Tansley review

Mesophyll photosynthesis and guard cell metabolism impacts on stomatal behaviour

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Summary

Stomata control gaseous fluxes between the internal leaf air spaces and the external atmosphere. Guard cells determine stomatal aperture and must operate to ensure an appropriate balance between CO_2 uptake for photosynthesis (A) and water loss, and ultimately plant water use efficiency (WUE). A strong correlation between A and stomatal conductance (g_s) is well documented and often observed, but the underlying mechanisms, possible signals and metabolites that promote this relationship are currently unknown. In this review we evaluate the current literature on mesophyll-driven signals that may coordinate stomatal behaviour with mesophyll carbon assimilation. We explore a possible role of various metabolites including sucrose and malate (from several potential sources; including guard cell photosynthesis) and new evidence that improvements in WUE have been made by manipulating sucrose metabolism within the guard cells. Finally we discuss the new tools and techniques available for potentially manipulating cell-specific metabolism, including guard and mesophyll cells, in order to elucidate mesophyll-derived signals that coordinate mesophyll CO_2 demands with stomatal behaviour, in order to provide a mechanistic understanding of these processes as this may identify potential targets for manipulations in order to improve plant WUE and crop yield.

I. Introduction

Global food security is currently the greatest challenge facing plant scientists. With an increasing global population predicted to stabilize at *c.* 9 billion by the year 2050 (Cohen, 2003; Godfray *et al.*, 2010) political and scientific pressure is mounting to improve crop yield for future sustainable food and fuel production. Abiotic and biotic stresses cause considerable losses of crop yield

(Chrispeels & Sadava, 2003) and therefore crop improvements need to include the ability to cope with such stresses, particularly those associated with a predicted rapidly changing climate. Water availability is a major constraint of crop yield (Araus *et al.*, 2002; Chaves, 2002) and is often considered the single most important factor limiting food production, leading to yield losses for example of 40% in maize (Boyer, 1982; Harrison *et al.*, 2014). Currently agriculture accounts for 70–90% of all freshwater use (Morison *et al.*, 2008), and with average predictions for climate change including a mean annual temperature increase of between 1 and 3°C (depending on location and prediction scenario, IPCC, 2007) along with precipitation and evapotranspiration changes (IPCC, 2007), making agriculture sustainable will require a major reduction in crop water use in many areas (Morison *et al.*, 2008).

The major pathway for water loss from plants is through the stomatal pores that are found on the surfaces of the majority of the aerial parts of plants. These pores control the fluxes of gases between the outside atmosphere and the leaf interior, and therefore ultimately control the amount of CO_2 uptake by the leaf for photosynthesis (A) and, consequently, the amount of water lost by leaves through transpiration (E). At the leaf level, the ratio of CO_2 uptake to water loss (A/E) determines plant water use efficiency (WUE). Stomata must operate to ensure an appropriate balance between CO_2 uptake for Calvin cycle activity and autotrophic production of organic compounds with the plant's need for water to remain fully hydrated, and there is often a close correlation observed between photosynthetic rates and stomatal conductance (Wong *et al.*, 1979). Although the majority of water taken up by a plant (*c.* 97%) is not used in the biochemical reactions (Taiz & Zeiger, 1998), in order for a plant to expand and grow its cells must remain fully turgid (Schopfer, 2006). Any reduction in cell water volume and turgor pressure (and water potential) immediately decreases cell expansion and plant growth (Thompson, 2005). Stomata have long been considered a potential target for manipulation (Cowan & Troughton, 1971). However, the majority of improvements in WUE involving stomata to date have tended to reduce conductance (and water loss) at the expense of carbon gain (Lawson & Blatt, 2014) and therefore, although calculations of intrinsic water use efficiency (*I*WUE) may appear relatively high numerically, values of assimilation are low, potentially reducing productivity. Owing to our need to produce increased quantities of food and fuel, such traits are not entirely desirable. Although there is generally a close correlation between mesophyll photosynthetic rates and stomatal conductance over the long term, short-term perturbations in the environment (e.g. irradiance) often lead to temporal and spatial disconnections between stomatal conductance (g_s) and A (Kirschbaum *et al.*, 1988; Tinoco-Ojanguren & Pearcy, 1993; Lawson & Weyers, 1999; Lawson *et al.*, 2010). It would therefore be entirely plausible to hypothesise that improvements in the coordination and synchrony of stomatal responses and mesophyll photosynthetic rates with the dynamic environmental growth conditions could improve plant WUE over the long term (Lawson *et al.*, 2010, 2012; Lawson & Blatt, 2014).

Stomata respond directly to environmental stimuli. In response to changes in leaf external and internal environmental conditions, the guard cells that surround the stomatal pore adjust their volume resulting in adjustments of the pore aperture and, therefore, stomatal conductance to gas fluxes. It is generally well accepted that stomata open in response to increases in irradiance (with the exception of stomata in CAM plants), and low CO_2 concentrations within the intercellular air space (C_i) and close in darkness, high vapour pressure deficits (VPDs) and high CO_2 concentrations (Assmann, 1999; Outlaw, 2003) in order to balance the mesophyll demands for CO_2 against the need to maintain leaf water content. However, the natural growth environment for all plants is highly dynamic with changes in environmental stimuli on a variety of timescales. For example light can fluctuate on a timescale of seconds to minutes. Therefore, stomata perceive and respond to multiple signals simultaneously often in a hierarchical manner (Lawson *et al.*, 2010; Lawson & Blatt, 2014). Changes in external conditions affect photosynthetic carbon assimilation either directly (e.g. changing intensities of irradiance) or indirectly through the resulting impact on stomatal behaviour (e.g. VPD). A coordinated response of both stomata and mesophyll photosynthesis to changing stimuli helps the plant to maintain WUE (Lee & Bowling, 1995; Mott *et al.*, 2008) and results in the commonly observed correlation between stomatal conductance and photosynthesis (Wong *et al.*, 1979; Buckley *et al.*, 2003). However, the underlying mechanisms and signal that promote this relationship are currently unknown (Lawson *et al.*, 2010). The coordination between photosynthetic carbon gain and stomatal behaviour is key to determining plant WUE and productivity, as short leaf-level improvements in the ratio of carbon gain relative to water loss accumulate over a season and ultimately determine the amount of dry matter produced. As mentioned earlier, due to the fundamental role of stomata, manipulation of stomatal traits has been identified as a potential area for WUE improvements (Condon *et al.*, 1987; Fischer *et al.*, 1998; Masle *et al.*, 2005; Doheny-Adams *et al.*, 2012). However, mutations that increase WUE can do so at the expensive of carbon assimilation, reflecting the trade-off in CO_2 availability with reduction in stomatal water loss. For example, reduced stomatal conductance in Arabidopsis mutants with a loss of function in the vesicle trafficking protein SYP121 led to greater WUE, but only with reduced CO_2 assimilation which impaired growth (Eisenach *et al.*, 2012). Likewise, Antunes *et al.* (2012) showed that reductions of sucrose synthase 3 (SuSy3) in *Solanum tuberosum* led to an increase in WUE, with decreased g_s but this lowered g_s restricted CO_2 assimilation rate. Conversely, alterations in stomatal behaviour that increase photosynthesis can do so at the expense of water loss. This was demonstrated in the recent study by Tanaka *et al.* (2013), who showed that increased stomatal density (via manipulation of STOMAGEN) increased assimilation rates by 30%, whereas transpiration rates were increased by a greater amount (50%) leading to a 50% reduction in WUE.

Additionally, Antunes *et al.* (2012) in the same study mentioned above increased expression of SUC2 in guard cells specifically, and found enhanced g_s and assimilation rates but only with a parallel reduction in WUE. However, this is not always the case and several studies have demonstrated increased WUE and/or assimilation rate without compromising mesophyll CO_2 uptake. Laporte *et al.*

(2002) demonstrated that decreased expression of NADP-Malic enzyme decreased g_s, and although photosynthesis was not evaluated in this study, no effects on growth were observed. Yoo *et al.* (2010) illustrated high WUE, and a 25% reduction in transpiration rate and g_s with no effect on CO_2 assimilation rate in Arabidopsis loss of function GT-2 LIKE1 (GTL1) mutants. These studies illustrate the importance of considering the relationship between mesophyll CO_2 demands and stomatal behaviour when attempting to manipulate stomatal behaviour in order to improve WUE, and the importance of elucidating the mechanisms that coordinate mesophyll CO_2 demand with stomatal function. They also highlight the potential for improving WUE through manipulation of stomata. An excellent example of manipulations in stomatal metabolism to improve WUE has recently been published by Wang *et al.* (2014): this illustrated enhanced light-induced stomatal opening rates, greater photosynthesis and improved growth rates in Arabidopsis plants over expressing H^+-ATPase under the control of a guard cell-specific promoter. What is truly exciting about this work is that not only have these authors shown improved photosynthesis and growth through manipulation of guard cell metabolism, but also they have demonstrated that such results are possible by altering the functional dynamic responses of stomata to improve photosynthesis and WUE, an area that to date has received little attention, but could provide future targets for manipulation (see Lawson & Blatt, 2014).

An earlier review by Lawson & Blatt (2014) examined the size, speed and responsiveness of stomata and highlighted the potential impacts on WUE. Although this review mentioned the importance of coordinated stomatal responses with mesophyll demands for CO_2 and provided examples of limitation to *A* and WUE caused by nonsynchronized behaviour, the potential mechanisms that coordinate mesophyll behaviour with stomatal function were not covered. Therefore, the aims of this review are to first provide a synopsis of the coordination of stomatal responses with mesophyll function and then explore the evidence put forward for a mesophyll-driven signal that couples these responses, including the evidence for a role of sucrose and malate in coordinating photosynthesis with stomatal conductance. In doing this we will examine the origins of sucrose (including guard cell photosynthesis) and the impact on guard cell function of manipulating sucrose metabolism. In the last section we provide an overview of the potential for exploring genetic approaches to manipulate guard and mesophyll photosynthesis and how this may help elucidate routes to improving plant water use efficiency.

II. Coordination between mesophyll and stomata

The close correlation between g_s and *A* (Wong *et al.*, 1979; Farquhar & Wong, 1984; Mansfield *et al.*, 1990; Buckley *et al.*, 2003) has often been observed over a range of CO_2 concentrations and light intensities (Radin *et al.*, 1988; Hetherington & Woodward, 2003), and it was originally proposed that the concentration of CO_2 inside the leaf (C_i) helped maintain the coordination of the mesophyll photosynthesis with stomatal aperture (Fig. 1). C_i is determined not only by stomatal aperture and the flux of gas from the bulk atmosphere into the leaf, but also by the consumption of

CO_2 through mesophyll photosynthesis. Light induces photosynthetic consumption of internal CO_2 (C_i) which opens stomata. It is therefore an attractive hypothesis that C_i coordinates photosynthetic responses and mesophyll demand for CO_2 with stomatal conductance. However, several studies have suggested that stomatal responses to CO_2 (incl. C_i) are too small and therefore insufficient to account for the relatively large changes in g_s that have been observed in response to light (Raschke, 1975; Farquhar *et al.*, 1978; Sharkey & Raschke, 1981b; Farquhar & Sharkey, 1982; Morison & Jarvis, 1983; Ramos & Hall, 1983; Mott, 1988). This evidence, along with the observations that stomata respond to light and CO_2 in epidermal peels, led to the suggestion of direct perception and signal transduction in the guard cells themselves (Mott, 2009). However, there is no consensus within the literature, with often different stomatal responses to light and CO_2 concentration reported for intact leaves and for isolated epidermis. Additionally, differences in magnitude and speed of change in stomatal conductance have been observed in response to identical stimuli in the same species, but in different laboratories. For these reasons the underlying mechanisms and signals that coordinate and promote the close relationship between photosynthesis (*A*) and g_s have not been unequivocally established.

Most traditional stomatal literature assumes that stomatal responses to light and CO_2 primarily arise in the guard cells and that the mesophyll has little or no effect (Mott *et al.*, 2008); any mesophyll influence is driven only by the consumption of CO_2 and the resultant impact on C_i. It is now well established that stomatal opening responses to light have at least two components: the blue and the red light responses. The specific blue light response is independent of photosynthesis, saturating at a low fluence rate (Zeiger *et al.*, 2002) and involves the activation of a plasma membrane H^+-ATPase in the guard cells (Kinoshita & Shimazaki, 1999; Shimazaki *et al.*, 2007). The red light response, or photosynthesis-mediated response, is saturated at similar light intensities to that of mesophyll photosynthesis and is abolished by inhibitors of photosynthetic electron transport such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Kuiper, 1964; Sharkey & Raschke, 1981a; Tominaga *et al.*, 2001; Olsen & Junttila, 2002; Messinger *et al.*, 2006). It is this second response, linked with assimilation rate, that has often been assumed to be driven entirely by mesophyll consumption of CO_2 leading to a reduction in C_i (Mott, 1988; Roelfsema *et al.*, 2002). Early literature is full of examples demonstrating that stomatal aperture adjusts to maintain a constant $C_i : C_a$ ratio (see Mott, 1988), C_i being *c.* ⅔ atmospheric CO_2 concentration (Ball & Berry, 1982). However, as mentioned above, there are also many reports that argue against a C_i driven coordination of *A* and g_s, including those examples demonstrating that changes in C_i are too small to account for the observed stomatal responses to light. Indeed, more recent studies show a stomatal response to light even when C_i is held constant (Messinger *et al.*, 2006; Lawson *et al.*, 2008; Wang & Song, 2008).

III. The mesophyll signal

It has been suggested that guard cell responses are linked to a product of photosynthetic activity in the mesophyll, via a diffusible
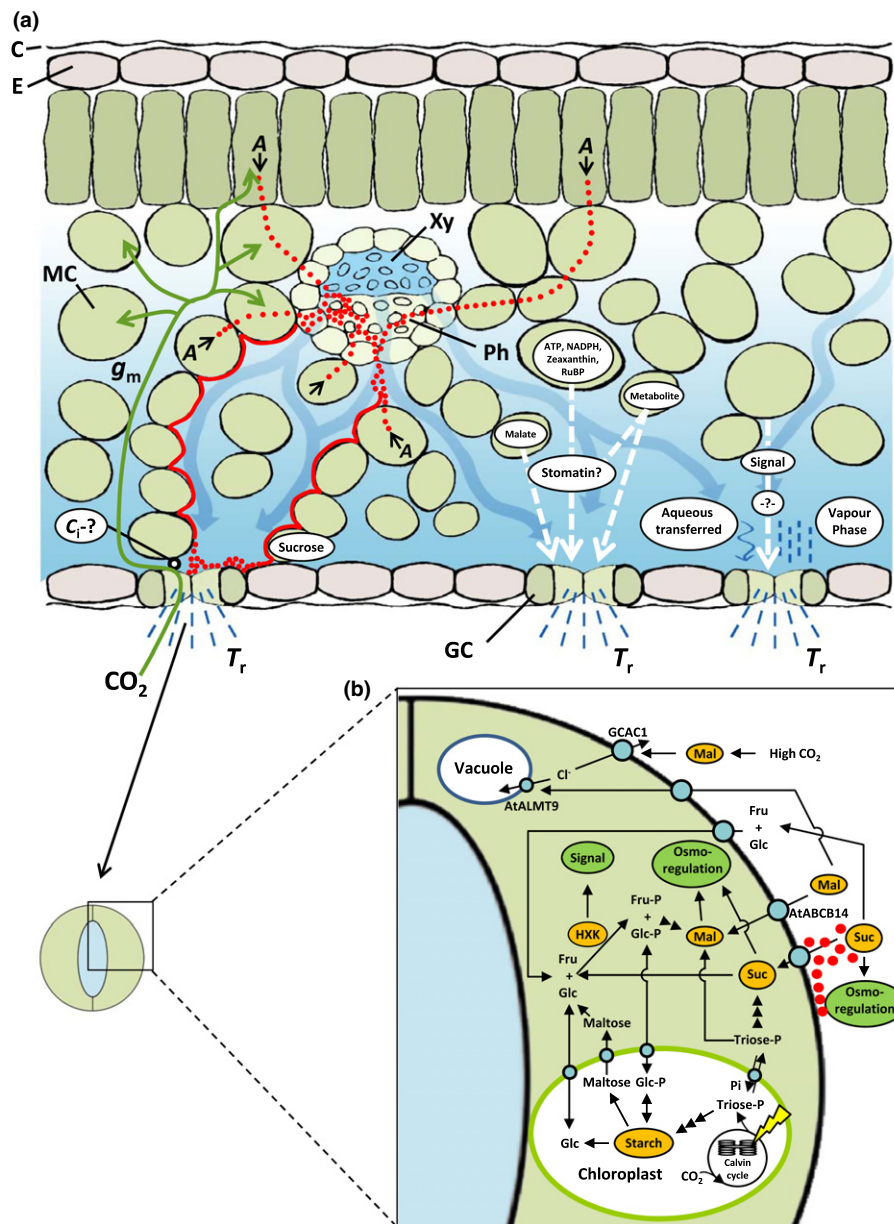
Fig. 1 Schematic diagram of a leaf cross-section showing possible mechanisms that connect mesophyll and guard cells and affect stomatal behaviour (a). CO_2 absorbed from the atmosphere through the stomatal pore diffuses towards the mesophyll cells (green line), where it is used for photosynthesis (*A*). Sucrose (Suc, represented by red dots) moves from the mesophyll cells (MC) toward the phloem (Ph). Water (blue arrows) coming out of the xylem (Xy) move toward the guard cells and evaporate (T_r) to the atmosphere. Some of the apoplastic Suc (red line) is carried by the transpiration stream toward the guard cells (GC) and accumulates at the GC. The diagram includes additional potential effectors (such as Ci, metabolites and aqueous- vs vapour-carried signals). (b) Schematic diagram of the primary metabolism in GC and the potential effects. Suc may accumulate at the GC cell wall (red dots), and may have an extracellular osmotic effect. Suc may enter the GC via Suc transporters or might be cleaved in the apoplast into glucose (Glc) and fructose (Fru) that also enter GC via a hexose specific transporter. In the cytosol, Suc may have an osmotic effect. Photosynthesis in GC yields triose-phosphates (triose-P) which may be converted to starch within the chloroplast or exported to the cytoplasm, where it might be converted to Suc or malate. Starch degradation may also contribute to Suc and malate accumulation. Malate may be produced in guard cells and/or arrive from mesophyll cells and enter the guard cells through malate transporters such as AtABCB14. Within guard cells malate may activate vacuolar Cl^- transporters such as AtALTM9, contributing to stomatal osmolarity and opening. High CO_2 concentration may increase concentrations of malate produced in the mesophyll stimulating anion efflux through channels such as GCAG1 and close stomata. The cytosolic Glc and Fru obtained from Suc cleavage or from starch degradation must be phosphorylated into Glc-P and Fru-P to be further metabolized. Sensing of Glc and Fru by hexokinases (HXK) may generate a signal that closes stomata. C, cuticle; E, epidermis; MC, mesophyll cell; GC, guard cell; *A*, photosynthesis; Xy, xylem; Ph, phloem; g_m, mesophyll conductance to CO_2; C_i, substomatal CO_2 concentration; T_r, transpiration; ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; RuBP, ribulose 1, 5-bisphosphate; Glc-P, glucose phosphate; HXK, hexokinase; Suc, sucrose; Triose-P, triose phosphate. White circles and white arrows, mesophyll-driven signals affecting stomatal movement; red dots and lines, sucrose paths; blue faded arrows, the flow of water from xylem toward the stomata; green arrow, CO_2 movement; blue circles, transporters; green circles, the effect on stomatal aperture.

factor, that is 'the mesophyll signal' (Wong *et al.*, 1979; Lee & Bowling, 1992; Mott *et al.*, 2008; Mott, 2009). The impetus for this hypothesis is the close relationship between mesophyll photosynthetic capacity and g_s, which is observed under a variety of conditions. The idea of a mesophyll-driven signal is not a new one. In 1954 Heath & Russell proposed that there was an indirect chemical or electrical signal transmitted either from the epidermal or mesophyll cells to influence stomatal behaviour. Subsequent studies suggested that the signal was a metabolite of photosynthesis (Wong *et al.*, 1979; Grantz & Schwartz, 1988) which balanced photosynthesis between Rubisco and electron transport limitation (Wong *et al.*, 1979; Messinger *et al.*, 2006). Support for the role of an active mesophyll driven signal in stomatal responses has come from experiments conducted on epidermal peels. These studies have demonstrated no effect of red light or CO_2 concentration on stomatal aperture (Lee & Bowling, 1992; Roelfsema *et al.*, 2002) or have reported a slower response often over several hours (Olsen & Junttila, 2002) and/or reduced sensitivity (Young *et al.*, 2006) compared with responses reported in intact leaves (Mott *et al.*, 2008). However, it should be noted that whilst several studies have reported little effect of light and CO_2 on stomatal responses in epidermal peels, there are many excellent studies and a plethora of literature that have illustrated significant stomatal responses to light and CO_2 in epidermal peels and guard cell protoplasts (see for example Meidner & Mansfield, 1968; Fitzsimons & Weyers, 1986; Mansfield *et al.*, 1990; Outlaw *et al.*, 1996; Webb *et al.*, 1996; Willmer & Fricker, 1996 Assmann & Shimazaki, 1999; Pei *et al.*, 2000 and reference within). Lee & Bowling (1992, 1993) demonstrated a stomatal response when the isolated epidermis was incubated in the presence of mesophyll cells or chloroplasts isolated from an illuminated leaf, but not when incubated without mesophyll or in the presence of chloroplasts isolated from dark adapted leaf material. Chloroplastic ATP, zeaxanthin, NADPH and Ribulose 1, 5-bisphosphate (RuBP) have all been put forward as a potential signal (Wong *et al.*, 1979; Farquhar & Wong, 1984; Lee & Bowling, 1992; Zeiger & Zhu, 1998; Tominaga *et al.*, 2001; Buckley *et al.*, 2003), but as yet the signal, named 'stomatin' by Lee & Bowling (1995), has remained elusive (Fig. 1). Recent studies have adopted a unique epidermis–mesophyll transfer experimental approach first used by (Mouravieff, 1956, 1957) and recently refined by Mott *et al.* (2008). In these experiments the epidermis is removed from the mesophyll and measured in isolation or replaced back onto the mesophyll belonging to the same or a different species. These studies demonstrated that stomatal responses to light and CO_2 concentration in isolated epidermis were not the same as those observed when the epidermis was placed back onto mesophyll (Mott *et al.*, 2008; McAdam & Brodribb, 2012; Fujita *et al.*, 2013) and, in general, responses tended to be slow and not of the same magnitude.

They also highlighted that the mesophyll signal was the same irrespective of species, but that some stomata were not responsive to the signal (Mott *et al.*, 2008). Mott *et al.* (2008) argued that stomata in *Vicia faba* epidermal peels do not respond to changes in light or CO_2 concentration, although many early studies observed responses in isolated *Vicia faba* peels (Brearley *et al.*, 1997), although the incubation media may have influenced these findings.

Recent studies in ferns and lycophytes showed that guard cells in these species were unresponsive to mesophyll signals, even though the mesophyll of these species still produced a signal that modern seed plants could respond to (McAdam & Brodribb, 2012). The nature of the signal has varied between different studies. Sibbernsen & Mott (2010) flooded the intercellular air space with water and used hydrophobic filters to conclude that the signal must be a vapour phase signal generated from the mesophyll, whilst Fujita *et al.* (2013) placed various sized cellophane and polyethylene spaces between the epidermis and mesophyll and concluded that there must be an aqueous apoplastic transferred signal from the mesophyll. The latter observations agree with Lee & Bowling (1992) who put forward the water soluble signal 'stomatin'. In a recent study Mott *et al.* (2013) used an electrode under the epidermis and monitored stomatal responses from the signal generated, and concluded that the signal must be a vapour phase ion that generated changes in pH of the epidermis.

Alternatively, it could be that guard cell photosynthesis (discussed later, see Fig. 1) may provide a metabolite signal (Wong *et al.*, 1979; Muschak *et al.*, 1999; Lawson, 2009) or the stomata could sense the redox state of the photosynthetic electron transport chain (Busch, 2013).

IV. Arguments against a mesophyll-driven signal other than C_i

There are many arguments against a mesophyll-driven signal that coordinates mesophyll photosynthesis with stomatal behaviour. Roelfsema *et al.* (2002, 2006) illuminated individual guard cells with and without mesophyll illumination, and albino areas of *Vicia faba* and variegated regions of *Chlorophytum comosum* leaves to demonstrate that guard cells only respond to red light if the underlying mesophyll was also illuminated. These studies support the idea of an active mesophyll-driven signal in stomatal responses, however, the authors assigned the response to mesophyll-driven changes in C_i. Further support for a C_i mediated stomatal response comes from work on the high temperature 1 (HT1) mutants, which carry a mutation in the gene encoding a protein kinase. Hashimoto *et al.* (2006) showed that stomata of *ht1* mutant responded to blue light but lacked both a red light and CO_2 response, therefore suggesting a coordination between red light and CO_2 stomatal responses, signifying a C_i driven coordination.

Experiments using transgenic plants with altered photosynthetic capacity have reported stomatal conductances that are equivalent (Quick *et al.*, 1991; Stitt, 1991; Hudson *et al.*, 1992; Lauerer *et al.*, 1993; Evans *et al.*, 1994; Price *et al.*, 1995; von Caemmerer *et al.*, 2004; Lawson *et al.*, 2008) or even greater (Muschak *et al.*, 1999; Lawson *et al.*, 2008) than wild-type controls. In these plants, the stomata opened in response to increasing light, despite having high C_i values (through reduced photosynthesis), suggesting that either light overrides a C_i response (Lawson *et al.*, 2008) or that stomata respond to external rather than internal CO_2 concentrations (von Caemmerer *et al.*, 2004). Such studies question both the role of photosynthesis (either mesophyll and/or guard cell) and C_i in stomatal responses to light, suggesting that signals not directly related to photosynthesis must be involved and demonstrating

that the environmentally induced correlation between g_s and A that is frequently observed can be broken (von Caemmerer *et al.*, 2004).

There are numerous examples in the literature of a close correlation between mesophyll photosynthetic rates and stomatal conductance, however, the mechanism(s) that coordinates these responses is not entirely known. The general consensus by many researchers that C_i maintains this balance does not account for the small change in stomatal aperture observed with changes in C_i concentration. This observation, in conjunction with the sluggish behaviour of stomata in isolated peels, supports a mesophyll-driven signal based on (1) a product of photosynthesis or electron transport, (2) the redox state of the tissue, (3) a metabolite in the transpiration stream, and (4) vapour phase ion and/or electrical signal. Although the existence and nature of a mesophyll-driven signal is controversial, it would make sense that stomatal behaviour is in some way coordinated with the mesophyll CO_2 demands or capacity. Stomata can respond and function in isolation but, equally, there is a general consensus that the fine-tuning of stomatal behaviour is under the influence of the mesophyll. This complex relationship could also explain the significant natural variation that exists in stomatal responses both between and within species (Weyers & Lawson, 1997; Lawson *et al.*, 1998; Lawson & Weyers, 1999), as well as the hierarchy of stomatal responses that are directed by both A and the environment. Such a hierarchal response would also validate why the close correlation between A and g_s can be broken in transgenic plants, and that light is the dominant signal and overrides any C_i response, resulting in stomata that remain open despite low A and high C_i (von Caemmerer *et al.*, 2004; Lawson *et al.*, 2008). Elucidating the signal and understanding the genetic bases and molecular signals behind any coordination between mesophyll photosynthetic carbon assimilation and stomatal control could be extremely lucrative for manipulating plants for improved WUE.

A recent paper using chlorophyll fluorescence imaging combined with gas exchange to show spatio-temporal decoupling of stomatal and mesophyll in response to cutting the leaf veins, suggesting that the two tissue types responded to two different signals (Hanson *et al.*, 2013).

V. Guard cell osmoregulation and evidence for a role of sucrose

Sucrose has been put forward as a potential metabolite involved in the coordination between mesophyll photosynthesis and guard cell osmoregulation. However, before exploring the potential mechanisms involving sucrose signalling, it is important to outline stomatal osmoregulation and the role and origin of sugars in guard cells and stomatal behaviour, as these also have the potential to provide a signalling or sensory mechanism/pathway.

Stomatal movements, regulated by a number of external and internal cues or signals are brought about by changes in osmotic potential, due to the loss or accumulation of solutes in the guard cells. There is a wealth of elegant biochemical and biophysical data describing the roles of potassium, calcium and ABA in these processes and mutants have been used to investigate the genetic basis of guard cell function (Assmann, 1993; Willmer & Fricker, 1996; Blatt, 2000; Evans & Hetherington, 2001; Schroeder *et al.*, 2001). Although for decades guard cell aperture was thought to be solely due to osmotic adjustment driven by the accumulation and loss of K^+ ions, with Cl^- and malate ions acting as counter ions (Schroeder *et al.*, 2001; Roelfsema & Hedrich, 2005; Pandey *et al.*, 2007), there has been a steady increase in evidence of a role for sucrose (Suc). Early hypotheses regarding guard cell osmoregulation involved a role for sugars. It has been proposed that at dawn sugars generated from the degradation of starch are the primary guard-cell osmolytes required for stomatal opening (Lloyd, 1908; Meidner & Mansfield, 1968). The role for sugar in guard cell osmoregulation and stomatal responses was abandoned with the discovery that K^+ in guard cells, with malate^{2-} and/or chloride (Cl^-) acting as the counter ion(s), correlated with stomatal opening (Imamura, 1943; Yamashita, 1952; Fischer, 1968; Fischer & Hsiao, 1968; Humble & Raschke, 1971; Allaway, 1973; Pearson, 1973; Outlaw & Lowry, 1977; Shimada *et al.*, 1979; Outlaw, 1983; Asai *et al.*, 2000). The role of K^+ and Cl^- ion channels in guard cell behavior was functionally confirmed (reviewed in MacRobbie, 1998; Schroeder *et al.*, 2001; Pandey *et al.*, 2007) and the starch-sugar hypothesis was replaced by the potassium-malate theory. The disappearance of starch in the guard cells throughout the day still fitted with this new theory, and the inverse correlation between guard cell starch concentration and stomatal aperture suggested that starch degradation contributes carbon skeleton for the synthesis of organic anions such as malate, to act as counter ions and support stomatal opening (Outlaw & Lowry, 1977; Schnabl, 1980, 1981). In guard cells, malate is thought to be synthesized via the reduction of oxaloacetate, formed from the carboxylation of phosphoenolpyruvate (PEP). The former step is catalysed by NADP- or NAD-dependent malate dehydrogenase (MDH), and the latter by phosphoenolpyruvate carboxylase (PEPC). The majority of enzymes necessary for malate synthesis from starch have been identified in guard cells (Rao & Anderson, 1983; Gotow *et al.*, 1985; Hedrich *et al.*, 1985; Robinson & Preiss, 1987; Raschke *et al.*, 1988; Shimazaki, 1989; Scheibe *et al.*, 1990; Parvathi & Raghavendra, 1997; Asai *et al.*, 2000).

A role for Suc in guard cell osmoregulation was revisited when studies proposed that K^+ and its counter ions malate could not provide all the osmotic required to support stomatal apertures in *Commelina communis* (MacRobbie & Lettau, 1980a,b), and it was suggested that soluble sugars account for the additional osmoticum required to support stomatal opening (MacRobbie, 1987; Talbott & Zeiger, 1993). A decline in K^+ concentrations concomitant with an increase in Suc concentrations throughout the day raised the hypothesis that K^+ is responsible for early morning opening of stomata, but that it is replaced later in the diel period by Suc which becomes the major osmolyte responsible for maintaining stomatal aperture from midday on (Amodeo *et al.*, 1996; Talbott & Zeiger, 1998; Schroeder *et al.*, 2001; Lawson, 2009). Pearson (1973), who assayed for sucrose in epidermal peels of *Vicia faba* and *Commelina cyanea*, found a consistent increase that peaked mid-afternoon, but the relationship between aperture and Suc content was quite weak, questioning the contribution of sugar to the guard cells osmoticum. Having said this, the role of Suc as a major osmoticum driving

stomatal responses is not universally accepted and many models examining guard cells fluxes across the plasma membrane and tonoplast concentrate on ion fluxes and tend to dismiss a major influence of Suc. However, several researchers have suggested that sucrose could play a key role in coordinating mesophyll and stomatal behaviour via the apoplast (Lu *et al.*, 1995, 1997; Outlaw & De Vlieghere-He, 2001; Outlaw, 2003; Kang *et al.*, 2007; Kelly *et al.*, 2013). The origin of Suc for guard cell movements is not entirely clear. Suc could be supplied to guard cells by three potential ways: (1) starch degradation in guard cells (Lloyd, 1908; Assmann, 1993); (2) guard cell photosynthetic carbon fixation; and (3) Suc imported from the mesophyll cells (Fig. 1) (Gotow *et al.*, 1988; Tallman & Zeiger, 1988; Poffenroth *et al.*, 1992; Talbott & Zeiger, 1993, 1996).

VI. Degradation of starch

Starch accumulates in guard cells at night and slowly disappears throughout the light period (Tallman & Zeiger, 1988). As mentioned above, this observation was the foundation of the starch-sugar hypothesis (Tallman & Zeiger, 1988), and later on for the starch-malate hypothesis (Willmer *et al.*, 1973; Reckmann *et al.*, 1990; Asai *et al.*, 2000). There is some evidence that suggests that these two metabolic processes, starch-sugar and starch-malate, depend on light quality, with blue light stimulating starch breakdown to Suc rather than malate production (Tallman & Zeiger, 1988). It is generally assumed that the starch found in guard cells originates from the end products of photosynthesis (e.g. Suc) in the underlying mesophyll (Pallas, 1964), which is imported into the guard cells. This in itself provides a long term link between mesophyll photosynthesis and guard cell function, but is temporally separated from one day to the next. Ritte *et al.* (1999) provided evidence of a guard cell-specific sugar transport by identifying activity of a monosaccharide-H$^+$ symporter (Ritte *et al.*, 1999). The expression of a H$^+$-monosacharide symporters *AtSTP1* in Arabidopsis was shown to be high at night (Stadler *et al.*, 2003), supporting the uptake of Suc at night for starch synthesis. However, it is interesting to note that transient diurnally regulated increases in the expression of *AtSTP1* observed around midday, suggested a role for osmoregulation (Stadler *et al.*, 2003). It should be noted, though, that starch is practically absent in the early morning in Arabidopsis guard cells (Stadler *et al.*, 2003) and therefore metabolism and stomatal responses may differ between species.

VII. Guard cell photosynthetic production of Suc

The majority of guard cells contain functional chloroplasts (Humble & Raschke, 1971; Willmer & Fricker, 1996); *Paphiopedilum* species is one of the exceptions to this rule, having no chloroplasts but functional stomata (Nelson & Mayo, 1975; Willmer & Fricker, 1996). It has also been known for many years that linear electron transport takes place in the guard cell chloroplasts (Hipkins *et al.*, 1983; Shimazaki & Zeiger, 1985; Willmer & Fricker, 1996; Cardon & Berry, 1992; Tsionsky *et al.*, 1997; Lawson *et al.*, 2002, 2003) although high numbers and activity of Photosystem I (Lurie, 1977) have been thought to

indicate high rates of cyclic electron flow and supporting the notion that ATP production potentially provides the energy required for plasma membrane proton pumps (Shimazaki & Zeiger, 1985; Tominaga *et al.*, 2001) required for ion uptake. Alternatively, the energy and reductant produced from electron transport (ATP & NADPH) could be used for the reduction of oxaloacetate (OAA) and malate production from starch degradation (Outlaw, 2003) which has been correlated with an increase in stomatal aperture (Imamura, 1943; Yamashita, 1952; Fischer, 1968; Fischer & Hsiao, 1968; Humble & Raschke, 1971; Allaway, 1973; Pearson, 1973; Outlaw & Lowry, 1977; Shimada *et al.*, 1979; Outlaw, 1983; Asai *et al.*, 2000). Several lines of evidence suggest a limited photosynthetic capacity in guard cells compared to mesophyll, and smaller numbers and sizes of chloroplasts in guard cells (Shimazaki *et al.*, 1982; Reckmann *et al.*, 1990; Gautier *et al.*, 1991; Outlaw & De Vlieghere-He, 2001; Vavasseur & Raghavendra, 2005). Using chlorophyll fluorescence imaging Lawson *et al.* (2002) proposed that electron transport in guard cells was 20% lower than the underlying mesophyll, but that both cells responded in a similar manner to environmental stimuli. (Lawson *et al.*, 2003). Although early reports suggested that there was no (or little) Calvin cycle activity in guard cells (Outlaw *et al.*, 1979, 1982; Outlaw, 1982, 1987, 1989; Tarczynski *et al.*, 1989), it is now generally accepted that all the Calvin cycle enzymes are present and functional in guard cells (see review by Lawson, 2009). It has also been shown that $^{14}CO_2$ uptake in guard cells can be incorporated into 3-PGA and RuBP (Gotow *et al.*, 1988) and perhaps used to produce Suc as a guard cell osmoticum for guard cell opening, specifically in response to red/photosynthetic light (Poffenroth *et al.*, 1992) and in the absence of starch breakdown (Talbott & Zeiger, 1993). However, other studies have reported malate as the primary fixation product (via PEPc), very little Calvin cycle metabolites (Willmer & Dittrich, 1974; Raschke & Dittrich, 1977) and low activity levels of Rubisco and Calvin cycle enzymes (Outlaw, 1982; Reckmann *et al.*, 1990). There is still controversy over the extent of guard cell photosynthetic CO_2 fixation, and whether Suc could be produced in any quantity that could be osmotically useful for guard cell behaviour (see Outlaw, 2003). The most widely accepted consensus is that even if CO_2 fixation via the Calvin cycle occurs in guard cells, the contribution to osmotic requirements for stomatal opening is minimal and too low for any significant function (see reviews by Outlaw *et al.*, 1982; Outlaw, 1989), with reports suggesting only a 2% contributions to guard cell osmotic adjustments (Reckmann *et al.*, 1990). However, despite decades of research the role of guard cell chloroplasts and their potential in providing energy or Suc for stomatal adjustments or as a signalling mechanism that enables coordination between mesophyll photosynthesis and stomatal behaviour is still unknown and requires further investigation.

VIII. Guard cell Suc imported from the mesophyll

Hite *et al.* (1993) suggested that guard cells might act as carbon sinks, taking up Suc via plasma membrane transporters. Suc and and hexose transporters were discovered in guard cells (Stadler *et al.*, 2003; Weise *et al.*, 2008; Bates *et al.*, 2012; Bauer *et al.*,

2013). Lu *et al.* (1995, 1997) have shown that mesophyll-derived Suc is accumulated at the guard cell apoplast and enters the guard cells of open stomata. It has been suggested that during times of high photosynthesis and transpiration rates, the apoplastic Suc concentration at the guard cells can be absorbed and replace potassium and malate as the osmoticum for the maintenance of stomatal opening (Ritte *et al.*, 1999). Outlaw and colleagues proposed that apoplastic Suc from the mesophyll cells was a source of Suc for guard cells and provided the osmoticum for stomatal opening (Lu *et al.*, 1997; Ewert *et al.*, 2000; Outlaw & De Vlieghere-He, 2001). They also claimed that Suc moving throughout the apoplast of a transpiring leaf may accumulate in an osmotically significant concentration in the guard cell wall and stimulate stomatal closure as a means to coordinate photosynthesis with transpiration (Ewert *et al.*, 2000; Outlaw & De Vlieghere-He, 2001; Kang *et al.*, 2007).

Apart from playing an osmotic role, the fate of sugars within guard cells is not yet known. As illustrated above, sugar might provide energy for stomatal opening and replenish carbohydrate stores such as starch. However, studies using transgenic plants with impairments in photosynthesis queried a role for photosynthetically produced Suc (from guard or mesophyll cells) in stomatal opening (Baroli *et al.*, 2008; Lawson *et al.*, 2008). In these studies, reduced photosynthesis through reduced activity of key enzymes in the Calvin cycle did not reduce stomatal conductance as would be expected if photosynthetically driven Suc opens stomata. On the contrary, plants with reduced photosynthesis due to a reduction in sedoheptulose-1,7-bisphosphatase (SBPase) activity (a key enzyme in the regeneration of RuBP that has a high control co-efficient on photosynthesis and therefore, Suc production), displayed a tendency toward greater stomatal conductance (Lawson *et al.*, 2008).

IX. Sugar sensing and metabolism

Suc is the most commonly transported photoassimilate in most plant species. Once in cells, Suc may be metabolized, stored in vacuoles or converted into starch in plastids. To metabolize Suc within the cell two pre-steps are required: (1) cleavage of Suc into its derivatives, glucose and fructose, by Suc cleaving enzymes (which may release UDP-glucose as well), and (2) phosphorylation of glucose and fructose by sugar phosphorylating enzymes, hexokinase (HXK) and fructokinases (FRK) (Dennis & Blakeley, 2000). The phosphorylated hexoses, glucose-P and fructose-P then serve as initial essential substrates for central metabolic processes, such as glycolysis, energy production and the formation of organic molecules (Dennis & Blakeley, 2000).

HXK, also expressed in guard cells (Arabidopsis eFP Browser http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?data-Source=Guard_Cell, and Bates *et al.* (2012)) is the only enzyme that can phosphorylate glucose and is therefore considered to play a pivotal metabolic role in most, if not all, living tissues (Granot *et al.*, 2013). In addition to its hexose phosphorylation catalytic activity, it is now well accepted that HXK is a dual functional enzyme that also holds a sugar sensing role (Jang *et al.*, 1997; Moore *et al.*, 2003). As part of its sensing role, HXK monitors glucose concentrations in

photosynthetic tissues and inhibits the expression of photosynthetic genes when sugar concentrations are sufficiently high, thus coordinating sugar production (photosynthesis) with sugar concentrations (Jang *et al.*, 1997; Dai *et al.*, 1999; Xiao *et al.*, 2000; Moore *et al.*, 2003; Rolland *et al.*, 2006; Kelly *et al.*, 2012). It is now well established that the sugar sensing role of HXK is mediated by ABA, so that HXK stimulates expression of ABA related genes, and ABA deficient mutants do not exhibit the HXK-related sugar effects (Zhou *et al.*, 1998; Laby *et al.*, 2000; Leon & Sheen, 2003; Rolland *et al.*, 2006; Rognoni *et al.*, 2007; Ramon *et al.*, 2008). However, the molecular mechanism by which HXK promotes ABA in response to sugars is still unknown.

Most of our knowledge on the sugar sensing role of HXK was obtained by manipulating HXK expression levels (see reviews by Ramon *et al.*, 2008; Granot *et al.*, 2013). Initial characterization of mature tomato and Arabidopsis plants expressing high levels of the Arabidopsis *HXK1* (*AtHXK1*), either expressed globally or specifically in guard cells, revealed that in both cases, transpiration and stomatal aperture size were significantly reduced (Kelly *et al.*, 2012, 2013). By contrast, the opposite behaviour was observed when assaying the *hxk1* mutant (Kelly *et al.*, 2013). As HXK activity is sugar-dependent, it is reasonable to assume that the sugars themselves, which are the substrate for HXK, trigger these effects. Outlaw and colleagues (Outlaw, 2003; Kang *et al.*, 2007) have shown that some of the apoplastic Suc generated in the mesophyll is carried to guard cells by the transpiration stream, and where the water evaporates, the Suc accumulates at the vicinity of the guard cells (Fig. 1). They suggested that the increase in Suc concentration outside the guard cells might impose an osmotic effect that may stimulate stomatal closure, thus coordinating photosynthesis with transpiration (Outlaw, 2003; Kang *et al.*, 2007). It was recently hypothesized that the Suc arriving at guard cells is cleaved in the apoplast to form glucose and fructose that enter the cells via hexose transporters, or enters the cell via sucrose transporters and is cleaved within the guard cells to form glucose and fructose, which are then sensed by HXK, stimulating stomatal closure (Fig. 1) (Kelly *et al.*, 2013). As HXK can sense increasing concentrations of sugar (Jang *et al.*, 1997), it was assumed that the closure effect on stomatal movement in response to increasing sugar concentrations entering the guard cells, coordinates photosynthesis with transpiration.

A functional approach has been taken to examine the above hypothesis, namely whether Suc stimulates stomatal closure and whether it happens via HXK. Exposing intact leaves or epidermal strips (composed of only guard and epidermis cells with the mesophyll removed) to Suc or to its derivates, glucose and fructose, stimulated stomatal closure (Kelly *et al.*, 2013). This effect was found to be enhanced when HXK was overexpressed and was partially revoked when a competitive inhibitor of HXK (*N*-acetylglucoseamine; Hofmann & Roitsch, 2000) was applied together with the Suc. This led to the conclusion that Suc stimulates stomatal closure and that this response is mediated by HXK (Kelly *et al.*, 2013). This recent observation is in line with the findings of Lee & Bowling (1992), who stated that incubating isolated epidermis with glucose and Suc prevent stomatal opening; this was surprising at the time, in view of the osmotic-opening role assigned

to sugars (Talbott & Zeiger, 1993, 1996; Amodeo *et al.*, 1996). This observation is also in line with the finding that plants with reduced rates of photosynthesis (and Suc production) displayed a tendency toward greater stomatal opening (Lawson *et al.*, 2008).

As already mentioned, there is solid evidence in the literature for an interaction between sugars, HXK and the hormone ABA. In general, the sugar-sensing effects mediated by HXK are known to be dependent on the production and signalling of ABA (Leon & Sheen, 2003; Rolland *et al.*, 2006; Rognoni *et al.*, 2007; Ramon *et al.*, 2008). It has been shown that sugar and HXK stimulate the ABA signalling pathway within guard cells (Kelly *et al.*, 2013) promoting stomatal closure, but it remains to be determined if sugar and HXK has any direct impact of ABA synthesis and ABA concentration in the guard cells. As initially suggested by Lu *et al.* (1997), the feedback inhibition of Suc generated by photosynthesis in the mesophyll and arriving at guard cells, potentially integrates three pivotal physiological processes: photosynthesis, transpiration and sugar translocation (Outlaw, 2003). It is the constant, on-going combination of the three that adjusts the amount of Suc streaming toward the stomata. When transpiration and photosynthesis rates are high and the mesophyll cells produce more sugar than can be uploaded into the phloem, surplus Suc is carried toward the stomata by the transpiration stream and stimulates stomatal closure, thus reducing water loss. Initially this link may seem counter-intuitive as conditions or situations that result in increased photosynthesis (and therefore Suc) are not normally associated with reduced stomatal aperture. However, increased apoplastic Suc concentrations would only arise if sink capacity was limited and phloem loading was saturated. Additionally, the mechanism might operate over the longer diel period – for example, reduced stomatal apertures are often observed in the afternoon despite environmental conditions being similar to those found in the morning.

Many plant species are apoplastic loaders in which Suc produced in mesophyll cells is exported to the intercellular space before being loaded into the phloem (Rennie & Turgeon, 2009). It is easy to see how apoplastic intercellular Suc carried by the transpiration stream to the guard cells would form a feedback mechanism that closes stomata (Lu *et al.*, 1997; Outlaw & De Vlieghere-He, 2001; Outlaw, 2003; Kelly *et al.*, 2013) that appears to be related to the type of phloem loading strategy (either apoplastic or symplastic) (Kang *et al.*, 2007). It is intriguing to study whether sugars have similar effects in symplastic loaders as well (Kang *et al.*, 2007). Because guard cells are capable of carrying out photosynthesis and producing sugars (see Lawson, 2009), it is possible that guard cell-produced sugars might also stimulate stomatal closure. Namely, the guard cell-produced sugars may also be sensed by the guard cell HXK in both apoplastic and symplastic loading species. In addition, the specific conditions under which sugars accumulate also remains to be elucidated and the threshold or dosage-dependency that stomata respond to is also unknown. To thoroughly elucidate the role of sugars in stomatal movement and the coordination of g_s with A, and to distinguish between external and internal sugar effects, the amount of Suc needs to be measured directly in guard cells in response to externally supplied sugars alongside stomatal aperture measurements. Diurnal measurements of photosynthetic production of Suc, phloem loading

and apoplastic Suc in the vicinity of guard cells along with guard cell Suc concentration, HXK activity and stomatal aperture are all needed to elucidate the entire mechanism.

There are several additional studies that support stomatal closure as a result of sugar concentration. The girdling technique, in which the outer phloem is removed restricting shoot-to-root sugar transport via phloem, results in endogenous accumulation of glucose and Suc in shoots along with a significant reduction in g_s (Setter & Brun, 1980; Else *et al.*, 1996; Urban & Alphonsout, 2007; Domec & Pruyn, 2008). Fruit load experiments provide additional observations of how stomata are affected by sugar content. A recent study conducted in avocado (*Persea americana*) compared fruit loaded trees with nonfruited. High fruit load, which leads to lower sugar concentrations in leaves, was found to be correlated with higher g_s values and higher water intake, whereas removing the fruits displayed an opposite behaviour; high sugar content and lower g_s and water uptake (Silber *et al.*, 2013). However, both of these experimental findings could equally be explained by reduced sink availably for photosynthate and a downregulation of photosynthesis and which in turn would result in a reduced stomatal aperture.

X. The importance of malate as a mesophyll-driven signal

In addition to sucrose, it is well established that malate acts as an osmoticum for the opening and closing of stomata providing a counter ion for K^+ ions (Imamura, 1943; Yamashita, 1952; Fischer, 1968; Fischer & Hsiao, 1968; Humble & Raschke, 1971; Allaway, 1973; Pearson, 1973; Outlaw & Lowry, 1977; Shimada *et al.*, 1979; Outlaw, 1983; Asai *et al.*, 2000). The production of malate in the guard cells has also been associated with electron transport and starch degradation in these cells (Outlaw & Lowry, 1977; Schnabl, 1980, 1981). Early studies suggested that the energy and reductant produced from electron transport within guard cells could support the production of malic acid via phosphoenolpyruvate carboxylase (PEPC) CO_2 fixation (Willmer & Dittrich, 1974; Raschke & Dittrich, 1977), with the breakdown of starch through the day providing the required carbon skeletons (see Outlaw & Manchester, 1979; Asai *et al.*, 2000). Increases in PEPc activity with irradiance have been reported, in combination with increased NADP- or NAD-dependent malate dehydrogenase activity (Rao & Anderson, 1983; Scheibe *et al.*, 1990) resulting in the accumulation of malate in the guard cells and stomatal opening (Allaway, 1973; Pearson, 1973; Pearson & Milthorpe, 1974; Vavasseur & Raghavendra, 2005). Inhibition of malate synthesis using the phosphoenolpyruvate carboxylase (PEPC) inhibitor 3,3-dichloro-2-dihyroxyphosphinoylmethyl-2-propenoate (DCDP) in epidermal strips of *Vicia faba* prevented stomatal opening in response to illumination due to a decrease in malate. When applied in the light, DCDP reduced stomatal aperture along with decreases in guard cell malate concentrations (Asai *et al.*, 2000) confirming the osmotic role for malate in guard cell opening. Further support for the involvement of PEPc activity comes from Cousins *et al.* (2007) who showed reduced rates of stomatal opening in PEPc-deficient *Amaranthus edulis* mutants compared

with wild-type controls (Cousins *et al.*, 2007), whilst *Solanum tuberosum* plants over-expressing PEPc had greater stomatal opening rates compared with plants with reduced PEPc (Gehlen *et al.*, 1996).

The above studies demonstrate that malate plays a key role in stomatal regulation as an osmoticum and as a sink for the end-products of guard cell electron transport. However, more recently the importance of malate as a regulator of stomatal responses to external CO_2 concentration has been demonstrated (Hedrich & Marten, 1993; Hedrich *et al.*, 1994; Roelfsema *et al.*, 2002; Lee *et al.*, 2008; Fernie & Martinoia, 2009). These and other studies illustrated that malate acts as a signal that coordinates stomatal behaviour with mesophyll photosynthetic demands (Hedrich & Marten, 1993; Lee *et al.*, 2008; Fernie & Martinoia, 2009; Araújo *et al.*, 2011). Below we provide a summary of reports providing evidence for the importance of malate in guard cell signalling and links with mesophyll photosynthesis.

Substantial evidence for malate acting as the mesophyll-derived signal influencing stomatal behaviour came from Araújo *et al.* (2011), who demonstrated greater stomatal conductance (and also photosynthesis) in *Solanum lycopersicum* plants with reduced expression (driven by the constitutive *35S* promoter) of *SISDH2-2* gene which encodes for the iron sulphur subunit of the succinate dehydrogenase protein. Succinate dehydrogenase catalyses the formation of fumarate from succinate in the tricarboxylic acid (TCA) cycle and therefore antisense plants exhibited a reduction in TCA metabolites including fumarate and malate. However, when repression was driven by a guard cell-specific promoter, no effect on stomatal conductance nor photosynthesis was apparent, signifying that changes in guard cell malate depended upon supply from the surrounding mesophyll cells and not from the guard cells themselves. These findings provided considerable evidence demonstrating that adjustments to mesophyll mitochondrial metabolism affect stomatal function via the regulation of organic acid concentrations (Fernie & Martinoia, 2009). Following the formation of fumarate from succinate, fumarate is metabolised into malate by Fumarase (fumarate hydratase; E.C. 4.2.1.2.) in a reversible hydration reaction (Nunes-Nesi *et al.*, 2007). In constitutive antisense fumarase *Solanum lycopersicum* plants photosynthesis was reduced due to low stomatal conductance and a 50% decrease in CO_2 uptake under atmospheric conditions was observed (Nunes-Nesi *et al.*, 2007). In these plants lower amounts of fumarase fed back to reduce mitochondrial activity, characterised by a mild reduction in flux through the TCA cycle in the light and a reduction in dark respiration resulting in increased malate concentrations, which in turn stimulated stomatal closure. These authors furthermore demonstrated that these changes led to a reduction in overall biomass, which they attributed to a deficiency in stomatal function (marked reduction in stomatal conductance).

The findings outlined above support earlier patch clamp studies which suggested that malate was the guard cell CO_2 sensor, whereby apoplastic malate concentration regulates anion efflux in guard cells through a malate-sensitive anion channel GCAC1 (Hedrich & Marten, 1993). GCAC1 (*Guard Cell Anion Channel*

1) also named QUAC1/ALMT12 (*QUick-activating Anion Channel 1 (R-type)*), is highly expressed in guard cells, targeted to the plasma membrane and is permeable to chloride and nitrate (Meyer *et al.*, 2010; Sasaki *et al.*, 2010). QUAC1 mutants are impaired in malate-induced anion currents (Meyer *et al.*, 2010), and it has been demonstrated that QUAC1 predominantly transports malate and sulphate (Hedrich & Marten, 1993; Hedrich *et al.*, 1994; Frachisse *et al.*, 1999; Meyer *et al.*, 2010) and is thus well suited for release of malate from guard cells as previously shown for *Vicia faba* (Keller *et al.*, 1989; Dietrich & Hedrich, 1994). It has been concluded that the QUAC1 complex represents an ABA-dependent anion-selective (Mal^{2-} and SO_4^{2-}) plasma membrane channel transporting malate and sulphate in a voltage-dependent manner (Mumm *et al.*, 2013; for a more in-depth review, see Negi *et al.*, 2014).

Hedrich and co-workers showed that changes in ambient CO_2 concentration modified extracellular malate which promoted stomatal closure through the activation of voltage-dependent properties of these anion-release channels in the guard cell plasma membrane, (Hedrich & Marten, 1993; Hedrich *et al.*, 1994). These studies provided further convincing evidence that malate functions as a CO_2 sensor in guard cells and thus provided support for a mechanism that links mesophyll photosynthesis and guard cell function. Additionally, high CO_2 induces stomatal closure and has been shown to be enhanced in plants lacking the ABC transporter AtABCB14 (Lee *et al.*, 2008). Lee *et al.* (2008) demonstrated that this ABC transporter is highly expressed in guard cells and functions as a malate importer protein, aiding in malate accumulation in the guard cells by transporting it from the apoplast, where it increases the osmotic pressure causing the stomata to open. These authors suggest that this mechanism allows a recycling of malate in response to elevated CO_2 concentrations and supports the theory that malate directly modulates stomatal responses to CO_2 and therefore functions as an important regulator of guard cell function (Lee *et al.*, 2008).

A more recent study has accentuated the importance of malate in stomatal function, identifying AtALMT9 as a vascular chloride channel that is activated by cytosolic malate concentration. ALMT9 belongs to the *AL*uminium-activated *M*alate *T*ransporter family (De Angeli *et al.*, 2013; Mumm *et al.*, 2013), and plasma membrane located ALMTs are involved in dicarboxylic acid excretion and the influx of inorganic and organic ions during stomatal closure (De Angeli *et al.*, 2013) – for example ALMT12/QUAC1 is expressed in guard cells transporting malate (see above). Arabidopsis AtALMT9 knockout mutants have been shown to have compromised stomatal opening, demonstrating that AtALMT9 is required for proper regulation of stomata (De Angeli *et al.*, 2013). These findings support the work of Wang & Blatt (2011), who showed that carboxylates play an important regulatory role inhibiting chloride fluxes across the plasma membrane of *Vicia faba* guard cells. Araújo *et al.* (2011) concluded from their study, along with the evidence for malate transporters, that malate provides a mechanism linking mesophyll and stomatal function, supporting the hypothesis of a signal coordinating stomatal responses with mesophyll (Mott, 2009; Lawson & Blatt, 2014). These data provide strong evidence that malate concentration in guard cells has

a significant influence on stomatal function and support the theory of mesophyll regulation of stomatal behaviour (Lee *et al.*, 2008; Mott *et al.*, 2008; Mott, 2009; Sibbernsen & Mott, 2010).

It should be remembered that here we have focused on signals that promote possible coordination between mesophyll photosynthetic demands and stomatal function and that there are many other signals to which stomata respond that have been shown to be equally if not more important than those discussed here, including ABA and other hormones, as well as redox signals which like malate also interact strongly with guard cell ion channel (for a more in depth review see Negi *et al.*, 2014).

XI. Role of aquaporins

In addition to the potential roles of sugars/malate and ion channels in regulating stomatal movements, aquaporins have also been implicated in the control of stomatal aperture. Aquaporins encode the water channels of intracellular and plasma membranes and play a crucial role in water conservation. In stomata, water flow is a crucial aspect of guard cell turgor and changes in water content can have wider implications for the osmotic potential of these cells (Maurel *et al.*, 2002). Moreover, some aquaporins have also been described as having a role in the transport of CO_2, H_2O_2, boron and silicon in addition to carbohydrate routing and are thus implicated in diverse functions such as carbon fixation, nutrient translocation and cell signalling (Terashima & Ono, 2002; Ma *et al.*, 2004; Flexas *et al.*, 2006; Heckwolf *et al.*, 2011), thereby playing a role in interactions between mesophyll and stomatal conductance.

One group of aquaporins, the plasma membrane intrinsic proteins (PIP) correspond to proteins found abundantly in vascular and plasma membranes and have been described as playing an important role in regulating stomatal aperture. Uehlein *et al.* (2003) generated transgenic tobacco plants that showed antisense inhibition and over-expression of the tobacco PIP1 homologue NtAQP1. A thorough characterization of these plant lines indicated that the mesophyll conductance to CO_2 was positively correlated to NtAQP1 expression, although it was equally demonstrated that two additional physiological parameters, stomatal conductance and net photosynthetic capacity, were also linked to NtAQP1 expression levels (Flexas *et al.*, 2006). Taken together, it was determined by these authors that NtAQP1 serves as a CO_2 pore in tobacco leaves (Uehlein *et al.*, 2003, 2012; Flexas *et al.*, 2006). Furthermore, in both tobacco and *Solanum lycopersicum*, the constitutive over-expression of NtAQP1 increased net photosynthesis (A_N), mesophyll CO_2 conductance (g_m), stomatal conductance (g_s) and increase root hydraulic conductivity under stress (Kelly *et al.*, 2014; Sade *et al.*, 2010, 2014).

Additionally, ABA mediates the regulation of aquaporins gene expression, protein abundance and/or activity in response to environmental constraints including drought and salt stress (Zhu *et al.*, 2005; Maurel *et al.*, 2008) providing an additional mechanism linking mesophyll CO_2 demands with stomatal behaviour. These data further attest to the complex, interconnected roles of multiple components implicated in the regulation of guard cell

aperture and highlights the importance of mesophyll-driven signals in stomatal responses.

XII. Guard cell manipulation and possible future directions

In the attempt to understand the link between mesophyll and guard cell metabolism genetic engineering provides a unique opportunity to dissect not only stomatal physiology and function (Nilson & Assmann, 2007), but also the specific role of each cell type in governing stomatal metabolism, stomatal opening and water use efficiency. Lawson & Blatt (2014) reviewed the complexity of alteration in stomatal numbers and sizes for improving WUE and highlighted that guard cell function can counterbalance anatomical changes. However, they also singled out manipulation of guard cell metabolism as a potential target for increasing WUE while maintaining photosynthesis rate. The aim of this section is to highlight the potential ways of manipulating guard cell metabolism and stomatal response, using the currently available tools.

Over the last 10 yr significant strides have been made in understanding the underlying mechanisms governing stomatal opening/closing and the proteins intrinsically linked to guard cell functions and mesophyll–stomatal interactions. Combined with the availability of a number of promoters permitting the specific expression of transcripts in different cell types, the idea of manipulating guard cell metabolism or specific stomatal traits has the real potential to elucidate mesophyll–stomatal interactions with the overall goal of delivering plants with improved WUE and yield. Table 1 compiles a list of promoters functionally evaluated for expression in different cells type, that is mesophyll, guard cells, both and neither (Muller-Rober *et al.*, 1995; Francia *et al.*, 2008; Galbiati *et al.*, 2008; Cominelli *et al.*, 2011; Kelly *et al.*, 2013) providing an effect toolbox to explore cell-specific interactions.

Guard cell-specific promoters, combined with organelle-specific transit peptides, may also allow the elucidation of the role of specific transcripts implicated in photosynthesis, electron transport, carbohydrate biosynthesis and ion channel function to be evaluated on a cell-by-cell basis. Moreover, the ability to induce transient expression in guard cells (Rusconi *et al.*, 2013) opens up the new, exciting possibility to study guard cell signalling and the transduction pathways involved. These tools would furthermore allow the role of specific transcripts in the coordination of guard cell behaviour and mesophyll–stomatal interactions to be determined experimentally.

For example, the over-expression or suppression of key photosynthetic transcripts such as SBPase, FBPaldolase in addition to the manipulation of key electron transport proteins can be exclusively targeted to guard cells or the mesophyll, to further elucidate the role of GC photosynthesis and electron transport on GC function, the role of mesophyll metabolism in stomatal behaviour and their role in a coordinated response to environmental stimuli that potentially involves GC photosynthesis and ion channels. In addition to these studies, the expression of CO_2 transporters could be used to evaluate the role of CO_2 fluxes on C_i and in turn evaluate the role C_i on the relationship between mesophyll/guard cell photosynthesis and stomatal aperture. This could be studied further by the over-

Table 1 Available promoters for transcript studies in guard and mesophyll cells

| Promoter	Gene ID	Localisation	References
CYTOCHROME P450 86A2 mono-oxygenase *CYP86A2*	At2g37300	High guard cell specificity	Francia *et al.* (2008) Galbiati *et al.* (2008)
CYTOCHROME P450 86A2 mono-oxygenase *CYP86A2* subfamily	At4g00360	High guard cell specificity	Galbiati *et al.* (2008)
AtMYB60 *transcription factor*	At1g08810	High guard cell specificity	Cominelli *et al.* (2011) Rusconi *et al.* (2013)
PP2C PROTEIN PHOSPHATASE(*AtPP2C*)	At1g03590	High guard cell specificity	Galbiati *et al.* (2008)
GC1	At1g22690	High guard cell specificity	Yang *et al.* (2008) Wang *et al.* (2014)
PLEIOTROPIC DRUG RESISTANCE 3 (AtPDR3) transporter	At2g29940	High guard cell specificity	Galbiati *et al.* (2008)
KST1 potato potassium channel (AtKAT1 homologue)	NP001275475	High guard cell specificity	Muller-Rober *et al.* (1995) Kelly *et al.* (2013)
FASCICLIN-LIKE gene	At5g44130	Stomata and mesophyll	Galbiati *et al.* (2008)
PHOTOSYSTEM II PROTEIN 12S SEED STORAGE CRA1 gene	At1g03600 At5g44120	Downregulated in guard cells compared to mesophyll cells	Galbiati *et al.* (2008)
PENTATRICOPEPTIDE REPEAT-CONTAINING PROTEIN	At2g37310	Was NOT detected either in stomata or mesophyll cells	Galbiati *et al.* (2008)

expression or downregulation of aquaporins in both guard cells and mesophyll cells. Aquaporins have been implicated in the control of stomatal aperture and have been proposed to play a role in CO_2 movement within guard cells. Given the availability of these tools, improving stomatal function and WUE through genetic manipulation presents itself as a viable option.

The role of sugars in guard cell function has also been heavily studied; however, the contrasting results remain controversial with regards to the origins of sugars in guard cells. The promoter of *KST1*, a guard cell-specific promoter (Muller-Rober *et al.*, 1995), has been used to drive expression of HXK in tomato guard cells. Net photosynthesis of the transformed *Solanum lycopersicum* plants remained unaffected, as well as plant growth, whereas transpiration was reduced relative to the wild-type control plants (Fig. 2). This improved the instantaneous water use efficiency (IWUE) of the plants (unpublished results). These results demonstrate that exclusive expression of HXK in guard cells might be an efficient way to improve the WUE of plants.

In this review, we have briefly discussed the role of ion channels in guard cell movements (for a more in depth review, see Negi *et al.*, 2014) and the current literature implies that a complex interaction between ion channels, sugars, malate and photosynthesis exists indicating that complex genetic changes touching on each of these areas could be used to alter guard cell–mesophyll coordinated responses and potentially improve WUE (see Lawson & Blatt, 2014). The role of some individual ion channels has been clearly elucidated even if the complex interactions of these channels have still to be clearly defined. Nevertheless, ion channels provide a number of targets to be identified for genetic manipulation to alter stomatal function or responses and therefore the link between stomatal behaviour and mesophyll photosynthesis. For example, it has been shown that disruption of GORK activity

in the mutant *gork-1*, results in the impairment of stomatal closure (Ache *et al.*, 2000; Hosy *et al.*, 2003). The loss of GORK activity also led to increased water consumption consistent with enhanced stomatal apertures. The GORK ion channels presents itself as an ideal candidate for manipulation and the over-expression of GORK could significantly impact guard cell function. However, GORK functions in tandem with the voltage-dependent K^+_{in} channels, such as KAT1 mediating potassium flow during guard cell opening and closing cycles. Although the over-expression of KAT1 had no effect on stomatal opening under experimental conditions (Wang *et al.*, 2014), disruption of KAT1 resulted in a more than 50% reduction in K^+_{in} conductance (Szyroki *et al.*, 2001). Gene stacking of GORK and KAT1 as a single expression cassette presents an interesting case study on the role of potassium flow on GC function. Furthermore, another ion channel mutant, *slac1* exhibits a reduced rate of stomatal opening in response to light, low CO_2 and high humidity, three physiological stimuli known to strongly activate stomatal opening (Laanemets *et al.*, 2013a,b; Merilo *et al.*, 2013). The manipulation of SLAC1 channel function through over-expression of the SLAC1 protein could therefore potentially accelerate stomatal opening in response to light and other stimuli. These studies have highlighted that manipulation of stomatal behaviour is possible through manipulating ions channels; however, what is still unknown is the role mesophyll signalling may play in these responses and activation of such ion channels.

In this review we have touched upon some of the key features regulating guard cell–mesophyll interactions. It is clear from the available studies that these relationships are complex involving multiple mechanisms and signalling responses. Therefore, an integrated approach will allow us to unravel the key components of each of these pathways and determine
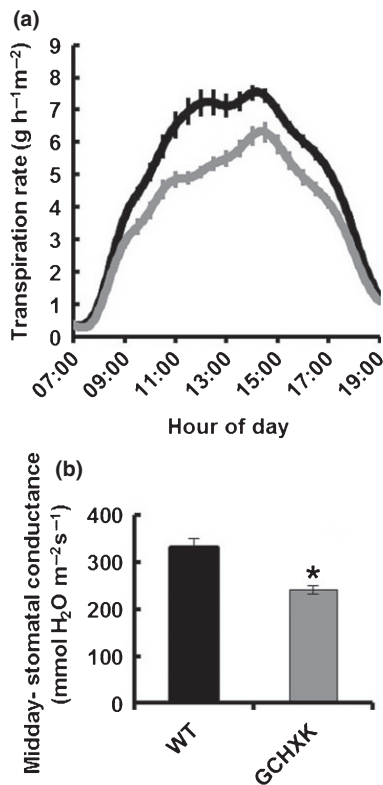
Fig. 2 Guard cell-specific expression of hexokinase reduces whole-plant transpiration and increases water-use efficiency (WUE) in *Solanum lycopersicum*. (a) The rates of transpiration, normalized to the total leaf area were monitored simultaneously and continuously throughout the day in wild-type (WT, black line) and transgenic plants expressing HXK specifically in guard cells (GCHXK, grey line) using the lysimeter system as described in (Kelly *et al.*, 2013). Data points (recorded every 3 min) are given as means of eight independent biological repeats. SE is displayed every 30 min. When not seen, SE is smaller than the symbol. (b) Stomatal conductance was monitored simultaneously and continuously throughout the day and was averaged between 11:00 h and 13:00 h to determine the midday stomatal conductance. Significant difference (*t*-test): *, $P < 0.01$.

how they inter-relate, providing possible potential targets for future manipulation.

Acknowledgements

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