). These results highlight that stomata from ferns and angiosperms have similar circadian rhythm in relative terms.

Ferns did not respond to ABA and display slower mannitol and sucrose induced stomatal closure

We next investigated the effect of exogenous application of ABA (5 μM), sucrose (25 mM) or mannitol (25 mM) on stomatal closure kinetics of the two ferns and in *V. unguiculata* . Given the similarity of both metabolic and stomatal responses throughout the diel course between both angiosperms species (Lima *et al.* 2019), the stomatal closure kinetics were carried out using only one representative angiosperm species

(*V. unguiculata*). Ferns stomata did not respond to ABA (Figures 2a,d). However, exogenous application of both mannitol and sucrose decreased g_s in all plants (Figures 2b,c), with no differences in the relative decrease in g_s between ferns and the angiosperm observed following the provision of sucrose and mannitol (Figures 2e,f).

The stomatal responses of the ferns to mannitol and the response of the fern *P. aureum* to sucrose were best fitted into a linear model, while the stomatal response of the fern *M. scolopendria* to sucrose and all stomatal responses of the angiosperm *V. unguiculata* reached steady state and were best fitted using an exponential decay model (Figures S2-S4). We next investigated the acceleration observed during stomatal closure by curve fitting the data using non-linear regressions. Linear responses exhibit constant speed and thus zero acceleration. With exception of the response of *M. scolopendria* to sucrose, the acceleration of the other treatments in ferns is zero. This approach also determines the plateau (in seconds) in which the acceleration starts to decrease. The plateau of the g_s response to sucrose was achieved at 2915 and 3681 seconds in *V. unguiculata* and the fern *M. scolopendria* , respectively. This analysis suggests that the stomatal response to sucrose is faster in *V. unguiculata* than *M. scolopendria* , as evidenced by the shorter time needed to reach the plateau. The plateau of *V. unguiculata* g_s response to ABA and mannitol was observed at 3461 and 3920 seconds, respectively (Figures S2-S4). The data was subsequently transformed following a maximum-minimum normalization (0-1 range) to allow time-series comparison. The results highlight that the sucrose-induced stomatal closure of the angiosperm was unique to reach V_{max} (Cluster 1), as compared to the other treatments, which were clustered separately (Figure S5).

We further analysed the slope of the stomatal closure kinetics, a parameter commonly used to estimate stomatal speediness (McAusland *et al.* 2016). Considering that the ferns used here did not respond to ABA in the time period analysed, the slope of stomatal closure kinetics under ABA was higher in *V. unguiculata* than both ferns. The results further highlight that ferns have slower g_s responses to mannitol than the angiosperm, whilst no statistical difference in the slope of stomatal closure kinetics induced by sucrose between ferns and angiosperms was observed (Figures 3a-c). We next compared these values with those previously reported during stomatal closure induced by dark or high CO_2 concentration (Lima *et al.* 2019). The fastest stomatal closure of *V. unguiculata* was observed under ABA and high CO_2, while these signals represent the slower stomatal closure in the fern *P. aureum* . No statistical difference among stomatal closure signals in the fern *M. scolopendria* was observed. Interestingly, the speed of stomatal closure induced by mannitol and sucrose are among the lowest in *V. unguiculata* , while sucrose-induced stomatal closure represents the higher average value in both ferns (Figures 3d-f). Taken together, these results highlight that an osmotic-mediated mechanism of stomatal closure is conserved among ferns and angiosperms and that the slower stomatal closure in ferns is associated to their limited capacity to respond to ABA and mesophyll-derived sucrose.

The diel course allocation toward primary and secondary metabolisms is distinct between ferns and angiosperms

Ferns and angiosperms have different evolutionary histories (Sussmilch *et al.* 2019), which may reflect in their metabolome. Furthermore, our previous results suggest that ferns and angiosperms have distinct allocation of the daily CO_2 assimilated toward the synthesis of primary/secondary metabolites (Lima *et al.* 2019), which may have consequences for both A -g_s and growth-stress tolerance trade-offs. We then next carried out a LC-MS-based metabolic fingerprinting analysis in leaf samples harvested throughout the diel course to better understand the metabolic differences between these plant groups. Metabolic fingerprinting is an untargeted metabolomics approach suitable to discriminate biological samples without metabolite identification, i.e. based in the intensity of the features (peaks found in the chromatograms) detected in the samples (Scholz *et al.* 2004; Kruger *et al.* 2008; Kosmides *et al.* 2013; Silveira-Sotelo *et al.* 2015; Perez de Souza *et al.* 2019). Given that the LC-MS platform used here mostly detects secondary metabolites (Tohge & Fernie 2010; Perez de Souza *et al.* 2021), metabolic fingerprinting analysis was next used to investigate how ferns and angiosperms differ with regard to their contents of secondary metabolites. Analysis of the chromatograms revealed an incredible metabolic diversity in ferns, as evidenced by the higher number of peaks solely detected

in ferns. However a high intensity of peaks for a given compounds was found in both plant groups (Figures 4a-d). Similarly, several mass-to-charge ratio (m/z) features obtained by MS analysis were solely found in ferns, especially at 5:00 h and 14:00 h (Figures 5a-d). A total of 19340, 7741, 9668 and 19598 features were statistically different ($P < 0.05$) between ferns and angiosperms at 5:00 h, 8:00 h, 14:00 h and 17:00 h, respectively. Several of these features had higher intensity in ferns, compared to angiosperms (Figures 6a-d). Hierarchical clustering analysis (HCA) (Figures 6a-d), orthogonal partial least squares discriminant analysis (orthoPLS-DA) (Figures 7a-d) or principal component analysis (PCA) (Figures S6a-d) of these features indicate that ferns and angiosperms differ substantially at the secondary metabolic level. Similarly, PCA using previously published GC-MS metabolite profiling data (Lima *et al.* 2019), which mostly refers to primary metabolites, also discriminate ferns and angiosperms (Figures S7a-d).

The results obtained here coupled to previous studies provide compelling evidence highlighting that fern stomata can respond to the circadian rhythm and close in response to sucrose, mannitol, dark, high VPD and high CO_2 concentration (Franks & Britton-Harper 2016; Hõrak *et al.* 2017; Lima *et al.* 2019; Cardoso *et al.*2019; Gong *et al.* 2021; Plackett *et al.* 2021). However, no ABA response was observed in the ferns used here (Figure 8a). These results indicate that the slower ferns stomatal closure is associated to their limited capacity to respond to ABA and to mesophyll-derived metabolites, especially sucrose, when compared to angiosperms. This idea is supported by the fact that the faster high CO_2-induced stomatal closure of angiosperms was associated to their higher capacity to produce sucrose (Figure 8b) (Lima*et al.* 2019), when compared to ferns. Furthermore, our metabolomics analyses suggest that ferns and angiosperms exhibit distinct patterns of allocation toward primary and secondary metabolisms throughout the diel course, which may affect the level of ROS in the guard cells of these species (Figure 8c) and ultimately have implications for the regulation of both$A$ -g_s and growth-stress tolerance trade-offs (Figure 9).

Discussion

The need for speed: fast stomatal closure requires a high ABA sensitivity

The fast stomatal responses of angiosperms confers a better capacity to respond to variations in environmental cues and thus provided a great competitive advantage to this group of plants during land plant colonization (Raven 2014). Despite the fact that the mechanisms by which stomatal speediness is regulated are unclear (Lawson & Vialet-Chabrand 2018), especially among different phylogenetic groups, our results strengthen the hypothesis that the slower fern stomatal response is associated to a reduced capacity to respond to ABA (Brodribb & McAdam 2011; McAdam & Brodribb 2012; Cardoso *et al.* 2019). This idea is supported by the results in which exogenous application of ABA rapidly reduced g_s in the angiosperm, whilst fern stomata did not respond to this phytohormone in the time period analysed. It is important to emphasize, however, that ferns stomata requires longer time to respond to stomatal closure stimulus such as dark and high CO_2 concentration (Franks & Britton-Harper 2016; Lima*et al.* 2019). Thus, we cannot currently conclude that the stomata of the ferns investigated here lack ABA responsiveness.

Interestingly, the stomata of the ferns used here responded to exogenous application of sucrose and mannitol, and to changes in the CO_2 concentration and during the light-to-dark transition. Furthermore, our results revealed that the diel course ofg_s is similar between ferns and angiosperms in relative terms, with a maximum g_s observed in the initial period of the day, as typically observed under tropical conditions (Antunes *et al.* 2012, 2017). This result indicates that fern stomata is able to respond to the natural circadian rhythm, which is controlled by a complex regulatory network associated to changes in environmental cues such as temperature, air humidity, VPD, CO_2 concentration and light quality and quantity (Gardner *et al.* 2006; Graf *et al.* 2010; Shalit-Kaneh*et al.* 2018). Taken together, these results strengthen the idea that fern stomata are responsive to environmental cues, although the velocity of these responses are much lower than those observed in angiosperms (Franks & Britton-Harper 2016). Given the discrepancy between ferns stomatal responses to ABA and sucrose, this suggests that ferns first acquired ABA-independent mechanisms for the regulation of stomatal speediness. In the next section, we discuss how mesophyll-derived metabolites may contribute to explaining the slower stomatal responses found in ferns and how fern stomata can respond to environmental cues such as darkness and high CO_2concentration with no or at least reduced ABA sensitivity.

Metabolism-mediated stomatal closure mechanisms contribute to explain the slower stomatal movements in ferns, compared to angiosperms

The speed of stomatal closure is 2.2 and 8.4-fold lower than stomatal opening in response to light and CO_2 transitions within ferns (Lima *et al.* 2019). This suggests that the mechanisms that coordinate stomatal closure are severely impaired in ferns, as compared to those controlling stomatal opening. Beyond morphological and genetic differences that aid to explain the evolution toward a highly responsive stomata in angiosperms (Cai *et al.* 2017; Sussmilch *et al.*2019; Harris *et al.* 2020; Gong *et al.* 2021), we put forward the hypothesis that metabolism-mediated mechanisms that coordinate the A -g_s trade-off may contribute to explain the rapid control of stomatal movements found in angiosperms. This idea relies in the fact that the faster high CO_2-induced stomatal closure found in angiosperms was positively correlated with leaf sucrose content (Lima *et al.*2019), a metabolite largely described as important to the$A$ -g_s trade-off regulation (Talbott & Zeiger 1998; Daloso *et al.* 2016a; Granot & Kelly 2019; Flütsch & Santelia 2021). This suggests that the higher photosynthetic capacity of angiosperms (Tosens *et al.* 2016; Gago *et al.* 2019), which results in higher sucrose production (Lima *et al.* 2019), is a key facilitator of rapid closure of the stomata. Indeed, exogenous application of sucrose and mannitol reduced g_s in both ferns, but in a lower speed, when compared to angiosperms. Although no difference in the slope of the g_s kinetic following provision of sucrose between ferns and angiosperms was observed (Figure 3c), this kinetic reached a maximum velocity ($Vmax$) only in *V. unguiculata* (Figure S5). Furthermore, the angiosperm reached a plateau earlier than the fern *M. scolopendria* , whilst the fern *P. aureum* did not reach a plateau (Figures S2i, S3i and S4i). These results indicate that *V. unguiculata* has a greater capacity to rapidly respond to the accumulation of sucrose.

Interestingly, both sucrose and mannitol induced stomatal closure in ferns and *V. unguiculata* (Figures 2b-c). However, the speed of sucrose-induced stomatal closure is higher than the mannitol treatment in all species. This is evidenced by the higher slope of stomatal closure kinetic under sucrose in *P. aureum* (Figure 3e), the plateau reached solely under sucrose treatment in *M. scolopendria*(Figures S2h-i), and the earlier plateau and the maximum velocity reached in *V. unguiculata* under sucrose (Figures S4h-i), when compared to the mannitol treatment within each species. These results suggest that stomata from ferns and *V. unguiculata* exhibit both osmotic and non-osmotic responses. In fact, it has been proposed that mesophyll-derived sucrose induces stomatal closure by two different mechanisms: (i) – by an osmotic mechanism, probably associated to the accumulation of sucrose and other osmolytes in the apoplastic space of guard cells (Lu *et al.* 1995, 1997; Kang *et al.* 2007a b); and (ii) by a signalling mechanism, in which these compounds would be perceived by guard cells and the stomatal closure triggered by signalling transduction pathways associated to ABA and hexokinase (Kelly*et al.* 2013, 2019; Lugassi *et al.* 2015). However, recent evidence highlights that the fern *Matteuccia struthiopteris* and the lycophyte *Selaginella moellendorffii* lack ABA-responsiveness due to a disruption in the ABA signalling pathway (Gong *et al.*2021). This work has demonstrated that these species have lower level of ROS and exhibits no increases in both nitric oxide (NO) and Ca^{+2} in their guard cells following provision of ABA (Gong *et al.* 2021). Therefore, whilst our results highlight that fern stomata do respond to exogenous application of both mannitol and sucrose at physiologically relevant concentrations, confirming that a metabolism-mediated stomatal closure mechanism is conserved among ferns and angiosperms, it remains unclear whether the sucrose-induced stomatal closure involves the hexokinase/ABA pathway described for angiosperms.

Our metabolic fingerprinting analysis suggests that the preferential allocation of the diel course CO_2 assimilated toward the secondary metabolism may also influence stomatal movement regulation in ferns. It has been shown that plants with higher accumulation of secondary metabolites have reduced levels of ROS in their guard cells (Watkins *et al.* 2014), which is associated to the capacity of these metabolites in removing ROS (Watkins *et al.* 2017; Delfin*et al.* 2019). It is thus reasonable to hypothesize that the higher allocation toward the secondary metabolism observed in ferns would leads to lower level of ROS in their guard cells, as compared to angiosperms (Figure 8c), which was indeed observed in a previous study (Gong *et al.* 2021). Therefore, a preferential carbon allocation toward the secondary rather than the primary metabolism would leads to both lower g_s throughout the diel course and slower stomatal closure responses in ferns, when compared to angiosperms.

Ferns preferentially use the daily carbon assimilated to the synthesis of secondary rather than primary metabolites

In order to better understand the metabolic differences between ferns and angiosperms, we performed an unprecedented LC-MS analysis of ferns leaves, which, combined with our previous published GC-MS-based metabolite profiling analysis (Lima *et al.* 2019), provided a clear depiction both regarding the metabolic differences among ferns and angiosperms and metabolic aspects underpinning the regulation of stomatal speediness. Multivariate analyses indicate that ferns and angiosperms differ substantially at metabolic level, as evidenced by their separation following HCA, orthoPLSDA and PCA. The LC-MS-based metabolic fingerprinting analysis further showed that several features are solely found in ferns (Figure 4), indicating a higher chemical diversity, compared to the angiosperms species studied here. Furthermore, several features found in both plant groups have higher level in ferns than angiosperms throughout the diel course (Figure 6), suggesting that ferns have a higher accumulation of secondary metabolites than angiosperms. This idea is further supported by the fact that amino acids precursors of secondary metabolism such as phenylalanine and tryptophan are present at higher levels in ferns than angiosperms throughout the diel course, while the majority of sugars and other amino acids have higher levels, throughout the diel course, in angiosperms, when compared to ferns (Lima *et al.* 2019). These results strongly indicate that ferns prioritize carbon allocation towards secondary rather than primary metabolism. Given the role of secondary metabolites for plant defense against (a)biotic stresses (Martins *et al.* 2014; Tohge *et al.* 2016; Austen *et al.* 2019; Li *et al.* 2021) and the evidence indicating that ferns have greater tolerance to different stress conditions, when compared to angiosperms (Proctor & Tuba 2002; C.H *et al.* 2017; Salachna & Piechocki 2020), it seems likely that the higher allocation of carbons toward the secondary metabolism could be a mechanism to improve stress tolerance in ferns, at expenses of reduced growth (Figure 9). These results contribute to answer the elusive question as to why the growth of ferns is slow and sheds light on the challenge that plant breeding programs face to produce stress tolerant genotypes in the absence of a major yield penalty.

Acknowledgments

This work was made possible through financial support from the National Council for Scientific and Technological Development (CNPq, Grant 428192/2018-1). We also thank the research fellowship granted by CNPq to DMD and the scholarships granted by CNPq to FBSF and the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES, Finance Code 001) to SAC-S and VFL.

Author contributions

SAC-S, VFL, JG and DMD designed the research and experiments. SAC-S and VFL performed the experiments. Mass spectrometry analysis was carried out by LPS, with the supervision of ARF. Analysis of metabolomics data was carried out by SAC-S. Data analysis and interpretation and the establishment of figures were carried out by SAC-S, VFL, FBSF and DMD. All authors contributed to writing the manuscript. DMD obtained funding and is responsible for this article.

Conflict of interest

The authors declare no potential conflict of interest.

References

Antunes W.C., Daloso D.M., Pinheiro D.P., Williams T.C.R. & Loureiro M.E. (2017) Guard cell-specific down-regulation of the sucrose transporter SUT1 leads to improved water use efficiency and reveals the interplay between carbohydrate metabolism and K+ accumulation in the regulation of stomatal opening. *Environmental and Experimental Botany* **135** , 73–85.

Antunes W.C., Provart N.J., Williams T.C.R. & Loureiro M.E. (2012) Changes in stomatal function and water use efficiency in potato plants with altered sucrolytic activity. *Plant, Cell and Environment***35** , 747–759.

Austen N., Walker H.J., Lake J.A., Phoenix G.K. & Cameron D.D. (2019) The Regulation of Plant Secondary Metabolism in Response to Abiotic Stress: Interactions Between Heat Shock and Elevated CO2.*Frontiers in Plant Science* **10** , 1–12.

Brodribb T.J. & McAdam S.A.M. (2011) Passive origins of stomatal control in vascular plants. *Science (New York, N.Y.)***331** , 582–5.

Brodribb T.J., Sussmilch F. & McAdam S.A.M. (2019) From reproduction to production, stomata are the master regulators. *Plant Journal* , 1–12.

C.H K., Krishnan M. & K M. (2017) Resilience of ferns: with reference to desiccation and rehydration stress offer new insights.*Kongunadu Research Journal* **4** , 89–94.

Cai S., Chen G., Wang Y., Huang Y., Marchant D.B., Wang Y., ... Chen Z.-H. (2017) Evolutionary Conservation of ABA Signaling for Stomatal Closure. *Plant Physiology* **174** , 732–747.

Cardoso A.A. & McAdam S.A.M. (2019) Misleading conclusions from exogenous ABA application: a cautionary tale about the evolution of stomatal responses to changes in leaf water status. *Plant Signaling & Behavior* **14** , 1610307.

Cardoso A.A., Randall J.M. & McAdam S.A.M. (2019) Hydraulics Regulate Stomatal Responses to Changes in Leaf Water Status in the Fern Athyrium filix-femina. *Plant Physiology* **179** , 533–543.

Ceciliato P.H.O., Zhang J., Liu Q., Shen X., Hu H., Liu C., ... Schroeder J.I. (2019) Intact leaf gas exchange provides a robust method for measuring the kinetics of stomatal conductance responses to abscisic acid and other small molecules in Arabidopsis and grasses. *Plant Methods* , 1–10.

Daloso D.M., dos Anjos L. & Fernie A.R. (2016a) Roles of sucrose in guard cell regulation. *New Phytologist* **211** , 809–818.

Daloso D.M., Williams T.C.R., Antunes W.C., Pinheiro D.P., Müller C., Loureiro M.E. & Fernie A.R. (2016b) Guard cell-specific upregulation of sucrose synthase 3 reveals that the role of sucrose in stomatal function is primarily energetic. *New Phytologist* **209** , 1470–1483.

Deans R.M., Brodribb T.J., Busch F.A. & Farquhar G.D. (2019) Plant water-use strategy mediates stomatal effects on the light induction of photosynthesis. *New Phytologist* **222** , 382:395.

Delfin J.C., Watanabe M. & Tohge T. (2019) Understanding the function and regulation of plant secondary metabolism through metabolomics approaches. *Theoretical and Experimental Plant Physiology***31** , 127–138.

Flütsch S., Nigro A., Conci F., Fajkus J., Thalmann M., Trtílek M., ... Santelia D. (2020a) Glucose uptake to guard cells via STP transporters provides carbon sources for stomatal opening and plant growth. *EMBO reports* **21** , 1–13.

Flütsch S. & Santelia D. (2021) Mesophyll-derived sugars are positive regulators of light-driven stomatal opening. *New Phytologist***230** , 1754–1760.

Flütsch S., Wang Y., Takemiya A., Vialet-Chabrand S.R.M., Klejchová M., Nigro A., ... Santelia D. (2020b) Guard Cell Starch Degradation Yields Glucose for Rapid Stomatal Opening in Arabidopsis. *The Plant Cell* **32** , 2325–2344.

Franks P.J. & Britton-Harper Z.J. (2016) No evidence of general CO2 insensitivity in ferns: one stomatal control mechanism for all land plants? *The New phytologist* **211** , 819–827.

Freire F.B.S., Bastos R.L.G., Bret R.S.C., Cândido-Sobrinho S.A., Medeiros D.B., Antunes W.C., ... Daloso D.M. (2021) Mild reductions in guard cell sucrose synthase 2 expression leads to slower stomatal opening and decreased whole plant transpiration in Nicotiana tabacum L.*Environmental and Experimental Botany* **184** , 104370.

Fujita T., Noguchi K., Ozaki H. & Terashima I. (2019) Confirmation of mesophyll signals controlling stomatal responses by a newly devised transplanting method. *Functional Plant Biology* **46** , 467–481.

Gago J., Carriquí M., Nadal M., Clemente-Moreno M.J., Coopman R.E., Fernie A.R. & Flexas J. (2019) Photosynthesis Optimized across Land Plant Phylogeny. *Trends in Plant Science* **73** , 1–12.

Gago J., Daloso D. de M., Figueroa C.M., Flexas J., Fernie A.R. & Nikoloski Z. (2016) Relationships of leaf net photosynthesis, stomatal conductance, and mesophyll conductance to primary metabolism: A multispecies meta-analysis approach. *Plant Physiology***171** , 265–279.

Gago J., Daloso D.M., Carriquí M., Nadal M., Morales M., Araújo W.L., ... Flexas J. (2020) The photosynthesis game is in the "inter-play": Mechanisms underlying CO2 diffusion in leaves.*Environmental and Experimental Botany* **178** , 104174.

Gardner M.J., Hubbard K.E., Hotta C.T., Dodd A.N. & Webb A.A.R. (2006) How plants tell the time. *Biochemical Journal* **397** , 15–24.

Gong L., Liu X.-D., Zeng Y.-Y., Tian X.-Q., Li Y.-L., Turner N.C. & Fang X.-W. (2021) Stomatal morphology and physiology explain varied sensitivity to abscisic acid across vascular plant lineages. *Plant Physiology* , 1–16.

Graf A., Schlereth A., Stitt M. & Smith A.M. (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night.*Proceedings of the National Academy of Sciences of the United States of America* **107** , 9458–9463.

Granot D. & Kelly G. (2019) Evolution of Guard-Cell Theories: The Story of Sugars. *Trends in Plant Science* **24** , 507–518.

Harris B.J., Harrison C.J., Hetherington A.M. & Williams T.A. (2020) Phylogenomic Evidence for the Monophyly of Bryophytes and the Reductive Evolution of Stomata. *Current Biology* , 1–12.

Hoagland D.R. & Arnon D.I. (1950) *The Water-Culture Method for Growing Plants without Soil. THE COLLEGE OF AGRICULTURE* , 2nd ed. The College of Agriculture, California, USA.

Hõrak H., Kollist H. & Merilo E. (2017) Fern stomatal responses to ABA and CO2 depend on species and growth conditions. *Plant Physiology***174** , 672–679.

Hotta C.T. (2021) From crops to shops: how agriculture can use circadian clocks. *Journal of Experimental Botany* .

Kang Y., Outlaw W.H., Andersen P.C. & Fiore G.B. (2007a) Guard-cell apoplastic sucrose concentration - A link between leaf photosynthesis and stomatal aperture size in the apoplastic phloem loader Vicia faba L.*Plant, Cell and Environment* **30** , 551–558.

Kang Y., Outlaw W.H., Fiore G.B. & Riddle K.A. (2007b) Guard cell apoplastic photosynthate accumulation corresponds to a phloem-loading mechanism. *Journal of Experimental Botany* **58** , 4061–4070.

Kelly G., Egbaria A., Khamaisi B., Lugassi N., Attia Z., Moshelion M. & Granot D. (2019) Guard-Cell Hexokinase Increases Water-Use Efficiency Under Normal and Drought Conditions. *Frontiers in Plant Science***10** .

Kelly G., Moshelion M., David-Schwartz R., Halperin O., Wallach R., Attia Z., ... Granot D. (2013) Hexokinase mediates stomatal closure. *Plant Journal* **75** , 977–988.

Kosmides A.K., Kamisoglu K., Calvano S.E., Corbett S.A. & Androulakis I.P. (2013) Metabolomic fingerprinting: Challenges and opportunities.*Critical Reviews in Biomedical Engineering* **41** , 205–221.

Kottapalli J., David-Schwartz R., Khamaisi B., Brandsma D., Lugassi N., Egbaria A., ... Granot D. (2018) Sucrose-induced stomatal closure is conserved across evolution. *PLoS ONE* **13** , 1–17.

Kruger N.J., Troncoso-Ponce M.A. & Ratcliffe R.G. (2008) 1H NMR metabolite fingerprinting and metabolomic analysis of perchloric acid extracts from plant tissues. *Nature Protocols* **3** , 1001–1012.

Lawson T. & Vialet-Chabrand S. (2018) Speedy stomata, photosynthesis and plant water use efficiency. *New Phytologist* .

Lê S., Josse J. & Husson F. (2008) FactoMineR : An R Package for Multivariate Analysis. *Journal of Statistical Software***25** , 253–258.

Li B., Fan R., Sun G., Sun T., Fan Y., Bai S., ... Song C. peng (2021) Flavonoids improve drought tolerance of maize seedlings by regulating the homeostasis of reactive oxygen species. *Plant and Soil* **461** , 389–405.

Li Y., Xu S., Gao J., Pan S. & Wang G. (2016) Glucose- and mannose-induced stomatal closure is mediated by ROS production , Ca^{2+} and water channel in Vicia faba. 252–261.

Lima V.F., Anjos L. dos, Medeiros D.B., Candido-Sobrinho S.A., Souza L.P., Gago J., ... Daloso D.M. (2019) The sucrose-to-malate ratio correlates with the faster CO_2 and light stomatal responses of angiosperms compared to ferns. *New Phytologist* , 1873–1887.

Lima V.F., Medeiros D.B., Dos Anjos L., Gago J., Fernie A.R. & Daloso D.M. (2018) Toward multifaceted roles of sucrose in the regulation of stomatal movement. *Plant Signaling & Behavior* **00** , 1–8.

Lisec J., Schauer N., Kopka J., Willmitzer L. & Fernie A.R. (2006) Gas chromatography mass spectrometry–based metabolite profiling in plants.*Nature Protocols* **1** , 387–396.

Lu P., Outlaw Jr W.H., Smith B.G. & Freed G.A. (1997) A new mechanism for the regulation of stomatal aperture size in intact leaves (accumulation of mesophyll-derived sucrose in the guard-cell wall of Vicia faba). *Plant physiology* **114** , 109–118.

Lu P., Zhang S.Q., Outlaw W.H. & Riddle K.A. (1995) Sucrose: a solute that accumulates in the guard-cell apoplast and guard-cell symplast of open stomata. *FEBS Letters* **362** , 180–184.

Luedemann A., Strassburg K., Erban A. & Kopka J. (2008) TagFinder for the quantitative analysis of gas metabolite profiling experiments.*Bioinformatics* **24** , 732–737.

Lugassi N., Kelly G., Fidel L., Yaniv Y., Attia Z., Levi A., ... Granot D. (2015) Expression of Arabidopsis Hexokinase in Citrus Guard Cells Controls Stomatal Aperture and Reduces Transpiration.*Frontiers in Plant Science* **6** , 1–11.

Martins S.C.V., Araujo W.L., Tohge T., Fernie A.R. & DaMatta F.M. (2014) In high-light-acclimated coffee plants the metabolic machinery is adjusted to avoid oxidative stress rather than to benefit from extra light enhancement in photosynthetic yield. *PLoS ONE* **9** , 1–11.

McAdam S.A.M. & Brodribb T.J. (2012) Fern and Lycophyte Guard Cells Do Not Respond to Endogenous Abscisic Acid. *The Plant Cell***24** , 1510–1521.

McAdam S.A.M., Duckett J.G., Sussmilch F.C., Pressel S., Renzaglia K.S., Hedrich R., ... Merced A. (2021) Stomata: the holey grail of plant evolution. *American Journal of Botany* **108** , 366–371.

McAdam S.A.M. & Sussmilch F.C. (2021) The evolving role of abscisic acid in cell function and plant development over geological time.*Seminars in Cell and Developmental Biology* **109** , 39–45.

McAusland L., Vialet-Chabrand S., Davey P., Baker N.R., Brendel O. & Lawson T. (2016) Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *The New phytologist***211** , 1209–1220.

Medeiros D.B., da Luz L.M., de Oliveira H.O., Araujo W.L., Daloso D.M. & Fernie A.R. (2019) Metabolomics for understanding stomatal movements.*Theoretical and Experimental Plant Physiology* **9** , 91–102.

Medeiros D.B., Perez Souza L., Antunes W.C., Araujo W.L., Daloso D.M. & Fernie A.R. (2018) Sucrose breakdown within guard cells provides substrates for glycolysis and glutamine biosynthesis during light-induced stomatal opening. *Plant Journal* **94** , 583–594.

Mott K.A. (2009) Opinion: Stomatal responses to light and CO2 depend on the mesophyll. *Plant, Cell and Environment* **32** , 1479–1486.

Outlaw W.H.J. (1995) Sucrose and stomata: a full circle. In *Carbon Partitioning and Source–Sink Interactions in Plants* . (eds M.A. Madore & W.J. Lucus), pp. 56–67. American Society of Plant Physiologists, Rockville, MD, USA.

Pang Z., Chong J., Li S. & Xia J. (2020) Metaboanalystr 3.0: Toward an optimized workflow for global metabolomics. *Metabolites***10** .

Papanatsiou M., Petersen J., Henderson L., Wang Y., Christie J.M. & Blatt M.R. (2019) Optogenetic manipulation of stomatal kinetics improves carbon assimilation,water use, and growth. *Science* **363** , 1456–1459.

Perez de Souza L., Alseekh S., Naake T. & Fernie A. (2019) Mass Spectrometry-Based Untargeted Plant Metabolomics. *Current protocols in plant biology* **4** , e20100.

Perez de Souza L., Alseekh S., Scossa F. & Fernie A.R. (2021) Ultra-high-performance liquid chromatography high-resolution mass spectrometry variants for metabolomics research. *Nature Methods* .

Plackett A.R.G., Emms D.M., Kelly S., Hetherington A.M. & Langdale J.A. (2021) Conditional stomatal closure in a fern shares molecular features with flowering plant active stomatal responses. *Current Biology* , 1–31.

Proctor M.C.F. & Tuba Z. (2002) Poikilohydry and homoihydry: antithesis or spectrum of possibilities? *New Phytologist* **156** , 327–349.

Qu M., Essemine J., Xu J., Ablat G., Perveen S., Wang H., ... Zhu X. (2020) Alterations in stomatal response to fluctuating light increase biomass and yield of rice under drought conditions. *Plant Journal***104** , 1334–1347.

Qu X., Peterson K.M. & Torii K.U. (2017) Stomatal development in time: the past and the future. *Current Opinion in Genetics & Development* **45** , 1–9.

R Core Team (2020) R: A Language and Environment for Statistical Computing.

Raven J.A. (2014) Speedy small stomata'. *Journal of Experimental Botany* **65** , 1415–1424.

Ruszala E.M., Beerling D.J., Franks P.J., Chater C., Casson S.A., Gray J.E. & Hetherington A.M. (2011) Land plants acquired active stomatal control early in their evolutionary history. *Current Biology***21** , 1030–1035.

Salachna P. & Piechocki R. (2020) Salinity Tolerance of Four Hardy Ferns from the Genus Dryopteris Adans. Grown under Different Light Conditions. *Agronomy* **11** , 49.

Sarda-Espinosa A. (2019) Time-series clustering in R Using the dtwclust package. *R Journal* **11** , 1–22.

Scholz M., Gatzek S., Sterling A., Fiehn O. & Selbig J. (2004) Metabolite fingerprinting: Detecting biological features by independent component analysis. *Bioinformatics* **20** , 2447–2454.

Shalit-Kaneh A., Kumimoto R.W., Filkov V. & Harmer S.L. (2018) Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. *Proceedings of the National Academy of Sciences* **115** , 7147–7152.

Silveira-Sotelo M., Chauvin A.L., Marsch-Martinez N., Winkler R. & de Folter S. (2015) Metabolic fingerprinting of Arabidopsis thaliana accessions. *Frontiers in Plant Science* **6** , 1–13.

Smith C.A., Want E.J., O'Maille G., Abagyan R. & Siuzdak G. (2006) XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. *Analytical Chemistry* **78** , 779–787.

Sussmilch F.C., Brodribb T.J. & McAdam S.A.M. (2017) What are the evolutionary origins of stomatal responses to abscisic acid in land plants? *Journal of Integrative Plant Biology* **59** , 240–260.

Sussmilch F.C., Schultz J., Hedrich R. & Roelfsema M.R.G. (2019) Acquiring Control: The Evolution of Stomatal Signalling Pathways. *Trends in Plant Science* **24** , 342–351.

Talbott L. & Zeiger E. (1998) The role of sucrose in guard cell osmoregulation. *Journal of Experimental Botany* **49** , 329–337.

Tohge T. & Fernie A.R. (2010) Combining genetic diversity, informatics and metabolomics to facilitate annotation of plant gene function. *Nature Protocols* **5** , 1210–1227.

Tohge T., Wendenburg R., Ishihara H., Nakabayashi R., Watanabe M., Sulpice R., ... Fernie A.R. (2016) Characterization of a recently evolved flavonol-phenylacyltransferase gene provides signatures of natural light selection in Brassicaceae. *Nature Communications* **7** .

Tosens T., Nishida K., Gago J., Coopman R.E., Cabrera H.M., Carriqui M., ... Flexas J. (2016) The photosynthetic capacity in 35 ferns and fern allies: Mesophyll CO2diffusion as a key trait. *New Phytologist* **209** , 1576–1590.

Tsugawa H., Cajka T., Kind T., Ma Y., Higgins B., Ikeda K., ... Arita M. (2015) MS-DIAL: Data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nature Methods* **12** , 523–526.

Watkins J., Chapman J.M. & Muday G.K. (2017) *Abscisic acid-induced reactive oxygen species are modulated by flavonols to control stomata aperture* .

Watkins J.M., Hechler P.J. & Muday G.K. (2014) Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. *Plant Physiology* **164** , 1707–1717.

Hosted file

`Candido-Sobrinho_Figures.docx` available at https://authorea.com/users/356289/articles/541173-metabolism-mediated-mechanisms-underpin-the-differential-stomatal-speediness-regulation-among-ferns-and-angiosperms