

PEPc-mediated CO₂ assimilation provides carbons to gluconeogenesis and the TCA cycle in both dark-exposed and illuminated guard cells

Valéria Lima¹, David Medeiros², Silvio Cândido-Sobrinho¹, Francisco Freire¹, Nicole Porto¹, Alexander Erban³, Joachim Kopka³, Markus Schwarzländer⁴, Alisdair R. Fernie⁵, and Danilo Daloso¹

¹Universidade Federal do Ceara

²Max Planck Institute of Molecular Plant Physiology

³Max-Planck Institute of Molecular Plant Physiology

⁴Universität Bonn

⁵Max-Planck Institute for Molecular Plant Physiology

April 05, 2024

Abstract

Evidence suggests that guard cells have higher rate of phosphoenolpyruvate carboxylase (PEPc)-mediated dark CO₂ assimilation than mesophyll cells. However, it is unknown which metabolic pathways are activated following dark CO₂ assimilation in guard cells. Furthermore, it remains unclear how the metabolic fluxes throughout the tricarboxylic acid (TCA) cycle and associated pathways are regulated in illuminated guard cells. Here we used ¹³C-HCO₃ labelling of tobacco guard cells harvested under continuous dark or during the dark-to-light transition to elucidate principles of metabolic dynamics downstream of CO₂ assimilation. Most metabolic changes were similar between dark-exposed and illuminated guard cells. However, illumination increased the ¹³C-enrichment in sugars and metabolites associated to the TCA cycle. Sucrose was labelled in the dark, but light exposure increased the ¹³C-labelling into this metabolite. Fumarate was strongly labelled under both dark and light conditions, while illumination increased the ¹³C-enrichment in pyruvate, succinate and glutamate. Only one ¹³C was incorporated into malate and citrate in either dark or light conditions. Our results collectively suggest that the PEPc-mediated CO₂ assimilation provides carbons for gluconeogenesis, the TCA cycle and glutamate synthesis and that previously stored malate and citrate are used to underpin the specific metabolic requirements of illuminated guard cells.

PEPc-mediated CO₂ assimilation provides carbons to gluconeogenesis and the TCA cycle in both dark-exposed and illuminated guard cells

Valéria F. Lima¹, David B. Medeiros², Silvio A. Cândido-Sobrinho¹, Francisco Bruno S. Freire¹, Nicole P. Porto¹, Alexander Erban², Joachim Kopka², Markus Schwarzländer³, Alisdair R. Fernie², Danilo M. Daloso^{1*}

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

² Max-Planck-Institute of Molecular Plant Physiology, 14476 Potsdam-Golm, Germany.

³ Institute of Plant Biology and Biotechnology, Westfälische-Wilhelms-Universität Münster, D-48143 Münster, Germany.

*Corresponding author: daloso@ufc.br

Summary

Evidence suggests that guard cells have higher rate of phosphoenol pyruvate carboxylase (PEPc)-mediated dark CO₂ assimilation than mesophyll cells. However, it is unknown which metabolic pathways are activated following dark CO₂ assimilation in guard cells. Furthermore, it remains unclear how the metabolic fluxes throughout the tricarboxylic acid (TCA) cycle and associated pathways are regulated in illuminated guard cells. Here we used ¹³C-HCO₃ labelling of tobacco guard cells harvested under continuous dark or during the dark-to-light transition to elucidate principles of metabolic dynamics downstream of CO₂ assimilation. Most metabolic changes were similar between dark-exposed and illuminated guard cells. However, illumination increased the ¹³C-enrichment in sugars and metabolites associated to the TCA cycle. Sucrose was labelled in the dark, but light exposure increased the ¹³C-labelling into this metabolite. Fumarate was strongly labelled under both dark and light conditions, while illumination increased the ¹³C-enrichment in pyruvate, succinate and glutamate. Only one ¹³C was incorporated into malate and citrate in either dark or light conditions. Our results collectively suggest that the PEPc-mediated CO₂ assimilation provides carbons for gluconeogenesis, the TCA cycle and glutamate synthesis and that previously stored malate and citrate are used to underpin the specific metabolic requirements of illuminated guard cells.

Key words: ¹³C-labelling analysis, glutamate, metabolic network, metabolic regulation, phosphoenol pyruvate carboxylase.

1. Introduction

Guard cell metabolism has been studied for over a century. From the starch-sugar conversion hypothesis to the discovery that inorganic ions such as potassium (K⁺) modulate guard cell osmotic potential during light-induced stomatal opening (Lloyd 1908; Fischer 1968), the understanding of how guard cell metabolism regulates stomatal movements has evolved dramatically over the decades (Granot & Kelly 2019). Breakdown of sugars, starch and lipids within guard cells are important mechanisms to sustain the speed of light-induced stomatal opening (Antunes *et al.* 2012; Ni 2012; Daloso *et al.* 2015a, 2016b; Horrer *et al.* 2016; McLachlan *et al.* 2016; Medeiros *et al.* 2018b; Flütsch *et al.* 2020b). Given the sink characteristics of guard cells (Hite *et al.* 1993; Ritte *et al.* 1999), the import of ATP, organic acids and sugars from mesophyll cells contributes to the stomatal opening upon illumination (Hedrich & Marten 1993; Araújo *et al.* 2011b; Kelly *et al.* 2013; Wang *et al.* 2014a, 2019; Lugassi *et al.* 2015; Medeiros *et al.* 2016; Antunes *et al.* 2017; Flütsch *et al.* 2020a). Thus, light mediates stomatal opening by both, perception directly in the guard cells (Kinoshita *et al.* 2001; Wang *et al.* 2010; Ando & Kinoshita 2018) and indirectly through signals derived from mesophyll cells (Mott 2009; Fujita *et al.* 2019; Flütsch & Santelia 2021). For the latter mode the respiratory activity seems to be of great importance (Nunes-Nesi *et al.* 2007a; Araújo *et al.* 2011b; Violet-Chabrand *et al.* 2021). Taken together, these works have substantially improved our understanding of light-induced changes in guard cell metabolism. However, light is just one out of several stimuli that affects stomatal aperture and how guard cell metabolism operates in the dark remains unexplored.

As the main energy source for plants, folial illumination activates photosynthesis alongside other mechanisms that sustain plant metabolism (Buchanan 2016). Photosynthetic ATP production and CO₂ assimilation mediated by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) are pivotal for the functioning of photoautotrophic cells. This is especially true for C3 and C4 plants, in which the vast majority of carbon assimilation occurs in the light period (Hohmann-Marriott & Blankenship 2011; Matthews *et al.* 2020). Additionally, given the capacity of plant cells to store and transport photoassimilates, the remobilisation of photosynthesis-derived products such as starch and sucrose is an important mechanism that sustains both metabolism and growth during the night period (Sulpice *et al.* 2014; Apelt *et al.* 2017; Mengin *et al.* 2017). By contrast to C3- and C4-plants, a special group of plants that evolved Crassulaceae Acid Metabolism (CAM) can also fix CO₂ through the activity of phosphoenol pyruvate carboxylase (PEPc) in the dark (Ranson & Thomas 1960; O’Leary *et al.* 2011). In these plants, the stomata open in the dark and close in the light. Whilst the stomatal opening in the dark favours the PEPc-mediated CO₂ fixation, stomatal closure

in the light avoids water loss, leading to the highest water use efficiency (WUE) observed in plants. Curiously, several C3 and C4 plants maintain a substantial transpiration stream during the night period (Caird *et al.* 2007; Costa *et al.* 2015). Furthermore, nocturnal stomatal conductance (g_{sn} - the rate of stomatal opening in the night) was positively correlated with relative growth rate in a multi-species meta-analysis (Resco de Dios *et al.* 2019). However, the regulation of g_{sn} remains insufficiently understood (Gago *et al.* 2020). Given the role of guard cell metabolism in regulating stomatal opening (Daloso *et al.* 2017; Lawson & Matthews 2020), unveiling how guard cell metabolism operates in the dark will be a prerequisite for a mechanistic understanding of g_{sn} regulation.

Radiotracer experiments suggest that guard cells can incorporate CO₂ in the dark at higher rates than mesophyll cells (Gotow *et al.* 1988). Furthermore, ¹³C-labelling results using mesophyll and guard cell protoplasts highlight that guard cells have faster and higher ¹³C-incorporation into malate under illuminated conditions (Robaina-Estevez *et al.* 2017). These results suggest a high PEPc activity in guard cells under either dark or light conditions. Indeed, recent ¹³C-positional labelling analysis confirmed that guard cells are able to assimilate CO₂ in the dark, as evidenced by the increases in the ¹³C-enrichment in the carbon 4 (4-C) of malate (Lima *et al.* 2021), which is derived from PEPc activity (Abadie & Tcherkez 2019). High dark CO₂ fixation rates are typically found in CAM cell types (Cockburn 1983). However, the idea that guard cells have CAM-like metabolism is not fully supported by transcriptome studies (Wang *et al.* 2011; Bates *et al.* 2012; Bauer *et al.* 2013; Aubry *et al.* 2016). It remains therefore unclear whether the metabolic photosynthetic mode of guard cells most closely resembles that of C3, C4 or CAM cells. Alternatively, guard cells might not strictly fit into any of these classifications. If this is the case, a specific mode of regulation may be predicted in these cells. This idea is supported by the finding that illumination triggers specific responses observed in guard cells, but not in mesophyll cells, such as the degradation of lipids, starch and sugars and the activation of glycolysis (Hedrich *et al.* 1985; Zhao & Assmann 2011; Daloso *et al.* 2015a, 2016b; Horrer *et al.* 2016; McLachlan *et al.* 2016; Robaina-Estevez *et al.* 2017; Medeiros *et al.* 2018b; Flutsch *et al.* 2020b). Additionally, the metabolic fluxes throughout the TCA cycle and associated metabolic pathways seems to be differentially regulated in illuminated guard cells, when compared to mesophyll cells (Daloso *et al.* 2017; Robaina-Estevez *et al.* 2017). These observations raise the question how light influences the regulation of key metabolic pathways in guard cells. Here we addressed this question using ¹³C-HCO₃ labelling in guard cells subjected to either dark or light conditions. To establish cell type-specific metabolic regulation and obtain an advanced picture of the metabolic flux landscape of guard cells in the light we integrate our current data with those of illuminated *Arabidopsis* (*Arabidopsis thaliana* L.) rosettes following provision of ¹³CO₂ and with illuminated *Arabidopsis* guard cells following provision of ¹³C-sucrose (Szecowka *et al.* 2013; Medeiros *et al.* 2018b).

2. Material and methods

2.1 Plant material and growth condition

Seeds of *Nicotiana tabacum* (L.) were germinated and cultivated in a substrate composed by a mixture of vermiculite, sand and soil (2:1:0.5) for 30 days. Plants were kept well-watered and nourished with Hoagland nutrient solution every week (Hoagland & Arnon 1950) under non-controlled greenhouse conditions with natural 12 h of photoperiod, ambient temperature 30 ± 4 degC, relative humidity 62 ± 10% and photosynthetic photon flux density (PPFD) which reached a maximum value of 500 μmol photons m⁻² s⁻¹.

2.2 Guard cell isolation

A pool of guard cell-enriched epidermal fragments (simply referred here as guard cells) was isolated following a protocol that was optimized for metabolite profiling analysis (Daloso *et al.* 2015a). Guard cells were isolated at pre-dawn by blending approximately three expanded leaves per replicate in a Warring blender (Philips, RI 2044 B.V. International Philips, Amsterdam, The Netherlands), that contains an internal filter to remove excess mesophyll cells, fibres and other cellular debris. The viability of the guard cells was analysed by staining with fluorescein diacetate and propidium iodide dyes, as described earlier (Huang *et al.* 1986). This

analysis demonstrated that only the guard cells are alive in the epidermal fragments (Daloso *et al.* 2015a; Antunes *et al.* 2017). All guard cell isolations were carried out in the dark in order to maintain closed stomata and simulate opening upon illumination, following the natural stomatal circadian rhythm (Daloso *et al.* 2016b; Antunes *et al.* 2017).

2.3 ^{13}C -isotope labelling experiment

After isolation, guard cells were transferred to light or dark conditions and incubated in a solution containing 50 μM CaCl_2 and 5 mM MES-Tris, pH 6.15 in the presence of 5 mM ^{13}C - NaHCO_3 . The experiment was initiated at the beginning of the light period of the day. Guard cell samples were rapidly harvested on a nylon membrane (220 μm) and snap-frozen in liquid nitrogen after 0, 10, 20 and 60 minutes under light or dark conditions.

2.4 Metabolomics analysis

Approximately 30 mg of guard cells were disrupted and transformed into a powder by maceration using mortar, pestle and liquid nitrogen. The powder was then used for metabolite extraction. The extraction and derivatization of polar metabolites were carried out as described previously (Lisec *et al.*, 2006), except that 1 ml of the polar (upper) phase, instead of 150 μl , was collected and reduced to dryness. Metabolites were derivatized by methoxyamination, with subsequent addition of tri-methyl-silyl (TMS) and finally analysed by gas chromatography electron impact – time of flight – mass spectrometry (GC-EI-TOF-MS), as described previously (Lisec *et al.*, 2006). The mass spectral analysis were performed using the software Xcalibur® 2.1 (Thermo Fisher Scientific, Waltham, MA, USA), as described earlier (Lima *et al.* 2018). The metabolites were identified using the Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>) (Kopka *et al.* 2005). The metabolite content is expressed as relative to ribitol (added to each biological replicate during the extraction) on a fresh weight (FW) basis. The data is reported following recommendations for metabolomics analysis (Supplemental Data S1) (Fernie *et al.* 2011; Alseekh *et al.* 2021).

2.5 ^{13}C -enrichment analysis

The relative abundance of the isotopologues (RIA) (M, M1, M2...Mn) and the fractional ^{13}C -enrichment ($F^{13}\text{C}$) was calculated as described previously (Lima *et al.* 2018). For instance, in a fragment of three carbons, the sum of the intensity of the isotopologues M, M1, M2 and M3 was set to 100%, and the intensity of each isotopologue is then relative to this sum. In this hypothetical fragment, the $F^{13}\text{C}$ is calculated according to the following equation: $F^{13}\text{C} = ((M1*1)+(M2*2)+(M3*3))/3$. The relative ^{13}C -enrichment ($R^{13}\text{C}$) was obtained by normalizing the $F^{13}\text{C}$ by the time 0 of the experiment.

2.6 Metabolic network analysis

Correlation-based metabolic networks were created using metabolite profiling data, in which the nodes correspond to the metabolites and the links to the strength of correlation between a pair of metabolites. The correlation was calculated using debiased sparse partial correlation (DSPC) analysis using the Java-based CorrelationCalculator software (Basu *et al.* 2017). The networks were designed by restricting the strength of the connections to a specific limit of DSPC coefficient (r) ($-0.5 > r > 0.5$) using Metscape on CYTOSCAPE v.3.7.2 software (Shannon *et al.* 2003; Karnovsky *et al.* 2012). Network-derived parameters such as clustering coefficient, network heterogeneity, network density and network centralization were obtained as described previously (Assenov *et al.* 2008). We further determined the preferential attachment and the appearance of new hubs, which highlight the nodes that are considered hubs before the start of the experiment (time 0) and maintain its degree of connection and demonstrate nodes that become highly connected after the beginning of the treatments, respectively (Freire *et al.* 2021).

2.7 Statistical analysis

All data are expressed as the mean of four replicates \pm standard error (SE). Significant difference in the content or relative ^{13}C -enrichment throughout the time was determined by one-way analysis of variance (ANOVA) and Dunnett test ($P < 0.05$), using the time 0 min as control. The difference between dark and light treatments in each time point was determined by Student's t test ($P < 0.05$). These statistical analyses were carried out using SIGMAPLOT12 (Systat Software Inc., San Jose, CA, USA) or Minitab 18 statistical software (State College, PA: Minitab, Inc.). Principal component analysis (PCA) was carried out in square root transformed and auto-scaled (mean-centred and divided by the standard deviation of each variable) data using the Metaboanalyst platform (Chong *et al.* 2018).

3. Results

To distinguish dark and light-mediated metabolic changes in guard cells, we performed a ^{13}C - HCO_3 labelling experiment. Guard cells were harvested pre-dawn and subjected to 0, 10, 20 and 60 minutes of continuous darkness or following transfer to white light conditions ($400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). Primary metabolites were identified and used to compare the changes at both metabolite content and ^{13}C -enrichment. Whilst changes in the content of metabolites indicate alterations in the pool of them throughout the experiment, the ^{13}C -enrichment analysis demonstrates the ^{13}C distribution derived from CO_2 assimilation mediated by PEPc in the dark and by both PEPc and RuBisCO in the light.

3.1 The changes in metabolite content were similar between dark-exposed and illuminated guard cells

Overall, the metabolic changes were similar between dark-exposed and illuminated guard cells. Under both dark and light conditions, the accumulation of fumarate, pyruvate, aspartate and lactate and the degradation of sucrose, glucose, gamma-aminobutyric acid (GABA) and different amino acids was observed (Figure 1). However, also differential behaviour of specific metabolites was observed. After 60 min of illumination the content of both glutamate (Glu) and urea increased as compared to the time 0 of the experiment and fructose, sorbose and citrate levels decreased (Figure 1). Comparing dark and light treatments in each time point, decreased content of alanine, citrate, glucose, *myo*-inositol, sorbose and sucrose was observed in illuminated samples. Interestingly, the content of malate is lower at 10 and 60 min and higher at 20 min in the light, as compared to dark-exposed guard cells (Supplemental Figure S1). This analysis highlights that the dynamic of relative metabolite accumulation/degradation throughout time is slightly altered by light imposition. This idea is further supported by principal component analysis (PCA), in which no clear separation of dark and light treatments was observed (Figure 2).

3.2 Illumination triggers re-modelling of metabolic network topology in guard cells

We have recently shown that light-induced stomatal opening involves changes in the density and topology of the guard cell metabolic network (Freire *et al.* 2021). Here, no general pattern of increasing or decreasing network parameters such as clustering coefficient, centralization, density and heterogeneity was observed over time in metabolic networks created using metabolite content data (Table S1). However, the number of hub-like nodes, the preferential attachment and the appearance of hub-like nodes differ between dark and light samples. Whilst these parameters reduced to zero after 60 min in the dark, they increased in the light metabolic network over time (Table S1), in which malate, fumarate, GABA and glucose appear as important hubs of the metabolic network of illuminated guard cells (Figure 3). These results suggest that illumination alter the metabolic network structure of guard cells, with particularly strong impact on the pathways associated to the TCA cycle and sugar metabolism.

3.3 Guard cells have a highly active metabolism in the dark

Similar to the described for metabolite content data, dark-exposed and illuminated guard cells display similar trends in the relative ^{13}C -enrichment (R^{13}C) data. Alanine, fumarate, glucose, glycine, sucrose and valine were labelled with statistical significance ($P < 0.05$) under both conditions in at least one time point, as compared to the time 0 of the experiment (Figure 4). However, comparing light and dark treatments in each time point, increased ^{13}C -labelling in sucrose, fructose, sorbose, malate, pyruvate, succinate and glutamate

was observed in illuminated guard cells, especially at 60 min of labelling (Supplemental Figure S2). In fact, PCA indicates that dark and light treatments differ mainly at 60 min, as evidenced by the separation by the first component (Figure 5). Analysis of the contribution of each metabolite for the PCA highlights that certain sugars (sucrose, fructose and glucose), organic acids (citrate and pyruvate), amino acids (Glu, Ile) and *myo*-inositol are those that mostly contributed to the separation observed in PCA of 60 min (Supplemental Figure S3). These results indicate that several metabolic pathways are activated following PEPc-mediated CO₂ assimilation in the dark, including gluconeogenesis and the TCA cycle. However, the combination of CO₂ assimilation catalysed by both PEPc and RuBisCO leads to more dramatic changes in ¹³C-distribution over these and other metabolic pathways in illuminated guard cells.

3.4 Fumarate is equally and strongly labelled under both dark and light conditions, but succinate labelling is increased by light exposure

We investigated the R¹³C in the fragments *m/z*245 of malate, *m/z* 245 of fumarate and *m/z* 247 of succinate, that contain the four carbons (1,2,3,4-C) of these metabolites. Increased R¹³C in fumarate *m/z* 245 under both dark and light conditions was observed (Figure 4) and no difference in the R¹³C of this fragment was noticed after 60 min of labelling between dark and light conditions (Figure 6). By contrast, light exposure leads to higher R¹³C in succinate *m/z* 247, when compared to dark-exposed guard cells (Figure 6). Relative isotopologue analysis (RIA) suggests the incorporation of four ¹³C into succinate under light conditions (Supplemental Figure S4) (see red circles at the Figure 6). No significant ¹³C-enrichment in malate *m/z* 245 was observed in either light or dark conditions, when compared to the time 0 of the experiment (Figure 4). However, the R¹³C-enrichment in malate *m/z* 245 was higher after 60 min in the light, when compared to guard cells kept in the dark (Figure 6). Intriguingly, the labelling in malate does not match those observed in fumarate and succinate. We have previously shown that only the 4-C of malate was labelled under either dark or light conditions (Lima *et al.* 2021), which contributes to explain the lack of significant labelling in malate *m/z* 245. The labelling in malate could be also interpreted by a dilution in the ¹³C-labelling from previously stored, non-labelled malate, which corroborates the role of malate as a counter-ion of potassium (K⁺) in the vacuole of guard cells (Outlaw & Lowry 1977; Tallman & Zeiger 1988; Talbott & Zeiger 1993).

3.5 Glutamate synthesis in illuminated guard cells likely depends on PEPc CO₂ assimilation and previously stored citrate

Illumination leads to increased R¹³C in glutamate *m/z* 156 (Figure 4). This fragment contains the 2,3,4,5-C of the Glu backbone, which are derived from the TCA cycle (2,3-C) and from pyruvate dehydrogenase (PDH) activity (4,5-C) (Abadie *et al.* 2017). We next investigated the sources of carbon for Glu synthesis in illuminated guard cells by analysing both R¹³C and RIA data of Glu and metabolites of, or associated to, the TCA cycle. Increased R¹³C in pyruvate *m/z* 174 (1,2,3-C) was only observed in the light (Figure 4). However, increases in the M3 isotopologue (*m/z* 177) of pyruvate was observed at 10 min of labelling in dark-exposed guard cells, indicating the incorporation of three carbons labelled at this time point (Supplemental Figure S5). After 60 min of labelling, the R¹³C in pyruvate was 2.5-fold higher in the light, when compared to dark-exposed guard cells (Figure 6). No substantial difference was noticed in the intensity of citrate isotopologues of the *m/z* 273 fragment (1,2,3,4,5-C) in both dark and light samples over time. Although a slight but significant increase in the intensity of *m/z* 274 was noticed (Supplemental Figure S6), this neither led to an increased R¹³C over time in the dark nor in the light (Figure 4). Furthermore, no difference in the R¹³C of *m/z* 273 between dark-exposed and illuminated guard cells after 60 min of labelling was observed (Figure 6). This fragment contains the 1,2,3,4,5-C of the citrate backbone (Okahashi *et al.* 2019). Interestingly, the labelling in citrate does not match those observed in Glu. RIA data of the fragment *m/z* 156 (2,3,4,5-C) indicates that four ¹³C were incorporated into Glu after 60 min in the light, as evidenced by the increases in the isotopologues M1-M4 (*m/z* 157-160) of this fragment (Supplemental Figure S7). This leads to a higher R¹³C in Glu in the light, when compared to dark-exposed guard cells (Figure 6). These results suggest that the carbon labelled in citrate is probably derived from PEPc activity (Abadie *et al.* 2017) and that the labelling in citrate is diluted by the incorporation of stored, non-labelled compounds, as previously suggested in leaves (Cheung *et al.* 2014).

Given that Glu could be synthesized by the activity of both Ala and Asp aminotransferases (AlaAT and AspAT) and degraded toward GABA synthesis, we additionally investigated the ^{13}C -enrichment in these metabolites. An increased R^{13}C in Ala was observed in both conditions, whilst Asp was only labelled in the light, when compared to the time 0 of the experiment (Figure 4). However, no difference in the fractional ^{13}C -enrichment (F^{13}C) of these metabolites between dark and light conditions after 60 min of labelling was observed (Figure 6). No ^{13}C -enrichment in GABA m/z 174 was observed (Figure 6). However, assessment of recent data from Arabidopsis guard cells fed with ^{13}C -sucrose (Medeiros *et al.* 2018b), showed an increased F^{13}C in this metabolite during dark-to-light transition (Supplemental Figure S8). Taken together, these results suggest that whilst both AlaAT and AspAT might contribute to the synthesis of Glu, the reactions catalysed by these enzymes do not fully explain the labelling found in Glu. It seems likely that Glu synthesis in illuminated guard cells mostly depends on carbons from both PEPc-mediated CO_2 assimilation and previously stored citrate.

3.6 Guard cells have smaller photorespiratory activity than mesophyll cells

Despite the presence of RuBisCO-mediated CO_2 assimilation in guard cells (Daloso *et al.* 2015a), the photorespiratory activity of these cells remains insufficiently understood. To shed light on the relative importance of photosynthesis and photorespiration in guard cells, we next evaluated the ^{13}C -enrichment into sugars as metabolites of successful photosynthetic assimilation, and glycine, serine and glycerate as photorespiratory metabolites. Increased R^{13}C in sucrose m/z 319 (3,4,5,6-C) was observed under both dark and light conditions. However, increased R^{13}C in sucrose m/z 361 (1,2,3,4,5,6-C) was only observed in the light, when compared to time 0 (Figure 4). This resulted in a higher R^{13}C in both sucrose m/z 319 and m/z 361 in illuminated guard cells, as compared to dark-exposed guard cells after 60 min of labelling. Similarly, sorbose, fructose and glucose were preferentially labelled in the light (Figure 7).

Increased R^{13}C in glycine m/z 102 was observed in both illuminated and dark-exposed guard cells, whilst increased R^{13}C in serine m/z 204 over time was only observed in the light (Figure 4). However, no difference in the R^{13}C of these metabolites between the treatments after 60 min of labelling was observed. Additionally, glycerate was not labelled in either condition (Figure 7). We next compared the ^{13}C -enrichment in different fragments of both glycine and serine with those obtained in illuminated Arabidopsis rosettes following provision of $^{13}\text{CO}_2$ (Szecowka *et al.* 2013). The R^{13}C in glycine and serine was 2.1 and 4.4-fold higher in whole Arabidopsis rosettes (where mesophyll represents the dominant tissue type) than guard cells (Supplemental Figure S9), suggesting particularly low photorespiratory activity in guard cells.

4. Discussion

4.1 On the complex interplay among the metabolic pathways that regulate the homeostasis of sugars and organic acids in guard cells

Stomata are important regulators of water use efficiency (WUE) in illuminated leaves (Brodribb *et al.* 2019). Furthermore, a recent growing body of evidence suggests that nocturnal stomatal conductance (g_{sn}) plays an important role in WUE regulation (Violet-Chabrand *et al.* 2021; McAusland *et al.* 2021). However, whilst the signalling pathways that regulate stomatal opening in the light have been widely investigated (Inoue & Kinoshita 2017), the mechanisms that regulate g_{sn} remain unknown. Here, we provide insight as to how guard cell metabolism may contribute to the regulation of g_{sn} . Given that the trends observed in both metabolite level and ^{13}C -enrichment are similar between dark-exposed and illuminated guard cells, it could be that similar mechanisms regulate both g_{sn} and g_{s} . This idea is supported by the fact that sucrose breakdown and fumarate synthesis, two mechanisms that support stomatal opening in the light (Daloso *et al.* 2015a; Medeiros *et al.* 2016, 2017, 2018b; Granot & Kelly 2019; Flütsch *et al.* 2020b), were observed under both dark and light conditions. However, it is important to highlight that the metabolic changes were generally more pronounced in illuminated guard cells. Light exposure may contribute to the activation of several other stomatal regulatory mechanisms in guard cells, given the degradation of starch and lipids in illuminated guard cells (Horrer *et al.* 2016; McLachlan *et al.* 2016; Flütsch *et al.* 2020b). Thus, the separation observed in the PCA at 60 min of labelling and the differences in the metabolic networks

between dark-exposed and illuminated guard cells could explain the need for more dramatic changes in illuminated guard cells, which would ultimately lead to higher g_s values, when compared to g_{sn} . This idea is further supported by the higher degradation rate of sugars observed in the light, which supports findings in which reduced sucrose cleavage capacity of guard cells severely compromises light-induced stomatal opening (Antunes *et al.* 2012; Ni 2012; Freire *et al.* 2021).

Reverse genetic studies have indicated that alteration in sugar homeostasis in guard cells affects stomatal behaviour (reviewed in Daloso *et al.*, 2016a; Flütsch & Santelia, 2021). Additionally, reduced photosynthetic activity in guard cell chloroplasts has been demonstrated to disrupt light-induced stomatal opening (Azoulay-Shemer *et al.* 2015). Although this can be attributed to changes in the cofactor metabolism of ATP and NADPH (Roelfsema *et al.* 2006; Wang *et al.* 2014a), reduced plastidial photosynthetic activity may also compromise sugar homeostasis in guard cells. Indeed, the $R^{13}C$ into sugars was higher in the light, evidencing that the RuBisCO-mediated CO_2 assimilation contribute to sugar synthesis in illuminated guard cells. The ^{13}C -labelling incorporation into sugars in the dark suggests that gluconeogenesis is active in guard cells. This corroborates the high ^{13}C -enrichment observed in the 3,4-C of glucose under either dark or light conditions (Lima *et al.* 2021), which are proposed to be the glucose carbons preferentially labelled by gluconeogenesis (Leegood & ap Rees 1978; Beylot *et al.* 1993). These results highlight that gluconeogenesis may be another metabolic pathway that contributes to sugar homeostasis in guard cells, an elusive source of carbon for sugar synthesis in guard cells that has long been debated (Willmer & Ditttrich 1974; Outlaw & Kennedy 1978; Talbott & Zeiger 1998; Zeiger *et al.* 2002; Outlaw 2003; Vavasseur & Raghavendra 2005; Daloso *et al.* 2016a).

Relative isotopologue analysis indicates that three ^{13}C were incorporated into pyruvate in both dark-exposed and illuminated guard cells, as evidenced by the significant increases in pyruvate m/z 177 after 10 min of exposure to continuous dark or after dark-to-light transition (Supplemental Figure S5). The labelling in pyruvate in the light might occur by a combination of ^{13}C derived from both RuBisCO and PEPc CO_2 assimilation, while the labelling in this metabolite in the dark suggests the activity of phosphoenol pyruvate carboxykinase (PEPCK) and/or malic enzyme (ME), in which labelled OAA and malate would be rapidly converted into PEP and pyruvate, respectively. Additionally, glycolysis and the activity of pyruvate kinase (PK), that converts PEP to pyruvate, could also contribute to pyruvate labelling. Given the labelling observed in sugars in the dark, it seems that the carbon assimilated by PEPc is used to create a substrate cycle between gluconeogenesis and glycolysis, allowing the circulation of carbon between sugars and organic acids without important loss of assimilated carbon. Thus, PEPc activity would be important to re-assimilate the CO_2 lost by several decarboxylation reactions that occurs in chloroplast, mitochondria and cytosol (Sweetlove *et al.* 2013). Indeed, previous modelling results suggest that the flux of CO_2 from the chloroplast to the cytosol is 17-fold higher in guard cells than mesophyll cells and is largely re-assimilated by PEPc in the cytosol (Robaina-Estévez *et al.* 2017). According to this model, the carbon assimilated by PEPc is transported back to the chloroplast as malate, resulting in a net production of NADPH (Robaina-Estévez *et al.* 2017). These results collectively suggest that PEPc activity is important for both the carbon re-assimilation and the homeostasis of sugars and organic acids in guard cells. The maintenance of a flux of carbon between sugars and organic acids (gluconeogenesis and glycolysis) could be a mechanism to rapidly provide carbons for starch synthesis or for the TCA cycle and associated pathways during stomatal closure and opening conditions, respectively (Outlaw & Manchester 1979; Medeiros *et al.* 2018b).

4.2 Regulation of the TCA cycle and associated metabolic pathways in guard cells

The regulation of plant TCA cycle enzymes is strongly dependent on light quality and quantity (Nunes-Nesi *et al.* 2013). Several lines of evidence point to restricted metabolic fluxes through the TCA cycle in illuminated leaves (Tcherkez *et al.* 2009; Gauthier *et al.* 2010; Daloso *et al.* 2015b; Abadie *et al.* 2017; Florez-Sarasa *et al.* 2019), in which different non-cyclic TCA flux modes may contribute to maintain the oxidative phosphorylation system (OxPHOS) (Rocha *et al.* 2010; Sweetlove *et al.* 2010). The GABA shunt has been shown to be an important alternative pathway for the synthesis of succinate, the substrate of the complex II of OxPHOS (Nunes-Nesi *et al.* 2007b; Studart-Guimaraes *et al.* 2007). GABA can be synthesized in the

cytosol by glutamate decarboxylase or in the mitochondria by GABA transaminases, in which glutamate, 2-oxoglutarate or pyruvate serve as substrates (Bouché & Fromm 2004). Thus, GABA synthesis is closely associated with the TCA cycle, representing a hub for the C:N metabolic network (Fait *et al.* 2008). Additionally, recent evidence suggests that GABA is an important modulator of stomatal movements, acting as negative regulator of stomatal opening (Xu *et al.* 2021). Our previous data highlighted that the carbon derived from ^{13}C -sucrose is incorporated into GABA, but to a lesser extent than into Gln (Medeiros *et al.* 2018b). Here, GABA was degraded in a light-independent manner, which may supply OxPHOS with substrate by supporting succinate synthesis in mitochondria. However, no ^{13}C -enrichment in GABA was observed. In parallel, increased ^{13}C -enrichment in Glu in illuminated guard cells was observed. Therefore, while Glu and Gln seems to be important sinks of the carbons derived from CO_2 assimilation mediated by both RuBisCO and PEPc, the metabolic flux from Glu/Gln to GABA may be restricted during the dark-to-light transition as a mechanism to allow stomatal opening. This idea is supported by the fact that GABA is a negative regulator of ALTM9 (aluminium-activated malate transporter 9) (Xu *et al.* 2021; Siqueira *et al.* 2021), a key vacuolar anion uptake channel activated during stomatal opening (De Angeli *et al.* 2013; Medeiros *et al.* 2018a).

Illumination increased the metabolic fluxes throughout the TCA cycle and associated pathways in guard cells. This idea is supported by the higher F^{13}C observed in malate, succinate, pyruvate and Glu in the light, when compared to dark-exposed guard cells (Figure 6). Furthermore, increased R^{13}C in lactate and aspartate was only observed in the light. It is noteworthy that these results were obtained in guard cells with no K^+ in the medium, given that the presence of this ion strongly increased the ^{13}C -enrichment in TCA cycle metabolites, especially in fumarate and malate (Daloso *et al.* 2015a). Thus, one would expect that the light and dark metabolic differences may be higher *in vivo*, given that light stimulates the influx of potassium to guard cells (Hills *et al.* 2012; Wang *et al.* 2014b c). Surprisingly, the relative content and the R^{13}C in fumarate increased substantially under both dark and light conditions. The light-induced ^{13}C -enrichment in fumarate resembles previous ^{13}C -feeding experiments using $^{13}\text{C}\text{-HCO}_3$ (Daloso *et al.* 2015a, 2016b; Robaina-Estévez *et al.* 2017). This corroborates the facts that fumarate is the major organic acid accumulated in the light (Pracharoenwattana *et al.* 2010) and that plants with higher fumarate accumulation have higher g_s (Nunes-Nesiet *et al.* 2007a; Araújo *et al.* 2011b; Medeiros *et al.* 2016, 2017). Furthermore, fumarate emerged as an important hub for the guard cell metabolic network during dark-to-light transition, in agreement with previous observations in guard cells (Freire *et al.* 2021). However, no evidence has hitherto indicated that fumarate is neither the main organic acid accumulated nor can act as osmolyte under dark conditions (Gauthier *et al.* 2010; Araújo *et al.* 2011a; Cheung *et al.* 2014; Tan & Cheung 2020). Thus, whether the accumulation of fumarate in dark-exposed guard cells is a mechanism to sustain g_{sn} and/or to store carbon skeletons for the following light period remains unclear. Whilst the characterization of the dynamic of g_{sn} in plants with altered fumarate accumulation may be sufficient to understand whether fumarate acts as osmolyte in the dark, testing the second hypothesis will require more sophisticated metabolic experiments to determine the pattern and the subcellular accumulation of fumarate in guard cells during the diel cycle.

Our results suggest that previously stored, non-labelled organic acids are used to support the metabolic requirements of guard cell metabolism in the light, given that the ^{13}C -enrichment in citrate and malate is lower than in metabolites of the following steps of the pathway such as fumarate, succinate and Glu. Genome scale metabolic modelling suggests that citrate is the main organic acid accumulated in leaf vacuoles during the night period, which is released and used as substrate for Glu synthesis in the light (Cheung *et al.* 2014). A similar model build specifically for guard cell metabolism predicted that malate accumulates at high rate in the vacuole of guard cells, especially when K^+ accumulation was restricted by the model (Tan & Cheung 2020). Taken together, modelling and ^{13}C -labelling results indicate the importance of previously stored organic acids to support the TCA cycle and Glu synthesis in illuminated guard cells, resembling the mechanism of TCA cycle regulation observed in leaves (Sweetlove *et al.* 2010; Nunes-Nesi *et al.* 2013; da Fonseca-Pereira *et al.* 2021).

4.3 On the source of carbons for glutamate synthesis in illuminated guard cells

The connection between the TCA cycle and Glu metabolism is well-established (Araújo *et al.* 2012, 2013; O’Leary & Plaxton 2020). ^{13}C -NMR studies indicate that the synthesis of Glu in illuminated leaves strongly depends on stored compounds (Tcherkez *et al.* 2009; Gauthier *et al.* 2010; Abadie *et al.* 2017), which are presumably accumulated in the previous night period (Cheung *et al.* 2014). This is likely an alternative to overcome the light-inhibition of PDH (Tovar-Méndez *et al.* 2003; Zhang *et al.* 2021), an important source of carbons for the TCA cycle and associated pathways (Reid *et al.* 1977). The degradation of labelled pyruvate by PDH or PC would provide two and three labelled carbons into acetyl-CoA and OAA, respectively. Once synthesized, the acetyl-CoA could be used for citrate synthesis or exported from the mitochondria for fatty acid synthesis (Lonien & Schwender 2009). However, export of citrate for fatty acid synthesis is unlikely to hold true given that light exposure triggers fatty acid degradation in guard cells (McLachlan *et al.* 2016). It seems likely that fully labelled OAA is synthesized by the activity of both PEPc and PC, which is in turn simultaneously used to the synthesis of succinate through the activity of MDH and fumarase through the C4-branch of the TCA cycle and the synthesis of Glu through the C6(5)-branch of the TCA cycle. In parallel, PDH-derived acetyl-CoA would also contribute to the labelling of Glu through the C6(5)-branch of the TCA cycle.

This labelling pattern throughout the TCA cycle and associated pathways consider the incorporation of non-labelled carbons into malate and citrate. Alternatively, succinate, that is strongly labelled in the light, but not in the dark, could be used as a substrate for Glu synthesis. In this scenario, the TCA cycle would occur in a counterclockwise direction between succinate and 2-OG in illuminated guard cells. Although this possibility has never been confirmed in plants, reverse TCA cycle flux modes have been identified in certain bacteria, being a route to assimilate CO_2 through the activities of 2-oxoglutarate dehydrogenase (OGDH) and isocitrate dehydrogenase (IDH) in the counterclockwise direction (Evans *et al.* 1966; Buchanan & Arnon 1990; Mall *et al.* 2018; Steffens *et al.* 2021). Thus, beyond providing carbon skeletons for Glu synthesis, a reverse mode of operation of the TCA cycle would also contribute to increase the CO_2 assimilated in guard cells. However, this hypothesis is unlikely to occur given the biochemical characteristics of OGDH, that is improbable to occur in the reverse (counterclockwise) direction (Araújo *et al.* 2013), and the fact that this would depend on the activity of 2-OG synthase (E.C 1.2.7.3), that has only been described in bacteria’s (Buchanan & Evans 1965; Buchanan & Arnon 1990; Yoon *et al.* 1996, 1997; Dörner & Boll 2002; Yamamoto *et al.* 2010). Therefore, our results collectively suggest that the carbon derived from PEPc-mediated CO_2 assimilation is simultaneously used to support gluconeogenesis, the TCA cycle and Glu synthesis and that previously stored citrate and malate may also be an important source of carbons for the TCA cycle and associated pathways.

Acknowledgments

This work was made possible through financial support from the National Council for Scientific and Technological Development (CNPq, Grant 428192/2018-1). We also thank the research fellowship granted by CNPq to D.M.D and the scholarships granted by CNPq to F.B.S.F and by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES-Brazil) to V.F.L. and N.P.P.

Author contributions

VFL and DMD designed the experiment. VFL, FBSF, NPP and SACS performed the ^{13}C -labelling experiment. Mass spectrometry analysis was performed by DBM under the supervision of ARF. Data analysis was performed by VFL, FBSF, SACS, AE, JK and DMD. All authors contributed to write the final manuscript. DMD obtained funding and is responsible for this article.

Conflict of interest

The authors declare no potential conflict of interest.

References

Abadie C., Lothier J., Boex-Fontvieille E., Carroll A. & Tcherkez G. (2017) Direct assessment of the metabolic

origin of carbon atoms in glutamate from illuminated leaves using ^{13}C -NMR. *New Phytologist* **216** , 1079–1089.

Abadie C. & Tcherkez G. (2019) In vivo phosphoenolpyruvate carboxylase activity is controlled by CO_2 and O_2 mole fractions and represents a major flux at high photorespiration rates. *New Phytologist* **221** , 1843–1852.

Alseikh S., Aharoni A., Brotman Y., Contrepolis K., D’Auria J., Ewald J., ... Fernie A.R. (2021) Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. *Nature Methods* **18** , 747–756.

Ando E. & Kinoshita T. (2018) Red Light-Induced Phosphorylation of Plasma Membrane H^+ -ATPase in Stomatal Guard Cells. *Plant Physiology* **178** , 838–849.

De Angeli A., Zhang J., Meyer S. & Martinoia E. (2013) AtALMT9 is a malate-activated vacuolar chloride channel required for stomatal opening in Arabidopsis. *Nature Communications* **4** , 1804–1810.

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

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

Apelt F., Breuer D., Olas J.J., Annunziata M.G., Flis A., Nikoloski Z., ... Stitt M. (2017) Circadian, carbon, and light control of expansion growth and leaf movement. *Plant Physiology* **174** , 1949–1968.

Araújo W.L., Nunes-Nesi A. & Fernie A.R. (2011a) Fumarate: Multiple functions of a simple metabolite. *Phytochemistry* **72** , 838–843.

Araújo W.L., Nunes-Nesi A., Nikoloski Z., Sweetlove L.J. & Fernie A.R. (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. *Plant, Cell and Environment* **35** , 1–21.

Araújo W.L., Nunes-Nesi A., Osorio S., Usadel B., Fuentes D., Nagy R., ... Fernie A.R. (2011b) Antisense Inhibition of the Iron-Sulphur Subunit of Succinate Dehydrogenase Enhances Photosynthesis and Growth in Tomato via an Organic Acid-Mediated Effect on Stomatal Aperture. *The Plant Cell* **23** , 600–627.

Araújo W.L., Trofimova L., Mkrtchyan G., Steinhäuser D., Krall L., Graf A., ... Bunik V.I. (2013) On the role of the mitochondrial 2-oxoglutarate dehydrogenase complex in amino acid metabolism. *Amino Acids* **44** , 683–700.

Assenov Y., Ramírez F., Schelhorn S.-E., Lengauer T. & Albrecht M. (2008) Computing topological parameters of biological networks. *Bioinformatics* **24** , 282–284.

Aubry S., Aresheva O., Reyna-Llorens I., Smith-Unna R.D., Hibberd J.M. & Genty B. (2016) A specific transcriptome signature for guard cells from the C_4 plant *Gynandropsis gynandra*. *Plant Physiology* **170** , 1345–1357.

Azoulay-Shemer T., Palomares A., Bagheri A., Israelsson-Nordstrom M., Engineer C.B., Bargmann B.O.R., ... Schroeder J.I. (2015) Guard cell photosynthesis is critical for stomatal turgor production, yet does not directly mediate CO_2 - and ABA-induced stomatal closing. *Plant Journal* **83** , 567–581.

Basu S., Duren W., Evans C.R., Burant C.F., Michailidis G. & Karnovsky A. (2017) Systems biology Sparse network modeling and metscape-based visualization methods for the analysis of large-scale metabolomics data. **33** , 1545–1553.

- Bates G.W., Rosenthal D.M., Sun J., Chattopadhyay M., Peffer E., Yang J., ... Jones A.M. (2012) A Comparative Study of the Arabidopsis thaliana Guard-Cell Transcriptome and Its Modulation by Sucrose. *PLoS ONE* **7** .
- Bauer H., Ache P., Wohlfart F., Al-Rasheid K.A.S., Sonnewald S., Sonnewald U., ... Hedrich R. (2013) How do stomata sense reductions in atmospheric relative humidity? *Molecular Plant* **6** , 1703–1706.
- Beylot M., Previs S.F., David F. & Brunengraber H. (1993) Determination of the ¹³C-labeling pattern of glucose by gas chromatography-mass spectrometry. *Analytical Biochemistry* **212** , 526–531.
- Bouché N. & Fromm H. (2004) GABA in plants: Just a metabolite? *Trends in Plant Science* **9** , 110–115.
- Brodribb T.J., Sussmilch F. & McAdam S.A.M. (2019) From Reproduction to Production, Stomata are the Master Regulators. *The Plant Journal* , tpj.14561.
- Buchanan B.B. (2016) The carbon (formerly dark) reactions of photosynthesis. *Photosynthesis Research* **128** , 215–217.
- Buchanan B.B. & Arnon D.I. (1990) A reverse KREBS cycle in photosynthesis: consensus at last. *Photosynthesis Research* **24** , 47–53.
- Buchanan B.B. & Evans M.C. (1965) The synthesis of alpha-ketoglutarate from succinate and carbon dioxide by a subcellular preparation of a photosynthetic bacterium. *Proceedings of the National Academy of Sciences of the United States of America* **54** , 1212–1218.
- Caird M.A., Richards J.H. & Donovan L.A. (2007) Nighttime stomatal conductance and transpiration in C3 and C4 plants. *Plant Physiology* **143** , 4–10.
- Cheung C.Y.M., Poolman M.G., Fell D.A., George Ratcliffe R. & Sweetlove L.J. (2014) A diel flux balance model captures interactions between light and dark metabolism during day-night cycles in C3 and crassulacean acid metabolism leaves. *Plant Physiology* **165** , 917–929.
- Chong J., Soufan O., Li C., Caraus I., Li S., Bourque G., ... Xia J. (2018) MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research* **46** , W486–W494.
- Cockburn W. (1983) Stomatal mechanism as the basis of the evolution of CAM and C4 photosynthesis. *Plant, Cell & Environment* **6** , 275–279.
- Costa J.M., Monnet F., Jannaud D., Leonhardt N., Ksas B., Reiter I.M., ... Genty B. (2015) Open all night long: The Dark Side of Stomatal Control. *Plant Physiology* **167** , 289–294.
- Daloso D.M., dos Anjos L. & Fernie A.R. (2016a) Roles of sucrose in guard cell regulation. *New Phytologist* **211** , 809–818.
- Daloso D.M., Antunes W.C., Pinheiro D.P., Waquim J.P., Araújo W.L., Loureiro M.E., ... Williams T.C.R. (2015a) Tobacco guard cells fix CO₂ by both Rubisco and PEPcase while sucrose acts as a substrate during light-induced stomatal opening. *Plant Cell and Environment* **38** , 2353–2371.
- Daloso D.M., Medeiros D.B., dos Anjos L., Yoshida T., Araújo W.L. & Fernie A.R. (2017) Metabolism within the specialized guard cells of plants. *New Phytologist* **216** , 1018–1033.
- Daloso D.M., Müller K., Obata T., Florian A., Tohge T., Bottcher A., ... Fernie A.R. (2015b) Thioredoxin, a master regulator of the tricarboxylic acid cycle in plant mitochondria. *Proceedings of the National Academy of Sciences* **112** , E1392–E1400.
- Daloso D.M., Williams T.C.R., Antunes W.C., Pinheiro D.P., Müller C., Loureiro M.E. & Fernie A.R. (2016b) Guard cell-specific upregulation of sucrose synthase 3 reveals that the role of sucrose in stomatal function is primarily energetic. *New Phytologist* **209** , 1470–1483.

- Dörner E. & Boll M. (2002) Properties of 2-oxoglutarate:Ferredoxin oxidoreductase from *Thaueria aromatica* and its role in enzymatic reduction of the aromatic ring. *Journal of Bacteriology* **184** , 3975–3983.
- Evans M.C., Buchanan B.B. & Arnon D.I. (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. *Proceedings of the National Academy of Sciences of the United States of America* **55** , 928–934.
- Fait A., Fromm H., Walter D., Galili G. & Fernie A.R. (2008) Highway or byway: the metabolic role of the GABA shunt in plants. *Trends in Plant Science* **13** , 14–19.
- Fernie A.R., Aharoni A., Willmitzer L., Stitt M., Tohge T., Kopka J., . . . DeLuca V. (2011) Recommendations for Reporting Metabolite Data. *The Plant Cell* **23** , 2477–2482.
- Fischer R. (1968) Stomatal Opening: Role of Potassium Uptake by Guard Cells. *Science* **160** , 784–785.
- Florez-Sarasa I., Obata T., Del-Saz N.F., Reichheld J.-P., Meyer E.H., Rodriguez-Concepcion M., . . . Fernie A.R. (2019) The Lack of Mitochondrial Thioredoxin TRXo1 Affects In Vivo Alternative Oxidase Activity and Carbon Metabolism under Different Light Conditions. *Plant and Cell Physiology* **60** , 2369–2381.
- Flütsch S., Nigro A., Conci F., Fajkus J., Thalmann M., Trtílek M., . . . Santelia D. (2020a) Glucose uptake to guard cells via STP transporters provides carbon sources for stomatal opening and plant growth. *EMBO reports* **21** , 1–13.
- Flütsch S. & Santelia D. (2021) Mesophyll-derived sugars are positive regulators of light-driven stomatal opening. *New Phytologist* **230** , 1754–1760.
- Flütsch S., Wang Y., Takemiya A., Vialat-Chabrand S.R.M., Klejchová M., Nigro A., . . . Santelia D. (2020b) Guard Cell Starch Degradation Yields Glucose for Rapid Stomatal Opening in Arabidopsis. *The Plant Cell* **32** , 2325–2344.
- da Fonseca-Pereira P., Souza P.V.L., Fernie A.R., Timm S., Daloso D.M. & Araújo W.L. (2021) Thioredoxin-mediated regulation of (photo)respiration and central metabolism. *Journal of Experimental Botany* **72** , 5987–6002.
- Freire F.B.S., Bastos R.L.G., Bret R.S.C., Cândido-Sobrinho S.A., Medeiros D.B., Antunes W.C., . . . Daloso D.M. (2021) Mild reductions in guard cell sucrose synthase 2 expression leads to slower stomatal opening and decreased whole plant transpiration in *Nicotiana tabacum* L. *Environmental and Experimental Botany* **184** , 104370.
- Fujita T., Noguchi K., Ozaki H. & Terashima I. (2019) Confirmation of mesophyll signals controlling stomatal responses by a newly devised transplanting method. *Functional Plant Biology* **46** , 467–481.
- Gago J., Daloso D.M., Carriquí M., Nadal M., Morales M., Araújo W.L., . . . Flexas J. (2020) The photosynthesis game is in the “inter-play”: Mechanisms underlying CO₂ diffusion in leaves. *Environmental and Experimental Botany* **178** , 104174.
- Gauthier P.P.G., Bligny R., Gout E., Mahé A., Nogués S., Hodges M. & Tcherkez G.G.B. (2010) In folio isotopic tracing demonstrates that nitrogen assimilation into glutamate is mostly independent from current CO₂ assimilation in illuminated leaves of *Brassica napus*. *New Phytologist* **185** , 988–999.
- Gotow K., Taylor S. & Zeiger E. (1988) Photosynthetic Carbon Fixation in Guard Cell Protoplasts of *Vicia faba* L. *Plant Physiol* **86** , 700–705.
- Granot D. & Kelly G. (2019) Evolution of Guard-Cell Theories: The Story of Sugars. *Trends in Plant Science* **24** , 507–518.
- Hedrich R. & Marten I. (1993) Malate-induced feedback regulation of plasma membrane anion channels could provide a CO₂ sensor to guard cells. *The EMBO Journal* **12** , 897–901.

- Hedrich R., Raschke K. & Stitt M. (1985) A role for fructose 2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. *Plant physiology* **79** , 977–982.
- Hills A., Chen Z.-H., Amtmann A., Blatt M.R. & Lew V.L. (2012) OnGuard, a Computational Platform for Quantitative Kinetic Modeling of Guard Cell Physiology. *Plant Physiology* **159** , 1026–1042.
- Hite D.R.C., Outlaw W.H. & Tarczynski M.C. (1993) Elevated levels of both sucrose-phosphate synthase and sucrose synthase in Vicia guard cells indicate cell-specific carbohydrate interconversions. *Plant physiology* **101** , 1217–1221.
- Hoagland D.R. & Arnon D.I. (1950) The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* **347** , 1–32.
- Hohmann-Marriott M.F. & Blankenship R.E. (2011) Evolution of Photosynthesis. *Annual Review of Plant Biology* **62** , 515–548.
- Horrer D., Flütsch S., Pazmino D., Matthews J.S.A., Thalmann M., Nigro A., ... Santelia D. (2016) Blue light induces a distinct starch degradation pathway in guard cells for stomatal opening. *Current Biology* **26** , 362–370.
- Huang C.-N., Cornejo M.J., Bush D.S. & Jones R.L. (1986) Estimating viability of plant protoplasts using double and single staining. *Protoplasma* **135** , 80–87.
- Inoue S. & Kinoshita T. (2017) Blue Light Regulation of Stomatal Opening and the Plasma Membrane H⁺-ATPase. *Plant Physiology* **174** , 531–538.
- Karnovsky A., Weymouth T., Hull T., Tarcea V.G., Scardoni G., Laudanna C., ... Omenn G.S. (2012) Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data. *Bioinformatics* **28** , 373–380.
- Kelly G., Moshelion M., David-Schwartz R., Halperin O., Wallach R., Attia Z., ... Granot D. (2013) Hexokinase mediates stomatal closure. *Plant Journal* **75** , 977–988.
- Kinoshita T., Doi M., Suetsugu N., Kagawa T., Wada M. & Shimazaki K. (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414** , 656–660.
- Kopka J., Schauer N., Krueger S., Birkemeyer C., Usadel B., Bergmüller E., ... Steinhauser D. (2005) GMD@CSB.DB: The Golm metabolome database. *Bioinformatics* **21** , 1635–1638.
- Lawson T. & Matthews J. (2020) Guard Cell Metabolism and Stomatal Function. *Annual Review of Plant Biology* **71** , 273–302.
- Leegood R.C. & ap Rees T. (1978) Dark fixation of CO₂ during gluconeogenesis by the cotyledons of Cucurbita pepo L. *Planta* **140** , 275–282.
- Lima V.F., Erban A., Daubermann A.G., Freire F.B.S., Porto N.P., Cândido-Sobrinho S.A., ... Daloso D.M. (2021) Establishment of a GC-MS-based ¹³C-positional isotopomer approach suitable for investigating metabolic fluxes in plant primary metabolism. *The Plant Journal* .
- Lima V.F., de Souza L.P., Williams T.C.R., Fernie A.R. & Daloso D.M. (2018) Gas Chromatography–Mass Spectrometry–Based ¹³C-Labeling Studies in Plant Metabolomics. In *Plant Metabolomics* . pp. 47–58.
- Lloyd F. E. (1908) The physiology of stomata. *Publications of the Carnegie Institution of Washington* **82** , 1–42.
- Lonien J. & Schwender J. (2009) Analysis of Metabolic Flux Phenotypes for Two Arabidopsis Mutants with Severe Impairment in Seed Storage Lipid Synthesis. *Plant Physiology* **151** , 1617–1634.
- Lugassi N., Kelly G., Fidel L., Yaniv Y., Attia Z., Levi A., ... Granot D. (2015) Expression of Arabidopsis Hexokinase in Citrus Guard Cells Controls Stomatal Aperture and Reduces Transpiration. *Frontiers in Plant*

Science **6** , 1–11.

Mall A., Sobotta J., Huber C., Tschirner C., Kowarschik S., Bačnik K., ... Berg I.A. (2018) Reversibility of citrate synthase allows autotrophic growth of a thermophilic bacterium. *Science***359** , 563–567.

Matthews J.S.A., Vialet-Chabrand S. & Lawson T. (2020) Role of blue and red light in stomatal dynamic behaviour. *Journal of Experimental Botany* **71** , 2253–2269.

McAusland L., Smith K.E., Williams A., Molero G. & Murchie E.H. (2021) Nocturnal stomatal conductance in wheat is growth-stage specific and shows genotypic variation. *New Phytologist* **232** , 162–175.

McLachlan D.H., Lan J., Geilfus C.M., Dodd A.N., Larson T., Baker A., ... Hetherington A.M. (2016) The Breakdown of Stored Triacylglycerols is Required during Light-Induced Stomatal Opening. *Current Biology* **26** , 707–712.

Medeiros D.B., Barros K.A., Barros J.A.S., Omena-Garcia R.P., Arrivault S., Sanglard L.M.V.P., ... Araújo W.L. (2017) Impaired Malate and Fumarate Accumulation Due to the Mutation of the Tonoplast Dicarboxylate Transporter Has Little Effects on Stomatal Behavior. *Plant Physiology* **175** , 1068–1081.

Medeiros D.B., Fernie A.R. & Araújo W.L. (2018a) Discriminating the Function(s) of Guard Cell ALMT Channels. *Trends in Plant Science***23** , 649–651.

Medeiros D.B., Martins S.C.V., Cavalcanti J.H.F., Daloso D.M., Martinoia E., Nunes-Nesi A., ... Araújo W.L. (2016) Enhanced photosynthesis and growth in *atqua1* knockout mutants are due to altered organic acid accumulation and an increase in both stomatal and mesophyll conductance. *Plant Physiology* **170** , 86–101.

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

Mengin V., Pyl E.T., Alexandre Moraes T., Sulpice R., Krohn N., Encke B. & Stitt M. (2017) Photosynthate partitioning to starch in *Arabidopsis thaliana* is insensitive to light intensity but sensitive to photoperiod due to a restriction on growth in the light in short photoperiods. *Plant, cell & environment* **40** , 2608–2627.

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

Ni D.A. (2012) Role of vacuolar invertase in regulating *Arabidopsis* stomatal opening. *Acta Physiologiae Plantarum* **34** , 2449–2452.

Nunes-Nesi A., Araújo W.L., Obata T. & Fernie A.R. (2013) Regulation of the mitochondrial tricarboxylic acid cycle. *Current Opinion in Plant Biology* **16** , 335–343.

Nunes-Nesi A., Carrari F., Gibon Y., Sulpice R., Lytovchenko A., Fisahn J., ... Fernie A.R. (2007a) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. *Plant Journal* **50** , 1093–1106.

Nunes-Nesi A., Sweetlove L.J. & Fernie A.R. (2007b) Operation and function of the tricarboxylic acid cycle in the illuminated leaf. *Physiologia Plantarum* **129** , 45–56.

O’Leary B., Park J. & Plaxton W.C. (2011) The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological functions and post-translational controls of non-photosynthetic PEPCs. *Biochemical Journal* **436** , 15–34.

O’Leary B. & Plaxton W.C. (2020) Multifaceted functions of post-translational enzyme modifications in the control of plant glycolysis. *Current Opinion in Plant Biology* **55** , 28–37.

- Okahashi N., Kawana S., Iida J., Shimizu H. & Matsuda F. (2019) Fragmentation of Dicarboxylic and Tricarboxylic Acids in the Krebs Cycle Using GC-EI-MS and GC-EI-MS/MS. *Mass Spectrometry* **8** , A0073–A0073.
- Outlaw W.H. & Kennedy J. (1978) Enzymic and substrate basis for the anaplerotic step in guard cells. *Plant physiology* **62** , 648–652.
- Outlaw W.H. & Lowry O.H. (1977) Organic acid and potassium accumulation in guard cells during stomatal opening. **74** , 4434–4438.
- Outlaw W.H. & Manchester J. (1979) Guard cell starch concentration quantitatively related to stomatal aperture. *Plant physiology* **64** , 79–82.
- Outlaw W.H.J. (2003) Integration of Cellular and Physiological Functions of Guard Cells Integration of Cellular and Physiological Functions of Guard Cells. *Critical Reviews in Plant Sciences* **22** , 503–5229.
- Pracharoenwattana I., Zhou W., Keech O., Francisco P.B., Udomchalothorn T., Tschoep H., ... Smith S.M. (2010) Arabidopsis has a cytosolic fumarase required for the massive allocation of photosynthate into fumaric acid and for rapid plant growth on high nitrogen. *Plant Journal* **62** , 785–795.
- Ranson S.L. & Thomas M. (1960) Crassulacean Acid Metabolism. *Annual Review of Plant Physiology* **11** , 81–110.
- Reid E.E., Thompson P., Lyttle C.R. & Dennis D.T. (1977) Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. *Plant Physiology* **59** , 854–858.
- Resco de Dios V., Chowdhury F.I., Granda E., Yao Y. & Tissue D.T. (2019) Assessing the potential functions of nocturnal stomatal conductance in C3 and C4 plants. *New Phytologist* **223** , 1696–1706.
- Ritte G., Rosenfeld J., Rohrig K. & Raschke K. (1999) Rates of sugar uptake by guard cell protoplasts of *Pisum sativum* L. related to the solute requirement for stomatal opening. *Plant physiology* **121** , 647–656.
- Robaina-Estévez S., Daloso D.M., Zhang Y., Fernie A.R. & Nikoloski Z. (2017) Resolving the central metabolism of Arabidopsis guard cells. *Scientific Reports* **7** , 1–13.
- Rocha M., Licausi F., Araujo W.L., Nunes-Nesi A., Sodek L., Fernie A.R. & van Dongen J.T. (2010) Glycolysis and the Tricarboxylic Acid Cycle Are Linked by Alanine Aminotransferase during Hypoxia Induced by Waterlogging of *Lotus japonicus*. *Plant Physiology* **152** , 1501–1513.
- Roelfsema M.R.G., Konrad K.R., Marten H., Psaras G.K., Hartung W. & Hedrich R. (2006) Guard cells in albino leaf patches do not respond to photosynthetically active radiation, but are sensitive to blue light, CO₂ and abscisic acid. *Plant, Cell and Environment* **29** , 1595–1605.
- Shannon P., Markiel A., Owen Ozier 2, Baliga N.S., Wang J.T., Ramage D., ... Ideker T. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* , 2498–2504.
- Siqueira J.A., Oliveira H.O. de, Nunes-nesi A. & Araujo W.L. (2021) Guard cell regulation: pulling the strings behind the scenes. *Trends in Plant Science* .
- Steffens L., Pettinato E., Steiner T.M., Mall A., König S., Eisenreich W. & Berg I.A. (2021) High CO₂ levels drive the TCA cycle backwards towards autotrophy. *Nature* **592** , 784–788.
- Studart-Guimaraes C., Fait A., Nunes-Nesi A., Carrari F., Usadel B. & Fernie A.R. (2007) Reduced Expression of Succinyl-Coenzyme A Ligase Can Be Compensated for by Up-Regulation of the -Aminobutyrate Shunt in Illuminated Tomato Leaves. *Plant Physiology* **145** , 626–639.
- Sulpice R., Flis A., Ivakov A.A., Apelt F., Krohn N., Encke B., ... Stitt M. (2014) Arabidopsis coordinates the diurnal regulation of carbon allocation and growth across a wide range of Photoperiods. *Molecular Plant* **7** , 137–155.

- Sweetlove L.J., Beard K.F.M., Nunes-Nesi A., Fernie A.R. & Ratcliffe R.G. (2010) Not just a circle: Flux modes in the plant TCA cycle. *Trends in Plant Science* **15** , 462–470.
- Sweetlove L.J., Williams T.C.R., Cheung M. & Ratcliffe R.G. (2013) Modelling metabolic CO₂ evolution - a fresh perspective on respiration. *Plant, Cell & Environment* **36** , 1631–1640.
- Szewcowa M., Heise R., Tohge T., Nunes-Nesi A., Vosloh D., Huege J., ... Arrivault S. (2013) Metabolic Fluxes in an Illuminated Arabidopsis Rosette. *The Plant Cell* **25** , 694–714.
- Talbott L. & Zeiger E. (1998) The role of sucrose in guard cell osmoregulation. *Journal of Experimental Botany* **49** , 329–337.
- Talbott L.D. & Zeiger E. (1993) Sugar and organic acid accumulation in guard cells of *Vicia faba* in response to red and blue light. *Plant Physiology* **102** , 1163–1169.
- Tallman G. & Zeiger E. (1988) Light quality and osmoregulation in vicia guard cells : evidence for involvement of three metabolic pathways. *Plant physiology* **88** , 887–895.
- Tan X.L.J. & Cheung C.Y.M. (2020) A multiphase flux balance model reveals flexibility of central carbon metabolism in guard cells of C₃ plants. *The Plant Journal* **104** , 1648–1656.
- Tcherkez G., Mahe A., Gauthier P., Mauve C., Gout E., Bligny R., ... Hodges M. (2009) In folio respiratory fluxomics revealed by ¹³C isotopic labeling and H/D isotope effects highlight the noncyclic nature of the tricarboxylic acid “cycle” in illuminated leaves. *Plant Physiology* **151** , 620–630.
- Tovar-Mendez A., Miernyk J.A. & Randall D.D. (2003) Regulation of pyruvate dehydrogenase complex activity in plant cells. *European Journal of Biochemistry* **270** , 1043–1049.
- Vavasseur A. & Raghavendra A.S. (2005) Guard cell metabolism and CO₂ sensing. *New Phytologist* **165** , 665–682.
- Violet-Chabrand S., Matthews J.S.A. & Lawson T. (2021) Light, power, action! Interaction of respiratory energy- and blue light-induced stomatal movements. *New Phytologist* **231** , 2231–2246.
- Wang F.F., Lian H.L., Kang C.Y. & Yang H.Q. (2010) Phytochrome B is involved in mediating red light-induced stomatal opening in arabidopsis thaliana. *Molecular Plant* **3** , 246–259.
- Wang H., Yan S., Xin H., Huang W., Zhang H., Teng S., ... Lang Z. (2019) A Subsidiary Cell-Localized Glucose Transporter Promotes Stomatal Conductance and Photosynthesis. *The Plant cell* **31** , 1328–1343.
- Wang R.S., Pandey S., Li S., Gookin T.E., Zhao Z., Albert R. & Assmann S.M. (2011) Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. *BMC Genomics* **12** , 216.
- Wang S.W., Li Y., Zhang X.L., Yang H.Q., Han X.F., Liu Z.H., ... Chen Y.L. (2014a) Lacking chloroplasts in guard cells of crumpled leaf attenuates stomatal opening: Both guard cell chloroplasts and mesophyll contribute to guard cell ATP levels. *Plant, Cell and Environment* **37** , 2201–2210.
- Wang Y., Hills A. & Blatt M.R. (2014b) Systems Analysis of Guard Cell Membrane Transport for Enhanced Stomatal Dynamics and Water Use Efficiency. *Plant Physiology* **164** , 1593–1599.
- Wang Y., Noguchi K., Ono N., Inoue S., Terashima I. & Kinoshita T. (2014c) Overexpression of plasma membrane H⁺-ATPase in guard cells promotes light-induced stomatal opening and enhances plant growth. *Proceedings of the National Academy of Sciences of the United States of America* **111** , 533–8.
- Willmer C.M. & Ditttrich P. (1974) Carbon dioxide fixation by epidermal and mesophyll tissues of *Tulipa* and *Commelina*. *Planta* **117** , 123–132.
- Xu B., Long Y., Feng X., Zhu X., Sai N., Chirkova L., ... Gilliham M. (2021) GABA signalling modulates stomatal opening to enhance plant water use efficiency and drought resilience. *Nature Communications* **12** .

Yamamoto M., Ikeda T., Arai H., Ishii M. & Igarashi Y. (2010) Carboxylation reaction catalyzed by 2-oxoglutarate:ferredoxin oxidoreductases from *Hydrogenobacter thermophilus*. *Extremophiles* **14** , 79–85.

Yoon K.I.S., Ishii M., Igarashi Y. & Kodama T. (1996) Purification and characterization of 2-oxoglutarate: Ferredoxin oxidoreductase from a thermophilic, obligately chemolithoautotrophic bacterium, *Hydrogenobacter thermophilus* TK-6. *Journal of Bacteriology* **178** , 3365–3368.

Yoon K.S., Ishii M., Kodama T. & Igarashi Y. (1997) Carboxylation Reactions of Pyruvate: Ferredoxin Oxidoreductase and 2-Oxoglutarate: Ferredoxin Oxidoreductase from *Hydrogenobacter thermophilus* TK-6. *Bioscience, Biotechnology and Biochemistry* **61** , 510–513.

Zeiger E., Talbott L.D., Frechilla S., Srivastava A. & Zhu J. (2002) The guard cell chloroplast: A perspective for the twenty-first century. *New Phytologist* **153** , 415–424.

Zhang Y., Giese J., Mae-Lin Kerbler S., Siemiatkowska B., Perez de Souza L., Alpers J., ... Fernie A.R. (2021) Two mitochondrial phosphatases, PP2c63 and Sal2, are required for posttranslational regulation of the TCA cycle in *Arabidopsis*. *Molecular Plant* .

Zhao Z. & Assmann S.M. (2011) The glycolytic enzyme, phosphoglycerate mutase, has critical roles in stomatal movement, vegetative growth, and pollen production in *Arabidopsis thaliana*. *Journal of Experimental Botany* **62** , 5179–5189.

Hosted file

Figures.docx available at <https://authorea.com/users/440451/articles/712205-pepc-mediated-co2-assimilation-provides-carbons-to-gluconeogenesis-and-the-tca-cycle-in-both-dark-exposed-and-illuminated-guard-cells>