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VALÉRIA FREITAS LIMA

NEW INSIGHTS INTO THE REGULATION OF STOMATAL MOVEMENTS OBTAINED BY METABOLOMICS AND PHYSIOLOGICAL ANALYSES

FORTALEZA

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Dissertação apresentada ao Curso de Mestrado em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Bioquímica. Área de Concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Danilo de Menezes Daloso.

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BANCA EXAMINADORA

_____

Prof. Dr. Danilo de Menezes Daloso (Orientador)
Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Humberto de Henrique Carvalho
Universidade Federal do Ceará (UFC)

_____

Dra. Ana Karla Moreira Lobo
Universidade Federal do Ceará (UFC)

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes.”

(Marthin Luther King)

RESUMO

Estômatos são estruturas epidérmicas foliares constituídos de duas células-guarda (CG) que envolvem um poro, cuja abertura é ativamente regulada através de mudanças no acúmulo de diferentes osmólitos nessas células. A abertura e o fechamento deste poro regulam o influxo de CO_2 atmosférico para a fotossíntese, bem como o efluxo de água via transpiração. Durante a abertura estomática, há um aumento no volume das CG dirigido pela entrada e/ou geração intracelular de solutos, como potássio (K^+) e malato ($malato^{2-}$), que reduzem o potencial osmótico dessas células, criando, assim, um gradiente favorável para o influxo de água e a consequente abertura estomática. A teoria acerca da osmoregulação de CG sugere que o acúmulo de sacarose teria um papel osmorregulatório no simplasto destas células. No entanto, resultados recentes sugerem sacarose é degradada dentro das células-guarda durante abertura estomática induzida pela luz. Ademais, sabe-se que diferentes metabólitos oriundos das células mesofílicas, principalmente sacarose e malato, podem induzir mudanças na abertura estomática. Contudo, os mecanismos pelos o quais esses metabólitos influenciam os movimentos estomáticos permanecem obscuros. Além disso, não há um consenso a respeito da importância desses solutos orgânicos na regulação dos movimentos estomáticos, sobretudo em linhagens de plantas vasculares primitivas, como as samambaias. Portanto, este estudo teve o objetivo de investigar o papel da sacarose e malato nos processos de abertura e fechamento estomático. Neste contexto, o primeiro capítulo envolve uma atualização acerca dos múltiplos papéis da sacarose na regulação das CGs, destacando descobertas recentes a partir de estudos de metabolômica e biologia de sistemas. O segundo capítulo envolve um estudo do papel de metabólitos foliares, sobretudo, da relação sacarose/malato em samambaias e angiospermas frente a variações de luz e concentração de CO_2 atmosférica.

Palavras-chave: Células-guarda. Malato. Regulação estomática. Sacarose.

ABSTRACT

Stomata are leaf epidermal structures consisting of two guard cells (GC) that surround a pore, in which the opening is actively regulated by changes in the accumulation of different osmolytes in GCs. The opening and closure of this pore regulate the atmospheric CO_2 influx for photosynthesis as well as the water loss *via* transpiration. During stomatal opening, there is an increase in the volume of GCs caused by the intracellular entry and/or generation of solutes, such as potassium (K^+) and malate ($malate^{2-}$), which reduce the osmotic potential of these cells, creating a gradient favorable for the water influx and consequently, stomatal opening. The initial theory regarding GC osmoregulation suggested that the accumulation of sucrose had an osmoregulatory role in the symplast of GCs. However, recent results suggest that sucrose is degraded within guard cells during light-induced stomatal opening. In addition, it is also known that different metabolites from mesophyll cells, especially sucrose and malate, can induce changes in the stomatal opening. However, the mechanisms by which these metabolites influence stomatal movement remains unclear. Hence there is no consensus regarding the importance of these organic solutes in the regulation of stomatal movement, especially in primitive vascular plant groups such as ferns. Thus, this study aimed to investigate the role of the sucrose and malate in the stomatal opening and closure. In this context, the first chapter involves an update covering the multiple roles of sucrose in guard cell regulation, highlighting recent findings from metabolomic and systems biology studies. The second chapter involves an investigation of the role of leaf metabolites, specially, sucrose/malate ratio in responses to changes in light and atmospheric CO_2 concentration in ferns and angiosperms.

Keywords: Guard cells. Malate. Stomatal regulation. Sucrose.

CONTENTS

1 INTRODUCTION

Stomata are adjustable pores on leaf surfaces surrounding by two guard cells (GC) which enables gas exchange among plants and ambient. These leaf epidermal structures respond to a wide variety of endogenous and environmental stimulus such as light, temperature, humidity, atmospheric CO_2 concentration, phytohormones and different metabolites from mesophyll cells. Beyond the responses to different environmental stimuli, it is also known that specific mesophyll-derived metabolites may induce changes in stomatal movements. The most extensively studied organic osmolytes in GCs are sucrose and malate. However, how mesophyll-derived metabolites participate in the regulation of stomatal movements is still obscure.

In addition, stomatal responses to environmental signals differ substantially between ferns and angiosperms, but the mechanisms that lead to such responses remain unclear. Here we investigated whether leaf metabolic network coordinate the differential stomatal behaviour among ferns and angiosperms species, determining whether stomatal responses to light and CO_2 is an exclusively mechanism found in angiosperms or these responses are also observed in primitive vascular plants

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2 TOWARD MULTIFACETED ROLES OF SUCROSE IN THE REGULATION OF STOMATAL MOVEMENT

Valéria F. Lima[1], David B. Medeiros[2], Letícia dos Anjos[3], Jorge Gago[4], Alisdair R. Fernie[2], Danilo M. Daloso[1*]

[1] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará. Fortaleza-CE, 60451-970, Brasil.

[2] Central metabolism group, Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm 14476, Germany.

[3] Departamento de Biologia, Universidade Federal de Lavras. Lavras-MG, 37200-000, Brasil.

[4] Research Group on Plant Biology under Mediterranean Conditions. Departament de Biologia, Universitat de les Illes Balears) / Instituto de investigaciones Agroambientales y de la Economía del Agua (INAGEA), 07122 Palma de Mallorca, Illes Balears, Spain.

*Corresponding author

Tel: +55 85 33669821

E-mail address: daloso@ufc.br

Summary

Plant atmospheric CO_2 fixation depends on the aperture of stomatal pores at the leaf epidermis. Stomatal aperture or closure is regulated by changes in the metabolism of the two surrounding guard cells, which respond directly to environmental and internal cues such as mesophyll-derived metabolites. Sucrose has been shown to play a dual role during stomatal movements. The sucrose produced in the mesophyll cells can be transported to the vicinity of the guard cells via the transpiration stream, inducing closure in periods of high photosynthetic rate. By contrast, sucrose breakdown within guard cells sustains glycolysis and glutamine biosynthesis during light-induced stomatal opening. Here, we provide an update regarding the role of sucrose in the regulation of stomatal movement highlighting recent findings from metabolic and systems biology studies. We further explore how sucrose-mediated mechanisms of stomatal movement regulation could be useful to understand evolution of stomatal physiology among different plant groups.

Keywords: Guard cells, metabolic modelling, photosynthesis, stomatal conductance, stomatal movement, sucrose.

Introduction

Stomata are complexes composed by a stomatal pore surrounded by two guard cells, which are in many cases associated to subsidiary cells of the leaf epidermis (**Fig. 1**). Stomata appeared around 400 million years ago in the Plantae kingdom (CHATER et al., 2017) and, since then, changes in their morphology and behaviour have been greatly determined by changes in the atmospheric carbon dioxide (CO_2) concentration along the geological time scale. A progressive decrease in atmospheric CO_2 concentration created pressure for the selection of plants with higher capacity for CO_2 diffusive conductance, via reduction of stomatal size and the increase in leaf stomatal density (FRANKS; BEERLING, 2009). These changes in stomatal morphology were essential for land colonization by plants and, consequently, for the establishment of the different ecosystems on Earth. This is especially due to the fact that stomatal movement (aperture and closure) enables the exchange of gases between the plant and the environment. Stomatal aperture enables plants to import CO_2 from the atmosphere and release water (H_2O) vapour from the leaves in a process actively regulated by the surrounding guard cells. It is estimated that world terrestrial vegetation fix around 440×10^{15} g of CO_2 per year and 32×10^{15} kg of H_2O are lost per year in the warm forested tropical areas by transpiration through stomatal pores (HETHERINGTON; WOODWARD, 2003). Thus, stomatal movement has a pivotal importance in the maintenance of both CO_2 and H_2O biogeochemistry cycles. These facts already demonstrate the importance of understanding the regulation of stomatal movements. Beyond that, stomatal closure has been demonstrated to be one of the main responses of plants subjected to drought, the most limiting stress factor for crop productivity, as a mechanism to avoid excessive water loss by transpiration (NIELSEN; KEASLING, 2016). Therefore, understanding the particularities of guard cell metabolism and how these cells regulates stomatal movement will significantly contribute to better comprehend both ecosystem dynamics and the development of drought tolerant crop species (GAGO et al., 2014).

Stomatal movement is a result of changes in cell osmotic potential, which is tightly regulated by ions transport through guard cell plasma membrane, specially of potassium (K^+), nitrate (NO_3^-), and chloride (Cl^-) (LAWSON et al., 2014). Stomata respond to several endogenous signals, for instance, to phytohormones, mesophyll-derived metabolites, hydrogen peroxide (H_2O_2) and nitric oxide (NO), as well as to environmental signals such as light, air relative humidity, CO_2 concentration and presence of pathogens. Light-induced stomatal responses connect stomatal movement with mesophyll photosynthetic activity. Light induces

proton (H^+) efflux from guard cells, which is then used for the influx of ions and H_2O into guard cells, leading to stomatal opening and, ultimately, to the diffusion of CO_2 into the leaf. In parallel, light also enables the photosynthetic activity, prompting the fixation of CO_2 into sucrose molecules, notably in mesophyll cells (INOUE; KINOSHITA, 2017). Most of the excess sucrose produced in mesophyll cells is loaded into sieve elements and transported to sink tissues and cells via the phloem. However, part of the mesophyll-produced sucrose reaches the guard cell apoplastic space *via* the transpiration stream (**Fig. 2**) (KANG et al., 2007a, 2007b). Sucrose has long been thought to have an osmolytic role in the regulation of stomatal opening. However, recent findings, reviewed below, indicate that sucrose has a multifaceted role in guard cells, which includes the regulation of both stomatal opening and closure and of the photosynthesis (A)/stomatal conductance (g_s) trade-off.

Despite decades of research, several questions regarding the source and the function of sucrose in guard cells surprisingly remain unanswered. For instance, it is still questionable whether sucrose is capable to induce stomatal opening and even whether guard cells themselves can produce enough sucrose through the CO_2 fixation pathway mediated by ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) to support the regulation of stomatal movement. Furthermore, although the mechanisms by which light and K^+ induce stomatal opening have been well-documented, a potential role of guard cell sucrose during light and K^+-induced stomatal opening remains still obscure. A combination of different metabolomic, reverse genetic, and systems biology approaches were recently applied to provide insights into some of these unsolved questions. Most of the recent achievements is reviewed elsewhere (DALOSO et al., 2017; DALOSO; DOS ANJOS; FERNIE, 2016; SANTELIA; LAWSON, 2016). Here, we provide an update in this field based on the results obtained from the most recent published works. We highlight recent metabolic and systems biology studies that have improved our understanding of guard cell sucrose metabolism and that have contributed to solve the controversy story regarding the role of sucrose in the regulation of stomatal movement. We further explore how sucrose-mediated mechanisms can be used to understand the regulation of stomatal movement between the two distinct evolutionary groups of ferns and angiosperms.

Guard cell sucrose is a substrate during light-induced stomatal opening

Sucrose has been pointed out as an important metabolite for the regulation of both stomatal opening and closure. The sucrose-induced stomatal closure hypothesis suggests that mesophyll-derived and transpiration-linked sucrose would be a signal to induce stomatal

closure by its accumulation in the guard cell apoplastic space (**Fig. 2**) (KANG et al., 2007a). By contrast, recent evidence has indicated that exogenous sucrose induces stomatal closure *via* a hexokinase-dependent and ABA-mediated process within guard cells rather than via its osmolytic effect at the apoplastic space (KELLY et al., 2013; LUGASSI et al., 2015) (**Fig. 2**). Despite the controversy among the theories presented in these works, most of them suggest that mesophyll-derived sucrose tightly connects photosynthesis and stomatal movement, such that high photosynthetic rate (*A*) and sucrose accumulation would reduce g_s, most likely to decrease transpiration in C-replete conditions. It therefore seems likely that sucrose is an important metabolite for the induction of stomatal closure. However, a role of sucrose during stomatal opening has also been postulated.

Initial studies revealed that the rate of stomatal aperture is positively associated with sucrose content in guard cells in a diel course (AMODEO; TALBOTT; ZEIGER, 1996; TALBOTT; ZEIGER, 1996). This leads to the idea that sucrose may act as an osmolyte within guard cells for the maintenance of the stomatal opening during the afternoon period (TALBOTT; ZEIGER, 1998). However, they did not directly test the role of sucrose during stomatal opening, thus leaving still opened three simple questions. First, can sucrose induce stomatal opening? Second, is sucrose associated with K^+ and light-induced stomatal opening? Does it act as an osmolyte or as a substrate during light-induced stomatal opening? Aiming to address these questions, Medeiros et al. Recently investigated the effect of different concentrations of sucrose on Arabidopsis stomatal aperture in the light (MEDEIROS et al., 2018). Their results showed that (i) sucrose cannot induce stomatal opening *per se*, (ii) the presence of sucrose does not increase K^+ and light-induced stomatal opening rate, at least in the range of sucrose concentration tested (0.1 to 100 mm), and (iii) sucrose induces stomatal closure only at high concentrations (>10 mm), with higher impact at 100 mm. These results provide strong evidence indicating that sucrose does not act as an osmolyte during light-induced stomatal opening. Previous results have supported the idea that sucrose is not an osmolyte, but rather it is degraded within guard cells during K^+ and light-induced stomatal opening (DALOSO et al., 2016). However, only a missing experiment using ^{13}C-labelled sucrose could confirm the degradation of sucrose within guard cells as well as to indicate the fate of the C released from sucrose breakdown under this condition. Thus, in order to gain better insights into this issue, Medeiros et al. Used a method which combined stomatal opening assays with metabolomic analysis (DALOSO et al., 2015a) to investigate the fate of the C released by sucrose within guard cells during dark-to-light transition. The results showed a fast and high ^{13}C-enrichment in fructose and glucose during K^+ and light-induced stomatal opening. High

^{13}C-enrichment in glutamine (Gln) was also observed (MEDEIROS et al., 2018), suggesting activation of glycolysis and of the tricarboxylic acid (TCA) cycle in the light (**Fig. 3**). The activation of the TCA cycle and Gln metabolism has been constantly observed in different guard cell ^{13}C-kinetic labelling experiments (DALOSO et al., 2015a, 2016). Nevertheless, the biological meaning behind such phenomenon remains unclear. In the next section, we highlight the differences in light regulation of sucrose metabolism between mesophyll and guard cells and discuss potential interpretations for the observed flux of C from sucrose breakdown to sustain glycolysis and Gln biosynthesis in guard cells during light-induced stomatal opening.

Light regulation of sucrose metabolism differs between mesophyll and guard cells

Light is the environmental source of energy for the photosynthetic process that takes place in the chloroplasts. Mitochondria are other important organelles that produce energetic molecules such as adenosine triphosphate (ATP) but in this case through the process of respiration in a light-independent manner (DEL-SAZ et al., 2017). In C3 leaves, the C fixed by rubisco is mainly used to starch or sucrose synthesis for C storage or export, respectively, rather than to sustain glycolysis and mitochondrial metabolism (SZECOWKA et al., 2013). Indeed, recent results from a ^{13}C-nuclear magnetic resonance-based metabolic flux study indicate that leaf glutamate (Glu) synthesis does not depend on the C derived from rubisco-mediated CO_2 fixation (ABADIE et al., 2017). Instead, Glu synthesis seems to depend on one C fixed by phospho*enol*pyruvate carboxylase (pepc) enzyme and on previous stored carbons, most likely on vacuolar citrate nocturnal storages (CHEUNG et al., 2014). By contrast to mesophyll cells, it was shown that guard cells have a remarkable accumulation of fructose-2,6-bisphosphate in the light and that this accumulation leads to the activation of the glycolytic enzyme pyrophosphate-dependent phosphofructokinase (PFP) (**Fig. 3**) (HEDRICH; RASCHKE; STITT, 1985). Furthermore, Medeiros et al. Have recently showed that the ^{13}C released from [U-^{13}C]-sucrose was increasingly detected in Glu and Gln over time under dark-to-light transition (MEDEIROS et al., 2018). These results strongly indicate that both glycolysis and the TCA cycle are activated in the light in guard cells and suggest a putative specific role for Gln during stomatal opening, which must be addressed in future studies.

The discrepancy observed between leaves and guard cells may be due to differential light/dark regulation of key enzymes of their respective metabolic pathways. For instance, whilst leaf starch-synthesis enzymes are activated and starch degradation enzymes are inhibited in the light (STITT; ZEEMAN, 2012), guard cells synthesize starch in the beginning of the

night and degrade their starch reserves at the onset of illumination, by using different starch-enzyme isoforms (HORRER et al., 2016; SANTELIA; LUNN, 2017). Similarly, the light-inhibition of metabolic fluxes through leaf TCA cycle may be due to the fact that enzymes such as succinate dehydrogenase (*SDH*), fumarase (*FUM*), and pyruvate dehydrogenase (*PDH*) are deactivated under this condition (DALOSO et al., 2015b; TCHERKEZ et al., 2005, 2009). Although information regarding the regulation of guard cell TCA cycle enzymes is not available yet, results from ^{13}C-labelling kinetic experiments strongly indicate that this pathway is most likely not inhibited in the light (DALOSO et al., 2015a, 2016). Therefore, it remains unclear whether and which enzymes from sucrose, glycolysis, and mitochondrial metabolism are differentially regulated in guard cells compared to mesophyll cells. Biochemical characterization of guard cell TCA cycle-related enzymes may provide important insights concerning how this pathway is regulated in these cells.

Insights into leaf TCA cycle regulation was recently obtained from an elegant study that investigated the allosteric and redox regulation of the two fumarase isoforms located in the mitochondria (*FUM1*) and in the cytosol (*FUM2*) (ZUBIMENDI et al., 2018). Interestingly, this work demonstrated that the activity of both *FUM1* and *FUM2* is activated by Gln in the direction of fumarate synthesis. Although this regulation cannot yet be extrapolated to guard cells, one could hypothesize that the C derived from sucrose breakdown is directed toward Gln synthesis as a mechanism to stimulate *FUM* activity and fumarate accumulation (**Fig. 3**), an important organic acid for stomatal movement regulation (DALOSO et al., 2017). Fumarate and malate are the two main organic acids involved in the regulation of stomatal movement (ARAÚJO; NUNES-NESI; FERNIE, 2011; FERNIE; MARTINOIA, 2009). Similar to sucrose, they have also been pointed out as important metabolites for both stomatal opening and closure (ARAÚJO et al., 2011; MEDEIROS et al., 2016, 2017). Whilst the accumulation of these metabolites at the apoplastic space of guard cells would be a mechanism to regulate anion efflux channels (DE ANGELI et al., 2013; HEDRICH; MARTEN, 1993; MEYER et al., 2010), their accumulation within guard cell vacuoles act as counter-ion of K^+ during light-induced stomatal opening. Thus, Gln-mediated *FUM1* and *FUM2* activation could be a mechanism to increase the amount of fumarate to act as counter ion of K^+ (**Fig. 3**). Indeed, metabolite profiling results from Medeiros et al. Showed higher increase in the relative content of fumarate (~7 times increased) compared to malate (~2 times increased) after 60 min in the light (MEDEIROS et al., 2018), strengthening the idea of *FUM* activation preferentially in the direction of fumarate synthesis under this condition. Additional experiments aiming to confirm this hypothesis assumes paramount importance to understand how and why sucrose breakdown provides

substrates for the TCA cycle and Gln synthesis in guard cells. The use of systems biology approaches in guard cells will certainly contribute to resolve this query and to improve our understanding concerning the source and the function of sucrose in guard cell metabolism.

Systems biology approach unravels particular features of guard cell sucrose metabolism

Systems biology approaches have been shown to be of pivotal importance to understand the dynamic and regulation of metabolic networks (NIKOLOSKI; PEREZ-STOREY; SWEETLOVE, 2015). Guard cell has been shown to be a great model system to study and modelling abscisic acid (ABA) signalling pathway and plasma membrane ion transport (CHEN et al., 2012; HILLS et al., 2012; WANG; HILLS; BLATT, 2014). However, the complexity ruling the metabolism of this highly specialized cell type have hampered the integration of metabolomic data for the development of metabolic models (MEDEIROS et al., 2015). This gap was partially filled by a recent study that combined genome scale metabolic modelling and ^{13}C-labelling kinetic analysis in both mesophyll and guard cells (ROBAINA-ESTÉVEZ et al., 2017). This work took advantage of the aracore model (ARNOLD; NIKOLOSKI, 2014) created for *Arabidopsis thaliana* L. and of transcriptomic data from mesophyll and guard cells to establish the aracore reduced model (aracorered) that predicts differential metabolic fluxes between these two cell types. The model predicted higher metabolic fluxes through a futile cycle around sucrose composed by enzymes such as sucrose synthase, invertase, hexokinase, phosphoglucomutase, and UDP-glucosepyrophosphorylase in guard cells, as compared to mesophyll cells (ROBAINA-ESTÉVEZ et al., 2017). Futile cycles are a set of enzymatic reactions in which the energetic cost is zero or near to it. Sucrose futile cycles have been widely reported in plant tissues (ALONSO et al., 2005). The formation of such cycle in guard cells could represent a strategic mechanism to avoid starch accumulation during light-induced stomatal opening, given that this metabolite does not have any osmolytic effect and its synthesis requires a number of sequential enzymatic reactions to produce glucose moieties. In this scenario, the C would circulate around sucrose and be readily available for glycolysis when necessary. Although this prediction suggests exciting mechanistic hypothesis for carbon flux within guard cell, it still has to be experimentally tested.

The source of sucrose within guard cells has long been a subject of debate. Initial evidence suggested that guard cells did not possess rubisco and, thus, they could not produce sucrose by the photosynthetic C reduction pathway (OUTLAW et al., 1979). However, recent proteomic studies have frequently detected the presence of rubisco in guard cells (ZHAO et al., 2008; ZHU et al., 2009) and rubisco-mediated CO_2 fixation has been confirmed by ^{13}C-isotopic

experiment (DALOSO et al., 2015a). Moreover, the aracorered model predicts that sucrose can be synthesized by the photosynthetic C reduction pathway, although at lower rates than mesophyll cells (ROBAINA-ESTÉVEZ et al., 2017). This result corroborates the lower levels of chlorophyll and rubisco found in guard cells (RECKMANN; SCHEIBE; RASCHKE, 1990). Nevertheless, further studies are required to quantify the magnitude by which sucrose synthesis is supported by C derived from guard cell photosynthetic and gluconeogenic pathways as well as to determine the contribution of mesophyll cells to the overall guard cell sucrose content. This information will help to unravel the relative importance of the different sources of sucrose to guard cell metabolism. Beyond the gaps in our knowledge regarding the sources and exact functions of sucrose in guard cell metabolism and in stomatal movement regulation, it also remains unclear whether these mechanisms are conservative in different plant groups.

Are the sucrose-mediated mechanisms controlling stomatal movement conserved across plant evolution?

Current theories postulate that stomata firstly appeared in the group of bryophytes around 400 million years ago and that a transition from passive to active control of stomatal movement emerged after the divergence of vascular plants, at about 360 million years ago (BRODRIBB; MCADAM, 2011; RUSZALA et al., 2011). Although several controversies exist regarding when the active control of stomatal movement appeared during evolution, it is clear that stomatal morphology and physiology differ substantially between ferns and angiosperms. For instance, recent evidence suggests that fern guard cells are connected to subsidiary or epidermal neighbouring cells by functional plasmodesmata, whilst seed plants such a cytoplasmic connection is absent (VOSS et al., 2018). Differences in the capacity and velocity of stomatal responses to different stimulus such as ABA, CO_2 and light/dark shifts are also found across plant evolutionary groups and are a subject of intensive debate (BRODRIBB; MCADAM, 2017a). Whilst some studies suggest that stomata from ferns are insensitive to vapour pressure deficit (VPD) and ABA (BRODRIBB; MCADAM, 2011; MCADAM; BRODRIBB, 2012a), others suggest that fern stomata do respond to these stimulus (CREESE et al., 2014; HÕRAK; KOLLIST; MERILO, 2017). Recent studies indicate that stomata of primary species also responds to dark, ABA and changes in CO_2 concentration (CREESE et al., 2014; HÕRAK; KOLLIST; MERILO, 2017; MCADAM; BRODRIBB, 2012a), though the responses are much slower in ferns compared to angiosperms and range according to species and growth condition (FRANKS; BRITTON-HARPER, 2016; HÕRAK; KOLLIST; MERILO, 2017). Despite these controversies, it is clear that ferns have remarkable lower photosynthetic

rate (*A*) compared to angiosperms (GAGO et al., 2014; TOSENS et al., 2016a). This suggests that the production of photosynthesis-derived metabolites such as sucrose is lower in ferns, which raises the question whether the differences in *A* and in sucrose production may explain, at least in part, the contrasting behaviour of stomatal responses between ferns and angiosperms. Assuming that mesophyll-derived sucrose can induce stomatal closure (DALOSO; DOS ANJOS; FERNIE, 2016; GAGO et al., 2016; KANG et al., 2007a; KELLY et al., 2013), it is reasonable to hypothesize that angiosperms have faster control of high CO_2-induced stomatal closure (FRANKS; BRITTON-HARPER, 2016) because of their higher capacity to produce and transport sucrose and other metabolites from mesophyll to guard cells. This hypothesis resembles Mott's idea in which stomatal responses to light and CO_2 depends on signals from mesophyll cells (MOTT, 2009; MOTT; SIBBERNSEN; SHOPE, 2008). Although the identity of these signals remain to be determined, several studies have suggested that sugars and organic acids have an important role to the regulation of A-g_s trade-off (ARAÚJO et al., 2011; GAGO et al., 2016; NUNES-NESI et al., 2007). Further experiments using species from different groups are however needed to experimentally unravel whether the multifaceted role of sucrose in stomatal movement regulation is also present in primitive vascular plant groups.

Concluding remarks

Guard cells have the capacity to produce their own sucrose. However, given the low photosynthetic rate and the high expression and activity of sink-markers found in guard cells (BATES et al., 2012; DALOSO et al., 2015a; GOTOW; TAYLOR; ZEIGER, 1988), it seems that guard cells resemble sink rather than source cells. This idea is strengthened by a recent study which showed that reductions in the import of sucrose into guard cells leads to reduced g_s (ANTUNES et al., 2017), highlighting that mesophyll-derived sucrose is needed for guard cell functioning. It has been shown that sucrose has a dual role in the regulation of stomatal movement. During light-induced stomatal opening, sucrose seems to have an energetic role, in which its degradation stimulates C flux through glycolysis, the TCA cycle, and Gln biosynthesis. On the other hand, photosynthesis-derived sucrose seems to be also important to induce stomatal closure. In this scenario, sucrose acts as a metabolic link between mesophyll photosynthesis and stomatal movement, potentially as an apoplastic osmolyte and/or as a signal metabolite within guard cell that induces stomatal closure via ABA (**Fig. 3**). Despite the important advances obtained in this field in the last decades, several outstanding questions regarding the source of sucrose for guard cells and the role of sucrose during stomatal opening

and closure remain to be explored, deserving special attention in the near future. Reverse genetic approaches using guard cell specific promoters (KELLY et al., 2017; YANG et al., 2008) combined with physiological and metabolic flux analysis have provided important information to the field and thus may be further extrapolated to the manipulation of other genes of guard metabolism. In parallel, the establishment of new mathematical models and the improvement of those currently in use will certainly help to unravel key mechanisms that regulate stomatal movement.

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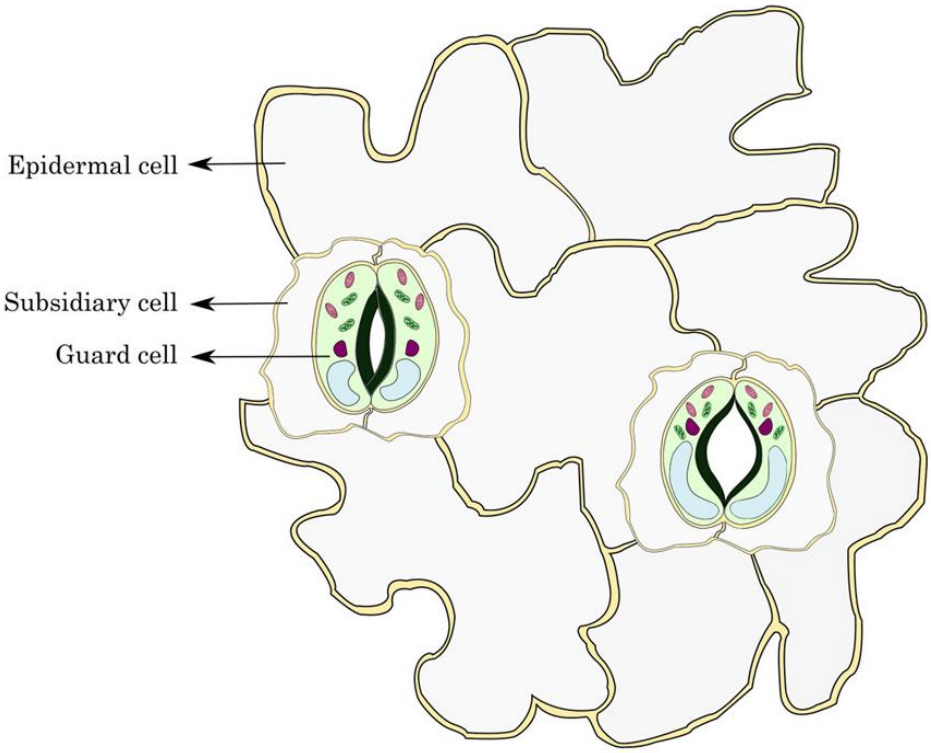

Figure 1. Schematic representation of a leaf epidermis containing epidermal cells and stomata complexes. The stomata demonstrated here are composed by two guard cells, that surround a stomatal pore, and two subsidiary cells, which may have different shapes and numbers nearby guard cells or not be present in certain species.

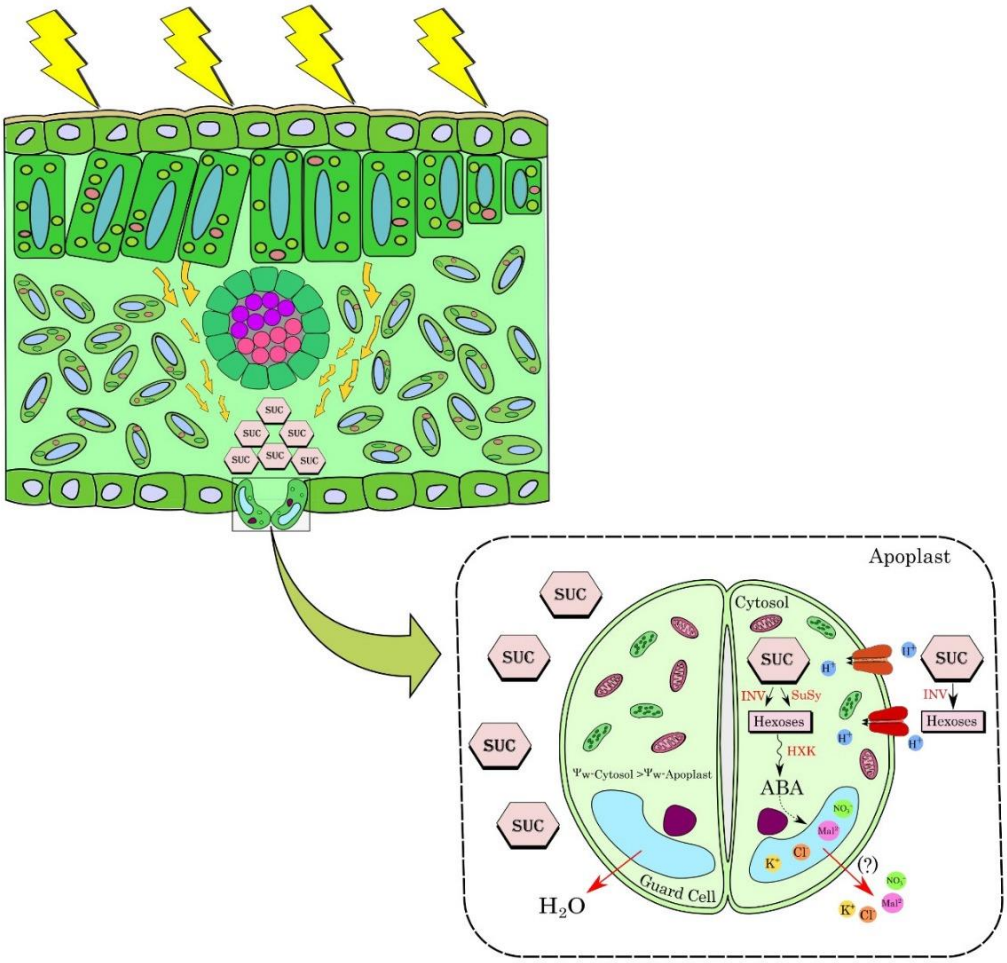

Figure 2. Sucrose-induced stomatal closure representation. The upper figure shows a leaf transversal section demonstrating how the sucrose derived from photosynthetic activity of mesophyll cells can reach stomata apoplastic space via the transpiration stream (LU et al., 1995a, 1997a). The lower figure, which is an augment of the stomata of the leaf transversal section, highlight two hypothesis regarding the mechanisms by which mesophyll-derived sucrose could induces stomatal closure. The first hypothesis, exemplified at the left guard cell of the stomata, suggest that the overaccumulation of sucrose in the apoplastic space would have an osmolytic effect in which the water potential (Ψ_w) of the apoplast would be reduced and become lower than the cytosolic Ψ_w leading to the efflux of water from guard cells and consequently stomatal closure (KANG et al., 2007b; LU et al., 1997a). Alternatively, as demonstrated in the right guard cell of the stomata, sucrose would have a non-osmolytic role during stomatal closure (KELLY et al., 2013; LUGASSI et al., 2015). This hypothesis suggest that sucrose is degraded by invertase (INV) and sucrose synthase (SuSy) within guard cells and induces stomatal closure in a mechanism dependent of hexokinase (HXK) and mediated by abscisic acid (ABA). It remains unclear whether sucrose is degraded by apoplastic INV, which enable hexose to enter guard cells through hexose transporters, as well as whether this HXK and ABA signalling pathway induces ion efflux from guard cells. Although the hypotheses of sucrose-induced stomatal closure are discussed separately, it is noteworthy that they do not exclude each other and thus can occur simultaneously. Abbreviations: Suc, sucrose. Ions in the right guard cell: K^+, potassium; Cl^-, chloride; NO_3^-, nitrate; Mal^{2-}, malate.

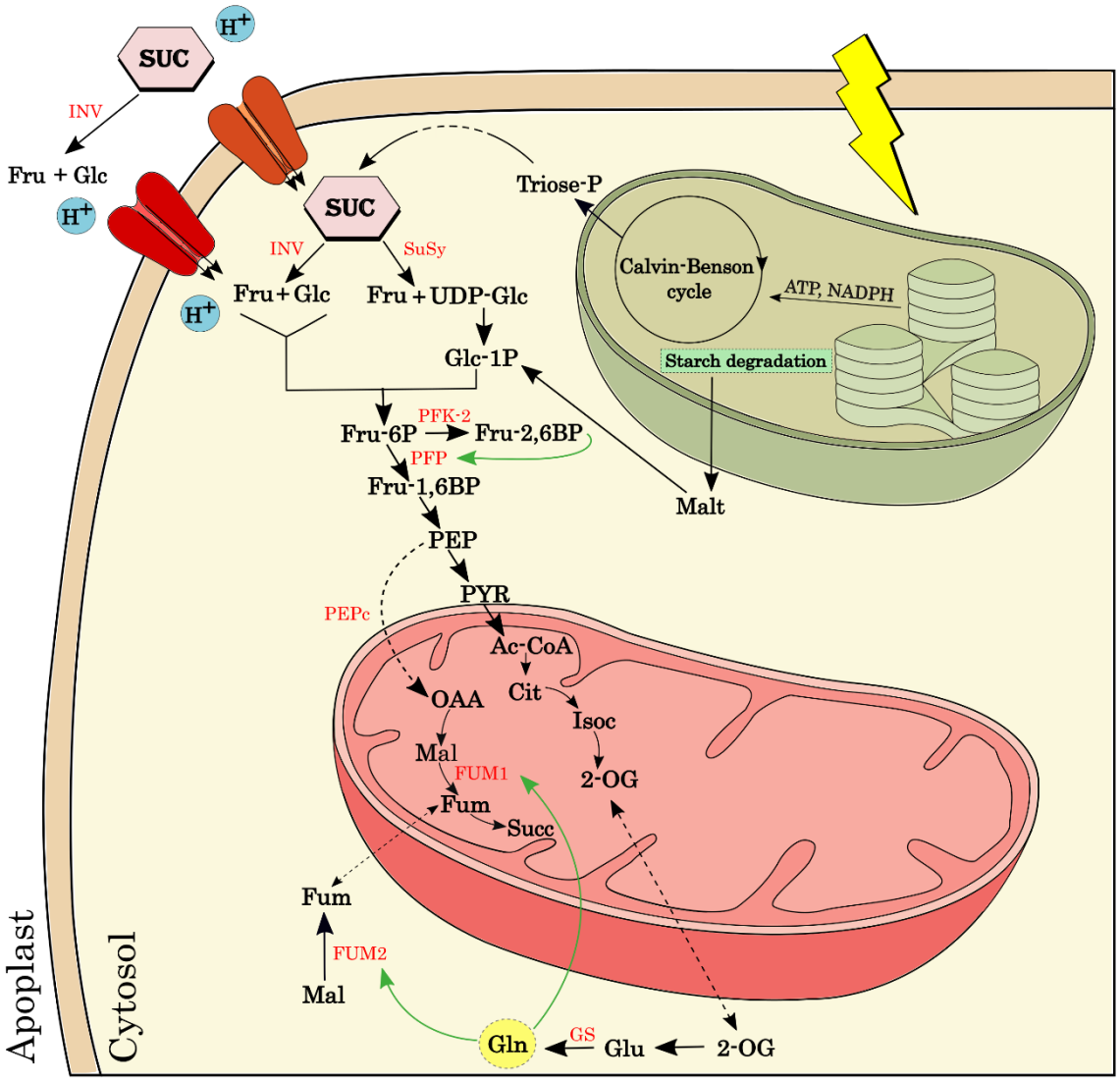

Figure 3. Illustration of metabolic changes induced by sucrose breakdown within guard cells during light-induced stomatal opening. Initial evidence showed that fructose-2,6-bisphosphate (Fru-2,6BP) accumulates in the light in guard cells and this leads to the activation of the glycolytic enzyme pyrophosphate-dependent phosphofructokinase (PFP) (HEDRICH; RASCHKE; STITT, 1985). In this scenario, glycolysis is activated in the light and use C from photosynthesis (DALOSO et al., 2015a; ROBAINA-ESTÉVEZ et al., 2017) and starch degradation (HORRER et al., 2016) within guard cells as well as by sugars imported as sucrose or hexoses from the apoplast (ANTUNES et al., 2017; RITTE et al., 1999). Recent results from labelling isotope experiment indicates that sucrose is degraded within guard cells during dark-to-light transition (MEDEIROS et al., 2018). This activates glycolysis, the tricarboxylic acid (TCA) cycle and glutamine (Gln) biosynthesis. It has been shown that Gln activate both cytosolic (FUM2) and mitochondrial (FUM1) fumarase activities in the direction of fumarate synthesis (ZUBIMENDI et al., 2018). We hypothesize that the C derived from sucrose breakdown would be directed toward Gln biosynthesis which would act as a signal to activate FUM activity and thus fumarate accumulation, a metabolite showed to accumulate in guard cells during dark-to-light transition (MEDEIROS et al., 2018). In addition, it has been shown that guard cells have higher anaplerotic CO_2 fixation catalysed by the enzyme phospho*enol*pyruvate carboxylase (PEPc) compared to mesophyll cells (DALOSO et al., 2017; ROBAINA-ESTÉVEZ et al., 2017) and that this reaction activate the left branch of the TCA cycle (DALOSO et al., 2015a). Taken together, these results suggest that left and right branches

of the TCA cycle are differentially activated depending on the source of the C, in which different non-cyclic modes of operation can be observed in this cycle. Abbreviations: 2-OG, 2-oxoglutarate; Ac-CoA, acetyl-CoA; Cit, citrate; Fru, fructose; Fru-1,6BP, fructose-*1,6-bisphosphate; Fru-6P*, fructose-6-phosphate; Fum, fumarate; Glc, glucose; Glc-1P, glucose-1-phosphate; Glu, glutamate; Isoc, isocitrate; Mal, malate; Malt, maltose; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PYR, pyruvate; Suc, sucrose; Succ, succinate; Triose-P, triose phosphate; UDP-Glc, UDP-glucose. Enzymes (in red): GS, glutamine synthetase; INV, invertase; PFK-2, phosphofructokinase-2; SuSy, sucrose synthase.

(Unpublished manuscript)

3 THE SUCROSE-TO-MALATE RATIO POSITIVELY CORRELATES WITH THE FASTER STOMATAL CLOSURE IN ANGIOSPERMS COMPARED TO FERNS

Valéria F. Lima[1], Letícia dos Anjos[2], David B. Medeiros[3], Leonardo P. Souza[3], Jorge Gago[4], Alisdair R. Fernie[3], Danilo M. Daloso[1*]

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará. Fortaleza-CE, 60451-970, Brasil.

[2]Departamento de Biologia, Setor de Fisiologia Vegetal, Universidade Federal de Lavras. Lavras-MG, 37200-000, Brasil.

[3]Central Metabolism group, Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm 14476, Germany.

[4]Research Group on Plant Biology under Mediterranean Conditions. Departament de Biologia, Universitat de les Illes Balears) / Instituto de investigaciones Agroambientales y de la Economía del Agua (INAGEA), 07122 Palma de Mallorca, Illes Balears, Spain.

*Corresponding author

Tel: +55 85 33669821

E-mail address: daloso@ufc.br

Summary

Stomata are leaf epidermal structures that respond to a wide variety of endogenous and environmental stimulus such as light, atmospheric CO_2 concentration, phytohormones and different metabolites from mesophyll cells. However, how mesophyll-derived metabolites participate in the regulation of stomatal movements remains unclear. Here we aimed to investigate the role of leaf metabolites in the stomatal opening and closure using four species, which represent a cross section of the evolution of terrestrial vascular plants, including ferns (*Microsorum scolopendria* and *Phlebodium aureum*) and angiosperms (*Vigna unguiculata* L. Walp and *Nicotiana tabacum* L.). We carried out different stomatal kinetics under dark-to-light transition and changes in CO_2 concentration and simultaneously determining leaf metabolite profile. Gas exchange parameters varied widely across species, however, ferns often showed lower absolute values of photosynthesis, stomatal conductance and transpiration rate than angiosperms. Stomata from angiosperms and ferns are responsive to changes in light or atmospheric CO_2 concentration, although both light and CO_2 stomatal responses are minor and slower in ferns. Malate was positively related to g_s while sucrose and sucrose/malate ratio were negatively related to g_s in all species, with a general tendency to increase this ratio during high CO_2-induced stomatal closure. These results suggest that sucrose/malate ratio is important to modulate g_s responses and can explain, at least partially, the difference in stomatal behaviour among ferns and angiosperms. The present study provides new important information that help to understand the mechanisms that regulate stomatal movements.

Keywords: guard cells, malate, stomatal control, stomatal evolution, sucrose.

Introduction

Stomata are adjustable pores on leaf surfaces surrounding by two guard cells (GC) which enables gas exchange among plants and atmosphere. They originated over a period of more than 400 million years, and since then, the stomata performance and morphology has been modified according to the changing environmental carbon dioxide (CO_2) conditions (FRANKS; BEERLING, 2009). Stomata evolution contributed to the adaptation of plants to a terrestrial environment, allowing the emergence of massive and widespread forests (CHATER et al., 2017; RAVEN, 2002). Stomata responds to several endogenous and environmental signals that coordinate stomatal aperture or closure (LAWSON et al., 2014). Stomatal conductance (g_s) – the rate of stomatal aperture/closure – control the balance of water loss through transpiration rate (E) and the CO_2 uptake for photosynthesis (A). Thus, stomata have a pivotal role for plants to maintain an optimum balance among transpiration and CO_2 assimilation (LIN et al., 2015). However, despite this widely recognized role, understanding the mechanisms behind it remains one of the major challenges of plant biology (BERRY; BEERLING; FRANKS, 2010; HETHERINGTON; WOODWARD, 2003)

The stomata open in response to multiple environmental influences such as blue light, red light and low concentrations of CO_2 (ROELFSEMA et al., 2002; SANTELIA; LAWSON, 2016; SHIMAZAKI et al., 2007). These environmental cues directly influence g_s *via* light and CO_2 perception by GCs and indirectly through the mesophyll photosynthetic activity. The blue light is perceived directly in GC by phototropins (KINOSHITA; DOI; SUETSUGU, 2001). After their autophosphorylation, the signaling pathway activate plasma membrane H^+-ATPases (KINOSHITA; SHIMAZAKI, 1999) which drives potassium ion (K^+) uptake with accumulation of malate^{2-}, chloride (Cl^-), and nitrate (NO_3^-) in the vacuole of GCs (SCHROEDER et al., 2001; SHIMAZAKI et al., 2007). This decreases guard cell water potential, causing water uptake, and consequently stomatal aperture. Conversely, the mechanisms by which red light induces stomatal opening are a matter of debate and the metabolic implications of the red-light perception and how mesophyll cells influence these response remains unclear (MOTT et al., 2014; SANTELIA; LAWSON, 2016).

Beyond the responses triggered by light, stomata are also sensitive to ambient CO_2 concentration (C_a) as it directly affects stomatal conductance *via* GC CO_2 perception and indirectly through mesophyll photosynthesis and the CO_2 concentration within the intercellular air space (C_i) (MOTT, 1988). Whilst elevated CO_2 induces stomatal closure (ENGINEER et al., 2016), low CO_2 promotes stomatal opening (NEGI et al., 2014). Experimental evidence

suggests that the low CO_2-induced stomatal opening is mediated by a complex crosstalk between different phytohormones (GENG et al., 2017), phototropins and HT1 (HIGH LEAF TEMPERATURE 1) (HIYAMA et al., 2017) as well as CBC 1 and 2 kinases (CONVERGENCE OF BLUE LIGHT and CO_2 1/2), which act as the convergence site for signals of blue light and low CO_2. The process of stomatal closure has also been extensively studied (HASHIMOTO et al., 2006; HU et al., 2010; TIAN et al., 2015; XUE et al., 2011). Closure of stomata is driven by anions released from GCs *via* S-type channels such as SLAC1 (SLOW ANION CHANNEL 1) and SLAH3 (SLAC1 HOMOLOGUE 3) (NEGI et al., 2008; VAHISALU et al., 2008). High CO_2 concentrations activate these anion channels as well as outward-rectifying K^+ channels (ENGINEER et al., 2016; ROELFSEMA; LEVCHENKO; HEDRICH, 2004), resulting in the membrane depolarization and thereby, stomatal closure. Indeed, a very recent study provides evidence that photoperiod length affects stomatal conductance responses to CO_2 concentration in WT and guard cell starch metabolism mutants (AZOULAY-SHEMER et al., 2018). Starch biosynthesis deficient AGPase (*ADG1*) Arabidopsis mutants under short-day growth conditions, but not starch degradation deficient *BAM1*, *BAM3* and *SEX1* mutants, were rate-limiting for CO_2-induced stomatal closure, which could indicate that, starch biosynthesis functions, during stomatal closure, as a sink for sugars and malate, thus promoting the necessary changes in GC turgor for water efflux. However, more studies are needed since the mechanisms underlying the CO_2 responses in guard cells are not fully understood.

Beyond the responses to different environmental stimuli, it is also known that specific mesophyll-derived metabolites may induce changes in stomatal movements. The most extensively studied organic osmolytes in GCs are sucrose and malate (DALOSO et al., 2017; FERNIE; MARTINOIA, 2009; LAWSON et al., 2014; MEDEIROS et al., 2016).These metabolites have been pointed out as important to modulate both stomatal opening and closure. The idea is that sucrose and malate may act as mesophyll to guard cell connectors, in which their synthesis by mesophyll photosynthetic activity and transport to guard cells may modulate g_s. It has been hypothesized that sucrose and malate overaccumulation at the apoplastic space of guard cells would be a mechanism to induce stomatal closure (OUTLAW, 2003). By contrast, malate accumulation and sucrose degradation within guard cells seems to be important mechanisms during stomatal opening (DALOSO et al., 2015a, 2016; MEDEIROS et al., 2018). Recent evidence from a multi-species meta-analysis study revealed that leaf malate and sucrose content have positive and negative correlation with g_s, respectively (Gago *et al.*, 2016).

However, the mechanisms by which mesophyll-derived malate and sucrose regulate g_s remains far from clear.

Guard cell has been proved to be a great model to understand how environmental signals are perceived and transmitted throughout the cell *via* different phytohormone and signaling pathway networks (ASSMANN; JEGLA, 2016). Furthermore, recent studies have extended the use of guard cells to understand the evolutionary origin of key components of both abscisic acid (ABA) and CO_2 signaling pathways and how they coordinate stomatal responses in plants from distant evolutionary origins such as ferns and angiosperms (BRODRIBB et al., 2009; BRODRIBB; MCADAM, 2017b; FRANKS; BRITTON-HARPER, 2016; HÕRAK; KOLLIST; MERILO, 2017; MCADAM; BRODRIBB, 2012b, 2012a; RUSZALA et al., 2011). These studies have provided important evidence concerning how plants acquired active control of stomatal responses (BRODRIBB; MCADAM, 2017b; FRANKS et al., 2017). It is clear therefore that further studies are needed to determine whether stomatal responses to light and CO_2 is an exclusively mechanism found in angiosperms or these responses are also observed in primitive vascular plants. Furthermore, it is important to highlight that no studies have investigated how mesophyll-derived metabolites combined with different external environmental stimulus influence stomatal movement, especially involving primitive vascular plants. Here, we carried out different experiments aiming to characterize stomatal and leaf metabolic responses to variations of light and CO_2 using different angiosperms and ferns species. We hypothesize that ferns do respond to light and CO_2 but in a lower velocity compared to angiosperms species, which may be related at least partially to a differential modulation of their metabolic networks.

Material and methods

Plant material and growth conditions

Two ferns and two angiosperms were evaluated in this study. The fern species *Microsorum scolopendria* (Burman) Copel. and *Phlebodium aureum* (L.) J. Sm. were obtained as adult plant from commercial suppliers and the angiosperms *Vigna unguiculata* (L.) Walp. and *Nicotiana tabacum* (L.) was propagated from seed obtained from the *Germplasm Bank* of the *Federal University* of Ceará, Fortaleza, CE, Brazil. In order to ensure seed sterilization, cowpea (*V. unguiculata*) seeds were soaked for 5 min in a 0.5% (v/v) sodium hypochlorite solution and washed five times with distilled water and germinated in 3.0 L pots containing a

substrate composed by a mixture of vermiculite, sand and vegetal soil (2:1:0.5) previously autoclaved. Tobacco (*N. tabacum*) seeds were germinated in 2.0 L pots containing the aforementioned substrate, and cultivated for 30 days. The resultant seedlings were transplanted to wider pots (3.0 L).

Plants were well watered and irrigated with Hoagland nutrient solution every week (HOAGLAND; ARNON, 1950). They were cultivated under greenhouse conditions with ambient temperature 30 ± 4 °C, relative humidity 62 ± 10% and photosynthetic photon flux density (PPFD) which reached a maximum value of 500 μmol photons m^{-2} s^{-1}, and 12 hours of photoperiod inside the greenhouse. Fully expanded leaves (1 and 2-month-old cowpea and tobacco plants, respectively) were used for photosynthetic and metabolomic analyses.

Photosynthetic light response curves

The photosynthetic analyses were performed using a portable infrared gas analyzer fitted with a 2 cm^2 leaf cuvette (LiCor 6400XT, Lincoln, NE, USA). A light response curve was measured using ten light intensities from high to low levels; in order, 2000, 1500, 1000, 800, 600, 400, 200, 100, 50 and 0 μmol photons m^{-2} s^{-1}, containing 10% blue light to optimize stomatal opening (FLEXAS et al., 2008). For these measurements, the reference CO_2 concentration was maintained in 400 ppm with block temperature at 26 °C and 1.5 kPa of vapour pressure deficit (VPD). Gas exchange was recorded after 2-3 min under each light intensity. All light response curves were measured between 8 a.m. and 12 p.m. of the daily course.

Light response curves were fitted using SIGMAPLOT 12 (Systat Software Inc., San Jose, CA, USA). A three-component exponential function equation was used to analyze the light responses: $A = a\ (1 - e^{-bx}) + c$, where A = photosynthetic rate, x = PPFD, and a, b, c are parameters estimated by the nonlinear regression (WATLING; PRESS; QUICK, 2000). Using the light response curves and the parameters from the non-linear regression mentioned above, light-saturated photosynthesis rate (A_{max}) was calculated as $a + c$ and quantum yield of photosynthesis (A_{qe}) as the initial slope at $A = 0$, calculated as $b\ (a + c)$. Dark respiration (R_d) was calculated at zero irradiance.

Stomatal responses over light transitions

The steady state leaf gas exchange parameters were measured to evaluate the stomatal response between light to dark transition (1000 to 0 μmol photons m^{-2} s^{-1}) and its subsequent

light return (0 to 1000 μmol photons m^{-2} s^{-1}). Block temperature, VPD and CO_2 concentration in the cuvette were maintained as described previously. Leaves were first left at 1000 μmol photons m^{-2} s^{-1} for a 40 min acclimation period in the cuvette, approximately, until both *A* and g_s present less than 5% changes during at least 10 min. Five minutes were recorded in this condition and after that, the light was turned off (0 μmol photons m^{-2} s^{-1}) and remained until steady state in darkness was reached. After that, light was set to 1000 μmol photons m^{-2} s^{-1} until steady state in light was reached. Data were regularly measured at 20 seconds intervals.

Each light dynamic was examined for 100 min, however the recorded time after light transitions from light to dark, and dark to light depended on the specie. The long periods of observation aimed to ensure that steady state was reached after each light transition. To ensure full hydration during the experiment, plants were irrigated with 20–30 mL deionized water, and to reduce the effect of circadian rhythms on the measurements, this dynamic was initiated no later than 8 a.m., and only two replicates were carried out per day.

Diel-course of stomatal conductance

To investigate the main metabolic factors that govern the regulation of stomatal movements throughout the day, gas exchange parameters (*A*, g_s and transpiration rate, *E*) were measured in leaves from pre-dawn until the end of the day in five different time points; in order 5 a.m., 8 a.m., 11 a.m., 2 p.m. and 5 p.m. Conditions in the cuvette were maintained as described (in *Photosynthetic light response curves*) and the light intensity was set to 1000 μmol photons m^{-2} s^{-1} (10:90% blue:red light). Subsequently, leaf discs were collected in each pre-selected time points (except at 11 a.m.) and immediately frozen to further metabolic analysis. The time point (11 a.m.) was not collected considering that the largest variations in *A* and g_s had been observed mainly at the beginning and at the end of the day.

Stomatal responses to changes in CO_2 concentration

Gas exchange was also measured following changes in atmospheric CO_2 (C_a) concentration between 200, 400 (ambient) and 800 ppm. The CO_2 concentration inside the leaf cuvette was controlled by the use of soda lime. Block temperature, VPD and light conditions were maintained as described (in *Diel-course of stomatal conductance*).

To evaluate the stomatal response over changes in C_a, leaves were subjected to a stabilization period (between 40-60 min) under controlled conditions and C_a of 400 ppm

(FRANKS; BRITTON-HARPER, 2016). Initially, gas exchange was recorded for 10 min. After that, C_a was changed to 800 ppm (high CO_2) until g_s reached steady state. Then, C_a was changed to 200 ppm (low CO_2), where g_s remained recorded until reached steady state. The time of exposure to the different concentrations of CO_2 was determined through previous tests with the selected species, and the recorded time after each CO_2 transitions also depended on the specie. All gas exchange parameters were automatically recorded every 20 seconds.

In order to investigate the metabolic changes in leaves over C_a changes, leaf discs were collected and immediately frozen after g_s reached steady state to subsequent metabolic analysis at four specific points over time: at steady state in 400 ppm ($g_{s\text{-}st400}$), 5 min after transition from 400 to 800 ppm ($g_{s\text{-}tr400\text{-}800}$), steady state in 800 ppm ($g_{s\text{-}st800}$) and 200 ppm ($g_{s\text{-}st200}$). The collected leaf material at 5 min after 400 to 800 transition ($g_{s\text{-}tr400\text{-}800}$) was intended to verify the changes which first occur at metabolites levels under high CO_2 concentration. The other time points were harvested in order to check which metabolites may rule steady state g_s under different C_a.

Quantitative analysis of gas exchange parameters and stomatal kinetics

Different parameters were determined from the gas exchange experiments performed in both CO_2 and light dynamics. The steady state g_s, A and E values were obtained by averaging the last ten points in each condition (ambient, high and low CO_2 concentrations, and for the light dynamic, light-dark-light conditions). Relative g_s, A and E in steady state were also calculated to investigate the proportional changes among the species studied here. Having established theses values, the difference observed in each parameter (absolute and relative) during the CO_2 concentration or light transitions was taken to check the changes in each parameter under stomatal dynamic variations. Furthermore, in order to verify the stomatal response speed in each specie during the imposed CO_2 or light transitions, the velocity of stomatal movements was estimated. For stomatal opening, this value was determined by the maximum slope of the linear zone after the increasing steps of g_s (800 to 200 ppm of C_a, and dark to light transitions), and for stomatal closure by the maximum slope of the linear zone after the decreasing steps of g_s (400 to 800 ppm of C_a, and light to dark transitions).

Extraction and analysis of leaf metabolites using GC-TOF-MS

Leaf discs of 2 cm^2 size were harvested at the time points indicated in each experiment described previously and immediately frozen in liquid nitrogen and stored at -80 °C. The

extraction and derivatization of polar metabolites were performed according to Lisec *et al.*, 2006. Approximately 50 mg of fresh leaf discs were grinded into powder, the frozen homogenate was shaken at 70 °C with methanol containing 30 μL of ribitol (0.2 mg mL^{-1}) as an internal quantitative standard for 15 min. The supernatant was collected after a centrifugation. The polar and apolar phases were separated by adding cold chloroform and water to the tube. After centrifugation, 150 μL of the resulting upper (polar) phase was taken and reduced to dryness for further derivatization and analyzed by gas chromatography coupled to time of flight mass spectrometry (GC-TOF-MS). Both chromatogram and mass spectral analysis were evaluated using the TagFinder program (LUEDEMANN et al., 2008) and Xcalibur™ 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA) according to Roessner-Tunali *et al.*, 2001.

Statistical analysis

All data are expressed as the mean of four or five replicates ± standard error (SE). Significant difference between means within each species was determined using one-way analysis of variance (ANOVA) and Tukey test ($P < 0.05$). Relationship between gas exchange and metabolites were examined by calculating Pearson correlation coefficients (r) and by performing linear regression analysis. These statistical analyses were carried out using SIGMAPLOT 12 (Systat Software Inc., San Jose, CA, USA) or Minitab 18 statistical software (State College, PA: Minitab, Inc.).

Results

Photosynthetic light response curves

Aiming to characterize the photosynthetic and stomatal conductance in response to increased light intensities, we first carried out photosynthetic light curves. The net photosynthetic rate (A) of angiosperms (*V. unguiculata* and *N. tabacum*) increased exponentially as PPFD increased from 0 to 400 μmol m^{-2} s^{-1}, whilst A of ferns (*M. scolopendria* and *P. aureum*) saturated near to 200 μmol m^{-2} s^{-1}, and then remained stable (Figure 1). The light response curves revealed that the maximum photosynthetic rate (A_{max}) of the angiosperms was greater than the ferns (Figure 2). Cowpea (*V. unguiculata*) and tobacco (*N. tabacum*) leaves had higher A_{max} (13.79 and 8.81 μmol m^{-2} s^{-1}, respectively), while the ferns (*M. scolopendria* and *P. aureum*) had significantly lower A_{max} (2.49 and 4.67 μmol m^{-2} s^{-1}, respectively). Dark

respiration (R_D) in angiosperms (2.70 and 2.50 μmol m^{-2} s^{-1}) was greater the ferns (1.11 and 1.13 μmol m^{-2} s^{-1}). However, no significant differences were found in the A_{max}/R_D ratio and quantum yield (A_{qe}) between the plants studied here.

Photosynthesis and stomatal conductance throughout the day

Stomatal aperture ranges substantially throughout the day according to the prevalent environmental condition. We thus decided to investigate the dynamic of stomatal conductance (g_s) and *A* in ferns and angiosperms along the diel course. Different patterns of photosynthetic and stomatal responses were observed along the day in each species. However, all of them demonstrated a typical pattern of tropical grown plants, in which a rapid increase in g_s in the first hours of the day is observed (Figure 3). Both *A* and g_s increased rapidly from 5 a.m. (pre-dawn) to 8 a.m. and remained high until 11 a.m. from which these parameters start do decrease till 5 p.m. (sunset). Cowpea (*V. unguiculata*) had almost the same pattern than tobacco (*N. tabacum*), but with higher values in both gas exchange parameters. The fern *M. scolopendria* showed an atypical g_s response in the daily course compared with the other species studied here, with a slight decrease in g_s observed near the midday and an absence of variation in *A* between 8 a.m. and 2 p.m. By contrast, the fern *P. aureum* showed a decrease in *A* from 8 a.m. until 5 p.m. Beyond providing information concerning the dynamic of g_s from angiosperms and ferns along the day, this experiment was also important to investigate the metabolic changes in leaf from the species studied. For this, leaf samples were immediately harvested and frozen after the gas exchange analysis in the 5 a.m., 8 a.m., 2 p.m. and 5 p.m. time points (discussed later).

Dynamic of stomatal conductance over light transitions

Aiming to improve our understanding concerning how ferns and angiosperms respond to light/dark transitions, we next analyzed the dynamic of g_s during dark-to-light and light-to-dark transitions. All species responded significantly to light changes, although the response pattern was species dependent (Figure 4). As expected, g_s decreased following a light-to-darkness transition and increased following a return to initial light conditions. The time to reach steady state in darkness ($g_{s\text{-st-Dark}}$) and in light conditions ($g_{s\text{-st-Light}}$) was different among species. Tobacco (*N. tabacum*) showed the shortest time, spending about 10 min to reach $g_{s\text{-st-Dark}}$, while the other species spent approximately 40 – 50 min. Nevertheless, the opposite was observed during the return to light, with tobacco showing the longest time to reach $g_{s\text{-st-Light}}$ (~ 40 min), while cowpea (*V. unguiculata*) and the ferns was almost similar, spending about 15 – 25 min.

In response to darkness, all plants showed a decrease in *A*, g_s and *E*. However, these parameters increased during the return to light, with angiosperms often presenting larger values than the ferns (Figure 5A,B and S1). The responses of ferns were not significantly different from each other, except in $g_{s\text{-}st\text{-}Dark}$. Given that the absolute gas exchange values of ferns are in general much lower than those from angiosperms, we next evaluated the *A*, g_s and *E* relative responses during dark-induced stomatal closure and light-induced stomatal opening. Ferns showed higher and lower relative stomatal and photosynthetic responses during dark-induced stomatal closure and light-induced stomatal opening, respectively, compared with angiosperms (Figure 5C,D). These differences were more pronounced for g_s than *A*, which means that stomata from ferns close and open less than stomatal from angiosperms during dark/light transitions. Interestingly, stomata that presented higher relative stomatal response to dark presented lower relative stomatal response to light within the groups of angiosperms and ferns. For instance, tobacco showed lower values of stomatal relative stomatal response during dark-induced stomatal closure and higher values of stomatal response during light-induced stomatal opening. The same applies for ferns, indicating a possible relationship between stomatal relative responsiveness within the groups. Indeed, there is an inverse relationship between these relative responses (Figure S2), confirming the idea that, within the groups, stomata with higher degree of stomatal closure in the dark are capable to open more when the light returns.

Dynamic of stomatal conductance under CO_2 concentration changes

The fern stomatal responses to changes in atmospheric CO_2 concentration C_a has theme of long debate given contradictory results found in the literature. We thus next investigated whether stomata from the ferns used in this study are capable to respond to changes in C_a. All angiosperms and ferns showed a reduction of g_s in response to elevated C_a (800 ppm), while g_s increased following a step decrease in C_a from 800 ppm to 200 ppm (Figure 6). This general pattern was observed in all replicates, however the increasing CO_2 concentration produced a different pattern of responses in which ferns typically showed a gradual and slower transition between steady state at 400 ppm ($g_{s\text{-}st400}$) and 800 ppm CO_2 ($g_{s\text{-}st800}$), whereas the transition in g_s for the angiosperms was faster. Also, the time to achieve the steady state after each transition were variable between species. Cowpea, tobacco and *M. scolopendria* and *P. aureum* spends approximately 10, 15, 170 and 80 min to achieve $g_{s\text{-}st800}$, respectively, but the time to achieve $g_{s\text{-}st200}$ was qualitatively similar across species, around 20-30 min. Thus, angiosperms had a shorter time to respond to both increased and decreased C_a, around ten times greater than the

ferns during high CO_2, and stomata of ferns showed a longer time to respond to increases in C_a, with *M. scolopendria* presenting the longest time, whilst a short response time to low CO_2 for both ferns, almost similar to angiosperms.

In cowpea (*V. unguiculata*), the absolute photosynthetic responses were larger than the other species, whilst the stomatal and transpiration responses was larger in both angiosperms than the ferns (except in tobacco (*N. tabacum*) at $g_{s\text{-}st800}$ (Figure 7A,B and S1). The responses of ferns were not significantly different from each other ($P > 0.05$). Interestingly, the relative stomatal responses to CO_2 resemble those from light/dark transitions, in which the relative closure responses were higher and lower in ferns during high CO_2-induced stomatal closure and low CO_2-induced stomatal opening conditions, respectively (Figure 7D). This also leads to an inverse and significant relationship between stomatal relative responsiveness within angiosperms and ferns (Figure S2).

Stomatal kinetic during dark/light and CO_2 transitions

The typical light and CO_2-induced stomatal responses (Figures 4 and 6) suggest that the speed of stomatal closure and opening differs among the species. To confirm that, we thus calculated the speed of stomatal movement in response to dark/light and CO_2 transitions. Angiosperms presented a faster control of stomatal movements when exposed to light-darkness-light transitions and different CO_2 concentrations than the ferns (Figures 8 A,D). The kinetics of stomatal closure speed of angiosperms were greater than the stomatal opening speed, whilst stomatal opening is faster than the closure in ferns. Notably, light-induced stomatal opening was negatively correlated with dark-induced stomatal closure within the groups (Figure 8 B,C), whereas no correlation between low CO_2–induced stomatal opening and high CO_2-induced stomatal closure was observed (Figure 8 E,F).

Species-specific metabolic responses throughout the day

Leaf samples were harvested at 5 a.m. (pre-dawn), 8 a.m., 2 p.m. and 5 p.m. (sunset) of the diel course experiment. The metabolite profile of these time points is demonstrated as a heat map normalized to the pre-dawn period (5 h) of each species to facilitate the comparison of the dynamic of the metabolites throughout day. This normalization also enables to compare the relative increase/decrease level of the metabolites among the species. In general, the four species behave differently. No clear pattern of metabolic alteration between angiosperms and ferns and even within these groups was observed (Figure 9). However, it is interesting to note

that angiosperms presented slight increases in the sucrose relative level in the first hours of light (5 a.m. and 8 a.m. and strong reduction at the end of day (5 p.m.), while slight variation in sucrose level was observed in ferns. Furthermore, the level of Glu, Gln, Tyr and pyroglutamate presented opposite behaviour among angiosperms and ferns, in which these metabolites increased over diel time in ferns and decreased in angiosperms. Intriguingly, increased level in the majority of the metabolites was observed in *P. aureum* (Figure 9).

The metabolite profile suggests that the metabolic dynamic throughout day differs substantially between the species studied here. We thus carried out a principal component analysis (PCA) to check the differences between the samples. PCA is a mathematical framework used to reduce large-scale data set into few clusters based in the multivariate similarity between the samples analysed. PCA analysis has been often used in metabolomics studies to discriminate plant genotypes or plants under different stress conditions (FIEHN et al., 2000; OBATA et al., 2015; WECKWERTH et al., 2004). Here, we applied PCA using all metabolite data set coupled with gas exchange data. The results showed a clear separation into three groups, in which both ferns were clustered together and separated of cowpea and tobacco by the first and second components, respectively (Figure 10), indicating that the dynamic of metabolism and gas exchange differs substantially between ferns and angiosperms, despite the similarities observed in specific metabolites throughout the day (Figure 9).

Species-specific metabolic responses during changes in CO_2 concentration

Leaf disks were harvested during different CO_2 transitions, from ambient (400 ppm) to high (800 ppm) and then to low (200 ppm) CO_2 conditions. We aim to investigate the metabolic responses during high CO_2-induced stomatal closure and low CO_2-induced stomatal opening. Five minutes under high CO_2 condition was sufficient to induce substantial alterations in the metabolite profile, especially in angiosperms species. Similarly, to the observed in the diel course experiment, no clear pattern of metabolic responses can be observed only with heat map analysis (Figure 11). However, PCA analysis of this experiment resemble those of the diel course experiment, in which ferns are clustered together and separated of cowpea and tobacco by the first and the second components, respectively (Figure 12). This reinforces the idea that ferns and angiosperms differ substantially at metabolic and gas exchange level and that both angiosperms cowpea and tobacco behave differently throughout the day and in response to changing CO_2 concentration.

Leaf metabolic responses during light-induced stomatal opening and high CO_2-induced stomatal closure conditions

Our PCA analysis indicates that substantial differences are found between ferns and angiosperms as well as within cowpea and tobacco. We thus next investigated metabolic responses of each species under two contrasting conditions; light-induced stomatal opening, using the data from 5 a.m. and 8 a.m. of the diel course, and high CO_2-induced stomatal closure, using the steady state data set from 400 ppm and 800 ppm. The relative change of each metabolite from each species during dark-to-light transition, from 5 a.m. to 8 a.m. of the diel course, is found at figure S3. This figure also highlights some differences between the relative metabolic changes in angiosperms and ferns species combined. Interestingly, several amino acids showed opposite behaviour between these groups, in which Asn, Glu, Gln, Met, Phe, Pro, Trp, Tyr and Asp increased in ferns and decreased in angiosperms. Fructose and glucose also showed contrasting behaviour, being increased in angiosperms and decreased in ferns (Figure S3).

The number of metabolites with significant changes differs substantially between angiosperms and ferns, despite the significant increase in A, E and g_s in all genotypes (Figure 13). For instance, only one metabolite (4-hydroxy-benzoic acid) changed significantly in *M. Scolopendria* and eight others changed in *P. aureum* (Figure 13). In contrast, more than 15 metabolites presented significant changes during light-induced stomatal opening in both angiosperms. Glycerate increased in *P. aureum* and in both angiosperms under this condition, while Ser increased in cowpea and *P. aureum* but decreased in tobacco. The difference between cowpea and tobacco demonstrated by the PCA analysis is also evident by the contrasting behaviour under dark-to-light transition. For instance, whilst the ratio between the sugars sucrose, glucose and fructose to succinate increased in tobacco, these ratios plus the ratio between these sugars to malate decreased in cowpea.

By contrast of the dark-to-light transition, the metabolic responses to changing CO_2 concentration were minor (Figure S4). Surprisingly, no metabolites showed significant difference during low CO_2-induced stomatal opening in all species. Similarly, no metabolites showed significant changes in *M. Scolopendria*, while only one in cowpea, five in tobacco and three in *P. aureum* showed significant changes. Glucose and Val in tobacco and the sucrose/malate ratio (Suc/Mal) in cowpea showed contrasting behaviour between light-induced stomatal opening (Figure 13) and high CO_2-induced stomatal closure (Figure 14).

Relationship between g_s and the content of sucrose and malate

Sugars and organic acids, especially sucrose and malate, have been suggested to be key players for the *A*-g_s regulation (GAGO et al., 2016; LAWSON et al., 2014). Indeed, we have observed significant differences in sugars and organic acids and the ratio between them in different analyses (Figures 13-15). We next decided to investigate the correlation between the accumulation of these metabolites with g_s and whether these can explain the differences observed between species and groups. However, it is important to highlight that the absolute values found in g_s and metabolites are in general much lower in ferns compared to angiosperms, except malate that present high levels in both ferns. This may not be suitable to correlation analysis data from angiosperms and ferns. Thus, caution should be taken when analysing this data set without normalization. Therefore, we solved this issue by analysing the relative changes in sucrose and malate within each species during light-induced stomatal opening, by normalizing the data of 8 a.m. to those found at 5 a.m. of the diel course, and high CO_2-induced stomatal closure by analysing the relative changes found in the steady state of 800 ppm compared to the steady state at 400 ppm of the dynamic of CO_2 changes experiment. When looking to the relative metabolic response per species, malate is positively related to g_s while sucrose and Suc/Mal ratio are negatively related to g_s (Figure S5). Furthermore, we observed a general tendency to increase the ratio between sucrose and the organic acids malate, succinate and fumarate during CO_2-induced stomatal closure, although this increase is higher and only significant in cowpea (Figure 15). Interestingly, these ratios decreased in cowpea during light-induced stomatal opening while the ratios between sucrose, glucose and fructose to succinate increased in tobacco (Figure 15). In this vein, it is clear that the ratio between Suc/Mal and sucrose/ succinate (Suc/Succ) is important to modulate g_s responses in cowpea, in which lower g_s is found as high are these ratios (Figure 15). Indeed, cowpea relative change in Suc/Mal ratio is higher during high CO_2-induced stomatal closure compared to all other species and lower during light-induced stomatal opening, although only significantly different in *P. aureum* (Figure 16).

Relationship between speed of stomatal closure and the relative changes in metabolites during high CO_2-induced stomatal closure

Several controversies are found regarding ferns g_s responses to CO_2. In one hand, initial studies suggested that fern's stomata are insensitive to changes in CO_2 concentration (BRODRIBB et al., 2009; BRODRIBB; MCADAM, 2011; MCADAM; BRODRIBB, 2012a).

However, recent studies have showed that fern's stomata are able to respond to CO_2 but in a much lower velocity (CREESE et al., 2014; FRANKS; BRITTON-HARPER, 2016; HÕRAK; KOLLIST; MERILO, 2017), fact corroborated by our results (Figure 8). We thus decided to explore whether the lower velocity of the high CO_2-induced stomatal closure observed in ferns compared with angiosperms may involve leaf metabolism. We have already mentioned that leaf primary metabolism presented several differences between ferns and angiosperms (Figure S4). We next correlated leaf primary metabolites with both speed of high CO_2-induced stomatal closure and the relative change in g_s during this condition. The results showed that caffeic acid (*cis* and *trans*) and guanidine are significantly different positively and negatively correlated speed of stomatal closure (Table S1). Interestingly, several sugars such as sucrose, raffinose, trehalose and rhammose presented high positive correlation to speed of high CO_2-induced stomatal closure, while the TCA cycle metabolites malate, fumarate, succinate and 2-oxoglutarate showed negative correlation (Figure 17; Table S1). Furthermore, the average response of these sugars is linearly related to the speed of high CO_2-induced stomatal closure ($R^2 = 0.99$; $P < 0.05$) (Figure S6). This suggests that sugar/organic acid ratio may at least partially explain the differences observed between angiosperms and ferns. Indeed, the ratios between Suc/Mal, sucrose/fumarate (Suc/Fum) and Suc/Succ are positively related to speed of stomatal closure (Figure 17). Notably, angiosperms presented higher percentage of changes in Suc/Mal ratio during high CO_2-induced stomatal closure (Figure 16). Interestingly, the opposite is observed during dark-to-light transition, although not significant in this case (Figure 16). Taken together, these results suggest that Suc/Mal ratio represent a mechanism by which leaf photosynthesis regulate g_s and may at least partially explain the differences in the speed of high CO_2-induced stomatal closure between ferns and angiosperms.

Relationship between relative stomatal response and relative metabolic changes during high CO_2-induced stomatal closure

Our previous analysis indicates that the kinetic of stomatal responses to high CO_2 may involve leaf metabolism. We next investigated whether leaf metabolism may be also involved in the regulation of the magnitude of g_s response from ambient (400 ppm) to high (800 ppm) CO_2 concentrations. Interestingly, Pearson correlation analysis showed exactly the opposite of the correlation analysis between metabolites and speed of stomatal closure, in which metabolites that are positively correlated to the speed of stomatal closure are negatively correlated to the relative changes in g_s during high CO_2-induced stomatal closure, and *vice versa*

(Figure S7; Table S2). Indeed, relative stomatal response is negatively correlated to relative stomatal decrease during high CO_2-induced stomatal closure (r = - 0.77; P = 0.232), suggesting that species with higher speed to close their stomata has smaller relative changes and this may be related to leaf metabolic signals.

It is interesting to highlight that there is a negative relationship between relative stomatal response to open and to close during dark/light and CO_2 transitions. In relative terms, stomata within angiosperm and fern groups that have higher relative rate of stomatal closure under dark and high CO_2 conditions presented subsequent lower increases in stomatal aperture induced by light and low CO_2 (Figure S2).

Discussion

Physiological differences between ferns and angiosperms

In the present study, photosynthesis (A) and stomatal conductance (g_s) were different between the studied species in all experiments performed. The photosynthetic light response curves showed that ferns had lower A_{max} values compared to angiosperms (Figures 1,2), which have been attributable to their much lower leaf vein density and hydraulic conductance, linking A_{max} of ferns to leaf water transport capacity (BRODRIBB; HOLBROOK, 2004; WATKINS; HOLBROOK; ZWIENIECKI, 2010; ZHANG et al., 2014). The response pattern of A and g_s were species dependent during the diel-course (Figure 3). In addition, ferns often showed lower absolute values of A, g_s and transpiration (E) than angiosperms during light and CO_2 concentration variations (Figures 4, 5A,B, 6, 7A,B and S1). The lower values of A in the ferns were mostly associated to lower g_s, although it is important to highlight that we haven't determined here whether mesophyll conductance (g_m) also contributes to the lower A in ferns. Previous studies indicate that fern photosynthesis is mainly constrained by both CO_2 diffusion resistance (g_s and g_m), while biochemical limitations constrained photosynthesis in angiosperms (CARRIQUÍ et al., 2015; GAGO et al., 2013; TOSENS et al., 2016b; XIONG; DOUTHE; FLEXAS, 2018). Although the gas exchange parameters mentioned above were higher in angiosperms in terms of absolute responses, they also differ in relative terms, as observed in the dynamics of light and CO_2 transitions (Figures 5C,D and 7C,D, respectively). In this case, we found that fern stomata have higher relative values after both dark and CO_2-induced stomatal closure, which means that the relative stomatal closure is lower in ferns. Similarly, angiosperm stomata's have higher response during stomatal opening, which leads to a negative correlation between relative stomatal closure and relative stomatal opening, especially under dark/light

transitions (Figure S2). A recent study noticed that ferns and angiosperms have some morphological particularities, in which ferns have larger stomata and lower stomatal density, while angiosperms have shorter stomata but higher stomatal density, suggesting that g_s responses increase with stomatal density, but declines with stomatal size across species (XIONG; DOUTHE; FLEXAS, 2018). In fact, this has been previously reported to be mediated by the CO_2 concentration along geological time (FRANKS; DRAKE; BEERLING, 2009). In view of these evidences, further morphological and anatomical analysis can provide important information to certify whether the differences between the two plant groups can explain the different gas exchange responses observed here.

Differential coordination of stomatal movements over light and CO_2 transitions

Light and atmospheric CO_2 concentration are key environmental signals that regulate stomatal movements. Aiming to understand how ferns and angiosperms respond to these signals, we evaluated the dynamic of g_s in ferns and angiosperms species using a stepwise of darkness followed by a return to the initial intensity of light as well as under different CO_2 concentrations (Figures 4,6). Stomata from all species closed when exposed to darkness or high CO_2 and opened when transferred to light or low CO2, supporting the idea that light and CO_2 stomatal responses are conserved among ferns and angiosperms (DOI; KITAGAWA; SHIMAZAKI, 2015). Fern stomatal responses to both dark/light and CO_2 transitions contrast with previous studies that consider fern stomata's insensitive either to light or CO_2 variations (BRODRIBB et al., 2009; MCADAM; BRODRIBB, 2012b). However, our results are in agreement with other works which showed that stomata of ferns are responsive to changes in light or atmospheric CO_2 concentration (CREESE et al., 2014; FRANKS; BRITTON-HARPER, 2016; HÕRAK; KOLLIST; MERILO, 2017). Interestingly, a common feature of all mentioned studies above is a conserved tendency to respond to CO_2, especially under low CO_2 condition (BRODRIBB et al., 2009; FRANKS; BRITTON-HARPER, 2016). Indeed, both the relative changes and the speed of stomatal opening are higher under low CO_2-induced stomatal opening than high CO_2-induced stomatal closure within ferns species (Figures 7,8). This suggests that stomata from ferns have higher impairment to close rather than to open during CO_2 transitions.

Despite the fact that fern stomata are able to respond to light and CO_2, it is important to note that substantial differences are found in the speed and in the relative changes of these responses among ferns and angiosperms. For instance, both stomatal closure and opening were

slower in ferns than in angiosperms during dark/light transitions and especially under high CO_2-induced stomatal closure condition, in which the slowest speed of stomatal responses was observed in ferns (Figure 8). These behavior are supported by previous investigations which indicates that regulation of stomatal movements differs between ferns and angiosperms (Brodribb *et al.*, 2009; Brodribb & McAdam, 2011; Mcadam & Brodribb, 2012; McAdam & Brodribb, 2012). Intriguingly, an inverse relationship between the speed to close and speed to open under light/dark variations within the groups was observed (Figure 8 B,C). Stomata with slower speed to close had higher speed to open. Furthermore, we also found that stomata that presented higher relative stomatal closure in response to dark and high CO_2 presented lower relative stomatal opening in response to light and low CO_2 (Figure S2). The molecular mechanisms that explain such observations are still unclear. We argue that this might also involves leaf metabolites, especially the ratio between sucrose and malate (discussed below in the section *Stomatal movement regulation by mesophyll-derived metabolites*).

It has been hypothesized that a passive mechanism mediated by leaf water potential (Ψ_w) is the main factor that regulates stomatal movements in response to environmental signals in ferns. This idea is supported by a recent study that showed when fern leaves are exposed to light and subsequently this light is turned off, the response of g_s is slow because the leaf water potential is maintained or even increased by the decrease in transpiration rate (XIONG; DOUTHE; FLEXAS, 2018). By contrast, stomatal movements in angiosperms are mostly regulated by complex signaling and metabolic networks (BRODRIBB; MCADAM, 2017a; SIERLA et al., 2016). For example, light induces stomatal opening by direct perception by the guard cells and by a decrease in the intercellular CO_2 concentration (C_i) due to enhanced *A* (FRANKS; BRITTON-HARPER, 2016; LAWSON; BLATT, 2014; MOTT; SIBBERNSEN; SHOPE, 2008; SHIMAZAKI et al., 2007). Additionally, high CO_2-induced stomatal closure involves a complex network composed by kinases, phosphatases, carbonic anhydrases, anion channels, ABA, Ca^{+2} and malate (ENGINEER et al., 2016). Thus, the differences in stomatal response speeds between ferns and angiosperms can be related to the differential stomatal regulation mechanisms found among these plant groups. The lack of some components of the signaling pathways that leads to stomatal opening and closure may impair a fast stomatal response in ferns. In addition, several studies have suggested that smaller stomata have faster speed of stomatal movements than large ones, and else the evolutionary trend from a few large stomata to high density stomata is assumed to represent greater efficiency in stomatal movement under natural conditions (DRAKE; FROEND; FRANKS, 2013; LAWSON et al., 2014; RAVEN, 2014). However, the mechanisms that explain the differential behavior

observed among ferns and angiosperms in this study remains unclear. Further studies using higher number of plant species from each group are needed to confirm those trends and maybe definitively conclude that these results indicate evolutionary patterns rather than species differences.

Stomatal movement regulation by mesophyll-derived metabolites

It is known that the magnitude of stomatal movements can range according to the accumulation of specific metabolites from mesophyll in the symplast and/or apoplast of guard cells. Furthermore, it has been proposed that this response depends on the concentration of the metabolite, that can induce stomatal opening or closure (SANTELIA; LAWSON, 2016). Here, we evaluated whether leaf metabolite levels can explain the differences observed between angiosperms and ferns under low and high CO_2 and during dark-to-light transition of the initial period of the diel course to better understand the regulation of stomatal movement. Notably, changes in many groups of metabolites, especially sugars, organic acids and amino acids, showed different trends in ferns and angiosperms and even between *V. unguiculata* and *N. tabacum* (Figure 13, 14). Metabolites such as sucrose, glucose, mannose, malate, fumarate and glutamate have been related to control of stomatal movements (ARAÚJO et al., 2011; KANG et al., 2007b; KELLY et al., 2013; LI et al., 2016; LU et al., 1997b; YOSHIDA et al., 2016). Our results showed significant changes in several other metabolites, including some of these above (Figure 15). It has been suggested that sugars and organic acids, especially sucrose and malate, play a key role in the A-g_s trade-off regulation (GAGO et al., 2016; LAWSON et al., 2014), coordinating mesophyll and stomatal behavior via the apoplastic transport from mesophyll to guard cells. Here, the relative data set using all species combined showed that sucrose and malate were negatively and positively related to g_s (Figure S5), in agreement with a recent multi-species meta-analysis study (GAGO et al., 2016). The accumulation of mesophyll-sucrose in the apoplast of guard cells or its import into the GC's have been proposed as mechanisms able to induce stomatal closure in phloem loader species (BEAN; OUTLAW; VLIEGHERE-HE, 2001; KANG et al., 2007b; KELLY et al., 2013; LU et al., 1995b; LUGASSI et al., 2015). In addition to sucrose, it is well established that malate acts as an osmolyte during stomatal opening, providing a counter ion for K^+ within guard cells (ALLAWAY WG, 1973; ASAI et al., 2000; FISCHER; HSIAO, 1968; HUMBLE; K. RASCHKE, 1971; OUTLAW; LOWRY, 1977). These results reinforce the idea that sucrose

and malate act as signals that coordinates stomatal behavior with mesophyll photosynthetic demands.

As stated above, the stomatal opening speed under low CO_2 is much higher than the speed to closure under high CO_2 in ferns and both responses are lower in ferns compared to angiosperms (Figures 8A,D). This fact might be explained for three potential ways: (1) due to an impaired CO_2 signaling pathway for stomatal closure in ferns; (2) lower sucrose/malate (Suc/Mal) ratio in these plants (Figure 16D), and (3) due to the accumulation of other metabolites highly correlated with g_s responses (Figures 17 and S7; Tables S1,S2). In the first case, an impaired CO_2 signaling pathway in ferns could hamper to fast close the stomata, whilst angiosperms acquire a faster control of stomata by the acquisition of some genes of the CO_2 signaling pathway (BRODRIBB; MCADAM, 2013; YOUNG et al., 2006). Here, we provide other evidence that might at least partially explain these differences in the differential speed of stomatal movement among ferns and angiosperms. The sucrose/malate (Suc/Mal) ratio have a negative relationship with g_s (Figure S5) and a positive relationship with the speed to close the stomata (Figure 17). This means that Suc/Mal ratio increase during stomatal closure, and decrease during stomatal opening. Beyond Suc/Mal ratio, Suc/Fum and Suc/Succ are also positively related to speed of stomatal closure (Figure 17), suggesting that the ratio between sugars/organic acids is important to modulate the g_s responses, although it is important to stress that only *V. unguiculata* significantly increased this ratio between sucrose and the organic acids malate or succinate during high CO_2-induced stomatal closure (Figure 14). Additionally, relative changes of other sugars such as trehalose, raffinose and rhammose also showed a positively correlation with stomatal closure speed (Figure 17), suggesting that not only sucrose but the pool of sugars may be important to regulate g_s and explain the differences between ferns and angiosperms. In fact, the average of these sugars linearly correlated with the speed of high CO_2-induced stomatal closure (Figure S6; Table S1). Interestingly, whilst these sugars are positively related to the speed of stomatal closure, the TCA cycle metabolites malate, fumarate, succinate and 2-oxoglutarate showed negative correlation (Figure 17; table S1), suggesting that the balance between sugar metabolism and the TCA cycle is a key point of stomatal movement regulation.

Sugars and organic acids are the most studied group of metabolites nominated to be key components of the metabolic network that regulates g_s. However, other metabolites have also been related to g_s regulation (Gago *et al.* 2016) and thus could explain the stomatal behavior and the difference in stomata closure speed between ferns and angiosperms. Our results demonstrated that metabolites that are positively correlated to the speed of stomatal closure are

negatively correlated to the relative changes in g_s during high CO_2-induced stomatal closure, and *vice versa* (Figure S7; table S2). This indicates that species with higher speed to close their stomata has smaller relative changes and this may be related to leaf metabolic signals. Interestingly, we observed a strong positive correlation between the amino acid serine (a metabolite related to photorespiration) and the relative response of g_s (Figure S7). Some studies have been reported an improved performance of plants with increased levels of photorespiratory enzymes. For example, the overexpression of glycine decarboxylase (GDC-H or GDC-L) in Arabidopsis resulted in an improvement of *A* and plant growth (TIMM et al., 2012, 2015). Furthermore, overexpression of serine hydroxymethyltransferase (SHMT) improved photosynthetic efficiency and plant productivity in rice (WU et al., 2015). These results suggest that the mitochondrial conversion of glycine to serine is somehow involved in the regulation of photosynthetic activity. In addition, it is known that serine may act as a metabolic signal for the transcriptional regulation of photorespiration (TIMM et al., 2013), and in a recent study involving some mutants with impaired activity of photorespiratory enzymes (phosphoglycolate phosphatase, serine hydroxymethyltransferase and glycerate kinase), were not able to adjust stomatal conductance under variations in external CO_2 (EISENHUT et al., 2017), supporting that serine and photorespiration may regulate photosynthesis and g_s.

Overall, it is important to note that in the current study, we only investigated two ferns and two angiosperms, which might not be representative of their entire groups. Nevertheless, these findings will support future works revealing, especially, the role of Suc/Mal ratio in the regulation of stomatal movements.

Concluding remarks

In agreement with previous studies, we find no evidence to support the idea that stomata of ferns are insensitive to variations in light or atmospheric CO_2 concentrations. All of the ferns showed significant changes in stomatal conductance during all experiments performed. However, these responses were always slower than angiosperms. Interestingly, stomatal kinetics experiments evidenced that ferns stomatal opening is faster than their closure. Although the reason behind such observation is still unclear, it suggests that angiosperms may have additional components of the high CO_2-induced stomatal closure signaling pathway that is not present in ferns, which can be a complex of genes and/or to incapacity of ferns stomata to respond mesophyll-derived metabolites such as sucrose and malate. The sucrose/malate ratio was negatively related to stomatal responses, but positively related to stomatal speed to closure,

suggesting that these metabolites are important to modulate stomatal responses. Moreover, sugar relative changes showed a positive correlation, whereas TCA cycle-derived organic acids showed a negative correlation, suggesting that the balance between sugar metabolism and the TCA cycle is a key point of stomatal movement regulation. Overall, our results provide new clues for understanding the coordination of stomatal conductance and leaf metabolites across different plant evolutionary groups.

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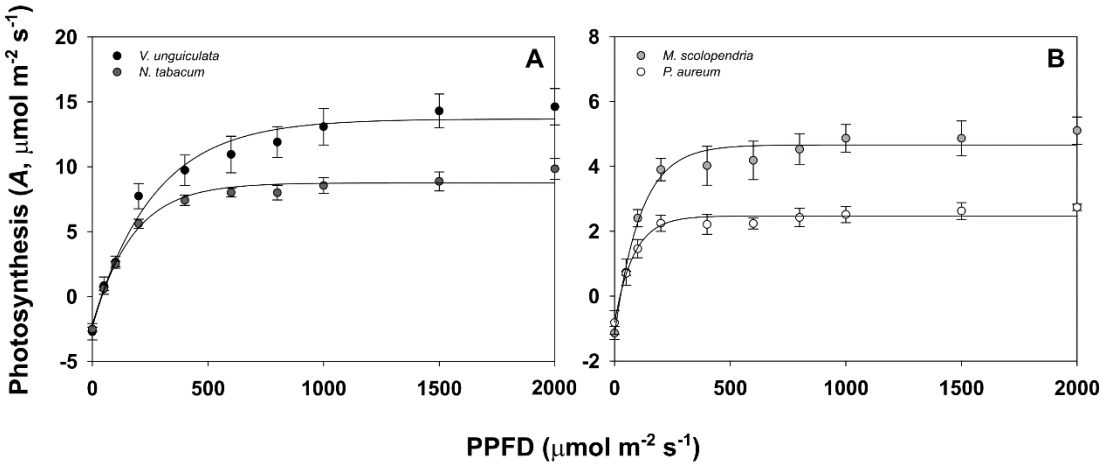

Figure 1. Photosynthetic light response curves of fully mature leaves of angiosperms (left figure) and ferns (right figure). Data are the means ± SE (n = 5). The regression line was determined using the function $A = a\ (1 - e^{-bx}) + c$ (Watling et al, 2000).

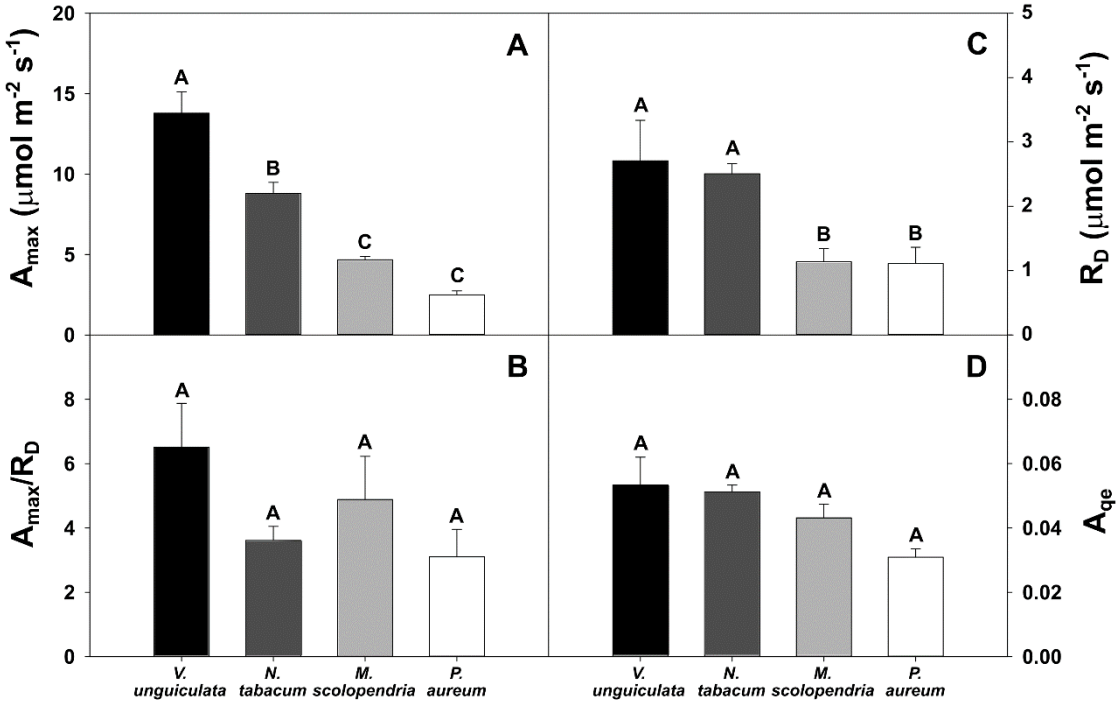

Figure 2. Maximum photosynthetic rate (A_{max}), respiration in darkness (R_D), maximum photosynthetic rate/respiration in darkness (A_{max}/R_D), and quantum yield of photosynthesis (A_{qe}) for each specie. Data are the means ± SE (n = 5).

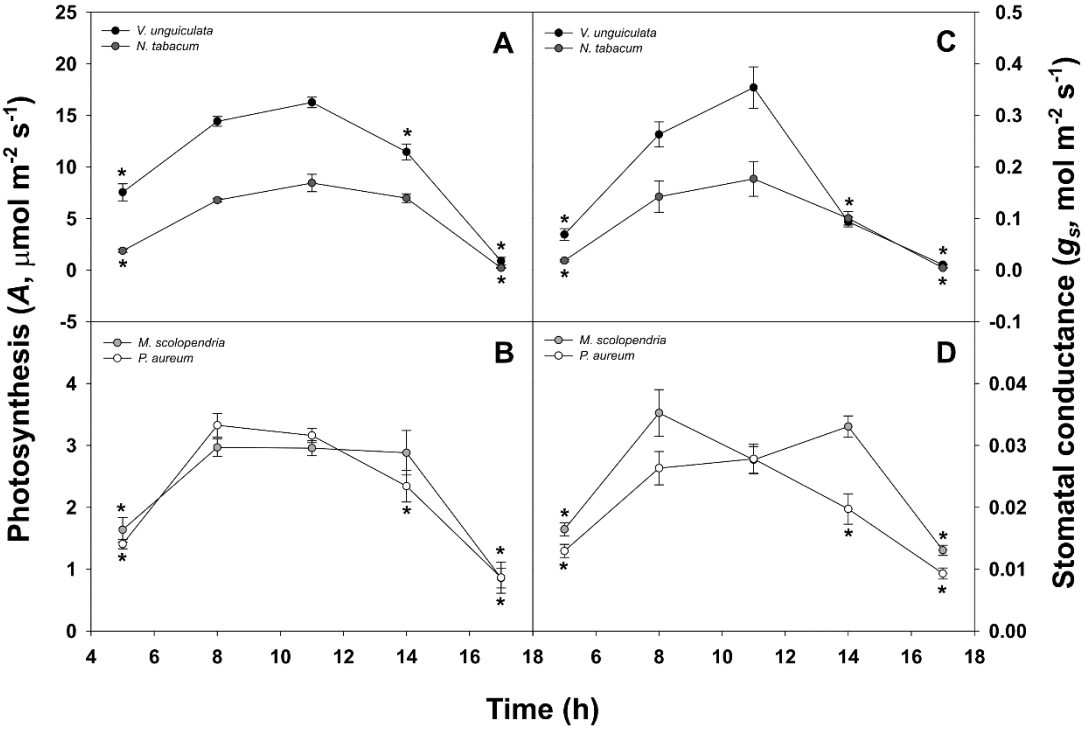

Figure 3. Diel-course of photosynthesis (figures A and B) and stomatal conductance (figures C and D) for each specie. All data were measured under 1,000 μmol m^{-2} s^{-1} PPFD. Data are the means ± SE (n = 5). At each time point, average points followed by asterisks differ statistically from 8 a.m.

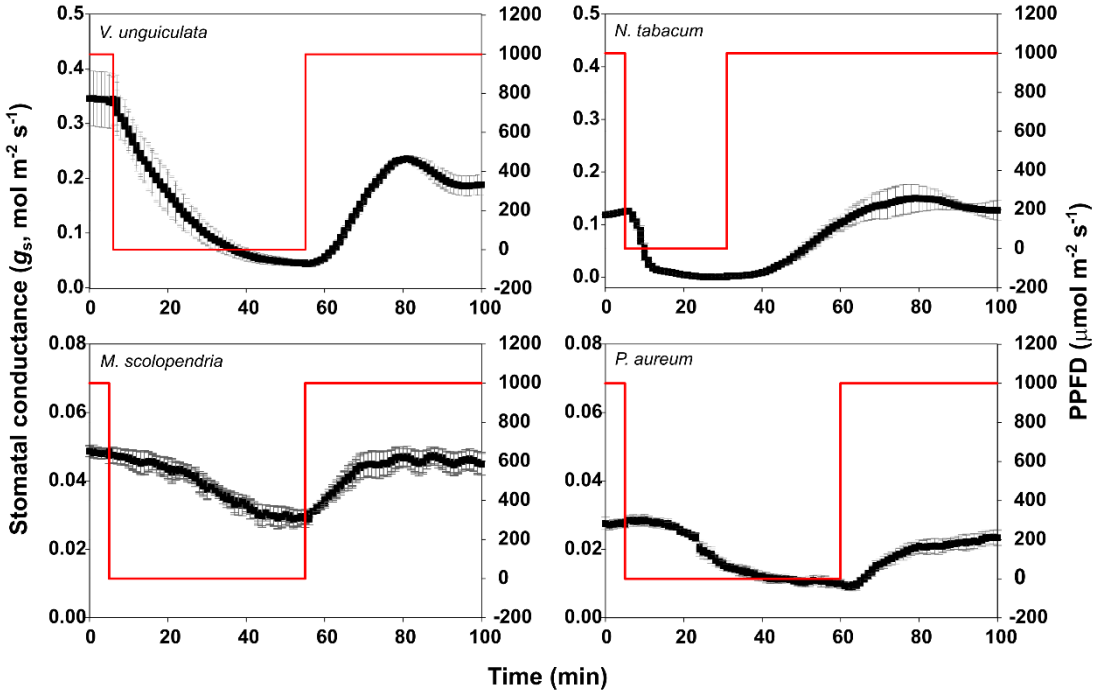

Figure 4. Typical time series responses (black data points) for each specie over a light-to-dark transition, followed by a dark-to-light transition (red line). Data are the mean ± SE (n = 4).

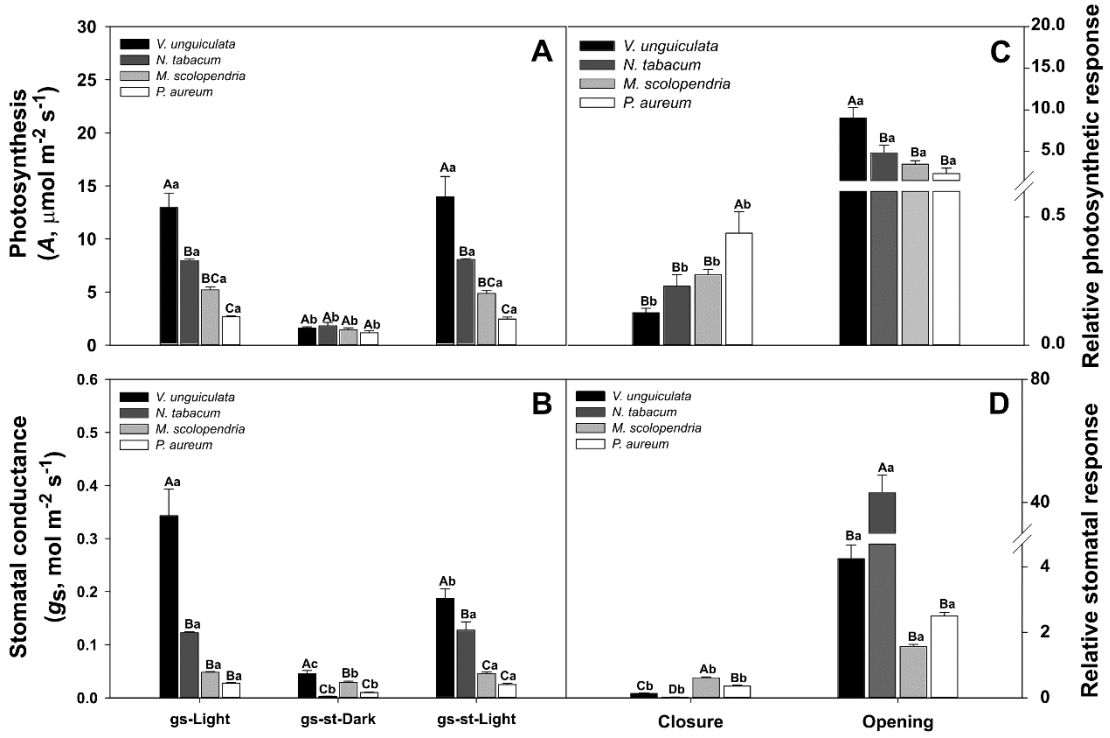

Figure 5. Changes in photosynthesis and stomatal conductance (figures A and B) and relative stomatal responses to open and to close during dark/light transitions (figures C and D). Relative stomatal closure refers to the difference observed during light-to-dark transitions, whilst relative stomatal opening refers to the difference observed during dark-to-light transitions. Identical letters, upper case among treatments and lower case between species in each treatment, do not significantly differ by Tukey's test ($P \leq 0.05$). Data are the mean ± SE (n = 4).

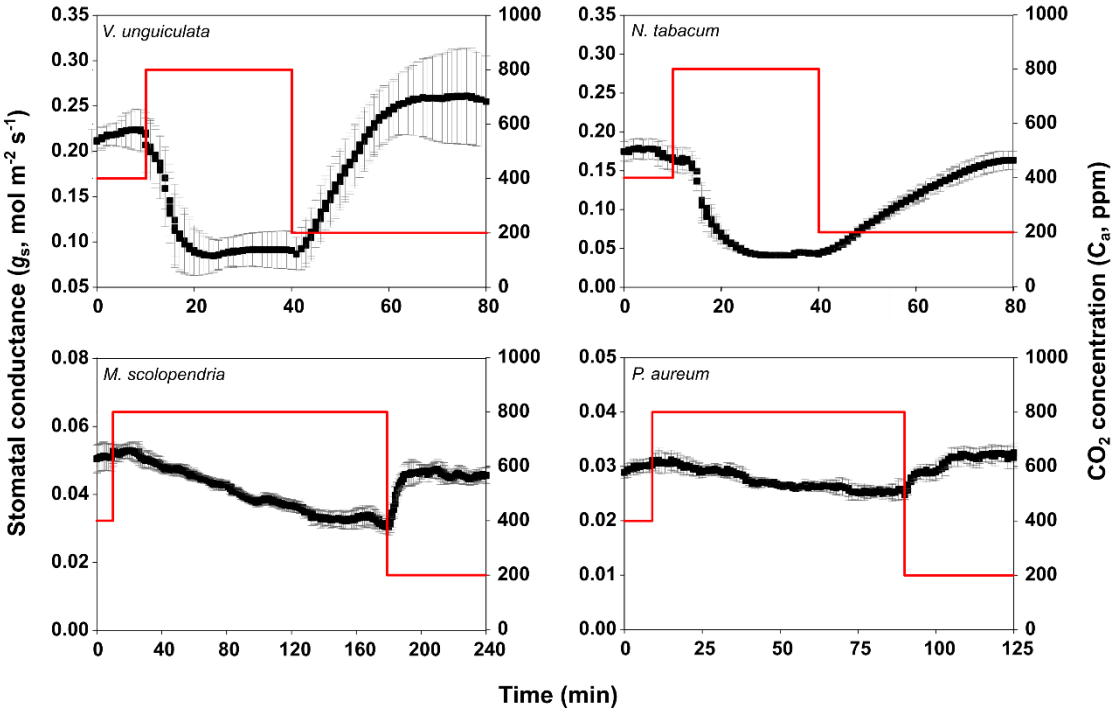

Figure 6. Typical time series responses (black data points) for each specie to a step increase in C_a from 400 to 800 ppm, followed by a decrease to 200 ppm (red line). Data are the means ± SE (n = 4).

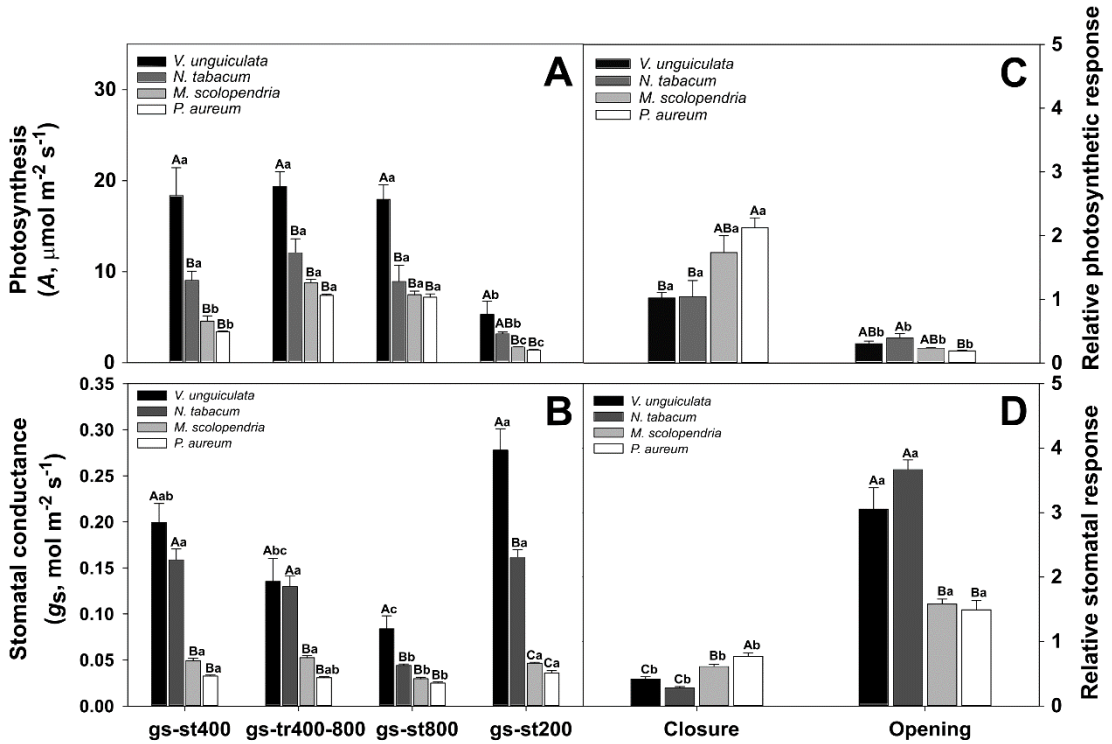

Figure 7. Changes in photosynthesis and stomatal conductance under CO_2 transitions (figures A and B) and relative stomatal responses to open and to close during changes in CO_2 concentrations (figures C and D). Relative stomatal closure refers to the difference observed during ambient (400 ppm) to high (800 ppm) CO_2 transitions, whilst relative stomatal opening refers to the difference observed during high (800 ppm) to low (200 ppm) CO_2 transitions. Identical letters, upper case among treatments and lower case between species in each treatment, do not significantly differ by Tukey's test ($P \leq 0.05$). Data are the mean ± SE (n = 4).

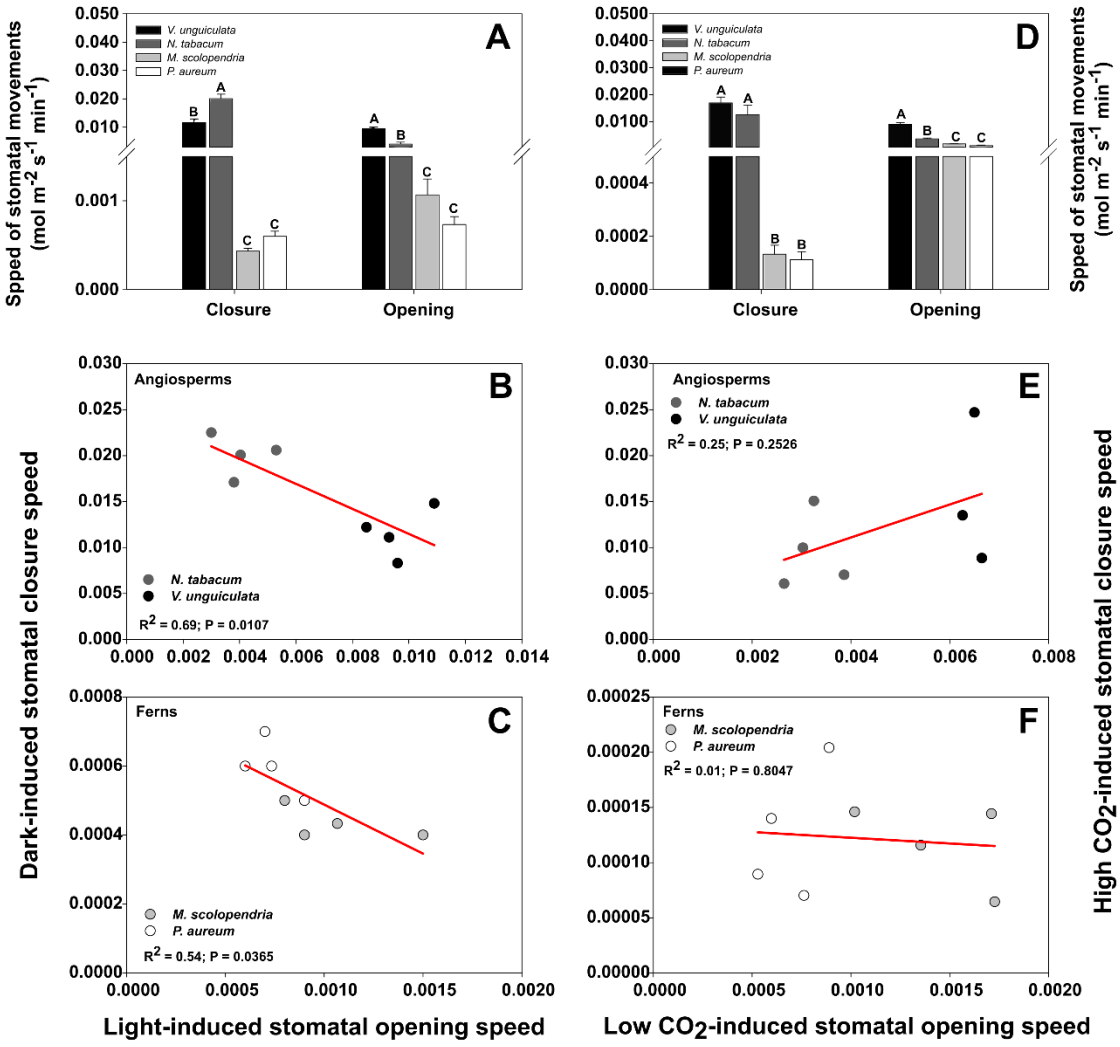

Figure 8. Relationship between the velocity of stomatal closure and stomatal opening induced by dark/light and CO_2 changes. Speed of stomatal closure was obtained during light-to-dark and ambient (400 ppm) to high (800 ppm) CO_2 transitions, whilst speed of stomatal opening was obtained during dark-to-light and high (800 ppm) to low (200 ppm) CO_2 transitions. Left figures were obtained during dark/light and right figures during CO_2 transitions. The speed of stomatal closure and opening was obtained through the linear step of the dark or high CO_2-induced stomatal closure and the light or low CO_2-induced stomatal opening, respectively (Figure 4 and 6).

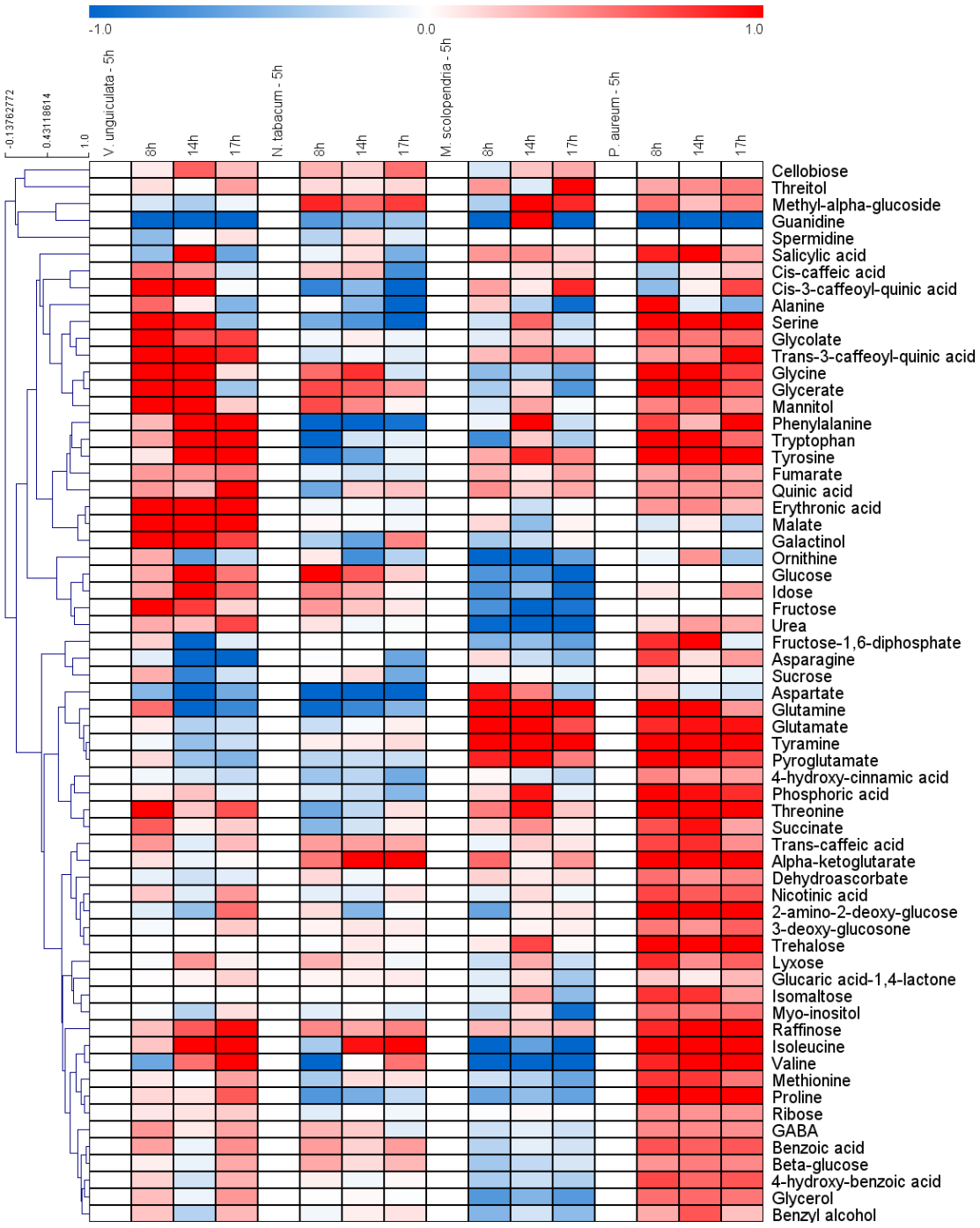

Figure 9. Metabolite profile of angiosperms (*V. unguiculata* and *N. tabacum*) and ferns (*M. scolopendria* and *P. aureum*) species during the diel course. Data are normalized according to the values found in the pre-dawn (5 a.m.) of each species. Heat map was carried out using MeV software.

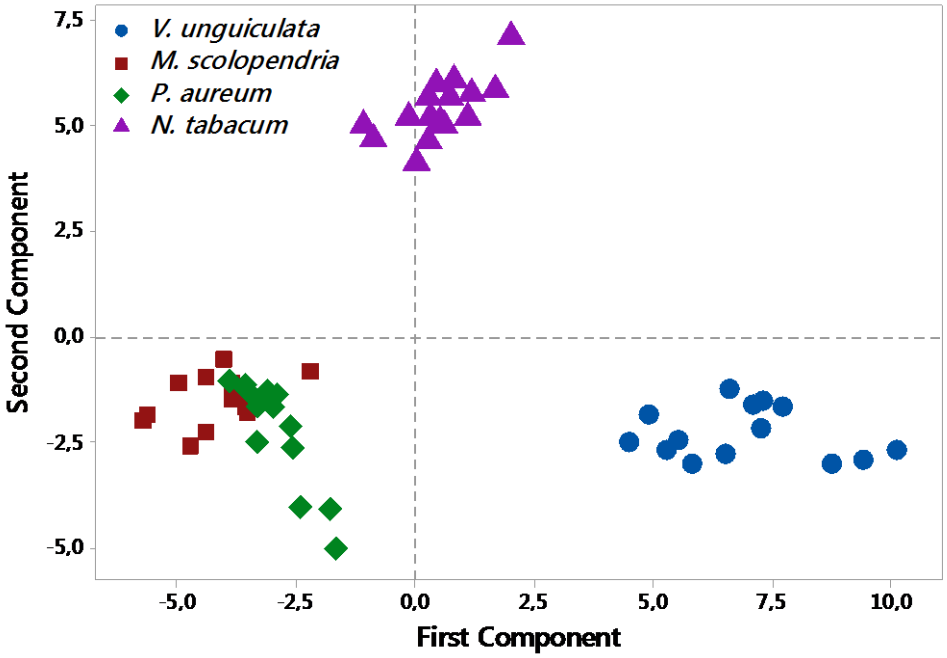

Figure 10. Principal component analysis (PCA) of metabolomic and gas exchange data from the diel course experiment. PCA analysis and graphic representation were carried out using Minitab 18 software.

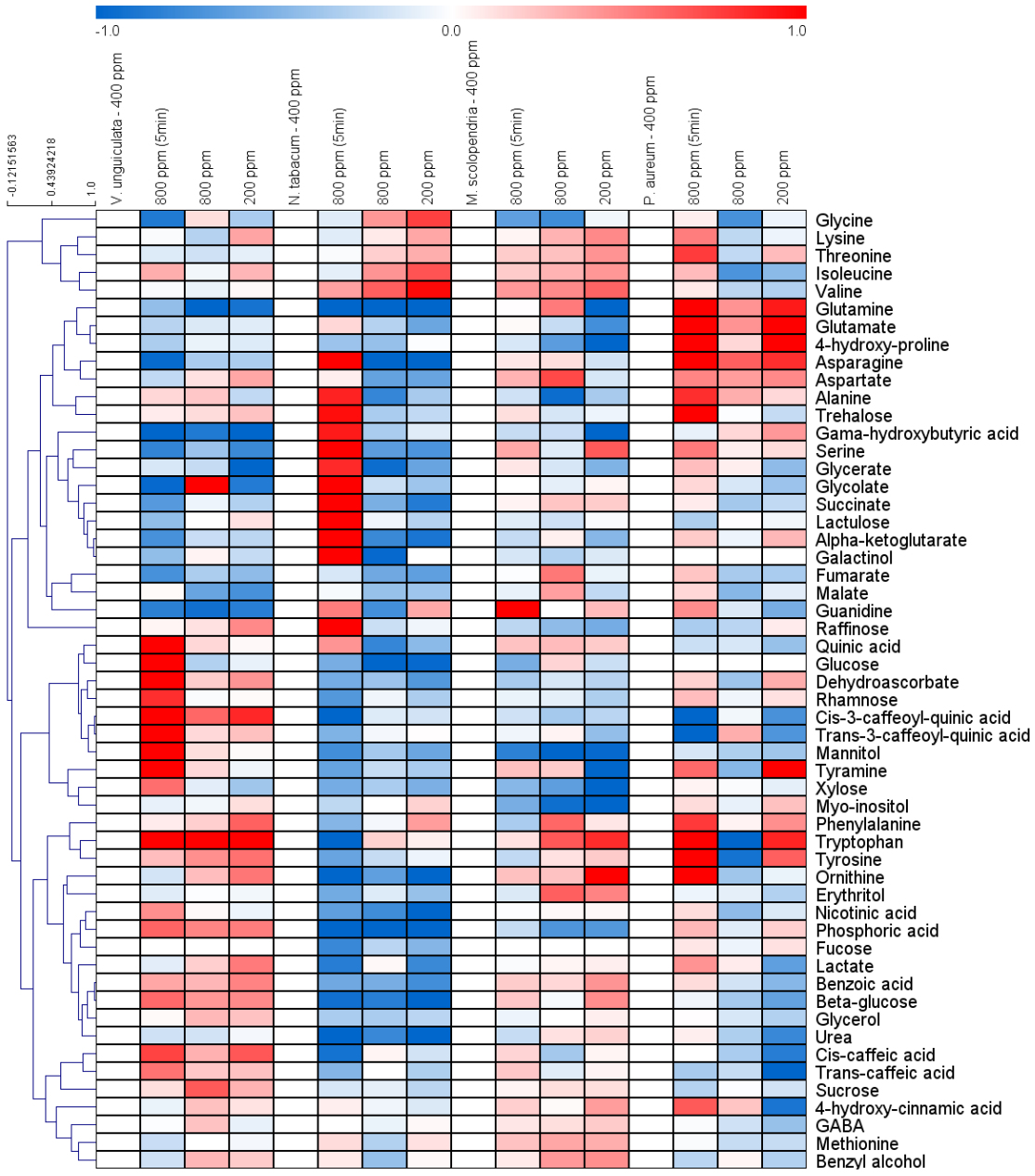

Figure 11. Metabolite profile of angiosperms (*V. unguiculata* and *N. tabacum*) and ferns (*M. scolopendria* and *P. aureum*) species during changes in CO_2 concentrations. Data are normalized according to the values found in the steady state 400 ppm ($g_{s\text{-}st400}$). Heat map was carried out using MeV software.

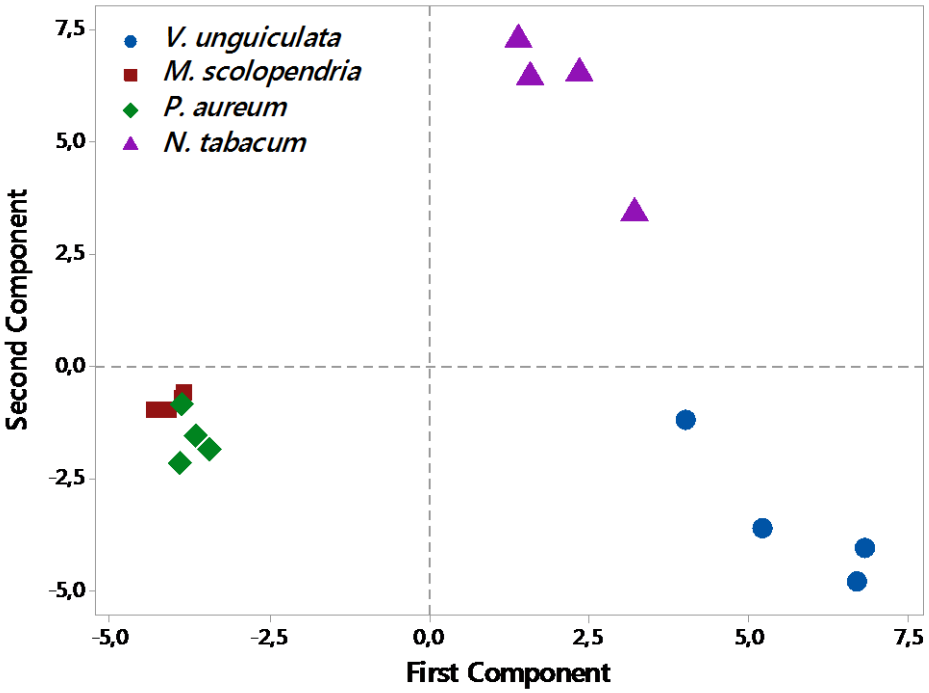

Figure 12. Principal component analysis of metabolomic and gas exchange data from the dynamic CO_2 of changes experiment. PCA analysis and graphic representation were carried out using Minitab 18 software.

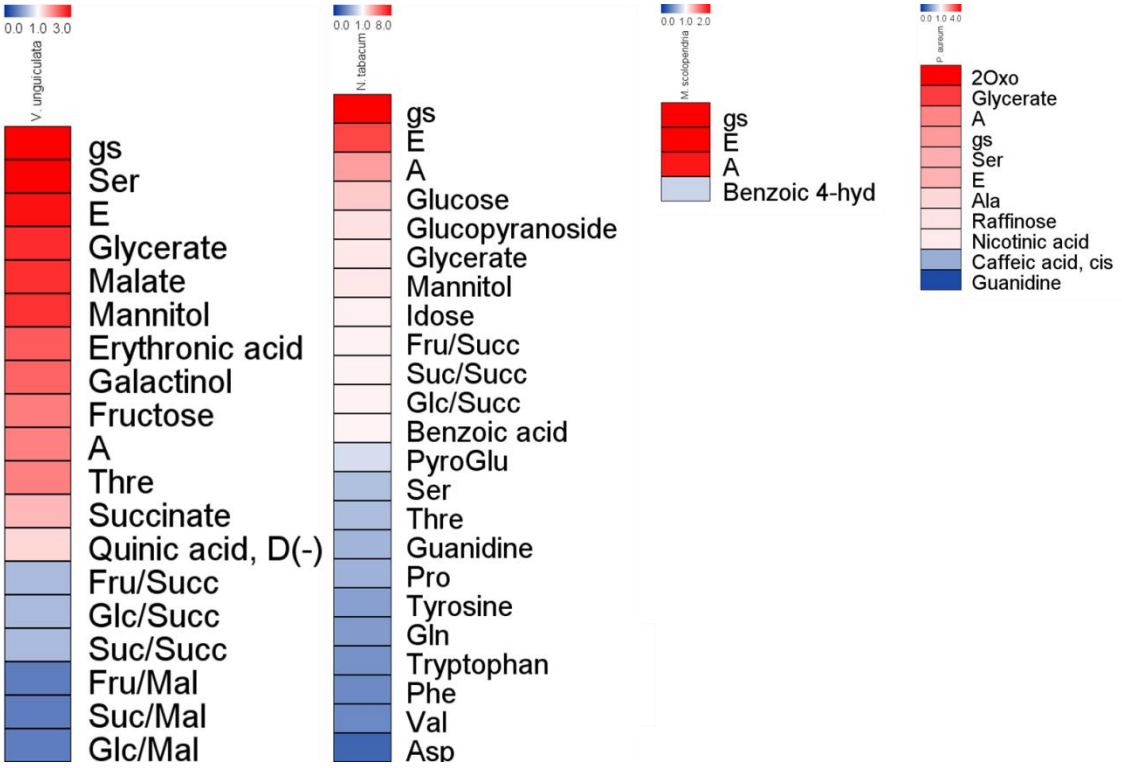

Figure 13. Metabolites and gas Exchange parameters with significant ($P < 0.05$) changes during dark-to-light transition. Data refers to the values found at 8 a.m. normalized to those of the pre-dawn (5 a.m.) in each species. Statistical by Student's *t* test.

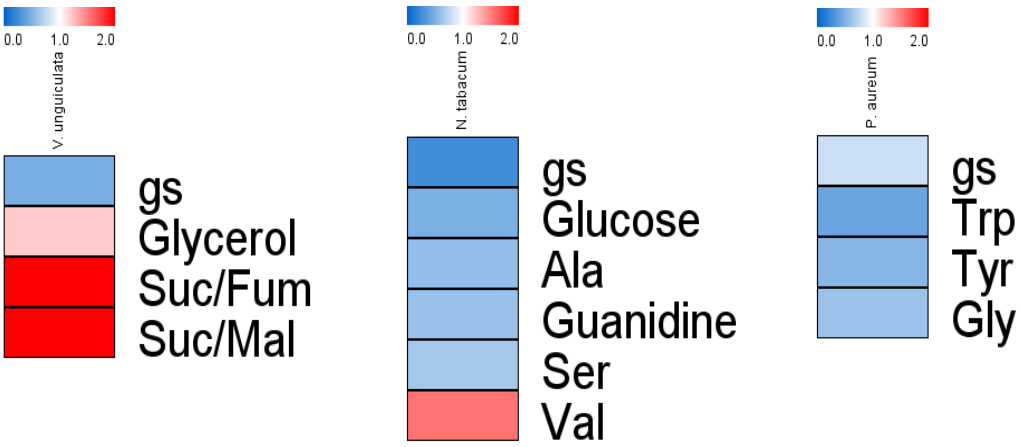

Figure 14. Metabolites and gas exchange parameters with significant ($P < 0.05$) changes during high CO_2-induced stomatal closure. Statistical by Student's *t* test. No metabolites or gas exchange parameters showed significant changes in the fern specie *M. scolopendria*.

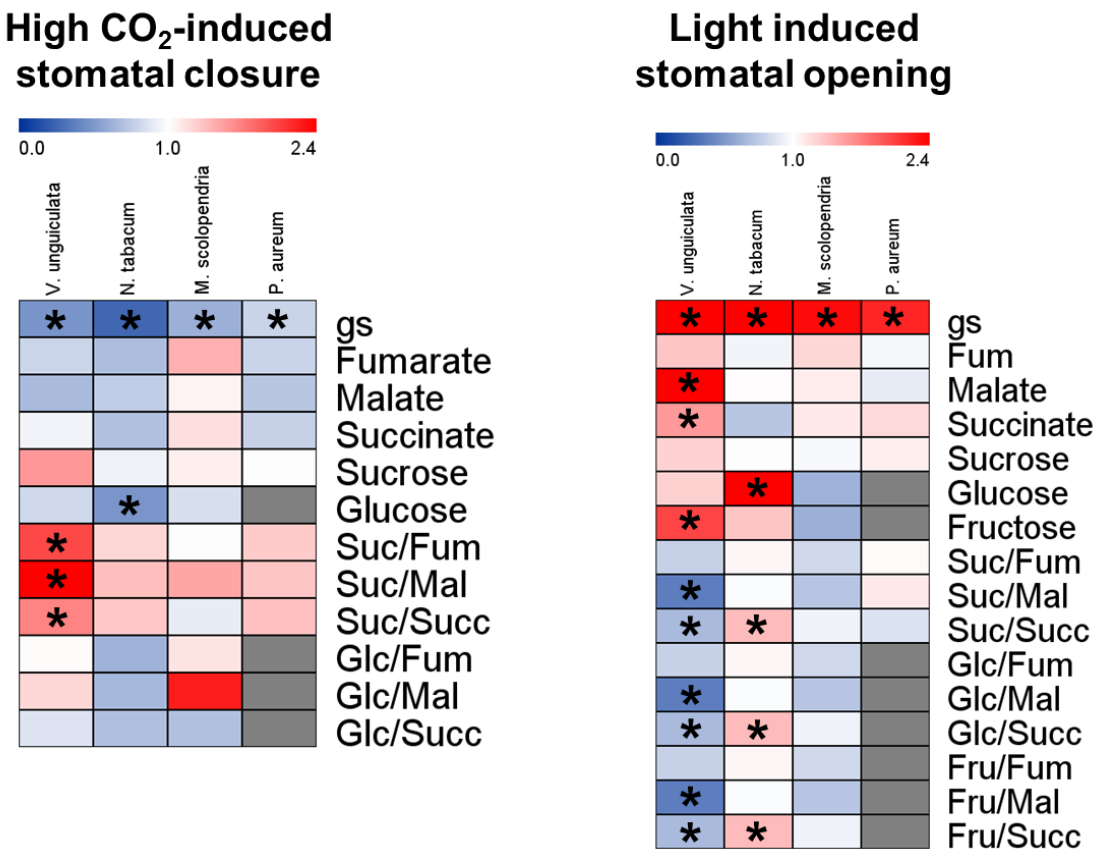

Figure 15. Changes in sugars and organic acid content during light-induced stomatal opening and high CO_2-induced stomatal closure. The gray color indicates undetectable metabolites, and asterisks indicates significant changes.

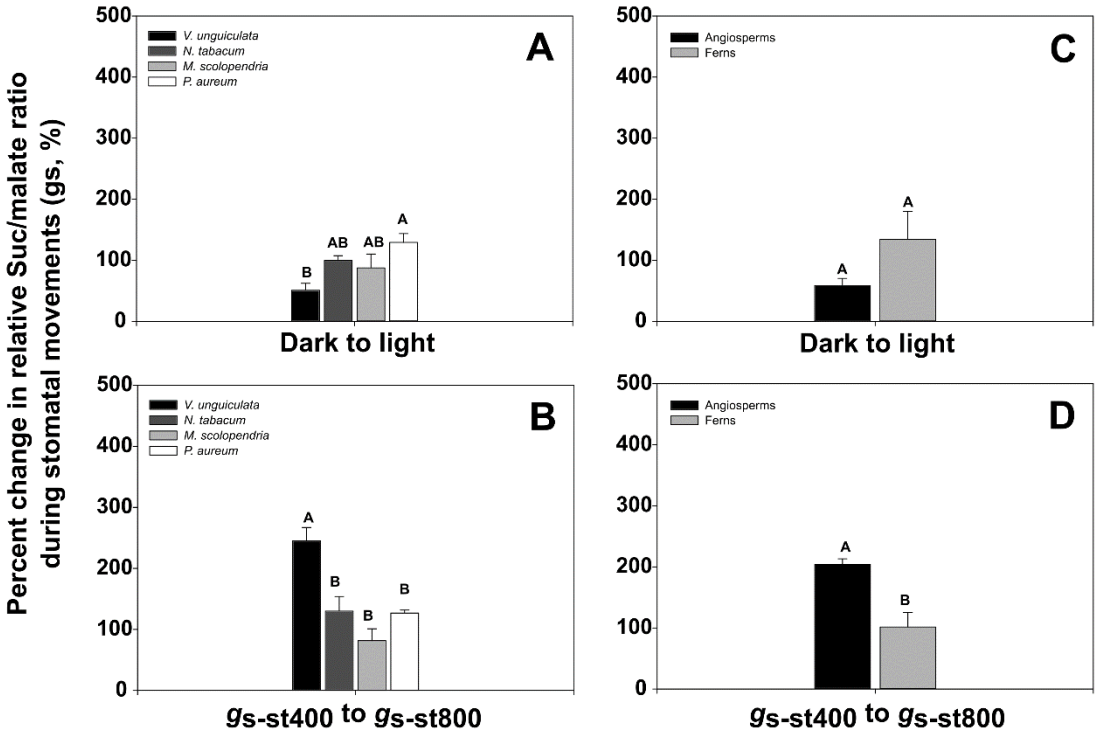

Figure 16. Sucrose to malate ratio (Suc/Mal) during dark-to-light transitions of the diel course (upper pannel) and ambient to high CO_2 concentrations ($g_{s\text{-}st400}$ to g_{sst800}).

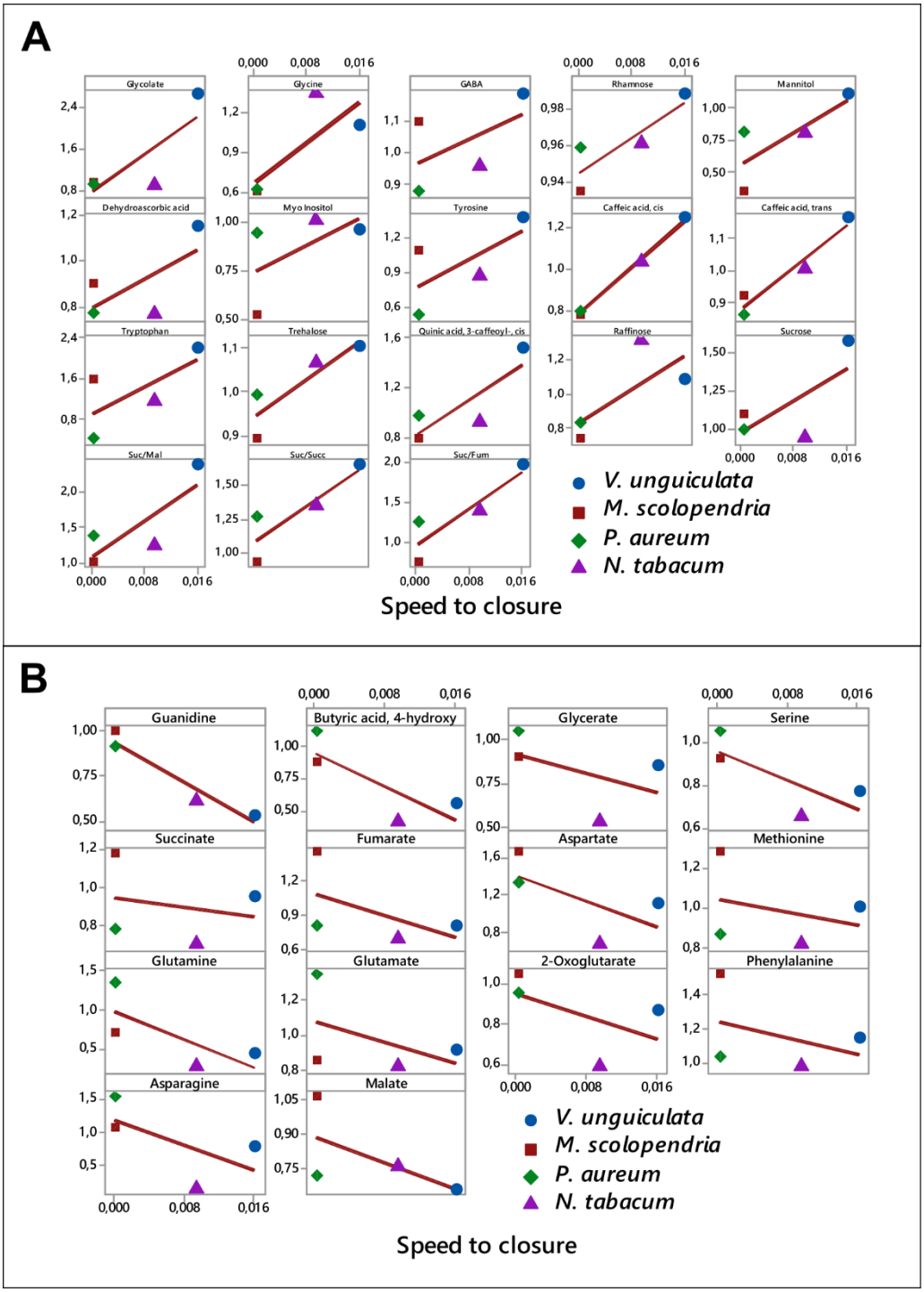

Figure 17. Relationship between relative metabolic changes and the speed of stomatal closure during high CO_2-induced stomatal closure. Data refers to the relative values obtained from all species in the time point $g_{s\text{-}st800}$ relative to $g_{s\text{-}st400}$. The speed of stomatal closure was obtained through the linear step of the high CO_2-induced stomatal closure kinetic, as demonstrated in the figure 8. In figure A were indicated metabolites with positive correlation and figure B metabolites with negative correlation. The Pearson correlation coefficient (r) of this analysis is found in the table S1.

Supplemental material

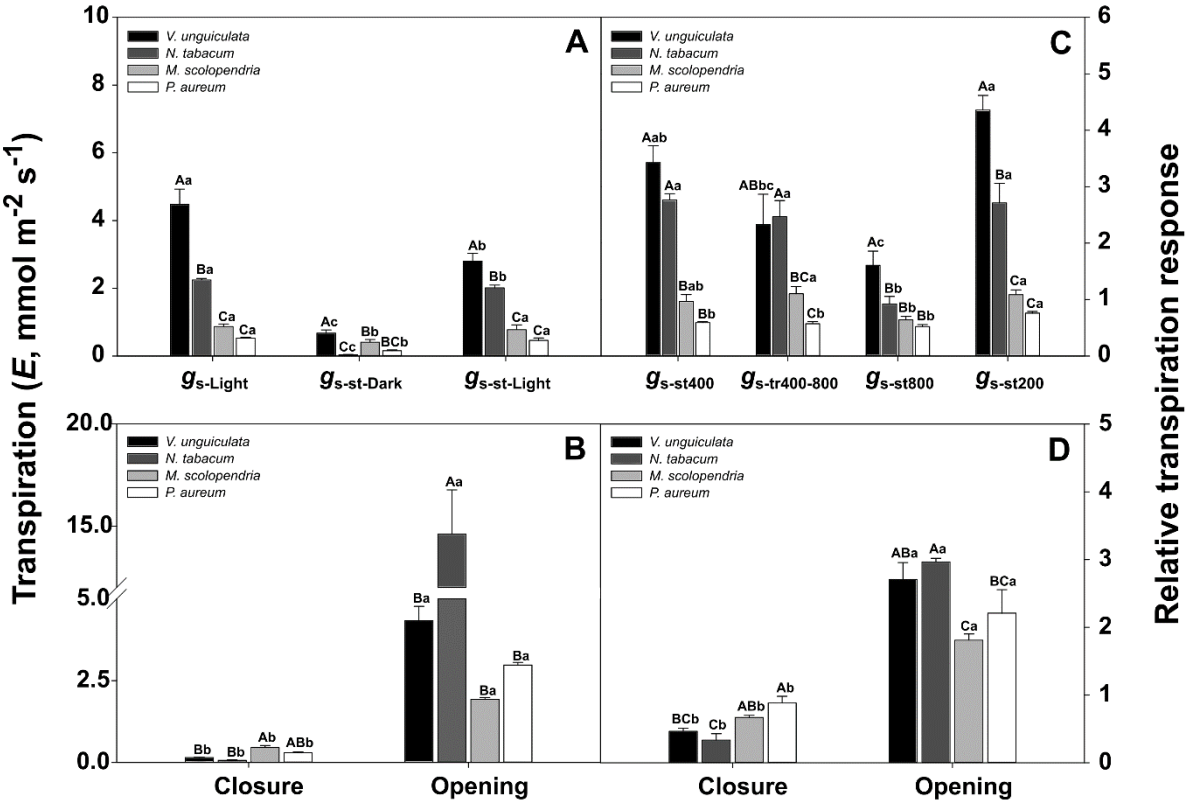

Figure S1. Changes in transpiration and relative stomatal responses to open and to close under light transitions (figures A and B) and under changes in CO_2 (figures C and D). Relative stomatal closure refers to the difference observed during light-to-dark and ambient (400 ppm) to high (800 ppm) CO_2 transitions, whilst relative stomatal opening refers to the difference observed during dark-to-light and high (800 ppm) to low (200 ppm) CO_2 transitions. Identical letters, upper case among treatments and lower case between species in each treatment, do not significantly differ by Tukey's test ($P \leq 0.05$). Data are the mean ± SE (n = 4).

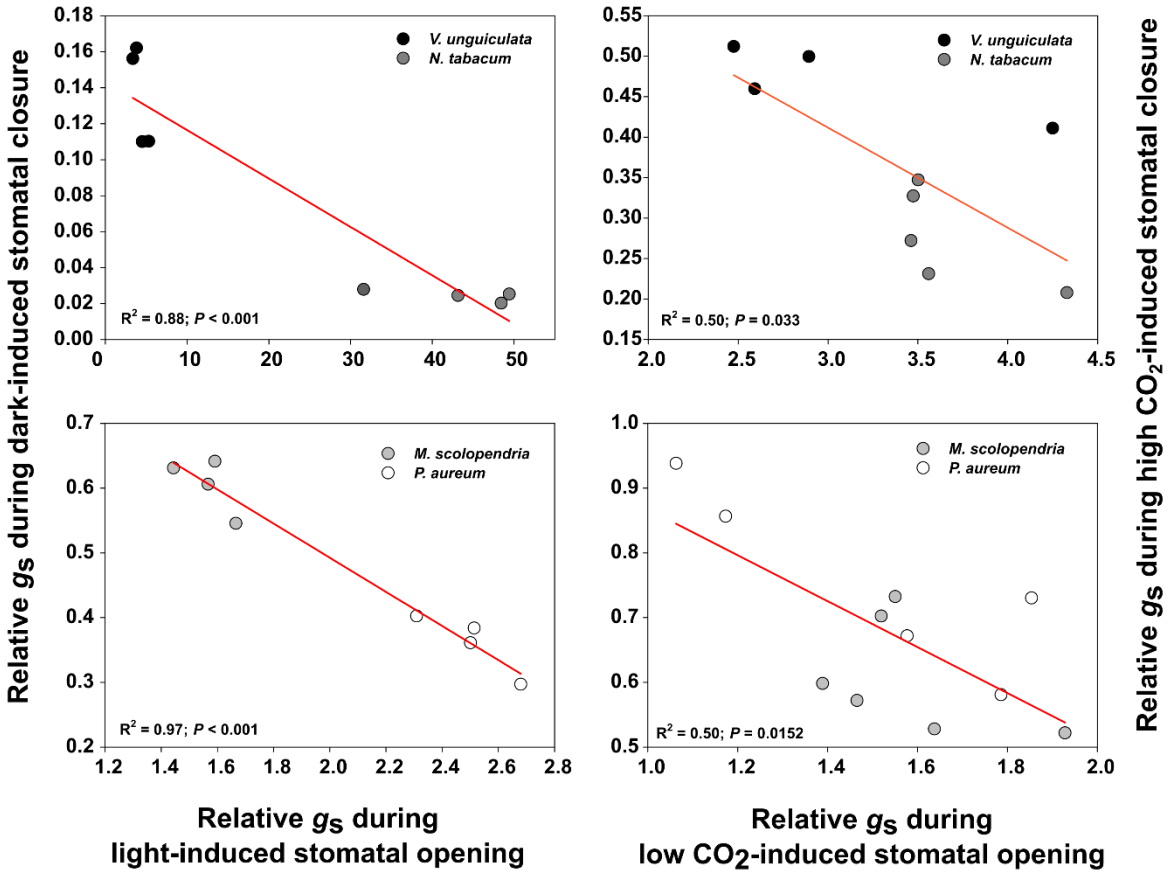

Figure S2. Relative stomatal responses to open and to close during dark/light transitions (left upper figure) and changes in CO_2 concentrations (right upper figure). The figures below demonstrate the relationship between the relative stomatal responses to open and to close under dark/light transitions and changes in the concentration of CO_2. Relative stomatal closure refers to the difference observed during light-to-dark and ambient (400 ppm) to high (800 ppm) CO_2 transitions, whilst relative stomatal opening refers to the difference observed during dark-to-light and high (800 ppm) to low (200 ppm) CO_2 transitions. Given the differences in scale between angiosperms and ferns, the scatterplot representation was carried out separately between these groups.

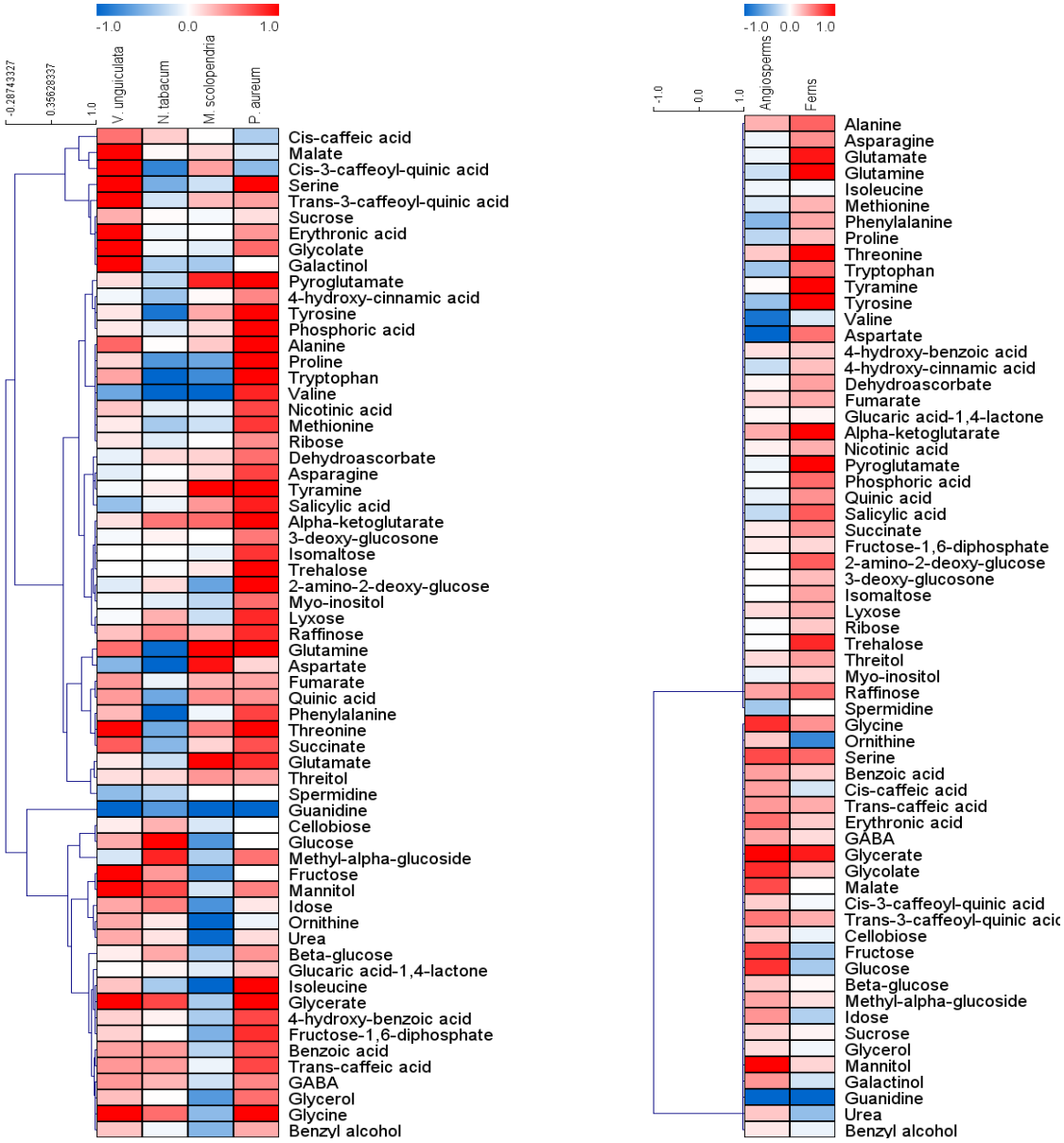

Figure S3. Metabolite profile of angiosperms (*V. unguiculata* and *N. tabacum*) and ferns (*M. scolopendria* and *P. aureum*) species during dark-to-light transition. Data are relative changes observed at 8 a.m. (light period) compared to 5 a.m. (dark period).

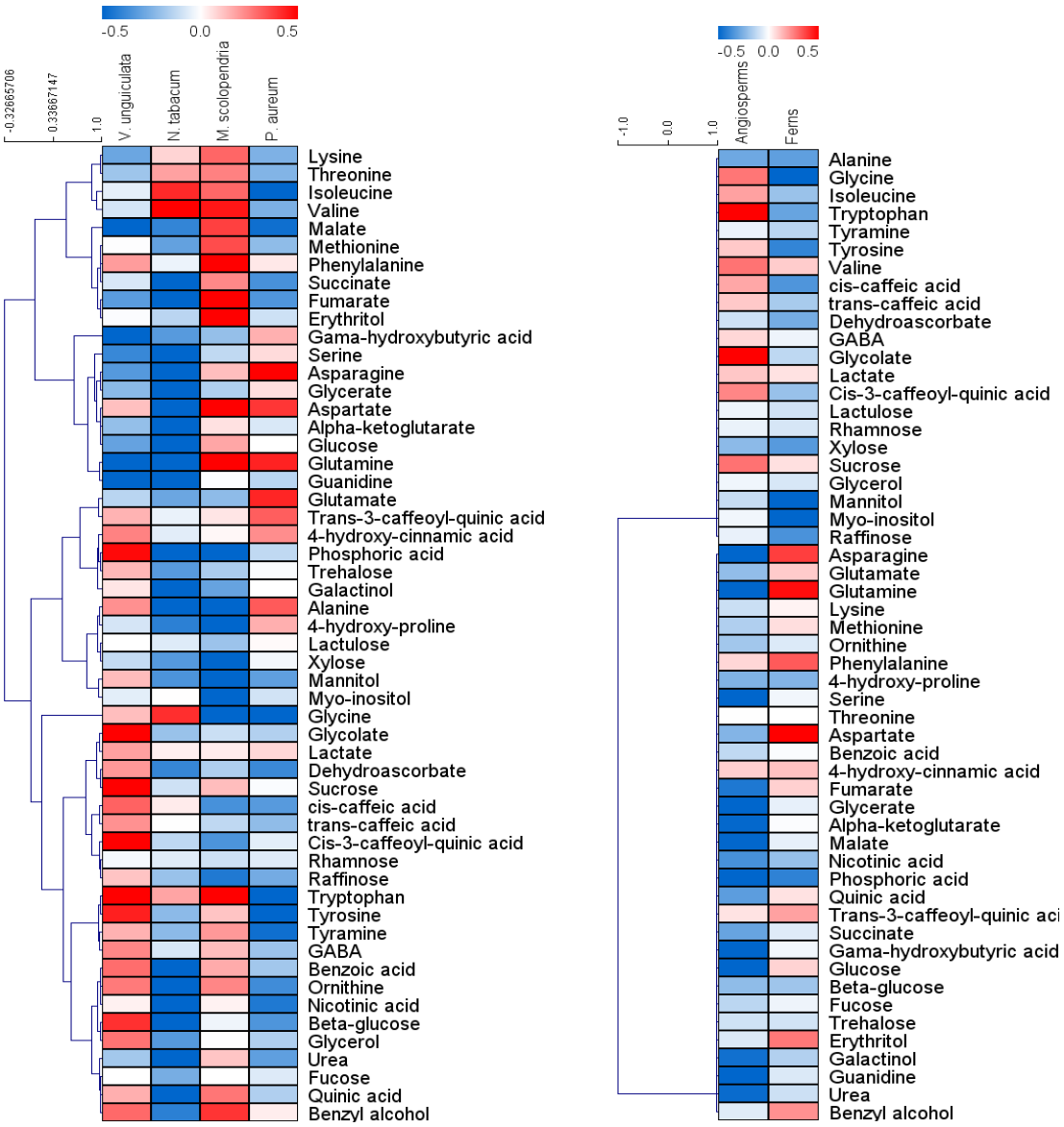

Figure S4. Metabolite profile of angiosperms (*V. unguiculata* and *N. tabacum*) and ferns (*M. scolopendria* and *P. aureum*) species during high CO_2-induced stomatal closure. Relative metabolic changes during ambient (400 ppm) to high (800 ppm) CO_2 condition per species (left) and among angiosperms and ferns (right).

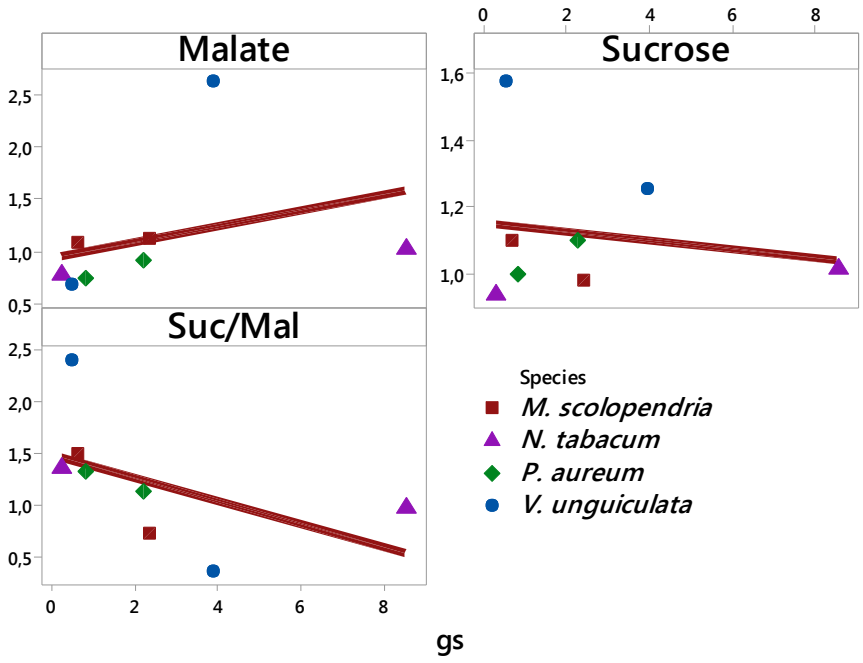

Figure S5. Relationship between sucrose, malate and Suc/Mal ratio using relative data from light-induced stomatal opening and high CO_2-induced stomatal closure. This relationship refers to the data presented in the figure 16.

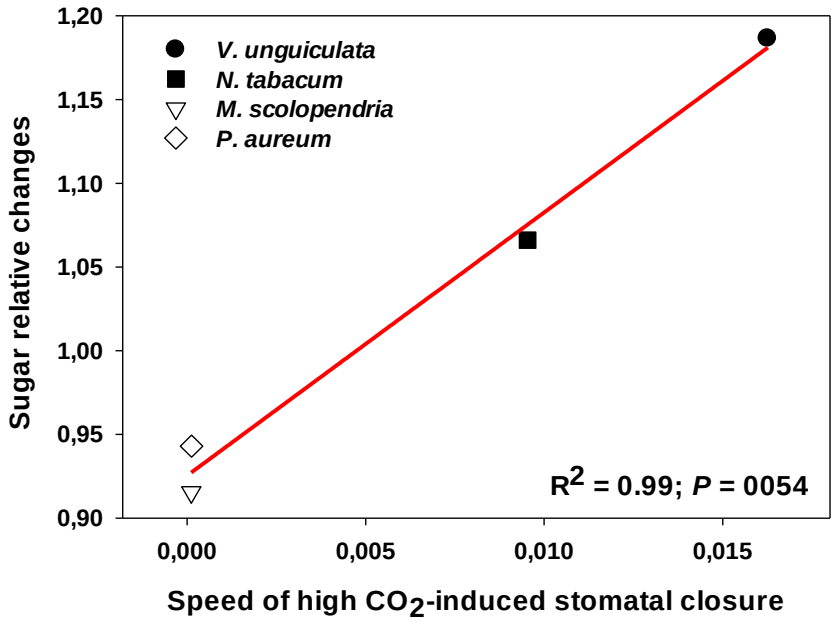

Figure S6. Relationship between sugar relative changes and the speed of stomatal closure during high CO_2-induced stomatal closure. Sugar relative changes refers to the average changes observed in the sugars sucrose, trehalose, raffinose and rhammose.

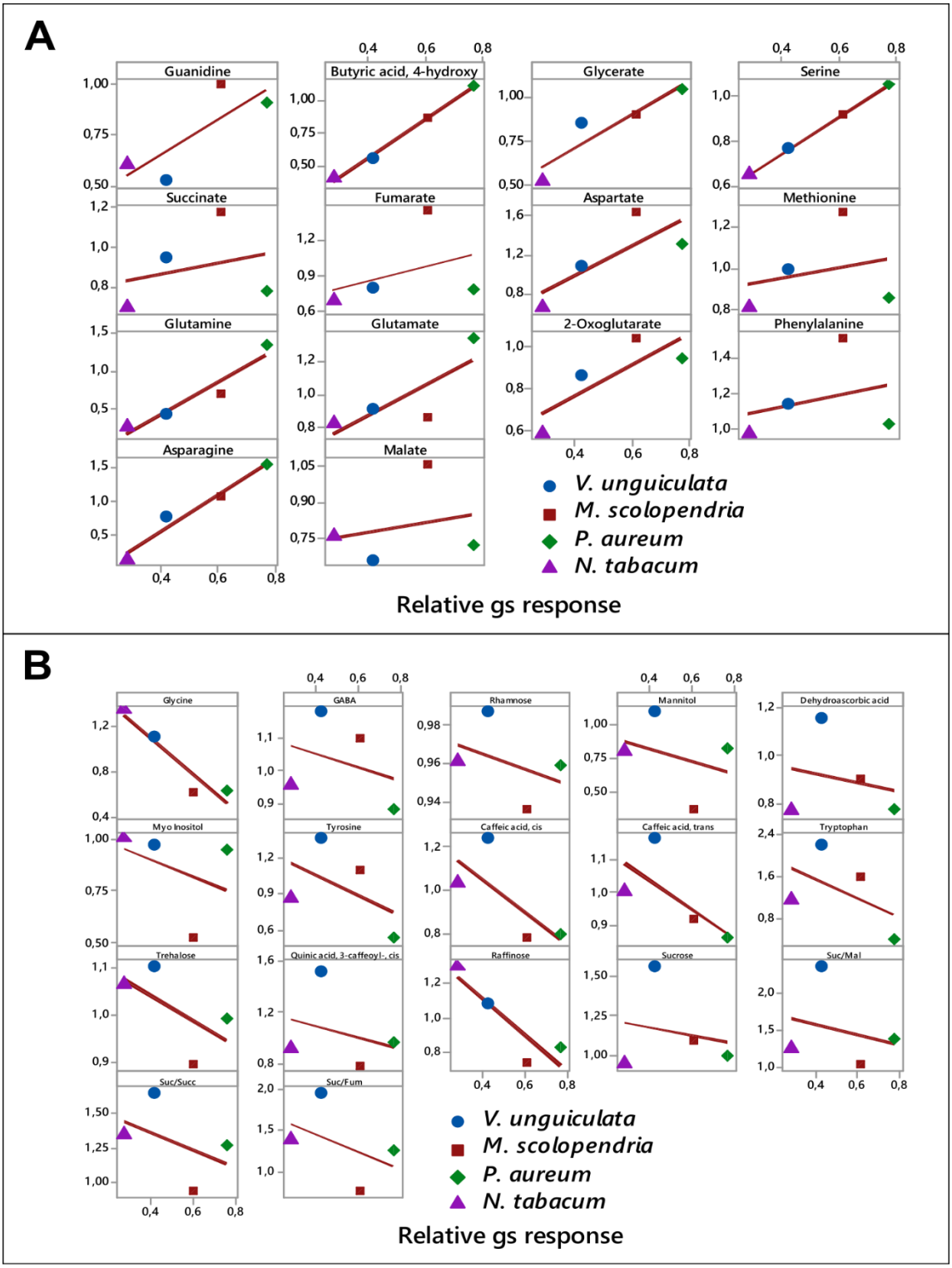

Figure S7. Relationship between relative metabolic changes and relative rate of stomatal closure during high CO_2-induced stomatal closure. Data refers to the relative values obtained from all species in the time point $g_{s\text{-}st800}$ relative to $g_{s\text{-}st400}$. In figure A were indicated metabolites with positive correlation and figure B metabolites with negative correlation. The Pearson correlation coefficient (r) of this analysis is found in the table S2.

Table S1. Pearson coefficient (*r*) between relative metabolic changes and speed of stomatal closure during high CO_2-induced stomatal closure. Metabolite data refers to the relative values obtained from all species in the time point $g_{s\text{-}st800}$ relative to $g_{s\text{-}st400}$. The speed of stomatal closure was obtained through the linear step of the high CO_2-induced stomatal closure kinetic, as demonstrated in the figure 8. The values in the table indicate the Pearson correlation coefficient (*r*) (in the same line of each metabolite) and the *P* value (below of *r*) for each metabolite-g_s correlation.

Relative metabolic changes *vs* speed of stomatal closure during ambient to high CO_2 transition			
Positive correlation		**Negative correlation**	
Glycolate	0.812	**Guanidine**	**-0.966**
	0.188		**0.034**
Glycine	0.811	Butyric acid, 4-hydroxy	-0.805
	0.189		0.195
GABA	0.547	Glycerate	-0.481
	0.453		0.519
Rhamnose	0.866	Serine	-0.758
	0.134		0.242
Mannitol	0.780	Succinate	-0.227
	0.220		0.773
Dehydroascorbic acid	0.673	Fumarate	-0.536
	0.327		0.464
Myo Inositol	0.583	Aspartate	-0.643
	0.417		0.357
Tyrosine	0.665	Methionine	-0.297
	0.335		0.703
Caffeic acid, cis	**0.998**	Glutamine	-0.724
	0.002		0.276
Caffeic acid, trans	**0.968**	Glutamate	-0.472
	0.032		0.528
Tryptophan	0.683	2-Oxoglutarate	-0.542
	0.317		0.458
Trehalose	0.888	Phenylalanine	-0.380
	0.112		0.620
Quinic acid, 3-caffeoyl-, cis	0.837	Asparagine	-0.626
	0.163		0.374
Raffinose	0.742	Malate	-0.626
	0.258		0.374
Sucrose	0.704		
	0.296		
Suc/Mal	0.817		
	0.183		
Suc/Succ	0.872		
	0.128		
Suc/Fum	0.892		
	0.108		

Table S2. Pearson coefficient (*r*) between relative metabolic changes and relative rate of stomatal closure during high CO_2-induced stomatal closure. Data refers to the relative values obtained from all species in the time point $g_{s\text{-}st800}$ relative to $g_{s\text{-}st400}$. The values in the table indicate the Pearson correlation coefficient (*r*) (in the same line of each metabolite) and the *P* value (below of *r*) for each metabolite-g_s correlation.

Relative metabolic changes *vs* relative stomatal closure during ambient to high CO_2 transition			
Positive correlation		**Negative correlation**	
Guanidine	0.813	Glycine	-0.948
	0.187		0.052
Butyric acid, 4-hydroxy	**0.997**	GABA	-0.323
	0.003		0.677
Glycerate	0.922	Rhamnose	-0.399
	0.078		0.601
Serine	**1.000**	Mannitol	-0.332
	0.000		0.668
Succinate	0.281	Dehydroascorbic acid	-0.224
	0.719		0.776
Fumarate	0.375	Myo Inositol	-0.380
	0.625		0.620
Aspartate	0.784	Tyrosine	-0.497
	0.216		0.503
Methionine	0.262	Caffeic acid, cis	-0.728
	0.738		0.272
Glutamine	0.961	Caffeic acid, trans	-0.696
	0.039		0.304
Glutamate	0.801	Tryptophan	-0.511
	0.199		0.489
2-Oxoglutarate	0.819	Trehalose	-0.623
	0.181		0.377
Phenylalanine	0.293	Quinic acid, 3-caffeoyl-, cis	-0.294
	0.707		0.706
Asparagine	0.979	Raffinose	-0.896
	0.021		0.104
Malate	0.246	Sucrose	-0.193
	0.754		0.807
		Suc/Mal	-0.258
			0.742
		Suc/Succ	-0.450
			0.550
		Suc/Fum	-0.455
			0.545

4 CONCLUSIONS

In summary, stomata from all species were responsive to changes in environmental stimuli, such as light/dark and CO_2 concentration. However, these responses were minor and slower in ferns than those observed in angiosperms. In both ferns and angiosperms, the sucrose-to-malate ratio was negatively related to stomatal responses and positively related to speed of stomatal closure. This suggests that these metabolites are important to modulate stomatal responses and explains, at least partially, the differential stomatal responses between angiosperms and ferns. Overall, our results provide important new information that helps to understand the metabolism-mediated mechanisms regulating stomatal movements across land plant evolution. Given that CO_2 stomatal responses are not strictly related to leaf metabolism, further studies aiming to identify whether ferns lack key components of the high CO_2-induced stomatal closure signalling network are of pivotal importance to improve our understanding concerning how plants acquired a better control of stomatal movement in response to CO_2 changes.

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