the plant journal
SEB Society for Experimental Biology

The Plant Journal (2018) 94, 583–594 doi: 10.1111/tpj.13889

Sucrose breakdown within guard cells provides substrates for glycolysis and glutamine biosynthesis during light-induced stomatal opening

David B. Medeiros[1,2], Leonardo Perez Souza[1], Werner C. Antunes[3], Wagner L. Araújo[2], Danilo M. Daloso[4,*] and Alisdair R. Fernie[1]

[1]*Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, Potsdam-Golm 14476, Germany,*
[2]*Max-Planck Partner Group, Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-900, Brazil,*
[3]*Departamento de Biologia, Universidade Estadual de Maringá, Maringá, Paraná, 87020-900, Brazil, and*
[4]*Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, Ceará, 60440-970, Brazil*

Received 15 December 2017; revised 13 February 2018; accepted 19 February 2018; published online 15 March 2018.
*For correspondence (e-mail daloso@ufc.br).

SUMMARY

Sucrose has long been thought to play an osmolytic role in stomatal opening. However, recent evidence supports the idea that the role of sucrose in this process is primarily energetic. Here we used a combination of stomatal aperture assays and kinetic [U-^{13}C]-sucrose isotope labelling experiments to confirm that sucrose is degraded during light-induced stomatal opening and to define the fate of the C released from sucrose breakdown. We additionally show that addition of sucrose to the medium did not enhance light-induced stomatal opening. The isotope experiment showed a consistent ^{13}C enrichment in fructose and glucose, indicating that during light-induced stomatal opening sucrose is indeed degraded. We also observed a clear ^{13}C enrichment in glutamate and glutamine (Gln), suggesting a concerted activation of sucrose degradation, glycolysis and the tricarboxylic acid cycle. This is in contrast to the situation for Gln biosynthesis in leaves under light, which has been demonstrated to rely on previously stored C. Our results thus collectively allow us to redraw current models concerning the influence of sucrose during light-induced stomatal opening, in which, instead of being accumulated, sucrose is degraded providing C skeletons for Gln biosynthesis.

Keywords: stomatal movements, sucrose, guard cell metabolism, TCA cycle, glycolysis, stable isotope labelling analysis.

INTRODUCTION

Stomata, microstructures found in the leaf epidermis, are composed of two guard cells with a pore between them. The opening of the stomatal pore is an active process that simultaneously enables the influx of CO_2 to the leaf and the efflux of water to the environment (Hetherington and Woodward, 2003). The ratio between the CO_2 assimilated by photosynthesis (A) and the magnitude of the stomatal movement, measured as stomatal conductance (g_s), is known as intrinsic water-use efficiency (A/g_s) and represents an important target for plant breeding (Condon *et al.*, 2004; Gago *et al.*, 2014; Lawson and Blatt, 2014; Flexas, 2016; Nunes-Nesi *et al.*, 2016). The regulation of stomatal movement, i.e. the opening and closing of the stomatal pore, is therefore of paramount importance for the regulation of photosynthesis and water-use efficiency. However, given the complexity of interacting regulatory endogenous and environmental factors, our knowledge of stomatal movements remains incomplete. Hence there is a growing demand for studies which aim to understand guard cell function and how the mesophyll cells and the external environment combine to influence stomatal movement.

More than a century of research indicates that stomatal movements are regulated by the osmotic potential of the surrounding guard cells (Lloyd, 1908; Imamura, 1943; Fischer, 1968). Turgid and flaccid guard cells induce stomatal opening and closure, respectively (Gao *et al.*, 2005). The accumulation of potassium (K^+) and its counter-ions chloride (Cl^-), nitrate (NO_3^-) and malate (malate^{2-}) is the best described model for the regulation of osmotic potential in guard cells (Inoue and Kinoshita, 2017; Jezek and Blatt, 2017). An influx of K^+ to guard cells has been demonstrated to occur following blue light perception and

© 2018 The Authors
The Plant Journal © 2018 John Wiley & Sons Ltd

stimulation of H^+ extrusion via H^+-ATPases (Hedrich, 2012). Although the signalling network downstream of the blue-light perception has been extensively studied in guard cells (Hiyama *et al.*, 2017), the associated metabolic changes have only recently been revealed. Blue light stimulates the breakdown of starch and lipid droplets in guard cells (Horrer *et al.*, 2016; McLachlan *et al.*, 2016). The authors suggest that this would be a mechanism to fuel mitochondrial metabolism to produce energy (ATP) via oxidative phosphorylation and/or for the accumulation of malate and other tricarboxylic acid (TCA) cycle-related metabolites. Similarly, sucrose breakdown has also been suggested as a mechanism to fuel the TCA cycle on the dark-to-light transition (Daloso *et al.*, 2015, 2016b). Despite these mechanisms having been postulated to be essential during stomatal opening, the fate of the C released from sucrose, starch and lipid breakdown remains unclear. A more complete understanding of which pathways are activated following the degradation of these storage molecules is important to unravel how guard cell metabolism contributes to the regulation of the stomatal opening process.

Based on the osmolytic features of sucrose coupled with the positive correlation between stomatal aperture and sucrose accumulation in guard cells (Talbott and Zeiger, 1993, 1996; Lu *et al.*, 1995; Amodeo *et al.*, 1996), sucrose has long been proposed to act as an osmolyte involved in stomatal opening. However, the capacity of sucrose to induce stomatal opening has, surprisingly, not yet been directly accessed. Moreover, recent evidence supports the idea that sucrose is degraded within guard cells during the dark-to-light transition in order to provide carbon skeletons for respiration (Daloso *et al.*, 2015, 2016b) and that high levels of exogenously applied sucrose (100 mM) can induce stomatal closure via a mechanism mediated by both abscisic acid (ABA) and hexokinase (HXK) (Kelly *et al.*, 2013). It has been proposed that HXK, a sugar-phosphorylating enzyme involved in sugar sensing, is able to mediate stomatal closure by coordinating photosynthesis and transpiration in both Arabidopsis and tomato (Kelly *et al.*, 2013). Furthermore, overexpression of the Arabidopsis HXK1 (AtHXK1) in citrus under the control of a guard cell-specific promoter, KST1, resulted in a reduced stomatal conductance and transpiration without impairing the rate of photosynthesis (Lugassi *et al.*, 2015). Taken together, these results suggest that guard cell sucrose metabolism has a key role in the regulation of water-use efficiency and that sucrose is likely to play a dual role in the regulation of stomatal movements.

Here we took advantage of a protocol which enables the rapid isolation of dark-adapted guard cell-enriched epidermal fragments in sufficient quantities to allow us to perform both stomatal aperture assays and metabolomic analyses (Daloso *et al.*, 2015). To clarify the effect of exogenously applied sucrose on stomatal opening, we further investigated the metabolic fate of ^{13}C-sucrose during light-induced stomatal opening. Our results not only demonstrate that a high level of sucrose induces stomatal closure, but also indicate that sucrose breakdown occurs during light-induced stomatal opening. We also provide evidence that sucrose degradation induces glutamine (Gln) biosynthesis during the dark-to-light transition. These collected findings are discussed within the context of current models of the metabolic influences on stomatal movement.

RESULTS

Sucrose-induced stomatal closure

Sucrose has long been proposed to act as an osmolyte during light-induced stomatal opening (Talbott and Zeiger, 1996). However, it has additionally recently been suggested that sucrose can induce stomatal closure at high concentrations (about 100 mM) (Kelly *et al.*, 2013). Here we first checked the capacity of sucrose to induce stomatal closure. Detached leaves from dark-adapted Arabidopsis plants were floated on opening buffer solution in the light for 2 h. This time was sufficient to induce stomatal opening. Following this, sucrose was added to the solution at diverse final concentrations and the stomatal aperture was determined after a further 2 h of incubation. Whilst the addition of 0.1 and 1 mM of sucrose has no effect on stomatal aperture, the two highest concentrations tested (10 and 100 mM) significantly reduced stomatal aperture (Figure 1a). By contrast, no difference in stomatal aperture between the opening buffer and osmotic control (100 mM mannitol) solutions was observed, suggesting that the effect of sucrose on stomatal aperture was not merely osmotic.

Can sucrose induce stomatal opening?

We have confirmed that sucrose can indeed induce stomatal closure in opened stomata. However, this result does not address the question of whether sucrose can induce stomatal opening. For this purpose, we directly assessed the dark-to-light transition, simulating the circadian rhythm of stomatal movements. To do this, Arabidopsis leaves were harvested in the pre-dawn and incubated in different solutions in the dark or light for a period of 2 h, whereafter the stomatal aperture was determined. The stomatal aperture was higher in all light treatments compared with the dark samples (Figure 1b). The largest stomatal aperture was observed in leaves under opening buffer solution containing KCl (Figure 1b). We also observed an increase in stomatal aperture in leaves floated on opening buffer without KCl solution, compared with the dark treatment, albeit to a lesser extent than in the solution containing KCl (Figure 1b). Interestingly, comparing sucrose treatments with their controls in the light, we observed no increase in

© 2018 The Authors

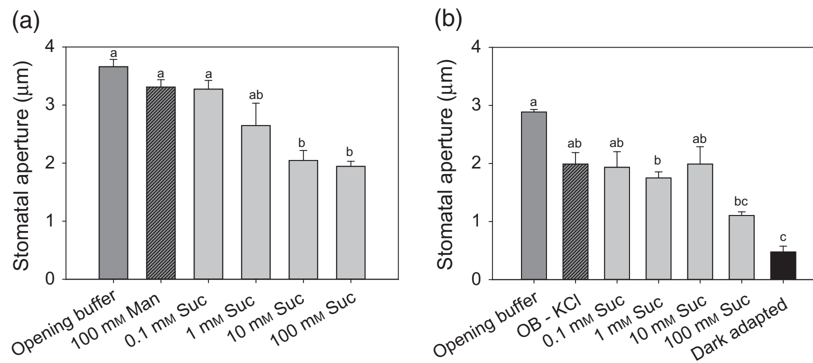

Figure 1. Sucrose-induced stomatal closure in 5-week-old Col-0 plants.
(a) Detached leaves (fifth totally expanded) from light-adapted Arabidopsis plants were floated on opening buffer under light condition for 2 h. Then sucrose was added to the solution to final concentrations of 0.1, 1, 10 and 100 mM. The stomatal aperture was determined after an additional 2 h of incubation. Opening buffer treatment was used as a control without sucrose (Suc) and 100 mM mannitol (Man) as an osmotic control. Four leaves were used per treatment and around 20 stomata were visualized, totallng at least 80 stomata per treatment.
(b) Detached leaves (fifth totally expanded) from dark-adapted Arabidopsis plants were floated under light conditions for 2 h on opening buffer (OB), OB without KCl (OB − KCl) and OB − KCl with different sucrose concentrations (0.1, 1, 10 and 100 mM). After 2 h of incubation the stomatal aperture was determined. All treatments were compared with OB − KCl. Four leaves were used per treatment and around 20 stomata were visualized, totalling at least 80 stomata per treatment. Identical letters among treatments do not significantly differ by Tukey's *t*-test ($P \leq 0.05$). Values are presented as mean ± SE ($n = 4$).

stomatal aperture following the addition of sucrose (Figure 1b). In contrast, the addition of 100 mM sucrose minimized light-induced stomatal opening, reinforcing the observation that sucrose induces stomatal closure at very high concentrations.

Can the presence of sucrose improve light- and potassium-induced stomatal opening?

Potassium is a well-known osmolyte that accumulates in guard cells during light-induced stomatal opening (Chen *et al.*, 2012). It is also known that K^+ influx to guard cells depends on a proton gradient created at the plasma membrane by H^+-ATPases (Inoue *et al.*, 2010). Thus, both light- and K^+-induced stomatal opening are characterized as ATP-dependent processes. Given that, we next tested whether sucrose, as a substrate for glycolysis and mitochondrial metabolism, could increase the rate of stomatal opening following induction by light and K^+. Detached leaves were incubated for 2 h under light in three different opening buffer solutions containing 0, 0.1 and 100 mM of sucrose, whereafter the stomatal aperture was measured. We tested the addition of 0.1 and 100 mM of sucrose given that these concentrations either previously showed no effect or induced stomatal closure (Figure 1), respectively. The stomatal aperture was higher in all opening buffer solutions compared with the dark treatment (Figure 2a). However, it was lower in the solution containing 100 mM of sucrose compared with opening buffer solution,

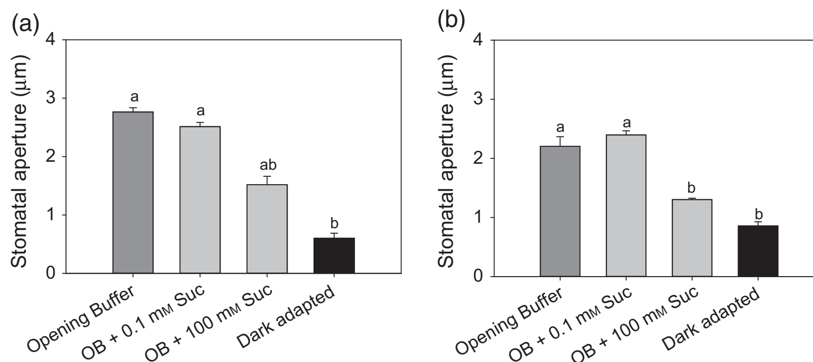

Figure 2. Sucrose-induced stomatal opening in 5-week-old Col-0 plants.
(a) Detached leaves (fifth totally expanded) or (b) guard cell-enriched epidermal fragments from dark-adapted Arabidopsis plants were floated under light conditions for 2 h on opening buffer (OB) or OB with two sucrose (Suc) concentrations (0.1 and 100 mM). After 2 h of incubation the stomatal aperture was determined. Four leaves were used per treatment and around 20 stomata were visualized, totalling at least 80 stomata per treatment. Identical letters among treatments do not significantly differ by Tukey's test ($P \leq 0.05$). Values are presented as mean ± SE ($n = 4$).

© 2018 The Authors

whereas no differences were observed between opening buffer and the solution containing 0.1 mM of sucrose (Figure 2a). In detached leaves the guard cells are not isolated but instead are in direct contact with the mesophyll, which could interfere with the fate of the exogenously applied sucrose. Therefore, we also tested the addition of 0.1 and 100 mM of sucrose in guard cell-enriched epidermal fragments, henceforth simply called guard cells (Figure 2b). This experimental system was further used for the feeding experiment, as described below. The stomatal apertures of guard cells in response to both concentrations of sucrose were similar to those observed in detached leaves (Figure 2b). Therefore, the results from both assays indicate that the addition of sucrose does not improve light- and K^+-induced stomatal opening.

We next directly evaluated the metabolic fate of sucrose by performing an unprecedented [U-^{13}C]-sucrose labelling kinetic experiment under light-induced stomatal opening conditions (a schematic representation of the workflow is provided in Figure S1 in the online Supporting Information). Given the amount of material needed to perform gas chromatography–mass spectrometry (GC-MS)-based metabolomics analysis, this experiment was performed using isolated guard cells following a protocol previously optimized for metabolite profiling in guard cells (Daloso *et al.*, 2015). We harvested guard cells in the pre-dawn and incubated them in an opening buffer solution in the presence or absence of 0.1 mM ^{13}C-sucrose in the light for 10, 30 or 60 min. We choose a sucrose concentration of 0.1 mM because this concentration does not affect light- or K^+-induced stomatal opening and is not able to induce stomatal closure (Figure 2). Our aim was to investigate the fate of the ^{13}C released from sucrose during light- and K^+-induced stomatal opening. To ascertain whether the guard cells remained functional following the isolation procedure, at each time point of the experiment samples were harvested in order to measure the stomatal aperture (Figure S1a). The results showed a continuous increase in stomatal aperture over time for both control samples and samples in opening buffer containing sucrose (0.1 mM) (Figure 3). As such they indicated that the guard cells remained functional after their isolation and that the effect

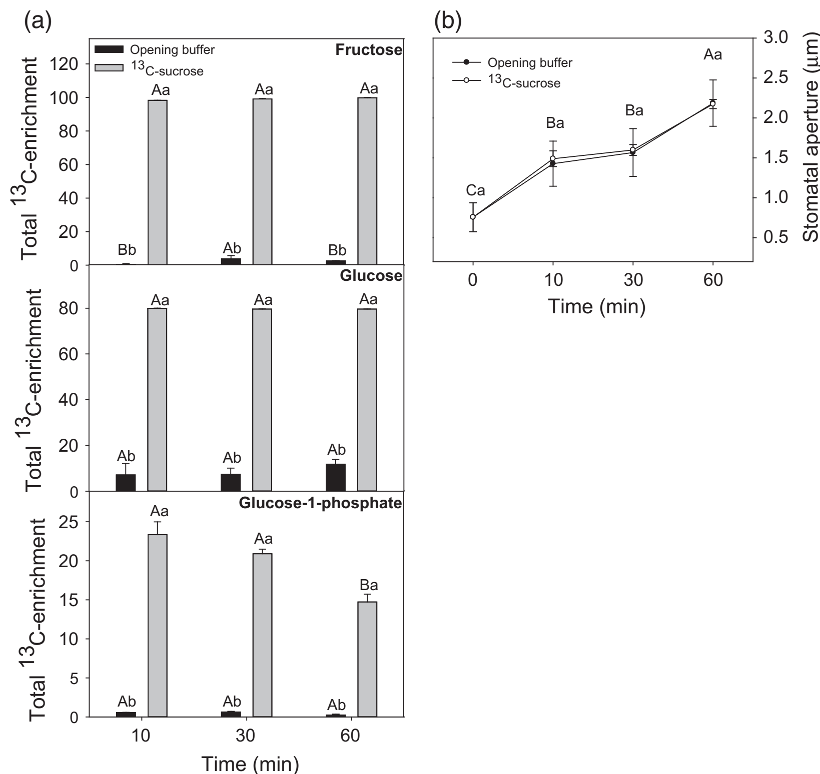

Figure 3. Total ^{13}C label enrichment into sugars.
(a) Total ^{13}C label enrichment of glucose, fructose and glucose-1-phosphate (G1P) following guard cell incubation. Dark-adapted guard cells were harvested in the pre-drawn and fed with [U^{13}C]-sucrose in opening buffer under light conditions for 2 h. Opening buffer without sucrose was used as a control. At 10, 30 and 60 min of incubation the guard cells were sampled and the enrichment into the metabolites were determined.
(b) Stomatal aperture of Col-0 guard cells during dark-to-light transition. The stomatal aperture was measured as the width of the stomatal pore. Identical letters, upper case among time points and lower case between treatments in each time point, do not significantly differ by Tukey's test ($P \leq 0.05$). Values are presented as mean ± SE (n = 4).

© 2018 The Authors

of 0.1 mM of sucrose on stomatal aperture did not change in the presence or absence of mesophyll cells.

What is the metabolic fate of sucrose during light-induced stomatal opening?

The ^{13}C-sucrose experiment was analysed by determining the relative ^{13}C enrichment and the total number of ^{13}C atoms incorporated into each fragment of each metabolite detected. The number of ^{13}C atoms incorporated in each fragment is evidenced by the shift in the mass-to-charge (*m*/*z*) ratio observed in the mass spectra (Figure S1b). For instance, the incorporation of one and two ^{13}C isotope(s) into a fragment of two carbons leads to an increase of the mass isotopomers M + 1 and M + 2, respectively (Figure S1b). We subsequently calculated the total ^{13}C enrichment for each fragment (Figure S1c) as previously described (Souza *et al.*, 2017).

On analysing the fate of the ^{13}C-sucrose we observed a high total ^{13}C enrichment in fructose and glucose. Fructose was almost 100% labelled after 10 min whereas glucose labelling reached 80% after 10 min, maintaining this proportion until 60 min (Figure 3). These results confirm that sucrose is degraded on light- and K^+-induced stomatal opening. The labelling pattern of glucose is highly stable over time with respect to the M + 1 and M + 2 isotopomers, while fructose displayed an increasing ^{13}C enrichment of the M + 3 ion over time (Figure S2). These labelling patterns suggest that the *m*/*z* 217 fragment of fructose was fully labelled while the *m*/*z* 160 fragment of glucose was not. It is important to mention that we cannot rule out that other minor glucose isotopomers were labelled, albeit at only negligible levels below the limit of detection of our GC-MS protocol. Moreover, the lower ^{13}C enrichment in glucose could be the result of unlabelled glucose residues resulting from starch breakdown, which are, by contrast, not incorporated in fructose (Stitt and Zeeman, 2012). Glucose-1-phosphate (Glc-1P) displayed a peak of labelling at 10 min (24%), following which the ^{13}C fractional enrichment decreased, down to 15% at 60 min (Figure 3). This decrease is probably best explained by the relative abundance of the isotopomers – with the fully labelled M + 3 ion displaying a peak of labelling at 10 min but being reduced until 60 min (Figure S2).

Photorespiratory metabolites such as glycolate and serine were also considerably labelled (Figure 4). Glycolate was clearly labelled across the experiment, whilst serine displayed a higher total ^{13}C enrichment only at 60 min compared with control samples. Relative abundance analysis of the respective isotopomers confirmed these conclusions, since for glycolate and serine the M and M + 1 ions were clearly decreased and increased, respectively (Figure S3). No increase was observed for M + 2 ion of serine (Figure S3), suggesting that only a single ^{13}C was incorporated into the photorespiratory metabolites.

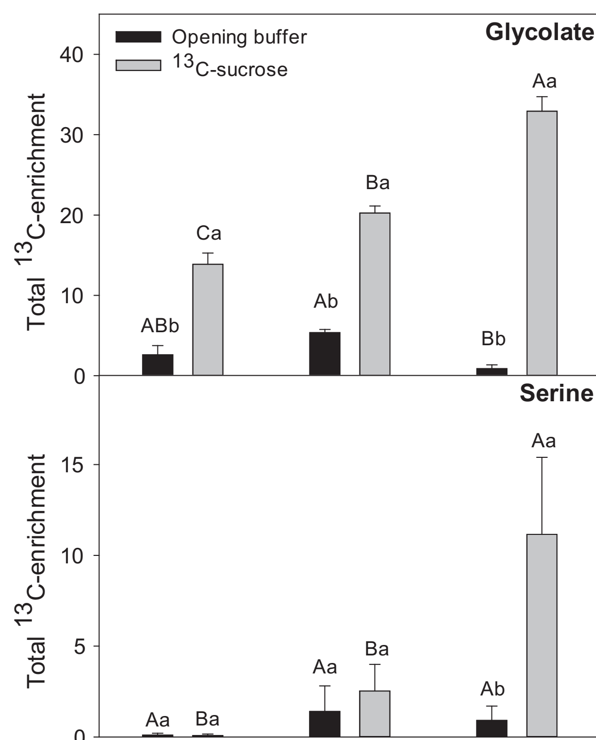

Figure 4. Total ^{13}C label enrichment into photorespiratory related metabolites following guard cell incubation.
Dark-adapted guard cells were harvested in the pre-drawn and fed with [U^{13}C]-sucrose in opening buffer under light conditions for 2 h. Opening buffer without sucrose was used as a control. At 10, 30 and 60 min of incubation the guard cells were sampled and the enrichment into the metabolites was determined. Identical letters, upper case among time points and lower case between treatments in each time point, do not significantly differ by Tukey's test ($P \leq 0.05$). Values are presented as mean ± SE ($n = 4$).

Enhanced ^{13}C enrichment in glutamate (Glu) and Gln was also observed over time, reaching maxima of 25% in Glu and 60% in Gln after 60 min of incubation (Figure 5a). Relative abundance analysis of the isotopomers suggests that Glu was substantially labelled only at M + 1 and M + 2. Decreases in the non-labelled Glu M ion were simultaneous with the increases in Glu M + 2, which reached 22% following 60 min of incubation (Figure 5b). By contrast, Gln displayed ^{13}C incorporation in the M + 3 and M + 4 ions. The relative intensity of the non-labelled M ion was considerably lower for Gln than Glu. Moreover, the relative intensity of the fully labelled Gln M + 4 ion increased linearly over time while that of the M + 3 ion decreased from 10 to 60 min (Figure 6b). This suggests that ^{13}C is continuously passing from sucrose to Gln via glycolysis and part of the TCA cycle (Figure 6). These data provide further evidence that sucrose is used as a respiratory substrate for glycolysis and the TCA cycle, which in turn provide 2-oxoglutarate (2-OG) for Gln biosynthesis (Figure 7).

It is important to highlight that increases in metabolic fluxes are not necessarily correlated with increases in the

© 2018 The Authors

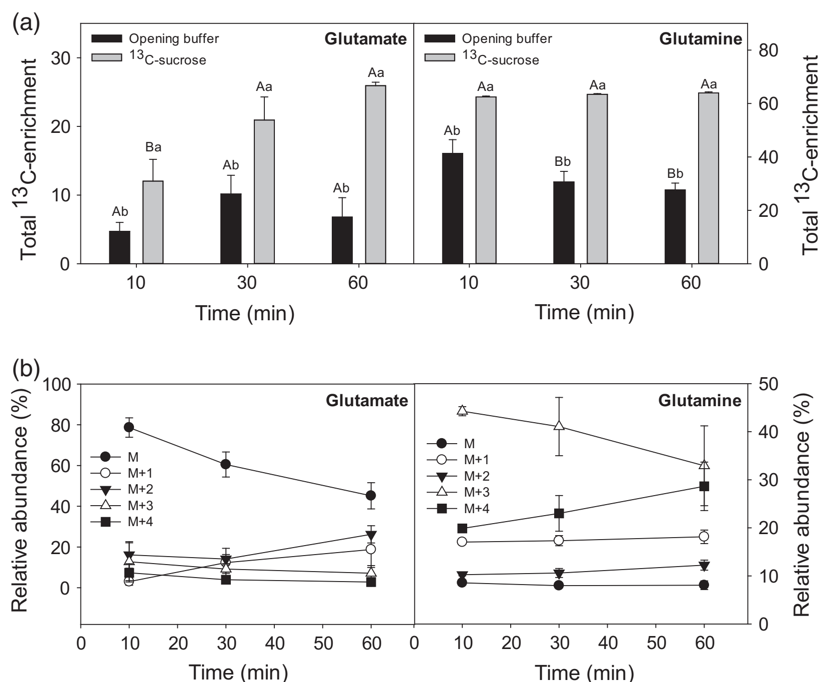

Figure 5. ^{13}C label enrichment of glutamate and glutamine.
(a) Total ^{13}C label enrichment into glutamate and glutamine. Dark-adapted guard cells were harvested in the pre-drawn and fed with [U^{13}C]-sucrose in opening buffer under light conditions for 60 min. Opening buffer without sucrose was used as a control.
(b) Relative abundance of mass isotopomers following guard cell incubation. Values are presented as mean ± SE (n = 4). Identical letters, upper case among time points and lower case between treatments in each time point, do not significantly differ by Tukey's test ($P \leq 0.05$). Values are presented as mean ± SE (n = 4).

level of the metabolites (Williams *et al.*, 2008). Thus, we further analysed the relative amounts of metabolites by integrating all the fragments detected for each metabolite (labelled with ^{13}C or not) via GC-MS. This analysis was performed in dark-adapted guard cells (used as a control for statistical comparison) as well as during the dark-to-light transition at 10, 30 and 60 min of incubation with opening buffer or ^{13}C-sucrose at 0.1 mM (Table S1). It revealed increases in the levels of Glu and Gln over time when samples treated with ^{13}C-sucrose are compared with dark-adapted guard cells. Interestingly, in addition to Gln displaying significant fractional enrichment of ^{13}C, its levels were also strongly increased following ^{13}C-sucrose supply. Significantly higher contents of phenylalanine (Phe) in the opening buffer treatment and lysine (Lys) in the ^{13}C-sucrose were also observed. By contrast, the levels of both fumarate and malate increased over time (although this was only significant in the case of fumarate), whilst supply of ^{13}C-sucrose reduced the levels of both organic acids during the incubation time. The fructose content was higher in both treatments at the three time points evaluated compared with dark-adapted samples, but it was strongly increased following incubation with ^{13}C-sucrose. Interestingly, the glucose levels in guard cells only incubated with opening buffer were significantly reduced over time, with the lowest level being at 60 min of incubation. However, after addition of ^{13}C-sucrose the glucose content was increased up to 30 min followed by a significant reduction at 60 min. Glc-1P was also significantly increased in a similar manner for both treatments.

DISCUSSION

The dual role of sucrose during stomatal opening and closure

Stomatal aperture assays carried out using different concentrations of sucrose indicate that the presence of sucrose at concentrations higher than 10 mM can induce stomatal closure under light conditions or minimize the magnitude of light-induced stomatal opening (Figures 1 and 2). Interestingly, exogenous application of sucrose at concentrations below 10 mM does not change stomatal aperture compared with the opening buffer control (Figures 1 and 2). Furthermore, the addition of 0.1 mM sucrose did not optimize light- or K^+-induced stomatal opening (Figure 2), suggesting that at low sucrose concentrations the stomatal pore opens in response to light but not in response to the availability of sucrose. Similar responses have previously been observed in isolated tobacco guard cells (Daloso *et al.*, 2015). These data indicate that guard cells can both

© 2018 The Authors
The Plant Journal © 2018 John Wiley & Sons Ltd,

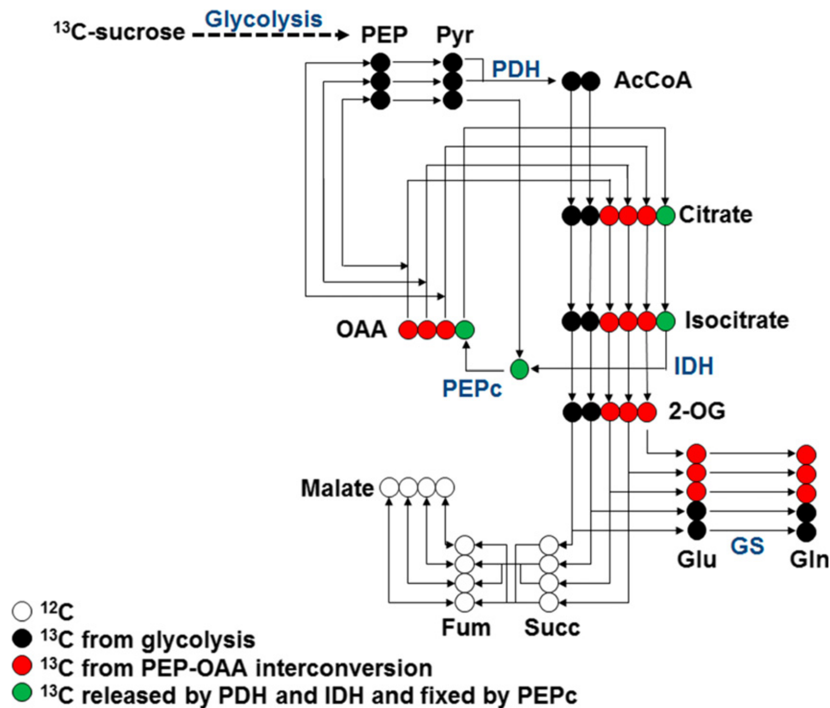

Figure 6. Schematic representation of stable isotope redistribution in guard cells during ^{13}C-sucrose labelling kinetic assay.
To detect fully labelled Gln it is assumed, firstly, that PEP is fully labelled by glycolysis, producing fully labelled Pyr and AcCoa. This assumption is supported by the experimental data given that hexoses and G1P were clearly labelled, indicating that the C from sucrose is used to sustain glycolysis and thus can produce fully labelled PEP. Following this idea, two of these carbons can be incorporated into Glu and Gln (follow black spheres from AcCoA). Secondly, the fully labelled PEP is also converted to OAA. This reaction can produce three labelled carbons (see red spheres) which are then incorporated into Gln. It is noteworthy that the PEPc fixation of the CO_2 released by PDH does not contribute to the ^{13}C enrichment observed in Gln, once this C is lost in the reaction catalysed by IDH. Enzymes are shown in blue. 2-OG, 2-oxoglutarate; AcCoa, acetyl CoA; Fum, fumarate; Gln, glutamine; Glu, glutamate; GS, glutamine synthetase; IDH, isocitrate dehydrogenase; PEP, phosphoenolpyruvate; PEPc, phosphoenolpyruvate carboxylase; Pyr, pyruvate; PDH, pyruvate dehydrogenase; Succ, succinate. [Colour figure can be viewed at wileyonlinelibrary.com].

produce the energy and the osmolytes required to open the stomata autonomously or import these compounds from mesophyll cells and store them as starch or lipids in the guard cell chloroplasts. Taken together, these results provide strong evidence to support the postulate that the function of sucrose in guard cells is not solely osmolytic. However, it is important to highlight that this fact does not mean that the presence of sucrose is not required during stomatal opening. Indeed, transgenic plants exhibiting decreased expression of the sucrose transporter *SUT1*, specifically in guard cells, displayed lower g_s than wild-type plants in the early morning (Antunes *et al*., 2017), the time of day during which the stomatal aperture seems to be mainly sustained by the accumulation of K^+ and its counter-ions (Talbott and Zeiger, 1998). Therefore, although our results most likely exclude the possibility that sucrose acts only as an osmolyte under light-induced stomatal opening, it is important to stress that, as in other cell types, sucrose may display a wide range of functions in guard cells beyond those related to osmotic potential regulation of the cell.

It was previously proposed that the accumulation of sucrose in the apoplastic space surrounding guard cells may be a mechanism to induce stomatal closure during periods of high photosynthetic rate in phloem-loading species (Lu *et al*., 1995, 1997; Kang *et al*., 2007a,b). Recent evidence also suggests that sucrose is sensed within guard cells and that the stomatal closure is mediated by ABA in a HKX-dependent manner (Kelly *et al*., 2013). Initial characterization of sucrose flux into the guard cell plasma membrane in *Vicia faba* indicates that it may saturate at about 25–40 mM and depends on the H^+ transmembrane gradient (Outlaw, 1995). Assuming that the import of sucrose to guard cells saturates at this concentration, it seems unlikely that 100 mM of sucrose (the concentration used by Kelly and co-workers) will be the concentration reached inside the guard cells, strengthening the idea that sucrose-induced stomatal closure could be mediated by an osmotic effect in the apoplastic space (Lu *et al*., 1995, 1997; Kang *et al*., 2007a,b) and/or by activating an ABA signalling network within the guard cells (Kelly *et al*., 2013). It is important to note that exogenous application of the osmotic control mannitol did not induce stomatal closure (Figure 1a), a fact also observed in tomato plants (Kelly *et al*., 2013). This suggests that whether or not there is an osmotic effect at the apoplastic space this is not strictly related

© 2018 The Authors

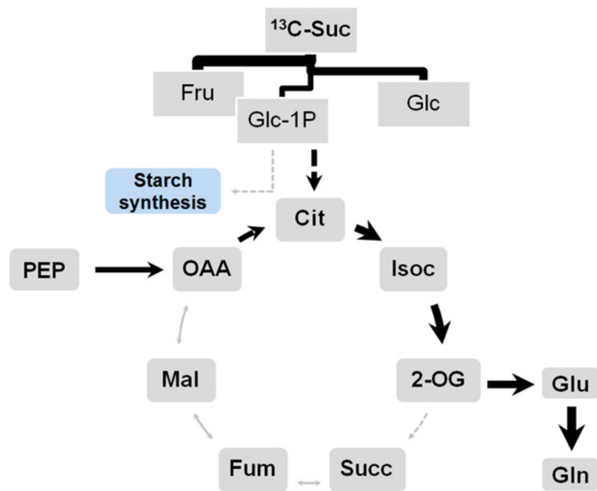

Figure 7. Schematic representation of the ^{13}C redistribution based on the results using [U-^{13}C]-sucrose in isolated guard cells under dark-to-light transition.

Arrow thickness represents possible fluxes, although it is important to stress that is currently not feasible to determine metabolic fluxes in guard cells. The experiment using ^{13}C-sucrose suggests higher fluxes through glycolysis, the tricarboxylic acid (TCA) cycle and the Glu/Gln pathway. The labelling found in Glu/Gln metabolites suggests a differential non-cyclic mode activity of the TCA cycle in guard cells under light conditions. Therefore, the ^{13}C from sucrose breakdown is used to sustain glycolysis and Gln biosynthesis via Cit, Isoc and 2-OG. 2-OG, 2-oxoglutarate; Cit, citrate; Fum, fumarate; Fru, fructose; Glc, glucose; Glc-1-P, glucose-1-phosphate; Gln, glutamine; Glu, glutamate; Isoc, isocitrate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Suc, sucrose; Succ, succinate. [Colour figure can be viewed at wileyonlinelibrary.com].

to sucrose accumulation. It could be due to an osmotic effect triggered by the accumulation of sucrose-derived products such as hexoses and/or by the induction of ABA signalling pathway by trehalose metabolism (Daloso *et al.*, 2017; Figueroa and Lunn, 2016). Indeed, it has been shown that glucose induces stomatal closure in *V. faba* in a dose- and time-dependent manner (Li *et al.*, 2016) and that ABA-induced stomatal closure is abolished in the *tre1* mutant that lacks the trehalase enzyme responsible for the conversion of trehalose to glucose (Van Houtte *et al.*, 2013). What remains unclear is whether the activation of the ABA signalling pathway observed in tomato, Arabidopsis and *V. faba* (Kelly *et al.*, 2013; Li *et al.*, 2016) also occurs under lower concentrations than 100 mM and what the connection is between sucrose and trehalose metabolism during stomatal closure.

Alternatively, the import of sucrose to guard cells, which follows Michaelis–Menten-type kinetics in a 1:1 H^+:-sucrose symport stoichiometry (Boorer *et al.*, 1996; Antunes *et al.*, 2017), may induce stomatal closure by plasma membrane depolarization and subsequent activation of outward-rectifying K^+ channels in the guard cells (e.g. AtGORK and AtKUP) (Jarzyniak and Jasiński, 2014). Whatever the mechanism by which sucrose induces stomatal closure, it seems clear that sucrose has a pivotal role in the trade-off between the regulation of photosynthesis and stomatal conductance (Gago *et al.*, 2016). The fact that only high concentrations of sucrose induce stomatal closure, and that this was not observed under high concentrations of mannitol, reinforces the idea that *in planta* this mechanism would occur only at high *A* as a mechanism to reduce water loss via transpiration in a non-carbon-limited condition. It thus seems likely that the differential accumulation and degradation of sucrose between the apoplastic space and within guard cells may act as a key point that tightly connects photosynthesis to stomatal movements (Daloso *et al.*, 2016a; Gago *et al.*, 2016).

The metabolic fate of ^{13}C-sucrose

Fructose and glucose, two of the three direct products of breakdown of plant sucrose, were rapidly and clearly labelled (Figures 3 and S2), demonstrating that sucrose has been effectively degraded during dark-to-light transition, as suggested in our previous experiments (Daloso *et al.*, 2015, 2016b). Fructose was fully labelled whilst glucose was not, indicating that the degradation of sucrose is probably occurring by both sucrose synthase (SuSy) and invertase (Inv) since an identical ^{13}C enrichment in the hexoses would be expected if it was mediated only by Inv. However, as we mentioned above, the remobilization of C reserves in starch could also produce asymmetric labelling of the hexoses. That said, a range of other evidence indicates that both SuSy and Inv are important players controlling sucrose metabolism in guard cells (Antunes *et al.*, 2012; Ni, 2012; Daloso *et al.*, 2016b), and further enzymatic characterization of these and others key enzymes of sucrose metabolism will bring important information on control of sugar metabolism in guard cells. Furthermore, Glc-1P was also rapidly and clearly labelled (Figure 3), suggesting that SuSy is indeed operational, in the degradative direction, within the guard cell (Kleczkowski *et al.*, 2004; Zeeman *et al.*, 2007). Irrespective of its origin, the total ^{13}C enrichment in Glc-1P reaches high values at 10 min then decreases until 60 min. The analysis of relative isotopomer abundance corroborates this observation, suggesting that Glc-1P is degraded over time (Figure S2). Glc-1P is a common substrate for glycolysis and starch metabolism, therefore its degradation could simultaneously activate these pathways (Stitt and Zeeman, 2012). However, it is important to mention that our experiment was carried out during the dark-to-light transition, simulating the circadian rhythm of the early morning stomatal aperture. Under this condition, starch has been shown to be rapidly degraded (Horrer *et al.*, 2016; Santelia and Lunn, 2017). Although we have not measured starch turnover under our experimental conditions, our data suggest that Glc-1P was mostly used to sustain glycolysis rather than starch synthesis. Our data

are furthermore consistent with the previous finding that increased contents of fructose 2,6-biphosphate were observed during dark-to-light transition (Hedrich *et al.*, 1985) as well as by the fact that TCA cycle-related metabolites such as Glu and Gln were highly labelled in our experiments (Figure 6). In keeping with this, it has been demonstrated that a double mutant lacking activity of the glycolytic enzyme phosphoglycerate mutase has a smaller blue light-induced stomatal aperture (Zhao and Assmann, 2011), suggesting that glycolysis is activated during light-induced stomatal opening. Taken together, these data suggest that guard cell metabolism, unlike that of the mesophyll, prioritises the activation of glycolysis above starch or sucrose synthesis during light-induced stomatal opening (Figure 7).

Substantial increases in the level of Gln coupled with a clear ^{13}C enrichment in Glu and Gln were observed following breakdown of ^{13}C-sucrose (Figure 5, Table S1), confirming that at least part of the guard cell sucrose pool is used as a substrate for glycolysis and the TCA cycle. This result it is in accordance with previous experiments which suggest that the glycolytic pathway in guard cell protoplasts is activated in the light (Hedrich *et al.*, 1985) and sheds light on the differential regulation of the central metabolism of guard cells compared with whole leaf metabolism, where respiration is inhibited under light (Tcherkez *et al.*, 2005, 2012) and the C used to sustain Glu/Gln biosynthesis in the light comes from previously stored C (Gauthier *et al.*, 2010; Cheung *et al.*, 2014; Abadie *et al.*, 2017). Interestingly, the total ^{13}C enrichment was higher in Gln than Glu and the relative abundance of the fully labelled $M + 4$ ion of Gln increased over time, while the $M + 4$ ion from Glu decreased (Figure 5), suggesting that Glu is being catabolised to produce Gln. Higher partitioning of ^{13}C derived from [U-^{13}C]-sucrose into Gln was recently demonstrated in sink leaves compared with source leaves (Dethloff *et al.*, 2017). It was shown that the sucrose-derived ^{13}C is preferentially used by GS/GOGAT and TCA cycle-related pathways in sink leaves, suggesting that regulation of glycolysis and the TCA cycle by light may differ between sink and source tissues (Dethloff *et al.*, 2017). In this vein, the metabolic distribution of ^{13}C throughout the central metabolism of guard cells seems to have much greater similarity with sink rather than source leaves, which fits well with the sink features already reported in guard cells such as low photosynthetic rate (Gotow *et al.*, 1988), higher activity of the sink marker enzyme sucrose synthase (Hite *et al.*, 1993; Daloso *et al.*, 2015), high activity of enzymes of catabolic pathways (Hampp *et al.*, 1982; Vani and Raghavendra, 1994) and a high mitochondrial respiration rate (Araújo *et al.*, 2011). This evidence, coupled with recent modelling results (Robaina-Estévez *et al.*, 2017), confirms the considerably different regulation of guard cell and mesophyll cell metabolism.

By contrast to what was observed in previous feeding experiments using ^{13}C-$NaHCO_3$ as a substrate (Daloso *et al.*, 2015; Robaina-Estévez *et al.*, 2017), here we were able to detect ^{13}C enrichment in metabolites related to the C6–C4 branch of the TCA cycle (Figure 6). Given that no ^{13}C labelling was observed here in malate and fumarate (Figure 7), the only explanations for detection of fully labelled Gln are: (i) phosphoenolpyruvate (PEP) is fully labelled by glycolysis, which produces fully labelled pyruvate and acetyl CoA (AcCoA) and that two of these C are then incorporated into Glu/Gln (follow the black spheres from AcCoA in the schematic of Figure 6); and (ii) the fully labelled PEP is also converted to oxaloacetate (OAA). This reaction can produce three labelled Cs which are then incorporated into Gln (follow the red spheres in the schematic of Figure 6). These findings suggest that the C fluxes following sucrose breakdown differ from those observed after anaplerotic CO_2 fixation catalysed by phosphoenolpyruvate carboxylase (PEPc) and that the TCA cycle is not working in a cyclic manner under our experimental conditions (Figure 7). In fact, the non-cyclic mode of operation of the TCA cycle has been documented in different conditions and plant tissues (Sweetlove *et al.*, 2010) and has also previously been suggested to occur in guard cells during dark-to-light transition under ^{13}C-$NaHCO_3^-$ treatment (Daloso *et al.*, 2015).

Collectively, our results provide direct evidence that the role of sucrose during light-induced stomatal opening is not primarily that of an osmolyte. This is supported by the fact that exogenously applied sucrose has no additive effect on light- and K^+-induced stomatal opening. Instead of merely being an osmolyte, our data directly prove that sucrose is used as a substrate for glycolysis and the TCA cycle in the light. This fact clearly discriminates guard cell metabolism from that exhibited by mesophyll cells. Perhaps equally importantly, our results provide evidence linking sucrose breakdown to Gln biosynthesis during the dark-to-light transition via a mechanism which has not yet been described in the literature on stomatal physiology. This finding suggests a regulatory role for Gln during light-induced stomatal opening. However, more detailed metabolic flux analyses are required in order to reveal the exact mode of operation of the TCA cycle in guard cells under different conditions. The application of approaches such as ^{13}C nuclear magnetic resonance-based metabolic flux analysis to guard cell metabolism may reveal important information regarding the regulation of the TCA cycle and GS/GOGAT pathways in guard cells as well as the biological relevance of the fact that the C released from sucrose is used to produce Gln during stomatal opening. Therefore, further studies on the reactions linking guard cell sucrose and Gln are a priority if we are to fully

© 2018 The Authors

understand the regulation of guard cell metabolism during light-induced stomatal opening.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

For all experiments described here we used the wild-type *Arabidopsis thaliana* Columbia-0 ecotype as plant material. Seeds were surface sterilized and imbibed for 2 days at 4°C in the dark on 0.7% (w/v) agar plates containing half-strength Murashige and Skoog (MS) medium (pH 5.7; Sigma-Aldrich, http://www.sigmaaldrich.com/). Seeds were subsequently germinated and grown at 22°C under short-day conditions (8-h light/16-h dark), 60% relative humidity with 150 μmol photons m^{-2} sec^{-1}. The fifth totally expanded leaves from 5-week-old plants were harvested and used for stomatal aperture assays. Whole rosettes grown in the same conditions were used for guard cell-enriched epidermal fragment isolation.

Stomatal aperture assays

For sucrose-induced stomatal closure assays light-adapted leaves were floated on stomatal opening buffer containing 10 mM KCl, 50 μM $CaCl_2$ and 5 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES)–2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) (pH 6.15) for 2 h under light (150 μmol m^{-2} sec^{-1}) in order to ensure fully open stomata. Then, opening buffer, mannitol (at a final concentration 100 mM) or sucrose at final concentrations of 0.1, 1, 10 or 100 mM were added. After an additional 2 h of incubation the stomatal aperture was evaluated. For sucrose-induced stomatal opening assays dark-adapted leaves were floated on opening buffer (in the presence or absence of KCl, as indicated in the figure legends) or opening buffer containing sucrose to a final concentration of 0.1, 1, 10 or 100 mM and incubated under light (150 μmol m^{-2} sec^{-1}) for 2 h, after which the stomatal aperture was evaluated. The leaves were gently dried and the adaxial epidermis was carefully fixed to autoclave tape. The abaxial surface of the leaves was subsequently peeled off following fixing and removal of an adhesive film (tesafilm® crystal clear; Tesa, https://www.tesa.com/) and microscopic images were taken immediately (Azoulay-Shemer *et al.*, 2015; Horrer *et al.*, 2016). The stomatal aperture was also determined in dark-adapted leaves and used as a control. Four leaves from different plants were evaluated and an aperture of at least 20 stomata per leaf was measured, resulting in a total of at least 80 stomatal aperture measurements per treatment. The images were taken with a digital camera (Axiocam MRc) attached to a microscope (Zeiss model AX10; https://www.zeiss.com/). Subsequent measurements were derived from the images using the image processing package FIJI with ImageJ software (Schindelin *et al.*, 2012, 2015).

Guard cell isolation for kinetic isotope labelling experiments

A pool of guard cell-enriched epidermal fragments (simply referred to as guard cells) was isolated following a protocol that was recently optimized for metabolite profiling analyses (Daloso *et al.*, 2015). Briefly, guard cells were isolated in the pre-dawn by blending approximately 10 Arabidopsis rosettes per replicate in a Waring blender (Philips RI 2044; https://www.philips.com/global) incorporating an internal filter to remove excess mesophyll cells, fibres and other cellular debris. All guard cell isolations were carried out in the dark in order to maintain closed stomata and simulate opening following the natural circadian rhythm.

^{13}C-sucrose isotope kinetic labelling experiment

The stomatal assays described above were essential in order to demonstrate that the effect of sucrose on stomatal opening/closure is concentration dependent. Having established this fact, we subsequently aimed to investigate the role of sucrose during light-induced stomatal opening. For this purpose, we decided to perform a ^{13}C-sucrose isotope experiment by providing sucrose at a concentration of 0.1 mM. Guard cells were isolated, as described above, and subsequently transferred to the light and incubated in opening buffer solution (10 mM KCl, 50 μM $CaCl_2$ and 5 mM MES–TRIS, pH 6.15) in the presence or absence of ^{13}C-sucrose. Guard cell samples were rapidly harvested on a nylon membrane (220 μm) and snap-frozen in liquid nitrogen following 10, 30 and 60 min of light prior to storage at −80°C before subsequent metabolic analyses. A single sample per treatment was additionally taken in order to ascertain the stomatal aperture at each time point.

GC-MS analysis. The extraction of polar metabolites and their derivatisation was carried out exactly as described previously (Lisec *et al.*, 2006). Briefly, approximately 30 mg of lyophilised guard cells were disrupted by shaking the cells in a tube together with metal balls. Extraction was subsequently performed in methanol, with shaking at 70°C for 1 h; 60 μl of ribitol (0.2 mg ml^{-1}) was added as an internal standard. Following centrifugation, the supernatant was taken and the polar and apolar phases were separated by adding chloroform and water to the tube. A new centrifugation was performed and 1 ml of the polar (upper) phase was taken and reduced to dryness for further derivatisation and analysed by gas chromatography coupled to time of flight mass spectrometry (GC-TOF-MS). For mass spectral analysis, the relative isotopomer abundance and the determination of the total ^{13}C enrichment was performed using Xcalibur® 2.1 software (Thermo Fisher Scientific, https://www.thermofisher.com/) and the CORRECTOR program (Huege *et al.*, 2014), as described in previous guard cell isotope labelling studies (Daloso *et al.*, 2015, 2016b).

Statistical analysis. Data were obtained from experiments using a completely randomized design. All data are expressed as the mean ± standard error (SE). Analysis of variance (ANOVA) and means comparison by the Tukey test ($P \leq 0.05$) were performed using the software GENES (Cruz, 2016).

ACKNOWLEDGEMENTS

This work was supported by funding from the Max-Planck Society, the National Council for Scientific and Technological Development (CNPq-Brazil), the Foundation for Research Assistance of Minas Gerais State (FAPEMIG-Brazil) and the Foundation for Support of Scientific and Technological Development of Ceará (FUNCAP). Scholarships granted by CNPq to LPS and by FAPEMIG to DBM (CRA-BDS-00020-16), CNPq grant 402511/2016-6 and FAPEMIG grants (APQ-01078-15, APQ-01357-14 and RED-00053-16) to WLA and FUNCAP grant AEP-0128-00092.01.00/17 to DMD are gratefully acknowledged. The authors also thank Professor Dr Thomas C. R. Williams for helpful discussions concerning guard cell sucrose metabolism.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

© 2018 The Authors

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Schematic representation of the workflow for the [U-^{13}C]-sucrose labelling kinetic experiment.

Figure S2. Relative abundance of mass isotopomers of glucose, fructose and glucose-1-phosphate following guard cell incubation.

Figure S3. Relative abundance of mass isotopomers of photorespiratory-related metabolites following guard cell incubation.

Table S1. Relative metabolite content in dark-adapted guard cells and after 10, 30 and 60 min of incubation in opening buffer in the presence or absence of ^{13}C-sucrose.

REFERENCES

Abadie, C., Lothier, J., Boex-Fontvieille, E., Carroll, A. and Tcherkez, G. (2017) Direct assessment of the metabolic origin of carbon atoms in glutamate from illuminated leaves using ^{13}C-NMR. *New Phytol.* **216**, 1079–1089.

Amodeo, G., Talbott, L.D. and Zeiger, E. (1996) Use of potassium and sucrose by onion guard cells during a daily cycle of osmoregulation. *Plant Cell Physiol.* **37**, 575–579.

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

Antunes, W.C., Daloso, D.M., Pinheiro, D.P., Williams, T.C.R. and 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. *Environ. Exp. Bot.* **135**, 73–85.

Araújo, W.L., Nunes-Nesi, A., Osorio, S., *et al*. (2011) 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. *Plant Cell*, **23**, 600–627.

Azoulay-Shemer, T., Palomares, A., Bagheri, A., Israelsson-Nordstrom, M., Engineer, C.B., Bargmann, B.O.R., Stephan, A.B. and Schroeder, J.I. (2015) Guard cell photosynthesis is critical for stomatal turgor production, yet does not directly mediate CO2- and ABA-induced stomatal closing". *Plant J.* **83**, 567–581.

Boorer, K.J., Loo, D.D.F., Frommer, W.B. and Wright, E.M. (1996) Transport mechanism of the cloned potato H^+/Sucrose cotransporter StSUT1. *J. Biol. Chem.* **271**, 25139–25144.

Chen, C., Xiao, Y.G., Li, X. and Ni, M. (2012) Light-regulated stomatal aperture in Arabidopsis. *Mol. Plant*, **5**, 566–572.

Cheung, C.Y.M., Poolman, M.G., Fell, D.A., Ratcliffe, R.G. and 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 Physiol.* **165**, 917–929.

Condon, A.G., Richards, R.A., Rebetzke, G.J. and Farquhar, G.D. (2004) Breeding for high water-use efficiency. *J. Exp. Bot.* **55**, 2447–2460.

Cruz, C.D. (2016) Genes Software – extended and integrated with the R, Matlab and Selegen. *Acta Sci.* **38**, 547–552.

Daloso, D.M., Antunes, W.C., Pinheiro, D.P., Waquim, J.P., Araújo, W.L., Loureiro, M.E., Fernie, A.R. and Williams, T.C.R. (2015) Tobacco guard cells fix CO_2 by both Rubisco and PEPcase while sucrose acts as a substrate during light-induced stomatal opening. *Plant, Cell Environ.* **38**, 2353–2371.

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

Daloso, D.M., Williams, T.C.R., Antunes, W.C., Pinheiro, D.P., Müller, C., Loureiro, M.E. and 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 Phytol.* **209**, 1470–1483.

Daloso, D.M., Medeiros, D.B., dos Anjos, L., Yoshida, T., Araújo, W.L. and Fernie, A.R. (2017) Metabolism within the specialized guard cells of plants. *New Phytol.*, **216**, 1018–1033.

Dethloff, F., Orf, I. and Kopka, J. (2017) Rapid in situ ^{13}C tracing of sucrose utilization in Arabidopsis sink and source leaves. *Plant Methods*, **13**, 87.

Figueroa, C.M. and Lunn, J.E. (2016) A tale of two sugars - trehalose 6-phosphate and sucrose. *Plant Physiol.* **172**, 7–27.

Fischer, R.A. (1968) Stomatal opening: role of potassium uptake by guard cells. *Science*, **160**, 784–785.

Flexas, J. (2016) Genetic improvement of leaf photosynthesis and intrinsic water use efficiency in C3 plants: why so much little success? *Plant Sci.* **251**, 155–161.

Gago, J., Douthe, C., Florez-Sarasa, I., Escalona, J.M., Galmes, J., Fernie, A.R., Flexas, J. and Medrano, H. (2014) Opportunities for improving leaf water use efficiency under climate change conditions. *Plant Sci.* **226**, 108–119.

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

Gao, X., Li, C., Wei, P., Zhang, X., Chen, J. and Wang, X. (2005) The dynamic changes of tonoplasts in guard cells are important for stomatal movement in *Vicia faba*. *Plant Physiol.* **139**, 1207–1216.

Gauthier, P.P.G., Bligny, R., Gout, E., Mahé, A., Nogués, S., Hodges, M. and Tcherkez, G.G.B. (2010) In folio isotopic tracing demonstrates that nitrogen assimilation into glutamate is mostly independent from current CO_2 assimilation in illuminated leaves of Brassica napus. *New Phytol.* **185**, 988–999.

Gotow, K., Taylor, S. and Zeiger, E. (1988) Photosynthetic carbon fixation in guard cell protoplasts of *Vicia faba* L.: evidence from Radiolabel Experiments. *Plant Physiol.* **86**, 700–705.

Hampp, R., Outlaw, W.H. and Tarczynski, M.C. (1982) Profile of basic carbon pathways in guard cells and other leaf cells of *Vicia faba* L. *Plant Physiol.* **70**, 1582–1585.

Hedrich, R. (2012) Ion channels in plants. *Physiol. Rev.* **92**, 1777–1811.

Hedrich, R., Raschke, K. and Stitt, M. (1985) A role for fructose 2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. *Plant Physiol.* **79**, 977–982.

Hetherington, A.M. and Woodward, F.I. (2003) The role of stomata in sensing and driving environmental change. *Nature*, **424**, 901–908.

Hite, D.R.C., Outlaw, W.H. and Tarczynski, M.C. (1993) Elevated levels of both sucrose-phosphate synthase and sucrose synthase in Vicia guard cells indicate cell-specific carbohydrate interconversions. *Plant Physiol.* **101**, 1217–1221.

Hiyama, A., Takemiya, A., Munemasa, S., Okuma, E., Sugiyama, N., Tada, Y., Murata, Y. and Shimazaki, K. (2017) Blue light and CO_2 signals converge to regulate light-induced stomatal opening. *Nature Comm.* **8**, 1284.

Horrer, D., Flütsch, S., Pazmino, D., Matthews, J.S.A., Thalmann, M., Nigro, A., Leonhardt, N., Lawson, T. and Santelia, D. (2016) Blue light induces a distinct starch degradation pathway in guard cells for stomatal opening. *Curr. Biol.* **26**, 362–370.

Huege, J., Goetze, J., Dethloff, F., Junker, B. and Kopka, J. (2014) Quantification of stable isotope label in metabolites via mass spectrometry. *Methods Mol. Biol.* **1056**, 213–223.

Imamura, S. (1943) Untersuchungen uber den mechanismus der turgorschwandung der spaltoffnungesschliesszellen. *Japanese J. Bot.* **12**, 82–88.

Inoue, S. and Kinoshita, T. (2017) Blue light regulation of stomatal opening and the plasma membrane H^+-ATPase. *Plant Physiol.* **174**, 531–538.

Inoue, S., Takemiya, A. and Shimazaki, K. (2010) Phototropin signaling and stomatal opening as a model case. *Curr. Opin. Plant Biol.* **13**, 587–593.

Jarzyniak, K.M. and Jasiński, M. (2014) Membrane transporters and drought resistance – a complex issue. *Front. Plant. Sci.* **5**, 687.

Jezek, M. and Blatt, M.R. (2017) The membrane transport system of the guard cell and its integration for stomatal dynamics. *Plant Physiol.* **174**, 487–519.

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

Kang, Y., Outlaw, W.H., Fiore, G.B. and Riddle, K.A. (2007b) Guard cell apoplastic photosynthate accumulation corresponds to a phloem-loading mechanism. *J. Exp. Bot.* **58**, 4061–4070.

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

Kleczkowski, L.A., Geisler, M., Ciereszko, I. and Johansson, H. (2004) UDP-Glucose pyrophosphorylase. An old protein with new tricks. *Plant Physiol.* **134**, 912–918.

© 2018 The Authors

Lawson, T. and Blatt, M.R. (2014) Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiol.* **164**, 1556–1570.

Li, Y., Xu, S., Gao, J., Pan, S. and Wang, G. (2016) Glucose- and mannose-induced stomatal closure is mediated by ROS production, Ca^{2+} and water channel in *Vicia faba*. *Physiol. Plant.* **156**, 252–261.

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

Lloyd, F. (1908) *The physiology of stomata*, 82nd edn. Washington: Carnegie Inst Washington Year Book.

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

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

Lugassi, N., Kelly, G., Fidel, L. *et al.* (2015) Expression of Arabidopsis hexokinase in citrus guard cells controls stomatal aperture and reduces transpiration. *Front. Plant. Sci.* **6**, 1114.

McLachlan, D.H., Lan, J., Geilfus, C.M. *et al.* (2016) The breakdown of stored triacylglycerols is required during light-induced stomatal pening. *Curr. Biol.* **26**, 707–712.

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

Nunes-Nesi, A., Nascimento, V.L., de Oliveira Silva, F.M., Zsogon, A., Araujo, W.L. and Sulpice, R. (2016) Natural genetic variation for morphological and molecular determinants of plant growth and yield. *J. Exp. Bot.* **67**, 2989–3001.

Outlaw, W.H.Jr (1995) Sucrose and stomata: a full circle. in *Carbon Partitioning and Sucrose-Sink Interactions in Plants* (Madore, MA and Lucus, WJ eds). Rockville, MD: American Society of Plant Physiologists, pp. 56–67.

Robaina-Estévez, S., Daloso, D.M., Zhang, Y., Fernie, A.R. and Nikoloski, Z (2017) Resolving the central metabolism of Arabidopsis guard cells. *Sci. Rep.* **7**, 8307.

Santelia, D. and Lunn, J.E. (2017) Transitory starch metabolism in guard cells: unique features for a unique function. *Plant Physiol.* **74**, 539–549.

Schindelin, J., Arganda-Carreras, I., Frise, E. *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, **9**, 676–682.

Schindelin, J., Rueden, C.T., Hiner, M.C. and Eliceiri, K.W. (2015) The ImageJ ecosystem, An open platform for biomedical image analysis. *Mol. Reprod. Dev.* **82**, 518–529.

Souza, L. P., Szecówka, M., Fernie, A.R. and Tohge, T. (2017) 13CO2 Labeling and Mass Spectral Analysis of Photorespiration. In *Photorespiration. Methods in molecular biology* (Fernie, A.R., Bauwe, H. and Weber, A.P.M. eds). New York, NY: Humana Press, pp. 157–166.

Stitt, M. and Zeeman, S.C. (2012) Starch turnover: pathways, regulation and role in growth. *Curr. Opin. Plant Biol.* **15**, 282–292.

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R. and Ratcliffe, R.G. (2010) Not just a circle: flux modes in the plant TCA cycle. *Trends Plant Sci.* **15**, 462–470.

Talbott, L.D. and Zeiger, E. (1993) Sugar and organic acid accumulation in guard cells of *Vicia faba* in response to red and blue light. *Plant Physiol.* **102**, 1163–1169.

Talbott, L.D. and Zeiger, E. (1996) Central roles for potassium and sucrose in guard-cell osmoregulation. *Plant Physiol.* **111**, 1051–1057.

Talbott, L. and Zeiger, E. (1998) The role of sucrose in guard cell osmoregulation. *J. Exp. Bot.* **49**, 329–337.

Tcherkez, G., Cornic, G., Bligny, R., Gout, E. and Ghashghaie, J. (2005) In vivo respiratory metabolism of illuminated leaves. *Plant Physiol.* **138**, 1596–1606.

Tcherkez, G., Boex-Fontvieille, E., Mahé, A. and Hodges, M. (2012) Respiratory carbon fluxes in leaves. *Curr. Opin. Plant Biol.* **15**, 308–314.

Van Houtte, H., Vandesteene, L., Lopez-Galvis, L. *et al.* (2013) Overexpression of the trehalase gene AtTRE1 leads to increased drought stress tolerance in Arabidopsis and is involved in abscisic acid-induced stomatal closure. *Plant Physiol.* **161**, 1158–1171.

Vani, T. and Raghavendra, A.S. (1994) High mitochondrial activity but incomplete engagement of the cyanide-resistant alternative pathway in guard cell protoplasts of pea. *Plant Physiol.* **105**, 1263–1268.

Williams, T.C.R., Miguet, L., Masakapalli, S.K., Kruger, N.J., Sweetlove, L.J. and Ratcliffe, R.G. (2008) Metabolic network fluxes in heterotrophic Arabidopsis cells: stability of the flux distribution under different oxygenation conditions. *Plant Physiol.* **148**, 704–718.

Zeeman, S.C., Smith, S.M. and Smith, A.M. (2007) The diurnal metabolism of leaf starch. *Biochem J.* **401**, 13–28.

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

© 2018 The Authors
The Plant Journal © 2018 John Wiley & Sons Ltd,