Received: 6 October 2021 | Revised: 12 November 2021 | Accepted: 14 November 2021

DOI: 10.1111/pce.14232

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

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

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Funding information

Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 428192/2018-1

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 (g_s) in ferns is missing. Here, we investigated the effect of exogenous application of abscisic acid (ABA), sucrose and mannitol on stomatal kinetics and carried out a metabolic fingerprinting analysis of ferns and angiosperms leaves harvested throughout a diel course. Fern stomata did not respond to ABA in the time period analysed. No differences in the relative decrease in g_s was observed between ferns and the angiosperm following provision of sucrose or mannitol. However, ferns have slower g_s responses to these compounds than angiosperms. Metabolomics analysis highlights that ferns have a 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 mechanisms underpin the differential stomatal speediness regulation among ferns and angiosperms, in which the slower stomatal closure in ferns is associated with the lack of ABA-responsiveness, to a reduced capacity to respond to mesophyll-derived sucrose and to a higher carbon allocation toward secondary metabolism, which likely modulates both photosynthesis-g_s and growth-stress tolerance trade-offs.

KEYWORDS

abscisic acid, metabolic fingerprinting, primary metabolism, secondary metabolism, stomatal evolution, stomatal movement regulation

1 | INTRODUCTION

Stomata are epidermal structures, each 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 particular losses in certain mosses and all liverworts during bryophyte evolution (Duckett & Pressel 2018; Harris et al., 2020; Renzaglia 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_2 between the leaf and the environment in aerial tissues covered by cuticle (Medeiros et al., 2019; Qu et al., 2017). 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 bryophytes and early tracheophytes such as lycophytes and ferns (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 (VPD), 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., 2017, 2012; Daloso, Williams et al., 2016; Flütsch, Wang et al., 2020; Freire et al., 2021; Kelly et al., 2019, 2013; Y. Li et al., 2016; Lugassi et al., 2015; Medeiros et al., 2018). These studies strengthen the idea that mesophyll-derived metabolites have a great influence on the regulation of stomatal movement (Fujita et al., 2019; Mott, 2009). Notably, reduced sugar import into guard cells compromises g_s, *A* and plant growth (Antunes et al., 2017) and substantially reduces stomatal speediness (Flütsch, Nigro et al., 2020). On the other hand, exogenous application of high concentrations of sucrose (≥100 mM) induce stomatal closure in a wide range of angiosperm species (Kelly et al., 2013; Kottapalli et al., 2018; Medeiros 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 ferns and, if so, whether they contribute to explain the slower stomatal responses found in ferns, when compared to angiosperms.

Ferns and angiosperms share certain common genetic components that control both stomatal formation and stomatal responses to light, CO_2 and abscisic acid (ABA; Harris et al., 2020; Sussmilch et al., 2017, 2019). 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 (Hõrak et al., 2017; Ruszala et al., 2011) and/or the ABA concentration used is too high (e.g., 100 μM) (Plackett et al., 2021). On the other side, evidence suggests that hydraulic and osmotic mechanisms independent of ABA are the major drivers of stomatal closure in ferns (Brodribb & McAdam, 2011; Cardoso & McAdam, 2019; Cardoso et al., 2019; McAdam & Brodribb, 2012a, 2012b). 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). However, this study was performed using an extremely high concentration of 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 (Gago et al., 2019; Tosens et al., 2016) and the capacity to produce ABA (Cardoso & McAdam, 2019). Further studies using relevant physiological concentrations of ABA and osmotic compounds are needed to better understand the evolution of stomatal 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 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 a 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., 2017, 2014), 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 on g_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.

2 | MATERIALS AND METHODS

2.1 | 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 h natural photoperiod, maximum photosynthetic photon flux density of 500 µmol m^{-2} s^{-1}, average ambient temperature of 30 ± 4°C, relative humidity 62 ± 2°C and VPD of 1.82 ± 0.06 kPa.

2.2 | 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). The gas exchange was recorded every 10 s for 40 min under 1000 µmol photons m^{-2} s^{-1}. After g_s stabilisation, 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 in angiosperms at this concentration (5 µM; Cardoso & McAdam, 2019), which was then used to confirm whether the stomatal kinetic approach is feasible for the species used here. The sucrose concentration was selected based on the facts that sucrose transport into guard cells saturate at c. 25–40 mM (Outlaw, 1995) and that sucrose concentration is estimated to range from 40 to 110 mM in the guard cell cytosol and can reach more than 150 mM at the apoplastic space of *Vicia faba* L. guard cells (Lu et al., 1997). These results ensure that the concentrations of ABA and sucrose used here are within the endogenous level found in angiosperms. 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 min. 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).

2.3 | 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, 08:00, 14:00 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 characterisation of ferns and angiosperms by carrying out an ultra-high-performance liquid chromatography high-resolution mass spectrometry (LC-MS) analysis, which mostly detects 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 were 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 (version 3.7.1) (Smith et al., 2006) and the MetaboanalystR package on R (Pang et al., 2020).

2.4 | Gathering previously published gas exchange and GC-MS data

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, 08:00, 14:00 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.

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

Angiosperms have higher absolute values of *A* and g_s (Franks & Britton-Harper, 2016; Gago et al., 2019; Lima et al., 2019; Tosens et al., 2016). 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 (0–1 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, 08:00, 14:00 and 17:00 h). The lowest average value of each variable was set to 0 and the highest to 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).

2.6 | 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 relative g_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).

3 | RESULTS

3.1 | 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 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 DTW analysis (Figure S1). Interestingly, both *A* and g_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* and g_s, but the relative changes in these parameters are identical between ferns and angiosperms throughout the diel course (Figure 1a–d). These results highlight that stomata from ferns and angiosperms have a similar circadian rhythm in relative terms.

3.2 | 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 angiosperm species (Lima et al., 2019), the stomatal closure kinetics were carried out using only one representative angiosperm species (*V. unguiculata*). Fern stomata did not respond to ABA (Figure 2a,d). However, exogenous application of both mannitol and sucrose decreased g_s in all plants (Figure 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 (Figure 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 s 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 s, respectively (Figures S2–S4). The data was subsequently transformed following a maximum–minimum normalisation (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, while no statistical difference in the slope of stomatal closure kinetics induced by sucrose between ferns and angiosperms was observed (Figure 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 (Figure 3d–f). Taken together, these results highlight that an osmotic-mediated mechanism of stomatal closure is observed in both ferns and angiosperms and that the slower stomatal closure in ferns is associated to their lack of ABA-responsiveness and to a slower response to mesophyll-derived sucrose.

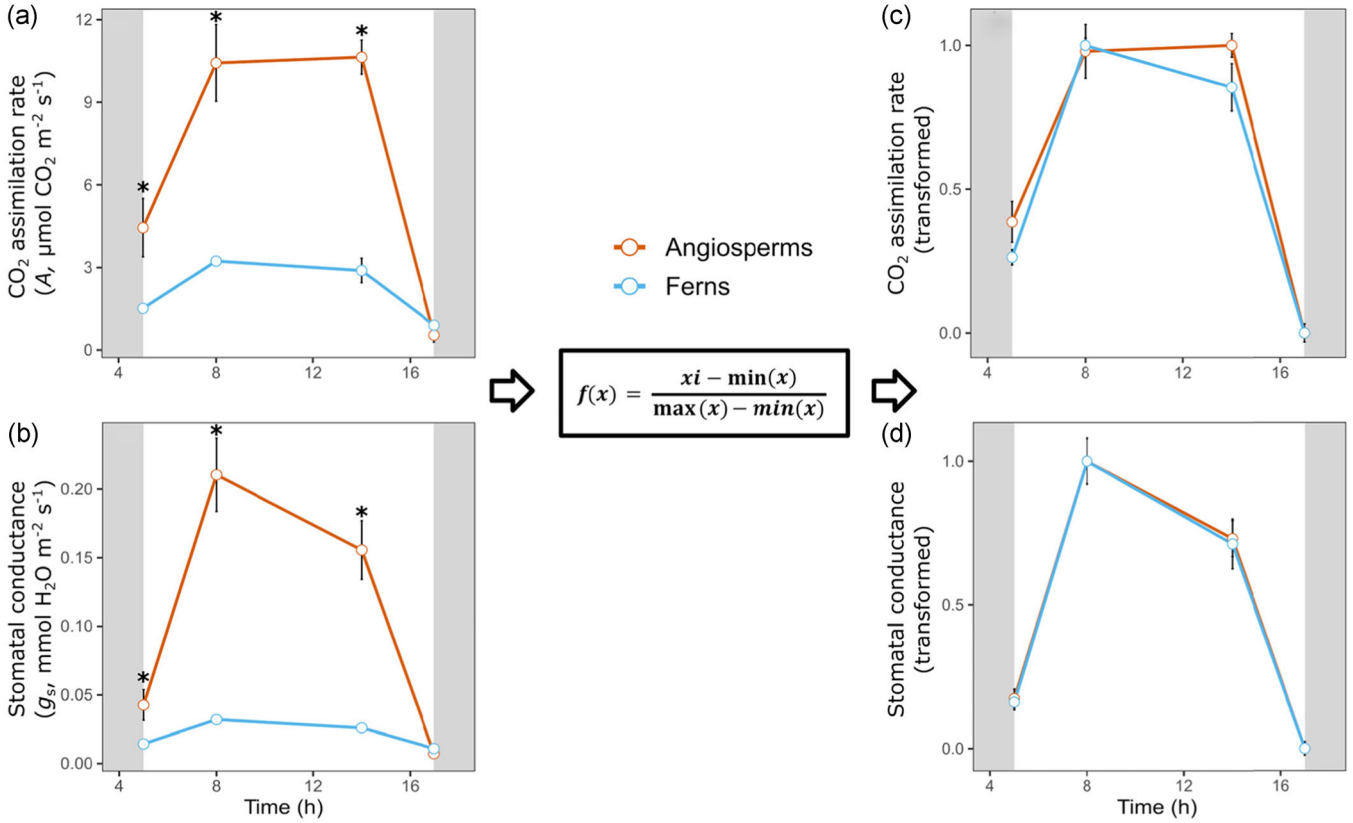

FIGURE 1 Leaf gas exchange throughout the diel course in ferns and angiosperms. CO_2 assimilation rate (*A*) (µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1}) were determined in leaves from ferns and angiosperms at 5:00, 8:00, 14:00 and 17:00 h of the day and are presented in absolute (a,b) and relative (c,d) terms. Grey areas in the graph represent the dark periods before the sunrise (5:00 h) and after the sunset (17:00 h). The relative data of *A* and g_s was obtained by a maximum–minimum transformation (0–1 range) according to the equation found in the middle of the figure, where *xi* is an observation of a gas exchange parameter. The lowest average value of each variable (*A* or g_s) was set to 0 and the highest to 1. The values in between were then proportionally normalized between the maximum and the minimum observed throughout the diel course. The lowest and the highest average values of both *A* and g_s were observed at 17:00 and 8:00 h of the day, respectively. Gas exchange data were retrieved from Lima et al. (2019). The data represents average ± SE (n = 4). *Statistical differences among ferns and angiosperms at each time point by Student's *t*-test (p < .05)

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

The origin of monylophyta and angiospermae plant groups is separated by over 160–200 Ma (Morris et al., 2018). Ferns and angiosperms have therefore different evolutionary histories (Sussmilch et al., 2019), which may reflect in their metabolome. Furthermore, our previous results suggest that ferns and angiosperms have a 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 an 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, that is, based on the intensity of the features (peaks found in the chromatograms) detected in the samples (Kosmides et al., 2013; Kruger et al., 2008; Perez de Souza et al., 2019; Scholz et al., 2004; Silveira-Sotelo et al., 2015). Given that the LC-MS platform used here mostly detects secondary metabolites (Perez de Souza et al., 2021; Tohge & Fernie, 2010), 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 (Figure 4a–d). Similarly, several mass-to-charge ratio (*m/z*) features obtained by MS analysis were solely found in ferns, especially at 5:00 and 14:00 h (Figure 5a–d). A total of 19 340, 7741, 9668 and 19 598 features were statistically different (p < .05) between ferns and angiosperms at 5:00, 8:00, 14:00 and 17:00 h, respectively. Several of these features had higher intensity in ferns, compared to angiosperms (Figure 6a–d). HCA (Figure 6a–d), orthoPLS-DA (Figure 7a–d) or PCA (Figure 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

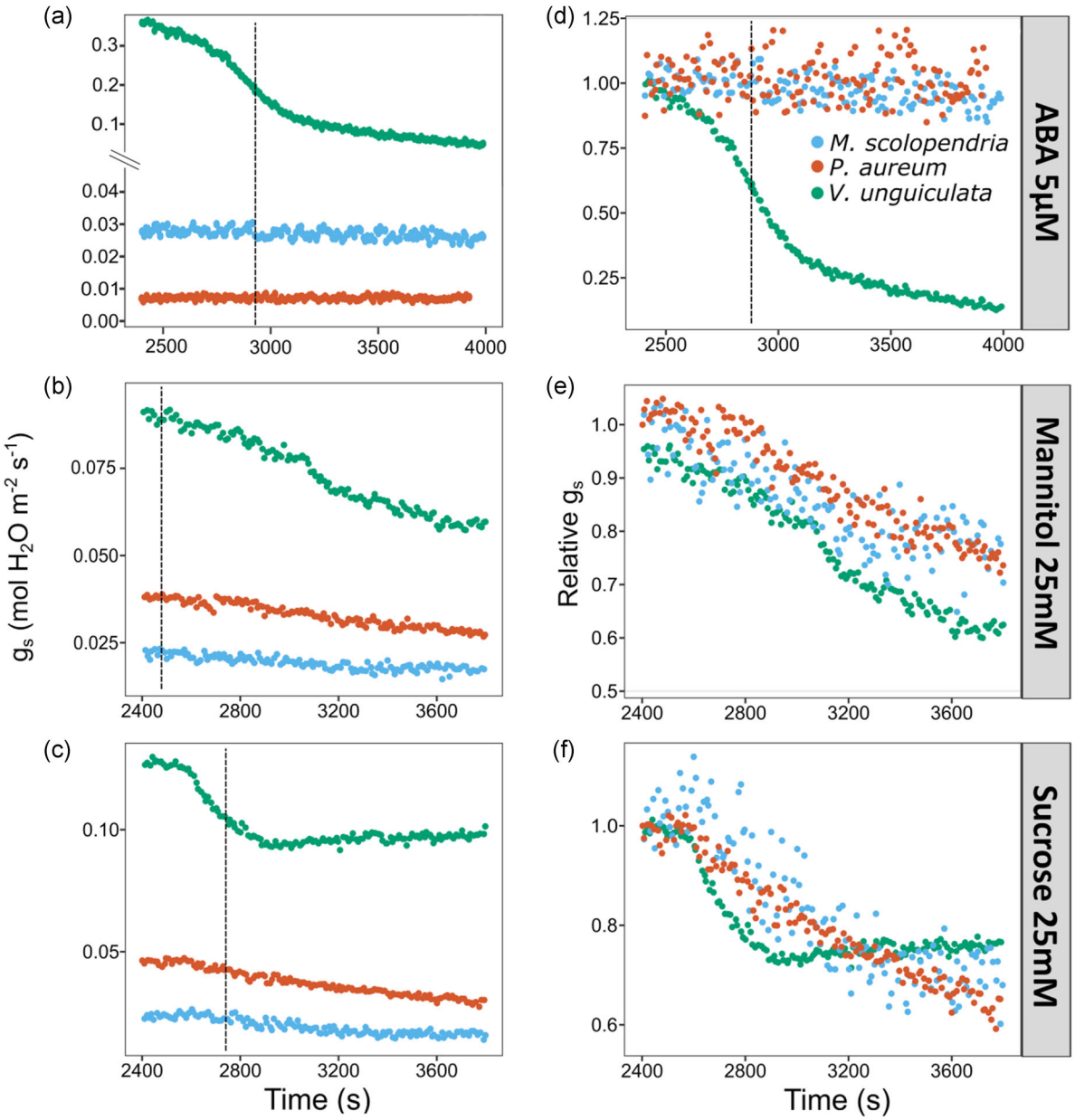

FIGURE 2 Effect of exogenous application of abscisic acid (ABA, 5 µM), mannitol (25 mM) and sucrose (25 mM) on stomatal closure kinetics in two ferns (*Microsorum scolopendria* and *Phlebodium aureum*) and one angiosperm species (*Vigna unguiculata*). (a–c) Absolute stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1}) values. (d–f) Relative g_s values, normalized according to the average of the last 10 min prior the application of the compounds. Both original and normalized data undergone Student's *t*-test for each time point. Vertical dashed lines indicate the time from each statiscal difference ($p < .05$) between angiosperm and ferns was observed. No statistical difference between the angiosperm and both ferns in relative g_s changes after application of mannitol and sucrose was observed. Data are expressed as an average of three biological replicates. Standard deviation was omitted for clarity ($n = 3$) [Color figure can be viewed at wileyonlinelibrary.com]

to primary metabolites, also discriminates ferns and angiosperms (Figures S7–S10).

The results obtained here coupled to previous studies provide compelling evidence highlighting that fern stomata can close in response to sucrose, mannitol, dark, high VPD and high CO_2 concentration and that the g_s dynamic throughout the day is similar between ferns and angiosperms in relative terms (Cardoso et al., 2019; Franks & Britton-Harper, 2016; Gong et al., 2021; Hõrak et al., 2017; Lima et al., 2019; 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 with 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 with their higher capacity to produce sucrose, when compared to ferns (Figure 8b) (Lima et al., 2019). Furthermore, our metabolomics analyses suggest that ferns and angiosperms exhibit distinct patterns of

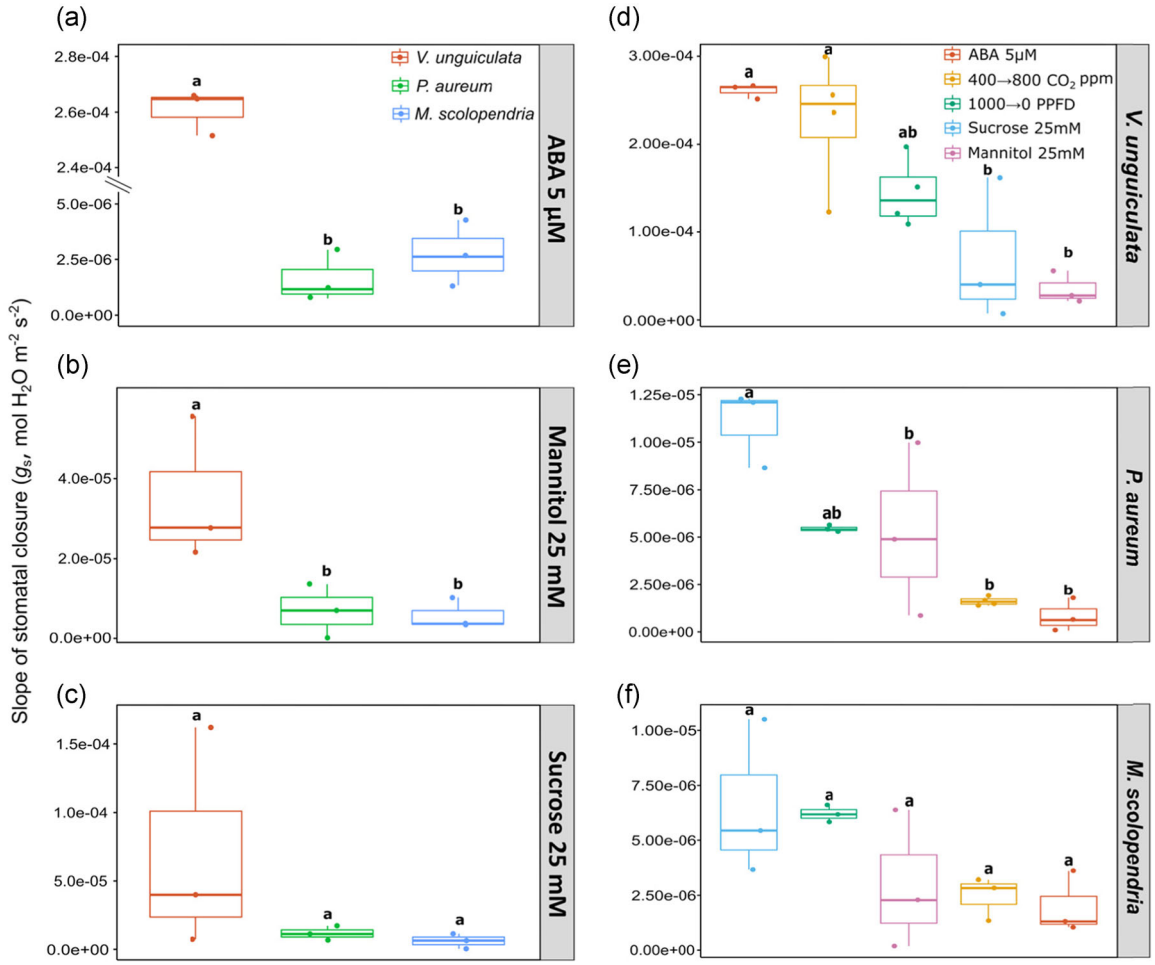

FIGURE 3 Slope of the kinetics of stomatal closure in two ferns (*Microsorum scolopendria* and *Phlebodium aureum*) and one angiosperm species (*Vigna unguiculata*). (a–c) Effect of exogenous application of abscisic acid (ABA, 5 µM), mannitol (25 mM) and sucrose (25 mM) on the slope of the kinetics of stomatal closure among species. (d–f) Comparison between the slope of the kinetics of stomatal closure after exogenous application of ABA, mannitol and sucrose with the stomatal closure induced by ambient to high CO_2 concentration (400 → 800 CO_2 ppm) and during light-to-dark (1000 → 0 PPFD) transitions, previously reported for these species (Lima et al., 2019). Box plots with different letters indicate statistical difference by analysis of variance and Tukey test ($p < .05$). All data are expressed as mean ± SE ($n = 3$ or $n = 4$) [Color figure can be viewed at wileyonlinelibrary.com]

allocation toward primary and secondary metabolisms throughout the diel course, which may affect the level of reactive oxygen species (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).

4 | DISCUSSION

4.1 | The need for speed: Fast stomatal closure requires a high ABA sensitivity

The fast stomatal responses of angiosperms confer 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 colonisation (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 with a reduced capacity to respond to ABA (Brodribb & McAdam, 2011; Cardoso et al., 2019; McAdam & Brodribb, 2012b). This idea is supported by the results in which exogenous application of 5 µM of ABA rapidly reduced g_s in the angiosperm, while fern stomata did not respond to this phytohormone in the time period analysed. Angiosperm stomata are highly sensitive to ABA. Exogenous application of low concentration (from 0.1 to 2 µM) of this phytohormone reduces g_s in more than 50% in a wide range of angiosperm species (reviewed in Cardoso & McAdam, 2019). By contrast, fern stomatal responses are slightly altered following the exogenous application of ABA (Cardoso et al., 2019; Hõrak et al., 2017; Ruszala et al., 2011). Interestingly, the concentration of leaf ABA increased up to 25-fold in ferns under

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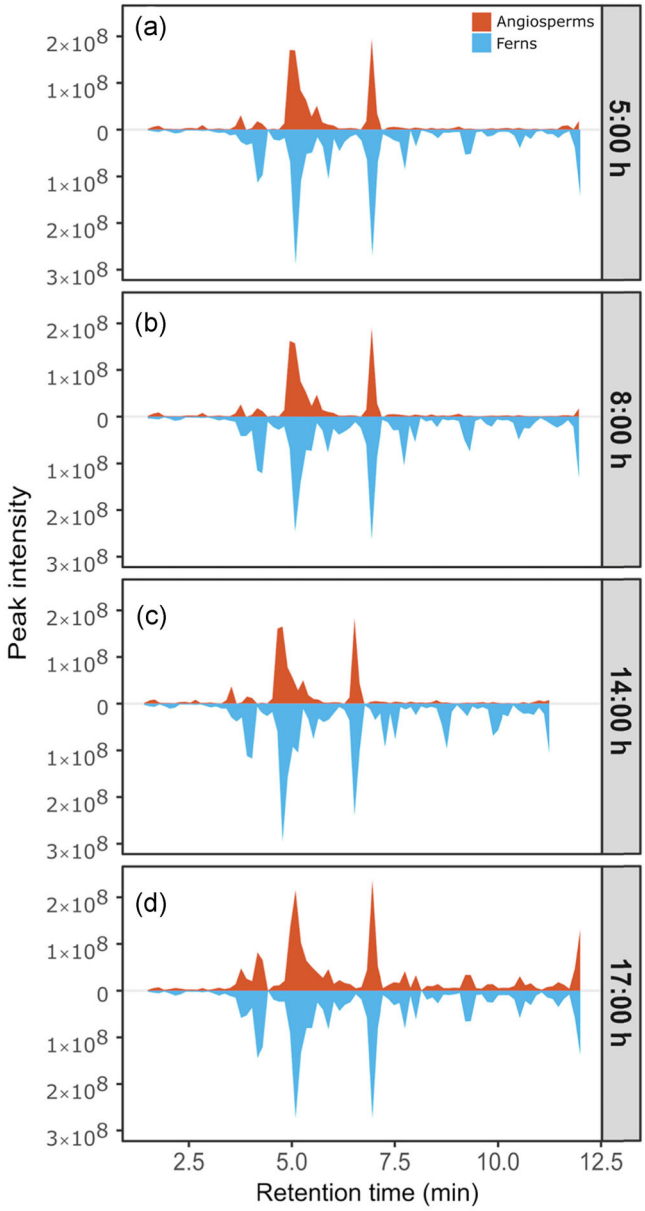

FIGURE 4 Peak intensities obtained by ultrahigh-performance liquid chromatography high-resolution mass spectrometry (LC-MS) analysis of leaf samples harvested at (a) 5:00, (b) 8:00, (c) 14:00 and (d) 17:00 h of the day. These figures highlight the intensity of the peaks (*y*-axis) according to their retention time (*x*-axis) in the chromatograms of LC-MS analysis. Note the lack and the reduced intensity of several peaks in angiosperms (red), when compared to the same peaks found in ferns (blue) [Color figure can be viewed at wileyonlinelibrary.com]

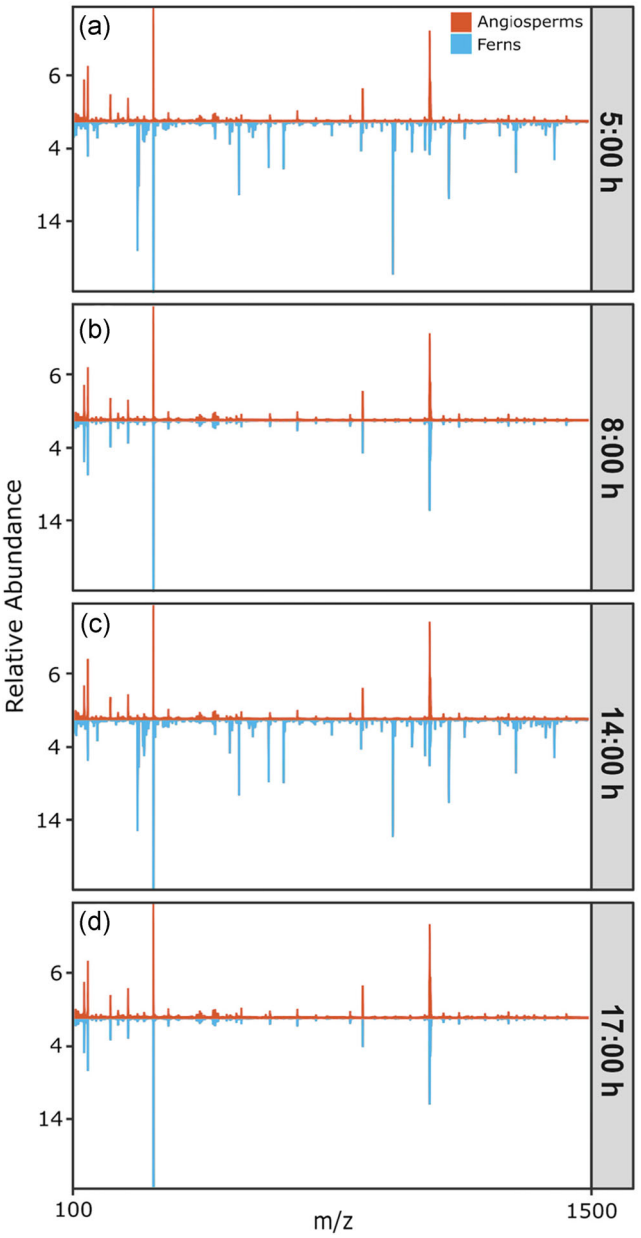

FIGURE 5 Relative abundance of mass fragments obtained by ultrahigh-performance liquid chromatography high-resolution mass spectrometry (LC-MS) analysis of leaf samples harvested at (a) 5:00, (b) 8:00, (c) 14:00 and (d) 17:00 h of the day. These figures demonstrate the relative abundance (*y*-axis) of different mass fragments according to their mass-to-charge ratio (*m/z*) (*x*-axis) in the mass spectral data obtained by LC-MS analysis. Note the presence and the higher intensity of several mass fragments in ferns (blue), when compared to angiosperms (red), especially at 5:00 and 14:00 h [Color figure can be viewed at wileyonlinelibrary.com]

drought (Cardoso et al., 2019; McAdam & Brodribb, 2012b). However, there is no evidence indicating that the increase in leaf ABA concentration leads to stomatal closure in ferns (McAdam & Sussmilch, 2021). Therefore, although fern 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), our results strengthen the hypothesis that fern stomata cannot respond to endogenous ABA (McAdam & Sussmilch, 2021).

The stomata of the ferns used here responded to exogenous application of sucrose and mannitol, to changes in the CO_2 concentration and during the light-to-dark transition. Furthermore, our results revealed that the diel course of g_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

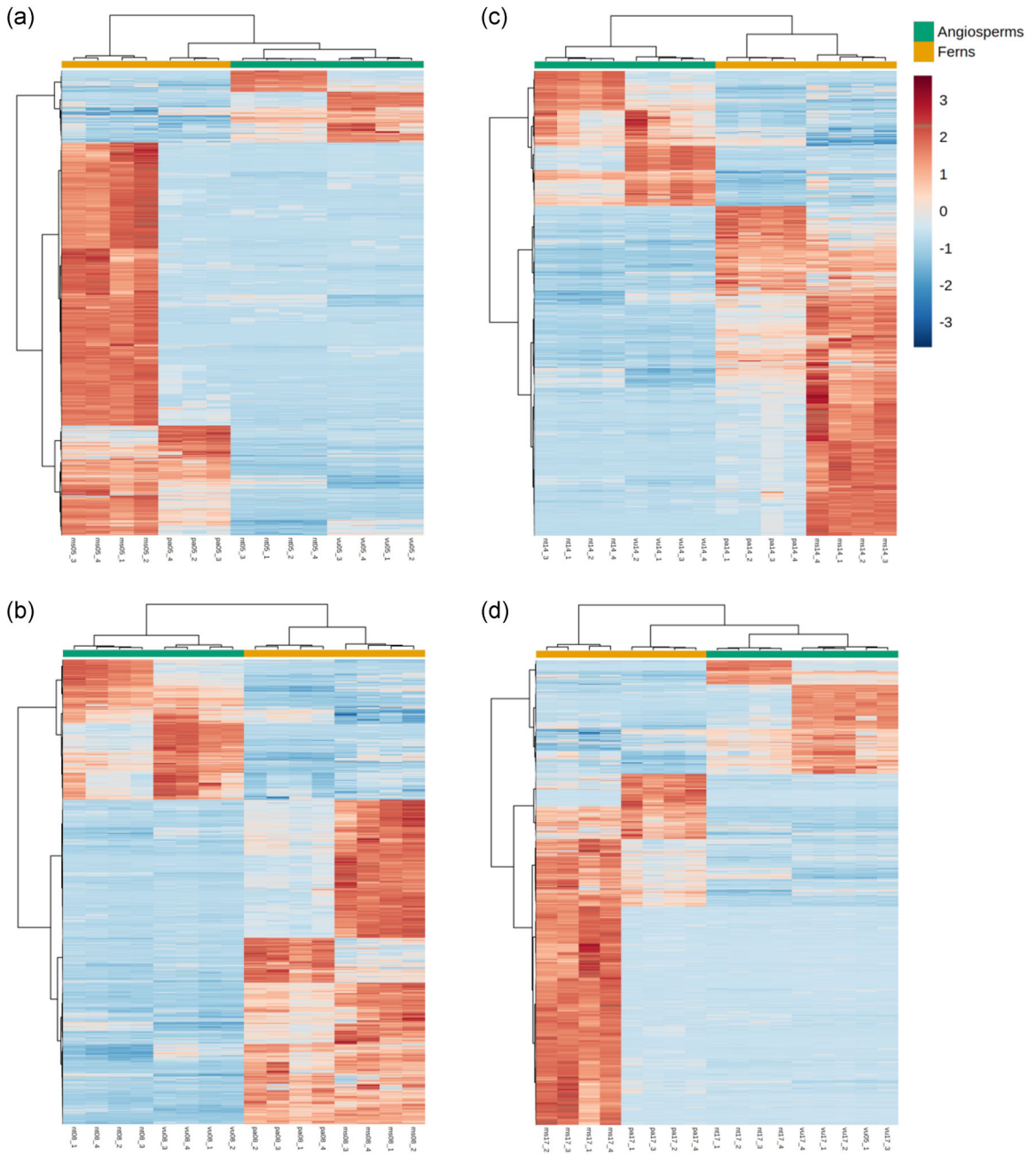

FIGURE 6 Heatmap representation of liquid chromatography coupled to mass spectrometry-based metabolic fingerprinting analysis in leaf samples harvested at (a) 05:00 and (b) 08:00 h, (c) 14:00 and (d) 17:00 h of the diel course. The relative level of the features is highlighted according to the colour scale at the top right of the figures. Hierarchical clustering analysis (HCA) clearly distinguished ferns (yellow) and angiosperms (green) and the species *Nicotiana tabacum* (nt), *Vigna unguiculata* (vu), *Microsorum scolopendria* (ms) and *Phlebodium aureum* (pa) within each plant group. Heatmap and HCA were carried out using Metaboanalyst (Chong et al., 2018) [Color figure can be viewed at wileyonlinelibrary.com]

(Antunes et al., 2017, 2012). It is important to highlight that the plants used here were grown in a greenhouse with no artificial light and no control of environmental cues. Thus, the g_s responses observed here suggest that fern stomata is able to respond to the natural circadian rhythm, which is controlled by a complex regulatory network associated to changes in different environmental cues (Gardner et al., 2006; Graf et al., 2010; Hotta, 2021; Shalit-Kaneh et al., 2018). However, further experiments using ferns and angiosperms subjected to different artificial light/dark cycles are important to understand at which extent fern stomata are able to adjust its behaviour according to the photoperiod.

Taken together, these results strengthen the idea that fern stomata are responsive to different environmental cues, although the velocity of these responses are much lower than those observed in angiosperms (Franks & Britton-Harper, 2016). Given the discrepancy between fern 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

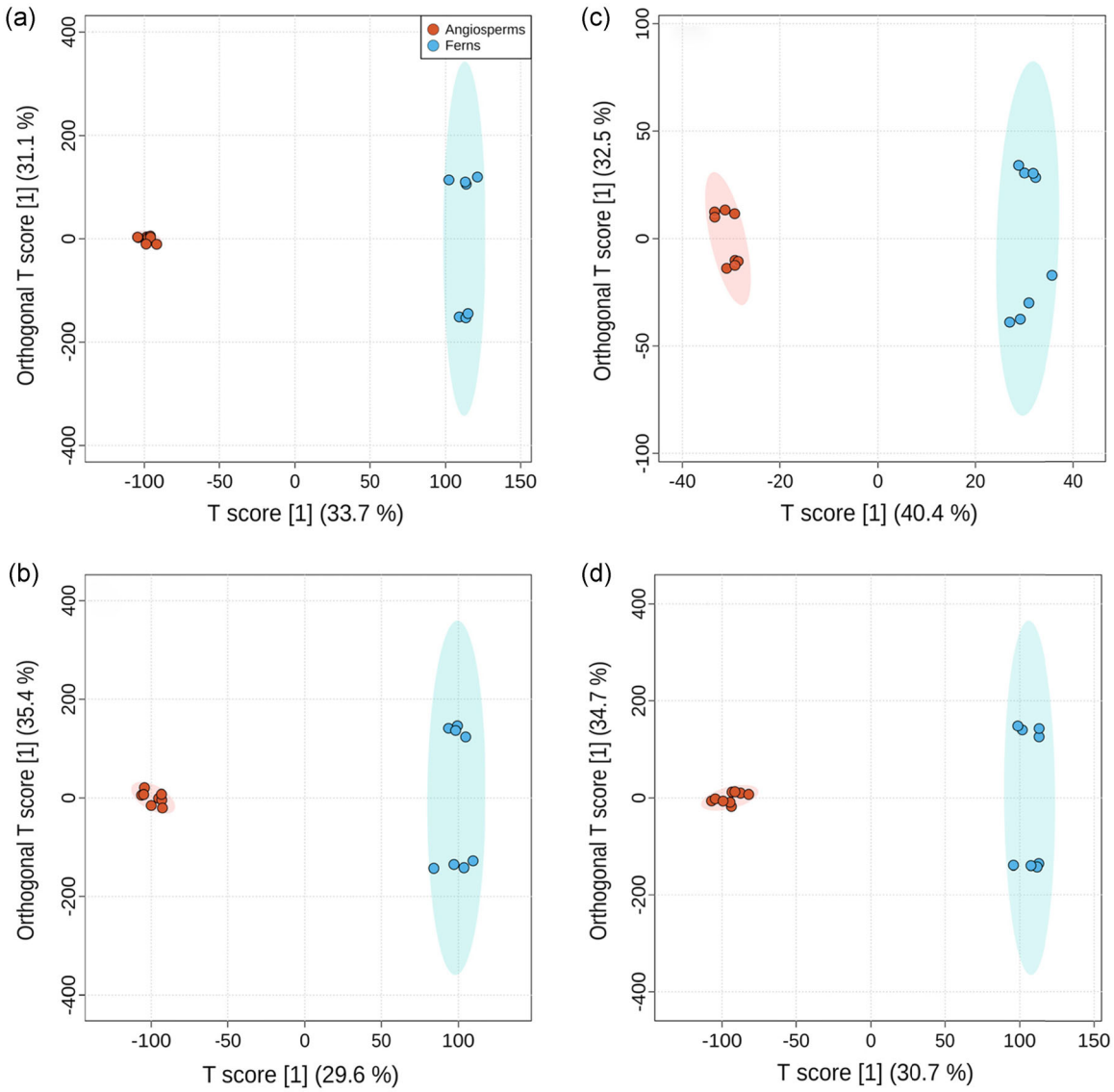

FIGURE 7 Orthogonal partial least squares discriminant analysis using liquid chromatography coupled to mass spectrometry-based metabolic fingerprinting data from leaf samples harvested at (a) 05:00 and (b) 08:00 h, (c) 14:00 and (d) 17:00 h of the diel course. The percentage variation explained by the components 1 and 2 is highlighted between parentheses in each axis of each figure. Light blue and light red colours refer to data from ferns and angiosperms, respectively. These analyses were carried out using Metaboanalyst (Chong et al., 2018) [Color figure can be viewed at wileyonlinelibrary.com]

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_2 concentration with no or at least reduced ABA sensitivity.

4.2 | 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; Gong et al., 2021; Harris et al., 2020; Sussmilch et al., 2019), 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 (Daloso, dos Anjos et al., 2016; Flütsch & Santelia, 2021; Granot & Kelly, 2019; Talbott & Zeiger, 1998). This suggests that the

higher photosynthetic capacity of angiosperms (Gago et al., 2019; Tosens et al., 2016), which results in up to 2.2-fold higher sucrose production in these plants during the dark-to-light transition (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 (V_{max}) 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–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* (Figure 2b,c). However, the speed of sucrose-induced stomatal closure is slightly higher than the mannitol treatment in all species. The higher speed of sucrose-induced stomatal closure 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* (Figure S2h,i), and the earlier plateau and the maximum velocity reached in *V. unguiculata* under sucrose (Figure S4h,i), when compared to the mannitol treatment within each species. These results suggest that stomata from ferns and especially from *V. unguiculata* exhibit both osmotic and non-osmotic responses. However, the difference between the speed of stomatal closure induced by sucrose and mannitol is low in ferns. Furthermore, it has been shown that the stomatal closure induced during high-to-low light transition is abolished in seed plants when the stomata are isolated from the mesophyll cells, and this was not observed in fern and lycophyte species (McAdam & Brodribb, 2012a). This suggests that fern and lycophyte stomata are not able to respond to mesophyll-derived signals that regulate stomatal closure. The transport of signals from mesophyll to guard cells has long been pointed as an important mechanism for the regulation of stomatal movements and WUE in angiosperms (Fujita et al., 2019, 2013; Lawson et al., 2014; Lee & Bowling, 1992, 1993, 1995; Mott, 2009). Thus, these results collectively suggest that an osmotic-mediated mechanism is the major driver of stomatal closure in ferns and that the metabolism of guard cell may be key to regulate this process.

It has been proposed that mesophyll-derived sucrose induces stomatal closure in angiosperms 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., 1997, 1995; Kang, Outlaw, Andersen et al., 2007; Kang, Outlaw, Fiore et al., 2007); 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., 2019, 2013; Lugassi et al., 2015). However, recent evidence highlights that the fern *Matteuccia struthiopteris* and the lycophyte

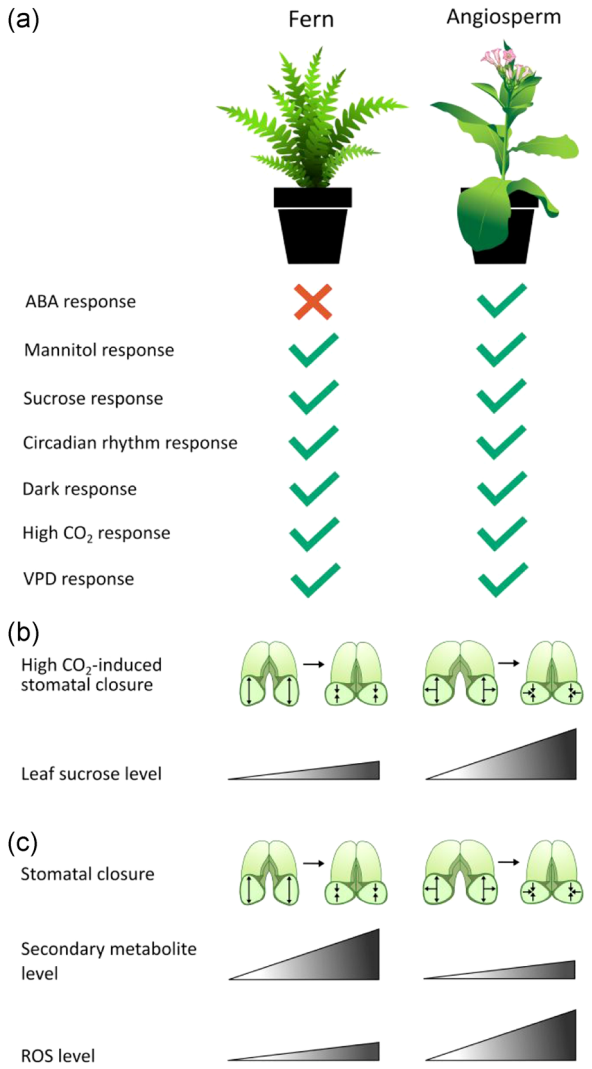

FIGURE 8 Schematic representation of stomatal responses and the mechanisms that differentially regulate stomatal speediness among ferns and angiosperms. (a) Stomatal responses of ferns and angiosperms to exogenous application of abscisic acid (ABA), mannitol, and sucrose, to the circadian rhythm, during light-to-dark transition, during ambient (400 ppm) to high (800 ppm) CO_2 and to vapour pressure deficit (VPD). Ferns and angiosperms respond to these compounds and environmental cues (√), with exception of ferns that did not respond to endogenous ABA (X). (b) Changes in leaf sucrose level during high CO_2-induced stomatal closure. The faster high CO_2-induced stomatal closure found in angiosperms was associated to a greater capacity to produce sucrose (Lima et al., 2019), as compared to ferns. (c) Our results highlight that ferns have a higher accumulation of secondary metabolites than angiosperms throughout the diel course. We hypothesize that the higher level of secondary metabolites leads to a lower level of reactive oxygen species (ROS) in fern guard cells. This idea is strongly supported by results showing that plants with a higher accumulation of secondary metabolites, such as flavonols, have lower level of ROS in their guard cells (Watkins et al., 2014) and that, in fact, ferns have a lower level of ROS in their guard cells than angiosperms. The (b) and (c) highlight the differences in the mechanics of stomatal closure between ferns and angiosperms, in which a lateral movement of guard cell walls is only observed in angiosperms (highlighted here by the arrows indicating the direction in which the guard cell wall moves during stomatal closure) [Color figure can be viewed at wileyonlinelibrary.com]

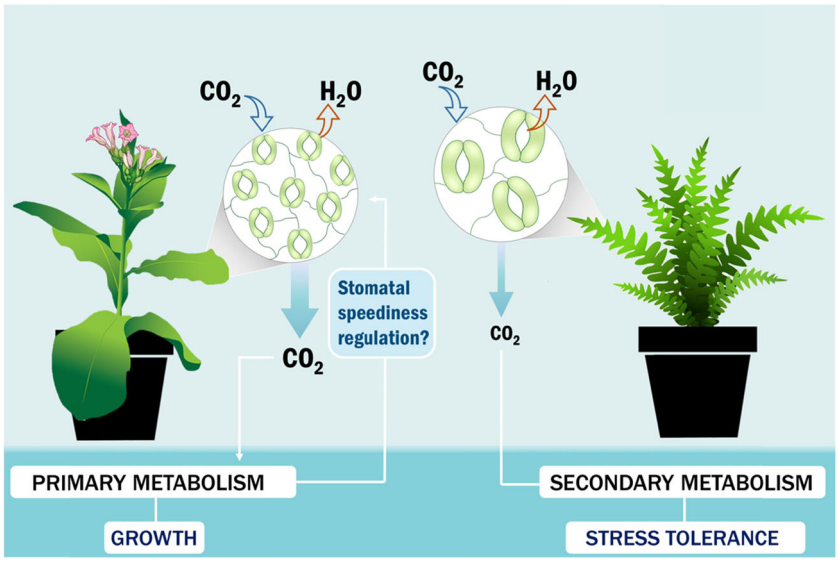

FIGURE 9 Schematic representation of the differential carbon allocation between ferns and angiosperms and its implication for the regulation of stomatal movement, growth and stress tolerance. Our metabolomics analyses suggest that angiosperms and ferns have a preferential carbon allocation toward primary and secondary metabolisms, respectively. Angiosperms have higher stomatal density and higher stomatal conductance (g_s) than ferns, which enable the entrance of more CO_2, providing more carbon for photosynthesis. Higher photosynthetic rate and a greater investment in primary metabolites favour plant growth in angiosperms. Given the faster stomatal responses observed in angiosperms, as compared to ferns, we postulate that a preferential accumulation of primary metabolites is an important mechanism to regulate stomatal speediness. By contrast, ferns have much lower g_s, higher water-use efficiency and higher accumulation of secondary metabolites, which is seemly associated to an improved stress tolerance [Color figure can be viewed at wileyonlinelibrary.com]

Selaginella moellendorffii lack ABA-responsiveness due to a disruption in the ABA signalling pathway (Gong et al., 2021). This study has demonstrated that these species have a lower level of ROS and exhibits no increases in both nitric oxide and Ca^{+2} in their guard cells following provision of ABA (Gong et al., 2021). Therefore, while 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 also observed in ferns, it seems likely that the sucrose-induced stomatal closure do not involves the ABA pathway in ferns.

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 a higher accumulation of secondary metabolites have reduced levels of ROS in their guard cells (Watkins et al., 2014), which is associated with the capacity of some of these metabolites in removing ROS (Delfin et al., 2019; Watkins et al., 2017). It is thus reasonable to hypothesize that the higher allocation toward the secondary metabolism observed in ferns would lead to a 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 lead to both lower g_s throughout the diel course and slower stomatal closure responses in ferns, when compared to angiosperms.

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

To better understand the metabolic differences between ferns and angiosperms, we performed an unprecedented LC-MS analysis of fern 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 the 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 a 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 defence against (a)biotic stresses (Austen et al., 2019; B. Li et al., 2021; Martins et al., 2014; Tohge et al., 2016) and the evidence indicating that ferns have greater tolerance to different stress conditions when compared to angiosperms (Proctor & Tuba, 2002; 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 the expense of slower stomatal responses and reduced growth (Figure 9). These results contribute to answering the elusive question as to why the growth of ferns is slow and shed light on the challenge that plant breeding programs face to produce stress-tolerant genotypes in the absence of a major yield penalty. In this vein, ferns represent an important genetic resource to improve crops resilience (Rathinasabapathi, 2006; Shukla et al., 2016). However, although the regulation of metabolic pathways of the secondary metabolism is relatively well-described in model plants such as *Arabidopsis* (Delfin et al., 2019; Saito et al., 2013; Tohge et al., 2016), it remains unclear what regulates the metabolic fluxes toward primary or secondary metabolisms and how the synthesis of important compounds for plant development and human nutrition such as flavonols are regulated in nonvascular and early vascular plants such as lycophytes and ferns (Tohge et al., 2013). Further metabolomics studies aiming to unveil the vast chemical diversity of ferns are important to improve our understanding concerning the regulation of both A–g_s and growth-stress tolerance trade-offs as well as may provide important information for plant metabolic engineer.

ACKNOWLEDGEMENTS

This study was made possible through financial support from the National Council for Scientific and Technological Development (CNPq, Grant No. 428192/2018-1). The authors also thank the research fellowship granted by CNPq to Danilo M. Daloso and the scholarships granted by CNPq to Francisco B. S. Freire and the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES, Finance Code 001) to Silvio A. Cândido-Sobrinho and Valéria F. Lima. Jorge Gago want to thank the project PID2019-107434GA-I00 'POLYSTRESS' funded by MCIN/AEI/10.13039/501100011033 and by the 'European Union NextGenerationEU/PRTR'.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its Supporting Information Materials. Please contact the corresponding author to obtain raw data from liquid chromatography–mass spectrometry analysis.

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How to cite this article: Cândido-Sobrinho, S.A., Lima, V.F., Freire, F.B.S., de Souza, L.P., Gago, J., Fernie, A.R. et al. (2022). Metabolism-mediated mechanisms underpin the differential stomatal speediness regulation among ferns and angiosperms. *Plant, Cell & Environment*, 45, 296–311. https://doi.org/10.1111/pce.14232