Review

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Metabolism within the specialized guard cells of plants

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Summary

Stomata are leaf epidermal structures consisting of two guard cells surrounding a pore. Changes in the aperture of this pore regulate plant water-use efficiency, defined as gain of C by photosynthesis per leaf water transpired. Stomatal aperture is actively regulated by reversible changes in guard cell osmolyte content. Despite the fact that guard cells can photosynthesize on their own, the accumulation of mesophyll-derived metabolites can seemingly act as signals which contribute to the regulation of stomatal movement. It has been shown that malate can act as a signalling molecule and a counter-ion of potassium, a well-established osmolyte that accumulates in the vacuole of guard cells during stomatal opening. By contrast, their efflux from guard cells is an important mechanism during stomatal closure. It has been hypothesized that the breakdown of starch, sucrose and lipids is an important mechanism during stomatal opening, which may be related to ATP production through glycolysis and mitochondrial metabolism, and/or accumulation of osmolytes such as sugars and malate. However, experimental evidence supporting this theory is lacking. Here we highlight the particularities of guard cell metabolism and discuss this in the context of the guard cells themselves and their interaction with the mesophyll cells.

I. Introduction

The stomatal complex consists of a pore surrounded by two specialized cells known as guard cells found in the epidermis of the leaves. The regulation of both stomatal development and movement are extremely sensitive to changes in the surrounding environment (Qu *et al.*, 2017). The aperture of the stomatal pore is actively regulated by the metabolism of the surrounding guard

cells, which is, in turn, influenced by both endogenous and environmental signals (Santelia & Lawson, 2016). The aperture of the stomatal pore simultaneously determines the amount of CO_2 that enters for photosynthesis (A) and the amount of water that is lost by transpiration (E). It has been shown that improvement of photosynthesis and/or water-use efficiency (WUE, the ratio between C gain per water transpired; Gago *et al.*, 2014), can be accomplished by modifying stomatal behaviour (Nunes-Nesi *et al.*, 2007; Araújo *et al.*, 2011; Antunes *et al.*, 2012, 2017; Penfield *et al.*, 2012; Daloso *et al.*, 2016b; Medeiros *et al.*, 2016). In general, improved photosynthesis has been achieved by increasing stomatal conductance (g_s) whilst increased WUE is commonly obtained in plants with lower g_s. However, recent results indicate that genetic manipulation of key genes that regulate the A–g_s trade-off leads to plants with improved A and WUE (Kelly *et al.*, 2013; Lugassi *et al.*, 2015). Thus, a better understanding of stomatal regulation and how it is influenced by the surrounding mesophyll cells and environmental conditions represents an important target for the development of plants with improved A and WUE (Lawson & Blatt, 2014; Flexas, 2016; Nunes-Nesi *et al.*, 2016).

Stomatal movements are associated with alterations in vacuolar volume of guard cells, which are, in turn, regulated by reversible changes in the accumulation of osmolytes in these cells (Gao *et al.*, 2005; Tanaka *et al.*, 2007; Eisenach & De Angeli, 2017). The stomatal aperture is associated with an increase in the volume of guard cells by 40–50%, which is due to the accumulation of solutes in the vacuole of guard cells (MacRobbie & Kurup, 2007). Potassium (K^+) is by far the best characterized osmolyte accumulating in guard cells. K^+ enters guard cells via inward-rectifying K^+ channels and accumulates in the vacuole following import via K^+–H^+ antiporters (Hedrich, 2012). The role of K^+ and its counterions malate, chloride and nitrate as osmolytes during light-induced stomatal opening has been extensively documented (Imamura, 1943; Ichida *et al.*, 1997; De Angeli *et al.*, 2013; Inoue & Kinoshita, 2017). It has been suggested that sucrose may have an osmolytic role in guard cells. This idea comes from studies carried out mostly using epidermal peels of *Vicia faba* (Talbott & Zeiger, 1993, 1996; Amodeo *et al.*, 1996). However, the results obtained in these studies have not been confirmed by recent works using transgenic plants with altered sugar metabolism specifically in guard cells, which provided compelling evidence that sucrose breakdown within guard cells is important in the control of stomatal movements (Antunes *et al.*, 2012; Kelly *et al.*, 2013; Daloso *et al.*, 2015, 2016b; Lugassi *et al.*, 2015). Thus, direct proof of a mechanistic role of sucrose as an osmolyte within guard cells that regulates stomatal movement is still lacking (Daloso *et al.*, 2016a).

In contrast to stomatal opening, the vacuolar volume of guard cells decreases during abscisic acid (ABA)-induced stomatal closure (Tanaka *et al.*, 2007). This process is associated with K^+ and anion efflux and subsequent loss of water from the guard cell to the apoplastic space (Hedrich, 2012). The ABA-induced stomatal closure has been investigated thoroughly using both electrophysiological and transcriptomic analyses. However, despite the fact that both the perception and the transduction of ABA signals in guard cells have received considerable research attention (Assmann & Jegla, 2016; Hõrak *et al.*, 2017), whether and if so how this signalling pathway it is connected to the guard cell metabolism remains unclear. It has been shown that shikimate is negatively correlated to stomatal conductance (g_s) (Gago *et al.*, 2016) and that Arabidopsis flavonol-less mutants have a more rapid response under ABA-induced stomatal closure condition (Watkins *et al.*, 2014). This suggests that flavonols may mediate ABA responses, most probably by modulating the amounts of reactive oxygen species in guard cells. Other metabolites such as sugars (sucrose, mannose and glucose), organic acids (malate and fumarate) and glutamate have been implicated in stomatal closure (Lu *et al.*, 1997; Kang *et al.*, 2007; Araújo *et al.*, 2011; Kelly *et al.*, 2013; Yoshida *et al.*, 2016; Li *et al.*, 2016). Beyond their role within guard cells, sugars and organic acids derived from mesophyll cells have been shown to be key metabolites that tightly regulate the A–g_s trade-off.

Guard cell metabolism has certain particularities meaning that it differs substantially from that of mesophyll cells (Santelia & Lawson, 2016). Transcriptome analyses reproducibly revealed that > 1000 genes are differentially expressed in guard cells compared to mesophyll cells (Leonhardt *et al.*, 2004; Yang *et al.*, 2008; Bates *et al.*, 2012; Bauer *et al.*, 2013a). Considerable differences were also observed in proteomic studies comparing mesophyll and guard cell protoplasts (Zhao *et al.*, 2008; Zhu *et al.*, 2009). These dissimilarities found at transcript and proteomic levels lead to a particular cell-specific metabolism in guard cells, as revealed by enzyme activity assays (Table 1) and studies developed in the 1970s and 1980s using radiolabelled techniques (Willmer & Dittrich, 1974; Outlaw & Kennedy, 1978; Brown & Outlaw, 1982; Hampp *et al.*, 1982; Gotow *et al.*, 1988). Recently, new insights into guard cell metabolism have been provided by targeted metabolomic studies on guard cell preparations focusing mainly in the central metabolism of guard cells (Jin *et al.*, 2013; Daloso *et al.*, 2015, 2016b; Misra *et al.*, 2015; Horrer *et al.*, 2016; McLachlan *et al.*, 2016). These recent studies, coupled with recent modelling approaches (Chen *et al.*, 2012c; Hills *et al.*, 2012; Sun *et al.*, 2014; Medeiros *et al.*, 2015; Vialet-Chabrand *et al.*, 2017), have enriched our understanding of guard cell function considerably. However, several pieces are still missing in the guard cell metabolism puzzle. In this review, we highlight the mechanisms that need to be elucidated to overcome the gaps regarding guard cell function. We start by reviewing how guard cells sense light and what are the roles and particularities of guard cell photosynthetic metabolism. After that, we provide an overview on what it is known regarding guard cell central metabolism, highlighting the particularities, regulation and function of glycolysis, gluconeogenesis, and both mitochondrial and starch metabolism. Following this, we discuss how mesophyll-derived metabolites influence stomatal movements and argue how malate and sucrose coordinate the A–g_s trade-off. To conclude, we outline the challenges and perspectives of stomatal physiology research.

II. Guard cell photosynthesis

1. Red light-mediated responses triggered by mesophyll and guard cells

Light provides not only the energy source for photosynthesis, but also a wealth of other information which is utilized in the

Table 1 Relative protein quantity and enzyme activities in mesophyll and guard cells

Enzyme	EC number	Relative protein quantity[A]		Enzyme activity					References
		Accession no.	GC/MC	GC	MC	GC/MC	Unit*	Species**	
RubisCO[B]	4.1.1.39	gi|8745521	0.38	131	446	0.29	1	*1*	Reckmann *et al.* (1990)
				140	460	0.30	1	*2*	Shimazaki & Okayama (1990)
PEPc	4.1.1.31	gi|30689081	5.63	2060	30	68.7	1	*2*	Shimazaki (1989)
				10.1	9.4	1.07	2	*1*	Vani & Raghavendra (1994)
NADP-MDH	1.1.1.82	gi|4995091	0.42	270	6	45.0	1	*2*	Gotow *et al.* (1985); Shimazaki (1989)
$NADP^+$-ME[C]	1.1.1.40	gi|27530932	0.42	357	113	3.16	1	*2*	Outlaw *et al.* (1981a)
ATP-PFK	2.7.1.11		–	6.3	–	–	3	*2*	Hedrich *et al.* (1985)
PFP[D]	2.7.1.90		–	10.2	–	–	3	*2*	Hedrich *et al.* (1985)
NAD^+-MDH	1.1.1.37		–	30 000	–	–	1	*2*	Gotow *et al.* (1985)
				1166	688	1.69	2	*1*	Vani & Raghavendra (1994)
Fumarase	4.2.1.2	gi|15226618	2.47	576	186	3.10	1	*2*	Hampp *et al.* (1982)
				115	61	1.89	2	*1*	Vani & Raghavendra (1994)
SDH	1.3.5.1	gi|15240075	2.14	2153	486	4.43	2	*1*	Vani & Raghavendra (1994)
Amylase	3.2.1.1, 3.2.1.2		–	2.75	–	–	2	*3*	Robinson & Preiss (1987)
SS	2.4.1.21	gi|15223331	0.19	0.13	–	–	2	*3*	Robinson & Preiss (1987)
SuSy	2.4.1.13		–	1.1	0.1	11.0	2	*2*	Hite *et al.* (1993)
				3600	120	30.0	2	*4*	Daloso *et al.* (2015)

All enzymatic assays were performed in protoplasts, except the data from Daloso *et al.* (2015) that was carried out using leaves and guard cell-enriched epidermal fragments.
[A]Data from differential proteomics using mesophyll and guard cell protoplasts of *Brassica napus* (Zhu *et al.*, 2009).
[B]The ratio between GC/MC in the absolute amount of Rubisco (pg $cell^{-1}$) is 0.002 according to Reckmann *et al.* (1990).
[C]Activated by light in guard cells.
[D]Activated by $Fru2,6P_2$ in the glycolytic direction in guard cells.
*Unity: 1, μmol mg^{-1} chl h^{-1}; 2, μmol mg^{-1} protein h^{-1}; 3, pmol $cell^{-1}$ h^{-1}.
**Species: 1, *Pisum sativum*; 2, *Vicia faba*; 3, *Commelina communis*; 4, *Nicotiana tabacum*.
GC, guard cells; MC, mesophyll cells; –, no data available.
MDH, malate dehydrogenase; ME, malic enzyme; PEPc, phospho*enol*pyruvate carboxylase; PFK, phosphofructokinase; PFP, pyrophosphate:fructose 6-phosphate phosphotransferase; RubisCO, ribulose-1,5-biphosphate carboxylase/oxygenase;SDH, succinate dehydrogenase; SS, starch synthase; SuSy, sucrose synthase.

optimization of plant growth. Two signalling pathways, triggered by blue or red light, are involved in the light regulation of stomatal movements (Shimazaki *et al.*, 2007). It is generally accepted that red- and blue-light responses are sensed by the chloroplast and by photoreceptors at the guard cell plasma membrane, respectively (Chen *et al.*, 2012a). The blue-light sensing by blue-light photoreceptors in guard cells (Kinoshita *et al.*, 2001) and the signalling pathway which culminates in the activation of H^+-ATPases at the plasma membrane have been relatively well-documented (check Section IV for details on the metabolic implications of guard cell blue-light perception) (Inoue *et al.*, 2010; Kinoshita & Hayashi, 2011; Chen *et al.*, 2012a; Inoue & Kinoshita, 2017). By contrast, it is still a matter of debate whether red-light is perceived by mesophyll cells (and subsequently transmitted as a signal to guard cells), or directly by the guard cells (Messinger *et al.*, 2006; Busch, 2014; Mott *et al.*, 2014). Experimental evidence indicates that it is likely that stomata respond to red light, but only at light intensities similar to that required to photosynthesis (Messinger *et al.*, 2006; Mott *et al.*, 2008; Mott, 2009). However, the metabolic implications of the red-light perception are far from clear.

Due to the close relationship between g_s and A, it was proposed that internal CO_2 (C_i) concentration rather than ambient [CO_2] could be a key player allowing the coordination between mesophyll photosynthetic activity and stomatal aperture (Mott, 1988). The generally accepted model for this coordination is that, in intact leaves, red light is sensed in mesophyll chloroplasts by chlorophylls, triggering a drop in C_i which, in turn, opens stomata (Mott, 1988; Hanstein *et al.*, 2001; Roelfsema & Hedrich, 2002; Roelfsema *et al.*, 2002, 2006; Fujita *et al.*, 2013). This idea is supported by the fact that stomata of albino leaf patches of *Vicia faba* and *Chlorophytum comosum* did not respond to red light, although blue light could still induce stomatal opening (Roelfsema *et al.*, 2006). These findings are in good accordance with the earlier demonstration that stomata of isolated epidermis incubated either without mesophyll cells or with dark-adapted chloroplasts are not able to fully open, whereas they open when the epidermis was placed either with illuminated mesophyll cells or chloroplast (Lee & Bowling, 1992). Additionally, it was more recently demonstrated that stomata of isolated epidermis are not able to completely respond to red light and CO_2, but when placed back with mesophyll cells from the same species or a different one they recovered their responsiveness (Mott *et al.*, 2008; McAdam & Brodribb, 2012; Fujita *et al.*, 2013). Collectively, these results clearly indicate the importance of mesophyll photosynthetic activity for stomatal opening.

Recent results suggests that photosynthesis in the guard cells may be critical for stomatal turgor production (Azoulay-Shemer *et al.*, 2015) and a range of evidence has also been discussed suggesting

that the stomatal red-light response signal may be provided by the redox state of photosynthetic electron transport chain components such as the redox state of plastoquinone and the production of ATP and NADPH (Schwartz & Zeiger, 1984; Tominaga *et al.*, 2001; Busch, 2014; Azoulay-Shemer *et al.*, 2015). Moreover, recent evidence suggest that plastid-generated 3′-phosphoadenosine 5′-phosphate may act as retrograde signal providing a form of communication between guard cell plastids and their nuclei in the regulation of stomatal closure (Pornsiriwong *et al.*, 2017). However, the role of this recently discovered signalling pathway in light-induced stomatal opening remains to be investigated. Detailed molecular characterization of the players responsible is therefore clearly required. In light of this, further studies aiming to elucidate the precise nature of the red-light signal and its interaction remains an exciting topic for future research, whether or not this includes a metabolic component.

2. Role of guard cell chloroplast electron transport chain in guard cell function

Guard cell chloroplasts contain the entire protein network required to autonomously produce ATP, NADPH and photoassimilates (Fig. 1) (Outlaw *et al.*, 1981b; Shimazaki *et al.*, 1989; Vani & Raghavendra, 1994; Lawson, 2009), yet little-to-nothing is known concerning the capacity of guard cell metabolism to regenerate ribulose-1,5-biphosphate (Baroli *et al.*, 2007; Lawson *et al.*, 2008). Guard cells have fewer and smaller chloroplasts (Willmer & Fricker, 1996), lower chlorophyll content, and lower content and activity of ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) than mesophyll cells (Table 1). It has been demonstrated that the photosynthetic capacity of guard cells is considerably lower in comparison to mesophyll cells (Lawson *et al.*, 2002, 2003; Reckmann *et al.*, 1990; Shimazaki & Okayama, 1990; Gotow *et al.*, 1988; Shimazaki, 1989). Nonetheless, it is important to stress that the lower photosynthetic capacity of guard cells does not mean that the guard cell chloroplasts and the products of the chloroplast electron transport chain (cETC) are unimportant for the functioning of this cell type.

The cETC has been proposed to be an additional source of ATP for guard cells (Fig. 1), besides that provided by glycolysis and mitochondrial respiration (Schwartz & Zeiger, 1984; Tominaga *et al.*, 2001; Wang *et al.*, 2014b). It was demonstrated that osmotic stress strongly impaired the photosynthesis in guard cell but not in mesophyll cells (Goh *et al.*, 2001). Furthermore, it was demonstrated that the activity of K^+-uptake channels at the guard cell plasma membrane as well as the photosynthetic electron transport in guard cell chloroplasts are dependent on cystolic ATP, providing evidence for ATP as important regulator linking guard cell photosynthesis and ion transport activity (Goh *et al.*, 2002). Moreover, the products of cETC are essential for blue-light-induced stomatal opening (Suetsugu *et al.*, 2014) and for the light activation of the guard cell NADP-malate dehydrogenase (NADP-MDH), the enzyme responsible for the interconversion of malate and oxaloacetate (OAA) in the chloroplast and cytosol (Gotow *et al.*, 1985), which presents a substantially higher activity in guard cells compared to mesophyll cells (Table 1). Moreover, reduced g_s has been observed in leaves with lower guard cell chlorophyll content (Azoulay-Shemer *et al.*, 2015). In contrast to mesophyll cells, in guard cells the light harvesting complex protein of complex II is found in the phosphorylated state in the dark (Kinoshita *et al.*, 1993), suggesting a differential dark/light regulation of the cETC in guard cells. Collectively, it seems likely that cETC is an important process for the generation of energy (ATP) and reducing power (NADPH) for guard cell function, but that the regulation of these processes is considerably different from that observed in mesophyll cells.

3. The black box of guard cell CO_2 fixation

The capacity of guard cell chloroplasts to fix CO_2 by RubisCO was historically thought to be insignificant (Outlaw *et al.*, 1979), particularly given the low content and activity of RubisCO found in this cell (Table 1) (Reckmann *et al.*, 1990; Shimazaki & Okayama, 1990). This view has changed considerably following ^{14}C-feeding experiments which provided compelling evidence for RubisCO-mediated CO_2 fixation (Gotow *et al.*, 1988; Shimazaki, 1989; Reckmann *et al.*, 1990). In parallel, several ^{14}C-feeding experiments indicated that guard cells have a high anaplerotic CO_2 fixation catalysed by phospho*enol*pyruvate carboxylase (PEPc) (Willmer & Dittrich, 1974; Outlaw & Kennedy, 1978; Schnabl, 1980; Gotow *et al.*, 1988; Parvanthi & Raghavendra, 1997), in accordance with the higher relative content and activity found in guard cells compared to mesophyll cells (Table 1). These findings have been confirmed recently by a mass spectrometry-based ^{13}C-isotope labelling study which indicates that guard cells are able to fix CO_2 by both RubisCO and PEPc (Daloso *et al.*, 2015). However, the proportion of C skeletons produced within guard cells and that imported from mesophyll cells is unclear.

Having solved the controversy of whether guard cells can or cannot fix CO_2 by RubisCO, the question raised is to what extent this pathway and those catalysed by PEPc contributes to the overall amount of C within guard cells. Moreover, several basic questions concerning guard cell physiology remain obscure and their answers are pivotal for guard cell modelling and metabolic engineering. Recent use of mutants and profiling approaches has considerably increased the knowledge and the particularities of guard cell photosynthetic metabolism. For instance, microarray-based transcriptomic studies revealed that several genes of the C_4 cycle are highly expressed in guard cells (Leonhardt *et al.*, 2004; Yang *et al.*, 2008; Wang *et al.*, 2011; Bates *et al.*, 2012; Bauer *et al.*, 2013a). Moreover, it has also been shown that guard cells from C_3 plants exhibit higher activities of the C_4 markers PEPc, MDH and malic enzyme (Table 1) (Outlaw *et al.*, 1981a; Gotow *et al.*, 1985), higher CO_2 fixation in the dark compared to mesophyll cells, and malate and aspartate are the main products after $^{14}CO_2$ fixation (Schnabl, 1980; Brown & Outlaw, 1982; Gautier *et al.*, 1991). Taken together, these results suggest that guard cells have a photosynthetic metabolism closer to C_4 and crassulaceae acid metabolism (CAM) than C_3. However, it is important to highlight that a recent RNAseq-based transcriptomic study revealed that the expression of these C_4-marker genes is actually higher in mesophyll compared to guard cells (Aubry *et al.*, 2016). It seems likely that the C_4-like

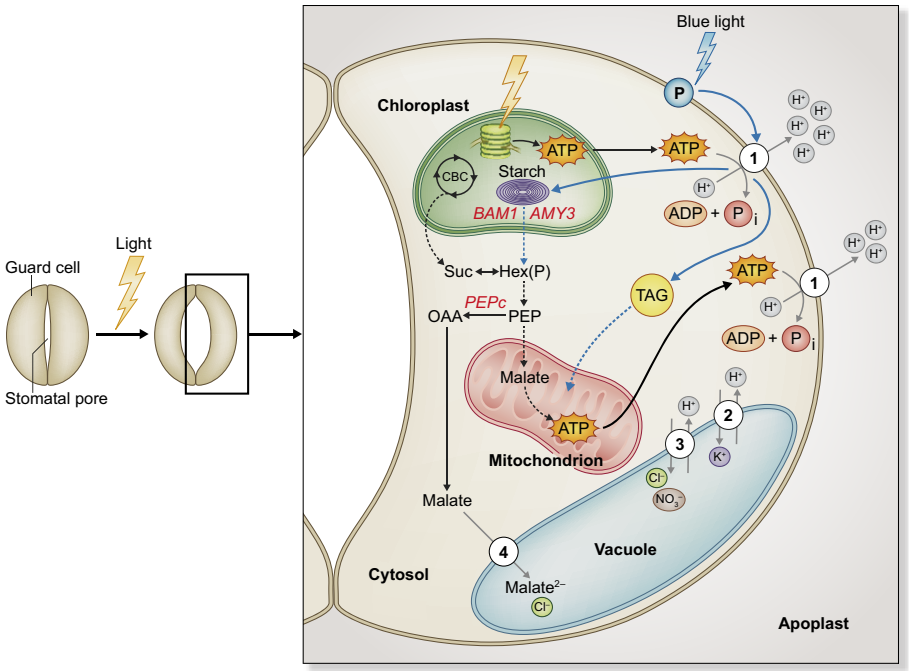

Fig. 1 Guard cell metabolic changes during light-induced stomatal opening. The stomatal opening is associated with the accumulation of potassium (K^+) and their counter-ions chloride (Cl^-), nitrate (NO_3^-) and malate ($malate^{2-}$) in the vacuole of guard cells. It has been shown that the process of stomatal aperture differs substantially between red and blue light. Whilst chloroplasts seem to be the location of red-light perception (See 'Guard Cell Photosynthesis'), phototropins (P) are the blue-light receptors at the plasma membrane of guard cells (Kinoshita *et al.*, 2001). Blue light stimulates H^+ extrusion via H^+-ATPases (1), which, in turn, allows the influx of K^+ and its accumulation into the vacuole (Inoue *et al.*, 2010). Furthermore, it seems that H^+ extrusion via H^+-ATPases is the signal to stimulates the breakdown of starch and lipid droplets (triacylglycerols – TAG) in guard cells – see blue arrows (Horrer *et al.*, 2016; McLachlan *et al.*, 2016). Given that the stomatal opening is an ATP-dependent process, and that guard cells contain few chloroplasts (Willmer & Fricker, 1996), this cell has certain particularities to overcome its energetic demand. Besides the ATP produced by the chloroplast electron transport chain, it has been shown that the guard cell has a high capacity to produce ATP by mitochondrial metabolism. This idea is supported by the fact that guard cells contain numerous mitochondria (Willmer & Fricker, 1996), high respiratory rate (Vani & Raghavendra, 1994; Araújo *et al.*, 2011), and that the breakdown of starch, sucrose and lipids seem to be important mechanisms for stimulating mitochondrial metabolism during light-induced stomatal opening (Daloso *et al.*, 2015; Horrer *et al.*, 2016; McLachlan *et al.*, 2016). Thus, glycolysis and β-oxidation are important pathways for the biosynthesis of malate, which can be respired to produce ATP, act as a counter-ion of potassium in the vacuole or act as a signalling molecule in the cytosol of guard cells (see Fig. 2 for details on the role of malate in guard cell regulation). ADP, adenosine di-phosphate; AMY3, alpha-amylase 3; ATP, adenosine tri-phosphate; BAM1, plastidic beta-amylase 1; CBC, Calvin–Benson cycle; CHLOROP, chloroplast; Hex(P), hexoses and hexoses phosphates; MITOC, mitochondria; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PEPc, phospho*enol*pyruvate carboxylase; Suc, sucrose. (P) phototropins. Transporters: (1) H^+-ATPases; (2) Tonoplast K^+-H^+ antiporter; (3) Anion-H^+ antiporters; (4) Aluminium-activated malate transporter (ALMT) members. Dashed arrows indicate steps involving several reactions.

features found in guard cells are an adaptation to produce malate due to the importance of this metabolite in the regulation of stomatal movements (Dittrich & Raschke, 1977; Hedrich *et al.*, 1994; Fernie & Martinoia, 2009). However, what type of metabolism – C_3, C_4 or CAM – is found in guard cells is still not completely clear. Further experiments aiming to characterize the magnitude of CO_2 fixation and respiration in the light and dark as well as the characterization of guard cell photosynthetic and (photo)respiratory mutants will be highly informative and must be performed. Only with this information in hand will we be able to better understand the intriguing guard cell photosynthetic metabolism.

III. Guard cell central metabolism

1. Glycolysis and gluconeogenesis

A transcriptome study revealed that 293 genes related to photosynthesis are differentially expressed between guard cells and mesophyll cells (Bates *et al.*, 2012). Interestingly, only five of those 293 genes were highly expressed in guard cells. These findings are in close agreement with other transcriptomic and proteomic studies (Leonhardt *et al.*, 2004; Yang *et al.*, 2008; Zhao *et al.*, 2008; Zhu *et al.*, 2009; Bauer *et al.*, 2013a). Two of those five genes highly expressed in guard cells; *FRUCTOSE-BIPHOSPHATE ALDOLASE* (*FBP ALDOLASE*, putative – At5g03690) and *PHOSPHOENOLPYRUVATE CARBOXYLASE-RELATED KINASE 2* (*PEPRK2* – At1g12680) are related to glycolysis and gluconeogenesis. The other three genes are: ferredoxin 3 (ATFD3–At2g27510), a putative chloroplast outer membrane protein (At5g20300), and an unknown gene. FBP aldolase is an enzyme responsible for the interconversion of fructose 1,6-biphosphate to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) in the glycolysis pathway. Early evidence indicates that guard cells possess a strong glycolytic capacity that it is activated in the light, as demonstrated by decreased hexose phosphate content and increased fructose 2,6-biphosphate content

after illumination (Hedrich *et al.*, 1985). Similarly, recent results highlight that sucrose is dissimilated within guard cells during dark-to-light transition, possibly as a mechanism to stimulate glycolysis and the mitochondrial metabolism (Daloso *et al.*, 2015). These results are opposed to those observed in mesophyll cells in the light, in which the metabolism is mainly in favour of sucrose and starch synthesis instead of activating glycolysis and mitochondrial metabolism (Tcherkez *et al.*, 2012; Santelia & Lunn, 2017). This suggests that the glycolytic pathway may have an important role for guard cell function in the light. In fact, Arabidopsis double mutants lacking phosphoglyrate mutase enzyme activity, a glycolytic enzyme, have reduced blue-light-induced stomatal opening (Zhao & Assmann, 2011). It seems likely that guard cell metabolism is fully adapted to supply the demand of energy for its own metabolism and ion transport, in which the activation of glycolysis in the light seems to be a mechanism to increase the rate of ATP production, possibly as an adaptation to the low chlorophyll content found in these cells (Willmer & Fricker, 1996).

Another enzyme related to glycolysis and gluconeogenesis highly expressed in guard cells is PEPRK2, one of the two PEPRKs found exclusively in plants (Hrabak *et al.*, 2003). This enzyme is supposed to be one of the regulators of PEPc activity, although its functional characterization in guard cells is still missing. Beyond PEPRK2, a recent meta-analysis of previous transcriptomic data revealed that several genes of gluconeogenesis such as *PEPc* (At3g14940, At2g42600), *PHOSPHOENOLPYRUVATE CARBOXYKINASE 1* (*PCK1* – At4g37870) and *PYRUVATE, ORTHODIPHOSPHATE DIKINASE* (*PPDK* – At4g15530) are highly expressed in guard cells (Daloso *et al.*, 2016a). Given the low CO_2 fixation by RubisCO found in guard cells (Gotow *et al.*, 1988), it suggests that gluconeogenesis has an important role in providing C skeletons for guard cell function. It has been shown that mutant plants lacking PCK1 activity, a key enzyme in the first steps of the gluconeogenesis pathway responsible for the conversion of OAA into PEP, has higher g_s and wider stomatal aperture compared to the wild-type (WT) (Penfield *et al.*, 2012), providing compelling evidence that gluconeogenesis is, in fact, important for stomatal aperture regulation. However, despite this work, no other study has used the genetic reverse approach to investigate the role and the importance of gluconeogenesis in guard cell metabolism. For instance, whether the recently described gluconeogenesis route that uses PPDK instead of PCK (Eastmond *et al.*, 2015) also occurs in guard cells has yet to be experimentally assessed. It is therefore tempting to speculate that further characterization of mutants or transgenic plants altered in the expression of genes related to gluconeogenesis will help us to understand the importance of this process both to guard cell metabolism and the regulation of stomatal movements.

2. The highly specialized guard cell mitochondrial metabolism

Guard cells contain an unusually large number of mitochondria (Willmer & Fricker, 1996), high expression of H^+-ATPases and ATPases (Yang *et al.*, 2008; Zhu *et al.*, 2009; Wang *et al.*, 2011; Bauer *et al.*, 2013a), higher activity of mitochondrial enzymes (Table 1) and higher respiratory rate compared to mesophyll cells (Mawson, 1993; Vani & Raghavendra, 1994; Araújo *et al.*, 2011). Additionally, a pioneer work showed that stomatal opening is promoted by the addition of NADH, ATP and succinate, and that light-induced stomatal opening is reduced in the presence of respiratory inhibitors (Raghavendra, 1981). Further studies have confirmed the role of ATP and other purine nucleotides in promoting stomatal opening (Clark *et al.*, 2011; Hao *et al.*, 2012; Wang *et al.*, 2014a). However, it is interesting to note that the role of ATP on stomatal aperture can be dose-dependent, given that low (< 35 μM) and high (> 150 μM) concentration of ATP-induced stomatal opening and closure, respectively (Clark *et al.*, 2011), although these results have not been confirmed by Hao and collaborators (Hao *et al.*, 2012). It has been shown that extracellular ATP acts as a signalling molecule and plays an important role in plant growth and stress tolerance in a mechanism mediated by the plasma membrane DORN1 receptor (Does not Respond to Nucleotides 1), and accumulation of Ca^{+2}, nitric oxide and hydrogen peroxide accumulation within the cells (Cao *et al.*, 2014; Choi *et al.*, 2014). Thus, given that these molecules are known to be signals for stomatal closure, it is reasonable to assume that ATP-induced stomatal closure would occur following this signalling pathway, yet experimental evidence supporting this idea is still lacking. Taken together, these findings suggest that guard cell ATP production via mitochondrial metabolism is important for regulation of stomatal movements (Fig. 1) (Araújo *et al.*, 2014).

Organic acid metabolism has long been reported to be involved in the regulation of stomatal function, especially due the importance of malate on stomatal aperture regulation (Outlaw & Lowry, 1977; Talbott & Zeiger, 1993; Fernie & Martinoia, 2009). Malate can be produced by guard cells or imported from mesophyll cells. In leaves, especially those from C_4 plants, the anaplerotic CO_2 fixation catalysed by PEPc is an important source of malate in the light, given that respiration is inhibited under this condition (Tcherkez *et al.*, 2005). In this mechanism, cytosolic PEPc catalyses the carboxylation of PEP yielding OAA, which is further reduced to malate via NADP-malate dehydrogenase (NADP-MDH) (Fig. 2). The operation of PEPc in tobacco guard cells in the light is supported by an isotope labelling experiment because the labelling enrichment from ^{13}C-$NaHCO_3$ into malate and fumarate increased to a much greater extent than that for succinate, consistent with label initially being incorporated into OAA through PEPc, following the malate and fumarate production by NAD^+-MDH and fumarase (Daloso *et al.*, 2015). These findings, coupled with the observation that glycolysis seems to be activated in the light (Hedrich *et al.*, 1985), suggest that guard cell metabolism is adapted to optimize the flux of C to the tricarboxylic acid (TCA) cycle in the light (Daloso *et al.*, 2015) and that PEPc activity may have an important role in providing OAA for malate synthesis. In fact, the activity of several malate-related enzymes such as PEPc, NAD(P)-MDH, malic enzyme, fumarase and succinate dehydrogenase (SDH) is higher in guard cells compared to mesophyll cells (Table 1).

It has been proposed that the breakdown of lipids, starch and sucrose is important to stimulate the flux of C to the TCA cycle, which in turn can be used for malate synthesis and/or for

mitochondrial ATP production (Fig. 1). This hypothesis is supported by the fact that the content of sucrose, starch and lipid droplets is reduced during dark-to-light transition, a condition in which the TCA cycle seems to be activated (as discussed above). However, it is important to note that malate can be synthesized independently of the TCA cycle (Fig. 2); therefore, further experiments are required to confirm this hypothesis. The idea that lipid breakdown is an important process to stimulate fluxes through β-oxidation and the TCA cycle comes from a recently published work, which revealed that the volume of lipid droplets is reduced during light-induced stomatal opening in WT plants and that this response was less pronounced in the blue-light photoreceptors *phot1*/*phot2* double mutant (McLachlan *et al.*, 2016). Moreover, it also has been demonstrated that the concentration of several triacylglycerols was reduced following the dark-to-light transition and that the light-induced stomatal opening is disrupted in triacylglycerol catabolism mutants (McLachlan *et al.*, 2016). These combined results highlight initial findings in which guard cell lipid profile was shown to be widely different from mesophyll cells (Sato, 1987; Sakaki *et al.*, 1995), suggesting that β-oxidation is a mechanism which contributes considerably to the overall production of ATP via guard cell mitochondrial respiration and that this mechanism is pivotal for the blue-light-induced stomatal opening (Fig. 1). Interestingly, β-oxidation as source of energy has precedent in the literature. Gland activity of *Venus flytrap* is powered by oil layers in cells with remarkable increased number of peroxisomes and mitochondria (Bemm *et al.*, 2016). Further experiments using ^{13}C-labelled substrates will, however, be required to confirm the physiological relevance of this observation in guard cells.

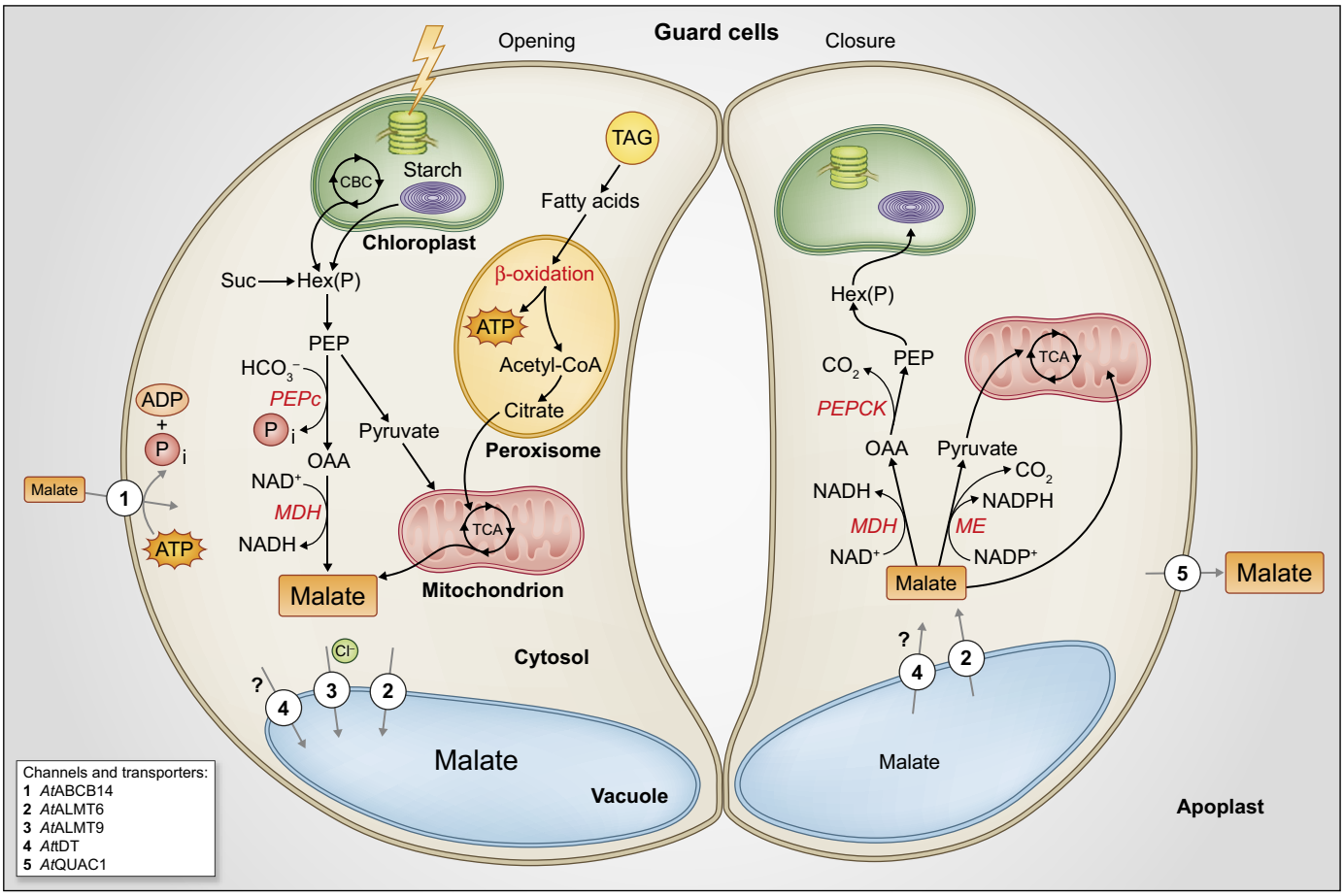

Fig. 2 Proposed pathways involved in the metabolism and transport of malate during stomatal movements. During stomatal opening, the accumulation of malate inside the guard cell occurs mainly due to the influx from the apoplast through the *At*ABCB14 (1), guard cell photosynthesis, mitochondrial activity and possibly from blue-light-induced starch and lipid breakdown. Cytosolic light-induced PEPc can catalyze the carboxylation of PEP yielding OAA, which is further reduced to malate via NAD^+-malate dehydrogenase (NADP-MDH). Malate is additionally transported into vacuoles through the channels *At*ALMT6 (2), *At*ALMT9 (3) and possibly through the transporter *At*tDT (4). In the guard cells, malate acts as an osmoregulator and counter ion for K^+ allowing the water inlet, and finally the stomatal aperture. Furthermore, cytosolic malate can increase the Cl^- currents through *At*ALMT9 (3), which is permeable to both Cl^- and malate. By contrast, during stomatal closing, the malate previously accumulated is released to the apoplastic space through *At*QUAC1 (5), or it can be metabolized via the decarboxylation by NADP+-malic enzyme ($NADP^+$-ME) or via NAD^+-MDH yielding pyruvate, which can be further metabolized through the TCA cycle and oxalacetate that can be partially converted into starch (not presenting osmotic activity) via the gluconeogenesis pathway. ALMT, Aluminium-activated malate transporters; CBC, Calvin–Benson cycle; Hex-P, hexose phosphate; Mal, malate; MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxalacetate; PEP, phospho*enol*pyruvate; PEPc, phospho*enol*pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; QUAC1, quick activating anion channel; Suc, sucrose; TAG, triacylglycerols; tDT, tonoplast dicarboxylate transporter.

IV. Guard cell starch metabolism differs from that of mesophyll cells and plays a key role in stomatal movement

1. Starch breakdown within guard cells as an important mechanism during blue-light stomatal opening

In the previous sections, we have demonstrated that the central metabolism differs substantially between mesophyll and guard cells. Here, the particularities of guard cell starch metabolism will be highlighted. The first record of a distinct starch metabolism in guard cells dates back over 100 years to when Lloyd (1908) observed that guard cell starch content is lower in the first hours of the day than in the previous night. This raised the hypothesis that guard cell photosynthesis is insufficient to support stomatal opening and starch hydrolysis plays an important role in energy supply. Nearly 60–80 years later, successive works based on iodine staining, histochemistry, and enzymatic and spectrophotometric assays have confirmed Lloyd's observation that starch accumulates in guard cells during the night and is degraded during the early morning. Meanwhile, an alternative hypothesis was raised that diurnal starch degradation in guard cell most probably occurs to provide C skeletons for the synthesis of osmoticum required for stomatal opening, particularly when concentrations of complementary mesophyll sugars in the apoplast are low (Pallas, 1964; Meidner & Mansfield, 1968; Outlaw & Manchester, 1979; Ritte *et al.*, 1999). This idea was based on the observation that stomatal aperture and starch content in guard cells are negative correlated, such that larger stomatal aperture is observed at low starch contents and *vice versa* (Outlaw & Manchester, 1979). Nevertheless, several parallel studies found no correlation between guard cell starch content and stomatal opening (Heath, 1947; Ogawa, 1981; Tallman & Zeiger, 1988). In the late 1980s there was a lack of consistency in the literature, which most probably is explained by differences among species, lack of a precise technique for isolation of absolutely pure guard cells, and/or by the light quality used in the experiments, given that recent works now show that starch degradation in guard cells is most likely triggered by blue and not red light (Santelia & Lunn, 2017).

The effect of blue light on stomatal opening and on starch hydrolysis for the synthesis of osmolytes in guard cells was first suggested by experiments using *Vicia faba* epidermal peels, which revealed an increase in maltose and malate contents in the first 30 min under blue light (Talbott & Zeiger, 1993). This result was lately corroborated by reverse genetic approaches. The first report showed that stomatal opening is lower in the starchless Arabidopsis *pgm* mutant than in the WT under blue light, whilst no difference is observed under red light (Lasceve *et al.*, 1997). More recently, reverse genetics coupled with high-resolution confocal microscopy have allowed the quantification of starch in guard cells of intact leaves in Arabidopsis mutants for key starch-degrading enzymes. This method has provided additional information on the diel time course of guard cell starch content and revealed that guard cell starch degradation starts slowly in the middle of the night and continues for up to 1 h into the light period (Horrer *et al.*, 2016). Interestingly, the rate of blue-light-induced starch degradation is markedly faster than the night-time degradation, such that guard cell starch content is nearly completely degraded after 30 min in the light, a result that corroborates earlier observations made by Talbott & Zeiger (1993). Rapid light-induced starch degradation also has been observed in guard cells harvested at the beginning of the day from tobacco plants grown in glasshouses under tropical conditions, at the exact time of day at which blue-light intensity is highest (Antunes *et al.*, 2017). No differences in starch content were observed in isolated guard cells illuminated by an incandescent lamp (Daloso *et al.*, 2015), which has a negligible amount of blue light (Sager & Mc Farlane, 1997). Taken together, these results indicate that light-induced starch degradation in guard cells may be a mechanism triggered only by blue light (Talbott & Zeiger, 1993; Lasceve *et al.*, 1997; Horrer *et al.*, 2016). However, what still remains unclear is the fate of the C released from starch degradation.

2. Guard cell starch synthesis as sink of C during stomatal closure

A recent work has provided indication that starch synthesis plays an essential role in CO_2-induced stomatal closure (Azoulay-Shemer *et al.*, 2016), presumably acting as a sink for C coming from malate degradation in the cytosol during guard cell osmotic adjustment (Dittrich & Raschke, 1977; Schnabl, 1980; Santelia & Lunn, 2017). In this scenario, during CO_2-induced stomatal closure upon illumination, malate would be degraded via gluconeogenesis and imported to guard cell chloroplasts as triose-phosphates (triose-P) via triose-phosphate/phosphate translocator (TPT). In the chloroplast, triose-P would enter the Calvin–Benson Cycle (CBC) to provide, together with cETC, substrates, energy and the reducing power required for ADP-glucose pyrophosphorylase (AGPase) activation and starch synthesis (Fig. 3). Nevertheless, the diel time course of guard cell starch content reveals that starch synthesis begins 1 h after dawn, is maintained through the day and, most interestingly, is continuous for up to 3 h into the night (Horrer *et al.*, 2016). In the absence of light, plastidial fructose-1,6-biphosphatase (pFBPase) and AGPase are not activated by the ferredoxin-thioredoxin reductase system, which uses electrons derived from cETC. Hence, triose-P cannot be converted to hexose-P in the chloroplast and further to ADP-Glc for starch synthesis. Thus, how starch synthesis occurs in the absence of light in guard cells and whether it occurs to support stomatal closure at night remains unclear.

Early evidence has shown that the TPT in the membrane of guard cell chloroplasts of *P. sativum* has an additional affinity for glucose-6-phosphate (Glc-6-P) that is as high as for 3-phosphoglycerate (3-PGA), similarly to the observed in leaves of CAM plants and in nongreen plastids (Fischer & Weber, 2002) and in contrast to mesophyll cells in which the affinity of this translocator is restricted to 3-PGA, dihydroxyacetone phosphate (DHA-P) and inorganic phosphate (Pi) (Overlach *et al.*, 1993). That said, it is reasonable to hypothesize that the import of Glc-6-P from cytosol into guard cell chloroplasts may directly feed the cross point that bypasses the reaction catalyzed by the plastidial glucose-6-phosphate dehydrogenase (pG6PDH) in the oxidative pentose phosphate pathway (OPPP) and the FBPase-independent starch

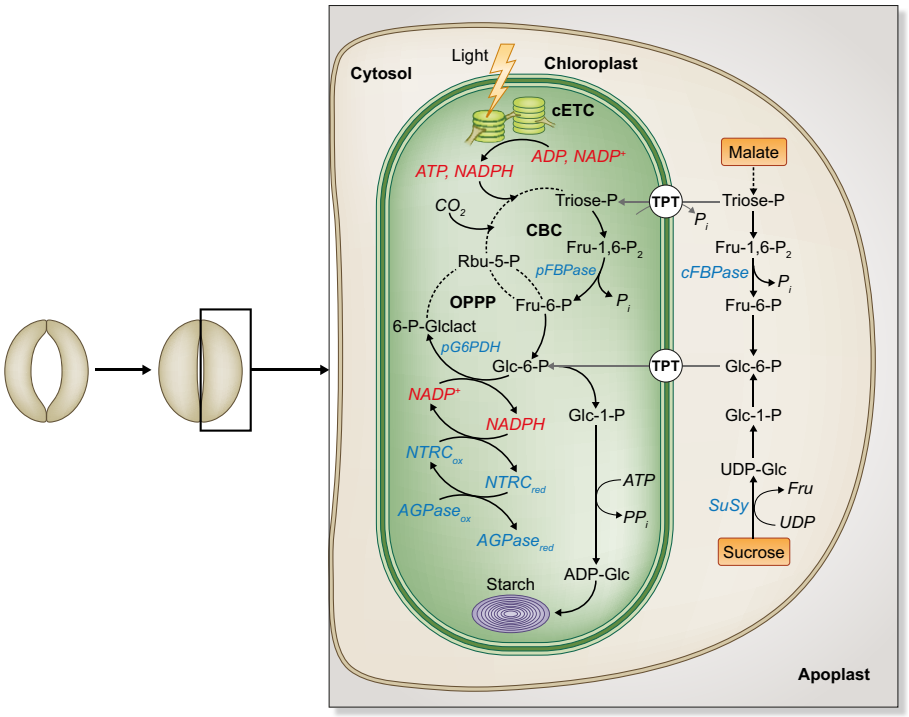

Fig. 3 Proposed pathways for starch synthesis during stomatal closure. It has been shown that starch synthesis in guard cell chloroplasts is an important mechanism during stomatal closure as a sink for C coming from malate, and possibly from sucrose, degradation in the cytosol (Outlaw & Manchester, 1979; Schnabl, 1980; Azoulay-Shemer *et al.*, 2016). Moreover, in contrast to mesophyll cells, guard cells have been implicated in the synthesis of starch, also in the dark (Horrer *et al.*, 2016). The proposed pathway described here provides a hypothesis by which starch could be synthesized under these conditions. During high CO_2-induced stomatal closure upon light, the vacuolar accumulated malate and the apoplast imported sucrose are converted mainly to triose-P (3-PGA and DHA-P) in the cytosol and transferred to the chloroplast in the exchange of P_i via triose-phosphate/phosphate translocator (TPT). In the chloroplast, triose-P enters the CBC to provide, together with cETC, substrates, energy and the reducing power required for AGPase activation and starch synthesis. By contrast, during dark-induced stomatal closure, the CBC and pFBPase are deactivated. Thus, the C required for starch synthesis is imported as Glc-6-P by TPT (Overlach *et al.*, 1993). It is noteworthy that Glc-6-P can also be imported by Glc-6-P/phosphate transporters (GPT) in nongreen tissues (Kammerer *et al.*, 1998); however, whether this mechanism also occurs in guard cells remains unclear. Beyond starch synthesis, Glc-6-P is also used as a substrate of pG6PDH in the OPPP, which is redox-activated in the dark (Née *et al.*, 2009), and it catalyzes a reaction that is the source of the NADPH required by the NTRC-mediated AGPase activation, a key point for starch synthesis (Michalska *et al.*, 2009). 3-PGA, 3-phosphoglycerate; 6-P-Glclact, 6-phosphogluconolactone; ADP-Glc, ADP-glucose; AGPase, ADP-Glucose Pyrophosphorylase; CBC, Calvin-Benson Cycle; cETC, chloroplastic electron transport chain; Chlorop, chloroplast; DHA-P, dihydroxyacetone phosphate; FBPase, fructose-1,6-biphosphatase; Fru, fructose; Fru-6-P, fructose-6-phosphate; Fru-2,6-P_2, fructose-2,6-biphosphate; Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Mal, malate; NTRC, NADPH-dependent thioredoxin reductase C; OPPP, oxidative pentose phosphate pathway; Pi, inorganic phosphate; Rbu-5-P, ribulose-5-phosphate; Suc, sucrose; SuSy, sucrose synthase; Triose-P, triose-phosphate; UDP-Glc, UDP-glucose. Enzymes are shown in blue. The redox state of both NTRC and AGPase enzymes is identified as reduced ($_{red}$) or oxidized ($_{ox}$). FBPase isoforms are identified as plastidial (pFBPase) and cytosolic (cFBPase). Dashed arrows indicate steps involving several reactions.

synthesis pathway in the dark (Fig. 3). This idea is further supported by the fact that pG6PDH is redox-activated in the dark (Née *et al.*, 2009), which provides the NADPH required for AGPase activation via plastid-localized NADPH-dependent thioredoxin reductase C (NTRC) (Michalska *et al.*, 2009; Lepistö *et al.*, 2013; Geigenberger *et al.*, 2017) (Fig. 3). Therefore, it seems likely that the substrate for guard cell starch synthesis is derived mainly from the import of triose-P from cytosol in the light and from the import of Glc-6-P in the dark and that the NADPH required for the NTRC-mediated AGPase-redox activation is provided by cETC in the light and by OPPP in the dark (Fig. 3). In this context, fructose-2,6-biphosphate (Fru-2,6-P_2), a well-established regulatory metabolite found in all eukaryotes (Nielsen *et al.*, 2004), would have a key role in guard cell cytosol, by providing rapid control of the cytosolic FBPase which will determine the fate of C during stomatal movements, stimulating glycolysis during stomatal opening (Hedrich *et al.*, 1985; Daloso *et al.*, 2015, 2016b) and gluconeogenesis during stomatal closure (Dittrich & Raschke, 1977; Van Kirk & Raschke, 1978; Schnabl, 1980; Penfield *et al.*, 2012). However, experimental evidence to support this theory is still missing. Moreover, the pathways and the exact regulatory mechanisms underlying guard cell starch metabolism, in particular the pathways involved in the rapid starch degradation observed upon light and the extension of starch synthesis after dusk, are yet to be fully elucidated.

V. Connectors between mesophyll and guard cells

1. Role of mesophyll-derived malate on stomatal movements

Malate has long been suggested as key regulator mechanistically involved in the guard cell response to environmental stimuli (Fernie & Martinoia, 2009). During stomatal opening, the accumulation

of malate inside the guard cells occur mainly based on its influx via a specific transporter at the plasma membrane or as a product of the starch breakdown or anaplerotic CO_2 fixation (Fig. 2) (Talbott & Zeiger, 1993; Lee *et al.*, 2008; Daloso *et al.*, 2015). On the other hand malate acts as an osmoregulator and counter ion for K^+ in guard cells allowing the entrance of water, and ultimately, stomatal opening. Moreover, it has been suggested that malate can act as a signalling metabolite during stomatal opening, because cytosolic malate accumulation increases currents through Aluminium-activated malate transporter 9 (ALMT 9) (Fig. 2) (De Angeli *et al.*, 2013). On the other hand, during stomatal closure, previously accumulated malate can be metabolized, partially converted into starch or released into the apoplastic space (Fig. 2) (Van Kirk & Raschke, 1978; Penfield *et al.*, 2012).

Over the last decade, three protein families have been identified and functionally characterized to be involved in the transport of malate at the guard cell plasma membrane and tonoplast (Emmerlich *et al.*, 2003; Lee *et al.*, 2008; Meyer *et al.*, 2010, 2011; Sasaki *et al.*, 2010). The transporter *At*ABCB14, a member of the ABC (*A*TP *B*inding *C*assette) family, is responsible for the transport of malate from the apoplast into guard cells (Fig. 4) (Lee *et al.*, 2008). This transporter was described as a negative modulator of stomatal closure induced by high CO_2 concentrations, supporting the assumption that malate acts as a CO_2 response regulator (Fig. 4). Additionally, a member of the ALMT family, *At*QUAC1 (Quick activating Anion Channel 1), was identified and characterized as an ABA-dependent anion-selective channel responsible for the efflux of malate from guard cells to the apoplast during the stomatal closing in a voltage-dependent manner (Meyer *et al.*, 2010; Sasaki *et al.*, 2010; Mumm *et al.*, 2013). It was shown that the SnRK2.6 kinase OST1 (Open Stomata 1) besides *At*SLAC1 (Slow anion channel 1) can also phosphorylate *At*QUAC1 (Fig. 4), providing the link between ABA signalling and transport of malate during stomatal closure (Imes *et al.*, 2013). Furthermore, the functional lack of *At*QUAC1 resulted in an impaired stomatal closing kinetic in response to dark and high CO_2 concentrations, reduced ABA-induced stomatal closure and changes in organic acid accumulation, as well as increases in both stomatal and mesophyll conductance (Meyer *et al.*, 2010; Medeiros *et al.*, 2016).

The vacuolar transport of malate also has been characterized and the transporter *t*onoplast *D*icarboxylate *T*ransporter (*At*tDT) was proposed to be the main transport system at the tonoplast being required for the proper accumulation of malate in Arabidopsis leaves (Emmerlich *et al.*, 2003; Hurth *et al.*, 2005), although its functional role in guard cells during stomatal movements is still unclear. Another ALMT member, *At*ALMT6, is involved in vacuolar malate transport being expressed at the vacuolar membrane of guard cells and regulated by cytosolic pH and malate concentrations (Meyer *et al.*, 2011). These two cellular features mediate the tonoplast potential and thus determine the functionality of *At*ALMT6, as a malate influx or efflux channel. Intriguingly, despite *atalmt6* knockout plants displaying reduced malate current in isolated vacuoles when compared to WT, no differences in stomatal movements were observed (Meyer *et al.*, 2011), suggesting the possible redundancy in the malate transport at the tonoplast.

Further evidence supporting the involvement of organic acid metabolism in leaves was demonstrated by increased stomatal conductance and photosynthesis mediated by the effect of organic acids on stomata in tomato (*Solanum lycopersicum*) plants with constitutively reduced expression of *SlSDH2-2*, which encodes the iron-sulphur subunit of succinate dehydrogenase (Araújo *et al.*, 2011). No effects were observed when the antisense construction for *SlSDH2-2* was expressed under the control of the guard cell specific *MYB60* promoter. By contrast, the constitutive inhibition of the mitochondrial fumarase in tomato plants decreased photosynthesis as a result of impaired stomatal function (Nunes-Nesi *et al.*, 2007). These studies indicate that organic acid leaf metabolism can modulate stomatal function. Additionally, it has been shown that apoplastic malate concentrations rise on average from 1.0 to 3.1 mM in response to high CO_2 concentrations, and that this is able to activate R-type anion channels at the plasma membrane (Hedrich & Marten, 1993; Hedrich *et al.*, 1994). These findings associated with those obtained for *ABCBB14* and *AtQUAC1* provide strong evidence that the apoplastic concentration of malate can drive stomatal movement. It therefore seems reasonable to propose these organic acids as signal metabolites during high CO_2-induced stomatal closure (Fig. 4).

2. Can mesophyll-produced sucrose coordinate the trade-off between photosynthesis and stomatal movements?

Guard cells have all the genes required to produce sucrose via photosynthesis, gluconeogenesis and starch breakdown. However, guard cells possess several characteristics of sink cells such as low photosynthetic rates (Gotow *et al.*, 1988), high and low activity of enzymes of catabolic and anabolic pathways, respectively (Hampp *et al.*, 1982; Vani & Raghavendra, 1994), high activity of sucrose synthase (Table 1) (Hite *et al.*, 1993; Daloso *et al.*, 2015), and high expression of sucrose synthase 3 and different hexose transporters (Stadler *et al.*, 2003; Leonhardt *et al.*, 2004; Yang *et al.*, 2008; Wang *et al.*, 2011; Bates *et al.*, 2012; Bauer *et al.*, 2013a). This suggests that guard cells import sucrose from mesophyll cells. In fact, the accumulation of mesophyll-derived sucrose in the guard cell apoplastic space or its dissimilation within the guard cell have been proposed as mechanisms for the induction of stomatal closure in phloem loader species (Lu *et al.*, 1995; Outlaw & De Vlieghere-He, 2001; Kang *et al.*, 2007; Kelly *et al.*, 2013; Lugassi *et al.*, 2015). However, recent evidence suggests that the dissimilation of sucrose within guard cells can induce stomatal opening or closure, depending on the growth conditions (Lugassi *et al.*, 2015). Thus, it seems likely that the differential accumulation of sucrose in mesophyll cells, apoplast and cytosol of guard cells is an important point that interconnects the processes of photosynthesis and stomatal opening. This hypothesis was further supported by a recent multi-species meta-analysis which revealed that the leaf sucrose content is positively and negatively correlated with A and g_s, respectively (Gago *et al.*, 2016), suggesting that the A–g_s trade-off maybe regulated, at least partially, by the amount of sucrose. This idea relies on the fact that, in periods of high A, sucrose may be a

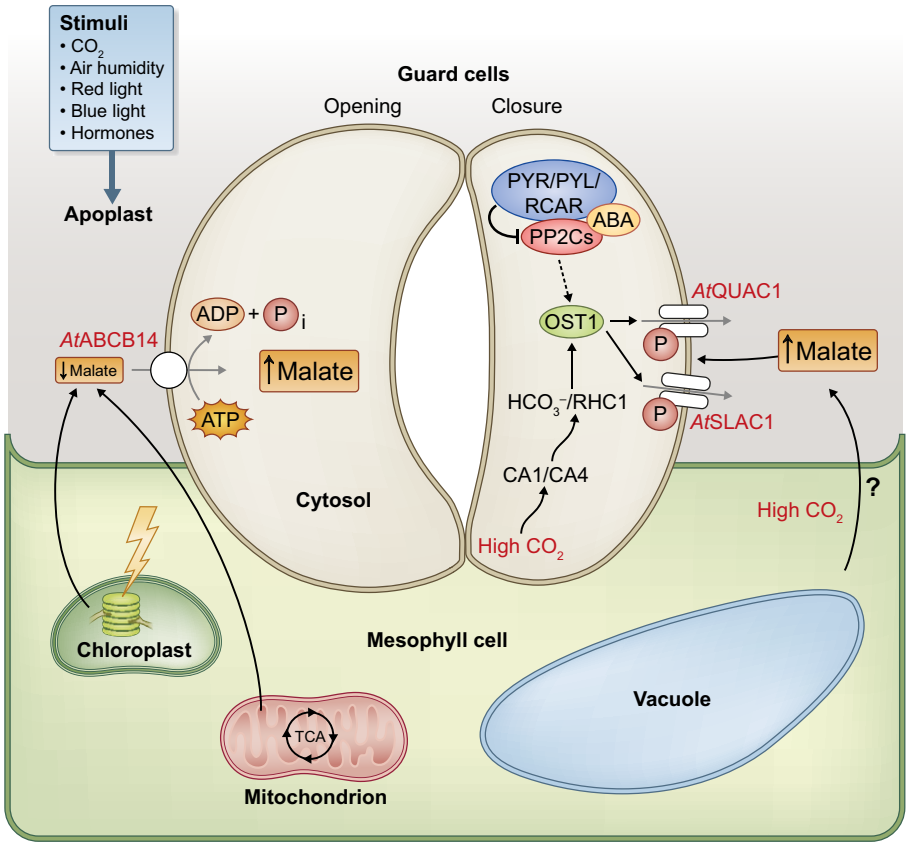

Fig. 4 Schematic representation of the role of malate connecting mesophyll and guard cells during stomatal opening (left panel) and closure (right panel). Mesophyll and guard cells are able to regulate their functioning and control the gain of CO_2 and the water loss by stomata in response to internal and environmental stimuli such as CO_2, air humidity, light quality, hormones, etc. During the stomatal opening, malate can be produced within guard cells and/or arrive from mesophyll cells and enters in guard cells through malate transporters such as *At*ABCB14. Malate is additionally transported into vacuoles, contributing to guard cell osmolality and water intake, and finally the stomatal opening (for details see Fig. 2). It has also been proposed that apoplastic malate concentrations can act as a signal and link between guard cell regulation and the mesophyll photosynthetic capacity in response to increased external CO_2 concentrations by activation of the R-type anion channels at guard cell plasma membrane, leading to stomatal closure. Recently, OST1 was shown to be able to phosphorylate both *At*QUAC1 and *At*SLAC1. Furthermore, OST1 is suggested as a convergence point for CO_2 and abscisic acid (ABA) signalling pathways also linking them to the malate transport during stomatal closure (Tian *et al.*, 2015). The proposed model involves the intracellular [HCO_3^-] driven by carbonic anhydrases CA1 and CA4 regulating the CO_2-induced stomatal responses, by activating the anion channels at plasma membrane required for stomatal closure (Hu *et al.*, 2010). Additional components of this signalling pathway includes different kinases such as HT1, GHR1 and MAPK kinases (Hashimoto *et al.*, 2006; Marten *et al.*, 2008; Hõrak *et al.*, 2016). For details regarding the guard cell signalling pathway during high-CO_2 induced stomatal closure, see Engineer *et al.* (2016). ABCB14, ATP binding cassette 14; CA1/CA4, carbonic anhydrases 1 and 4; GHR1, guard cell hydrogen peroxide-resistant 1; HCO_3^-, bicarbonate; HT1, high leaf temperature 1; Mal, malate; MAPK, mitogen-activated protein kinases; OST1, SNF1-related protein kinase 2.6 (SRK2E/SnRK2.6/OST1); PYR/PYL/RCAR, pyrabactin resistance/PYR-like/regulatory components of abscisic acid receptor; PP2Cs, protein phosphatases 2C; QUAC1, quick activating anion channel 1; RHC1, resistant to high CO_2; SLAC1, slow anion channel 1; TCA, tricarboxylic acid cycle.

signal from mesophyll cells to close the stomatal pore, as a mechanism to decrease transpiration in a nonlimiting C condition, which leads to increases in WUE. Taken together, these results indicate that sucrose has a pivotal role in mediating stomatal aperture, most probably via the mesophyll photosynthetic rate.

Between the genes of sucrose metabolism with great biotechnological potential, it has been shown that genes related to sucrose breakdown have great impact on stomatal movements (Antunes *et al.*, 2012; Kelly *et al.*, 2013; Lugassi *et al.*, 2015; Daloso *et al.*, 2016b). Furthermore, it is reasonable to assume that manipulation of sucrose transport through guard cell plasma membrane may impact on stomatal aperture (Stadler *et al.*, 2003; Antunes *et al.*, 2017). In this vein, there is no evidence to date showing whether guard cells have the capacity to export sucrose. Given the sink characteristics found in guard cells, it seems unlikely that sucrose would be exported from guard cells. However, it is important to note that different members of the *SUGARS WILL EVENTUALLY BE EXPORTED* (*SWEET*) family, *SWEET1, SWEET4, SWEET5, SWEET11* and *SWEET12*, are highly expressed in *Arabidopsis thaliana* guard cells compared to mesophyll cells (Mustroph *et al.*, 2009; Bauer *et al.*, 2013a,b). Although less characterized, it is known that SWEET proteins 1, 4 and 5 encode glucose transporters and were previously demonstrated to be expressed mainly in nonphotosynthetic tissues (Chen *et al.*, 2015). In leaves, SWEET11 and SWEET12 proteins are located at the plasma membrane and are responsible for the efflux of sucrose from the phloem parenchyma to the apoplastic space. However, it is noteworthy that these proteins as well as SWEET1 can also uptake

sucrose when expressed in *Xenopus* oocytes (Chen *et al.*, 2012b). Thus, we cannot exclude that the high expression of *SWEET1*, *SWEET11* and *SWEET12* in guard cells could be associated with the uptake of glucose and sucrose.

It seems likely that the balance between influx and efflux of sucrose to and from guard cells through SWEET and other sugar transporters can be a crucial point for the regulation of stomatal aperture. In fact, recent results from a study using transgenic plants containing an antisense construct against *SUCROSE TRANSPORTER 1* (SUT1, also called SUC2) specifically in guard cells showed that the reduction in the influx of sucrose to guard cells leads to altered stomatal dynamics and perturbation of K^+ accumulation and carbohydrate metabolism in guard cells (Antunes *et al.*, 2017). However, experimental evidence to support the idea that SWEET proteins are important to guard cell regulation and information pertaining to the general regulation of sucrose transport across guard cell membranes is currently lacking.

In summary, sucrose has been pointed out as a key metabolite that connects A and g_s, and consequently WUE. Manipulation of guard cell sucrose metabolism has high impact on stomatal movements, with great potential in obtaining plants with improved WUE (Kelly *et al.*, 2013; Antunes *et al.*, 2017). Although the mechanisms by which sucrose regulates stomatal aperture are unclear, it seems that sucrose may have a dual role in guard cells. During stomatal opening, the sucrose imported from mesophyll cells and that produced within guard cells may be broken down to sustain glycolysis and mitochondrial metabolism; however, only a missing isotope labelling experiment using ^{13}C-labelled sucrose can prove that this mechanism occurs during light-induced stomatal opening. By contrast, in periods of high A, the excess of sucrose derived from mesophyll cells would induce stomatal closure by two mechanisms; via an osmotic effect at the apoplast (Lu *et al.*, 1995, 1997; Kang *et al.*, 2007) and by activating the ABA signalling pathway within guard cells via hexokinase (Kelly *et al.*, 2013).

VI. Challenges and perspectives in understanding and modelling guard cell metabolism

It has been shown that mesophyll and guard cell protoplasts exhibit great differences in enzyme activities (Table 1) and in their metabolite profiles (Misra *et al.*, 2015). However, our knowledge of how the photosynthetic fluxes are distributed within guard cell metabolism remains rather fragmentary. Recent evidence from a study in tobacco guard cells combining ^{13}C-$NaHCO_3^-$ kinetic isotope labelling experiments and GC-MS analysis indicated that the ^{13}C redistribution of isolated guard cells is highly variant from that obtained from intact *A. thaliana* rosettes, which is determined predominantly by mesophyll cells (Szecowka *et al.*, 2013; Daloso *et al.*, 2015). The percentage of ^{13}C and the relative ^{13}C incorporated into sucrose and organic acids after 1 h of labelling differs substantially between mesophyll cells and guard cells (Table 2). Interestingly, whilst mesophyll cells have a higher percentage of ^{13}C incorporated into sucrose and malate, the relative ^{13}C incorporation analysed through organic acids-to-sucrose ratio is higher in guard cells (Table 2). Although these studies were performed using different approaches and even different ^{13}C-substrates ($^{13}CO_2$ for whole rosettes and ^{13}C-$NaHCO_3$ for guard cells), it is interesting to highlight that the data from the guard cell experiment suggest that the C fixed in guard cells is preferentially incorporated into organic acids rather than in sucrose, a product of CO_2 fixation mediated by RubisCO. These data are similar to what is observed in C_4 cell types and support previous ^{14}C-radiolabelled experiments carried out in isolated guard cell-enriched samples (Willmer & Dittrich, 1974; Schnabl, 1980; Brown & Outlaw, 1982). These combined observations suggest that guard cells do indeed display similarities to C_4 cells and that it needs to be taken into considered when modelling guard cell metabolism (Medeiros *et al.*, 2015). In this vein, the main challenges are to create experimental and mathematical approaches that allow a simultaneous comparison of the fluxes between mesophyll and guard cells.

Beyond the differences in the metabolic ^{13}C distribution observed in mesophyll and guard cells, there are many controversies regarding the extent by which guard cells are influenced by the surrounding mesophyll cells and further studies are clearly required to better understand the connections between both cell types. The establishment of a protocol to simultaneously isolate both mesophyll and guard cells from frozen leaves is desperately needed. Such an approach would allow the simultaneous analysis of both cells with no need of previous isolation or microdissection procedures. This protocol, together with the recently published methodologies to analyse metabolic fluxes in whole *A. thaliana* plants (Szecowka *et al.*, 2013), would enable quantitative investigation of the fate of mesophyll photosynthetic metabolites in guard cell metabolism. Given that guard cell metabolism harbours great potential for photosynthesis and WUE improvement (Flexas, 2016), it seems likely that all research effort directed towards improving our understanding of guard cell metabolism will help to enhance crop production marginal land. It is our contention that to fully explore and understand this fascinating system will ultimately require the development of better mechanisms for single cell analysis, as well as an increasing reliance on metabolic modelling. That said, as we demonstrate in this review, our understanding of

Table 2 Total ^{13}C-enrichment in sucrose and organic acids in whole-plant *Arabidopsis thaliana* (leaves) and isolated guard cells submitted to 60 min under $^{13}CO_2$ and ^{13}C-$NaHCO_3^-$, respectively

	Total ^{13}C enrichment	
Metabolite	Leaves[1]	Guard cells[2]
Sucrose	45	14
Malate	42	24
Fumarate	8	22
Succinate	0	7
Malate/Sucrose	0.9	1.7
Fumarate/Sucrose	0.17	1.6
Succinate/Sucrose	0	0.5

Although these values were obtained from studies using different methodologies and substrates, it is important to highlight that the comparison is made within the cell type.
[1]Data from Szecowka *et al.* (2013); [2]data from Daloso *et al.* (2015).

the interface between guard and mesophyll metabolism, their interaction and their individual function is becoming progressively richer.

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