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Stomatal responses to light and CO_2 in Epidermal Peels and Intact Leaves of C3 Plant of Broad Bean (*Vicia faba)* Species.

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Abstract
Stomata plays a vital role in gaseous exchange therefore, understanding the signal mechanisms in response to changing environmental conditions is critically important for sustainable agriculture that will require a major reduction in plant water use due to climate change. The effect of irradiance and CO_2 concentrations on mesophyll photosynthesis and stomatal conductance were conducted by physiological analysis using the novel epidermal peel experiment and infra-red gas analysis to monitor stomatal responses to dynamic environmental changes in the epidermal peel and the whole plant of *Vicia faba*. The approach used presented findings in the guard cell metabolism that possibly coordinate mesophyll CO_2 demands with stomatal behaviour towards crop improvement. Result showed that guard cell plays a role in stomatal function even though the degree of responsiveness is slower than when the mesophyll is present. More opportunities await guard cell exploitations for better crop improvement.

Keywords: Guard cells; Stomatal conductance; Gas exchange; Light; CO_2

Introduction

Climate change is predicted to lead to greater temperatures and reduced water availability which is anticipated to double by the end of this century (Keeling, Kortzinger, & Gruber, 2010; Keenan et al., 2014). Global surface temperature is predicted to rise by 2.6–4.8°C by the end of this century according to IPCC, 2013. This increase in temperature will lead to increases in evaporative demand which in turn will decreases soil moisture and subsequently results in reduced water availability for plants (Anderegg, Anderegg, Abatzoglou, Hausladen, & Berry, 2013; Park Williams et al., 2012). This subsequently may result in severe damages to crops which results in major yield losses (Zhu & Assmann, 2017). It has been reported that reduce water to plants causes more yield losses than any other single biotic or abiotic factor globally (Boyer, 1982). Therefore, in order to maintain crop productivity to feed the growing population, it has become necessary to develop new crop varieties or identify potential targets for manipulating plants for improved water use and productivity. Stomata and their behaviour have affected global fluxes of CO_2 and H_2O, with an estimated 300 x 10^{15} g of CO_2 and 35 x 10^{18} g of H_2O vapour passing through stomata of leaves every year (Hetherington & Woodward, 2003). As stomatal conductance determines the flux of gases between the inside of the leaf and the external atmosphere and therefore greatly influence photosynthetic carbon assimilation and water use, stomata are potential unexploited targets. A great deal of knowledge related to the structure, development, and physiology of stomata have so been acquired (Bergmann & Sack, 2007; Berry, Beerling, & Franks, 2010; Buckley, 2005) nevertheless, there are still many unanswered questions regarding signalling pathways, osmoregulation and the co-ordination between stomatal behaviour and mesophyll photosynthesis. For an optimal plant productivity and water use efficiency (defined at the leaf level as the ratio of carbon gained relative to water lost) stomata must open and close to environmental stimuli and internal signal to balance CO_2 uptake for photosynthesis (*A*) with water lost through transpiration (*E*) for optimal plant productivity and plant water use efficiency (WUE = *A*/E (Lawson & Blatt, 2014). A strong correlation between mesophyll photosynthesis (*A)* and stomatal conductance (g_s) has often been observed (Messinger, Buckley, & Mott, 2006; S. Wong,

Cowan, & Farquhar, 1979); however, the underlying mechanisms or possible signals that promote this relationship are not entirely understood and are currently being studied (Tracy Lawson & Michael R Blatt, 2014; Lawson, Simkin, Kelly, & Granot, 2014a; Lawson & Weyers, 1999; Terashima, Tang, & Muraoka, 2016). In view of this, the epidermal peel experiments provides a way to assess stomatal responses in the epidermis relative to intact leaves in order to find a possible mechanism coordinating stomata with mesophyll resulting in both improvement of WUE and plant productivity.

Stomatal response to environmental factors and internal cues

Stomata have a complex signal transduction networks which enables them to respond to endogenous and environmental signals promoting opening and closing of the stomatal pore within time scales of seconds to hours (Assmann & Jegla, 2016; Assmann & Wang, 2001).The balance between CO_2 uptake and transpiration rate depends on stomatal responses to these factors and is important for synchronizing stomatal behaviour relative to mesophyll demands for CO_2. Stomatal behaviour is influenced by variables such as light, [CO_2], humidity, pathogens, abscisic acid (ABA) and temperature (Kim, Goins, Wheeler, & Sager, 2004; Vavasseur & Raghavendra, 2005). Stomata respond rapidly and reversibly to both light and intercellular CO_2 concentration (C_i) (T. Fujita, K. Noguchi, & I. Terashima, 2013). Stomatal responses will therefore be focused on light and intercellular CO_2 because of their importance to photosynthesis.

Stomatal responses to light and [CO_2]

Stomata respond to light through the activation of pigments and photoreceptors. This happens through the absorption of light by these pigments in the guard cell chloroplasts resulting in proton extrusion thereby activating a specific plasma membrane proton pump and causing membrane hyperpolarization. This leads to ion transport K^+ influx and subsequent swelling of the guard cells resulting in stomatal opening (Shimazaki & Zeiger, 1985).

Stomatal conductance is also mediated by the CO_2 concentration inside the leaf (internal CO_2 concentration, C_i (Mott 1988) which is also determined by the photosynthetic rate. As a result, increased photosynthetic activities decrease C_i to which stomata respond by opening (Assmann & Shimazaki, 1999; K. A. Mott, 1988). Stomatal opening is stimulated when the internal CO_2 concentrations decreases for example, with increasing A, whilst increasing C_i induces stomatal closure (Assmann & Shimazaki, 1999; Mansfield, Hetherington, & Atkinson, 1990). This stomatal response to C_i has often been assumed to be the mechanism that co-ordinates A and g_s in order to balance CO_2 uptake to optimising mesophyll carbon demands without unnecessarily losing excess water (Lawson, 2009; T. Lawson & M. R. Blatt, 2014; K. A. Mott, 1988). It is assumed that stomata respond to a constant ratio of atmospheric CO_2 to C_i of about ⅔ atmospheric CO_2 (Mott, 1988). the challenge however is whether this signal is sensed directly by guard cells and/or by the mesophyll (Assmann & Shimazaki, 1999; Lawson, von Caemmerer, & Baroli, 2011) or whether a combination of the two contributes to the response.

Stomata also respond to changes in turgor pressure within the guard cells as a result of the accumulation or loss of potassium (K^+) ions and organic solutes such as malate or sucrose. Guard cells that regulate stomata also contain chloroplasts capable of performing electron transport (Lawson, Oxborough, Morison, & Baker, 2002, 2003) which could provide a source of energy (ATP) to drive stomata open or use this energy for CO_2 fixation leading to the production of sucrose as an osmoticum for stomatal movements. The fact that stomatal responses to light intensity and changes in CO_2 concentration in guard cells of detached leaf epidermis to be different from those in the intact leaves (Takashi Fujita, Ko Noguchi, & Ichiro Terashima, 2013; J. Lee & Bowling, 1992; Keith A Mott, Sibbernsen, & Shope, 2008; Schwartz, Ilan, & Grantz, 1988) suggesting that presence of the mesophyll greatly influences stomatal behaviour (J. Lee & Bowling, 1992; Keith A Mott et al., 2008; S. Wong, Cowan, IR, Farquhar GD, 1979). As a result, approaches have been made in an effort to unravel the mechanisms coordinating stomatal responses with mesophyll photosynthesis using a variety of approaches.

The epidermal peel-mesophyll transfer (Keith A Mott, Berg, Hunt, & Peak, 2013; Keith A Mott et al., 2008) is one such approach designed to unravel the influence or signals from the mesophyll that may play a role in coordinating stomatal behaviour with mesophyll demand for CO_2. Epidermal–mesophyll

transfer experiment, rely on removing the epidermis from one plant and placing it on mesophyll (with the epidermis removed) of another plant type or that of the same plant type but in which the mesophyll has been subjected to different environmental conditions (Keith A Mott et al., 2013; Keith A Mott et al., 2008). This novel experimental approach was the basis of this study in order to assess stomatal responses in the epidermis (where the under lying mesophyll influence is removed) and comparing it with the epidermis of a plant grafted on top of mesophyll of another plant. The aim was to determine whether the guard cells themselves have a mechanism that co-ordinate stomatal response with that of the mesophyll. The epidermal peel-mesophyll transfer approach exploited the photosynthetic pathways of the C3. In C3 plants, stomata open with light during the day and close at night or under conditions of high [CO_2]. Therefore using the peel and mesophyll from this plant type provides an ideal experimental platform to probe the influence of the mesophyll on stomatal behaviour. The experimental setup provides an additional tool kit for exploring the influence of mesophyll photosynthesis on stomatal behaviour.

Guard cell chloroplasts

Photosynthesis takes place primarily in the mesophyll tissue however, guard cells, which developed from protodermal cells, also contain photosynthetically active chloroplasts in most species (Kiyoshi Gotow, Scott Taylor, & Eduardo Zeiger, 1988; Outlaw, Mayne, Zenger, & Manchester, 1981; Shimazaki, Gotow, & Kondo, 1982; Zeiger, Armond, & Melis, 1981; Zemel & Gepstein, 1985). Guard cell chloroplasts has thylakoid network and chlorophyll contents but also has functional photosystems I and II, electron transport, oxygen evolution and photophosphorylation (Gotow, 1998; Lawson et al., 2002). Calvin cycle activity has also been demonstrated in guard cells. The role of guard cell chloroplasts and the amount and importance of guard cell photosynthesis is a controversial topic with different reports in different species and from different laboratories. This could be due to the complex and functional plasticity of guard cell osmoregulation and signalling pathways coupled with the possible multiple roles for which they vary depending on conditions or time of the day (Zeiger, Talbott, Frechilla, Srivastava, & Zhu, 2002). Therefore elucidating the role of the guard cell chloroplasts and guard cell photosynthesis presents a serious challenge for researchers in the field.

There have been several conflicting reports in the literature concerning the capacity of photosynthetic carbon reduction in guard cell chloroplasts and its importance in stomatal function (Shimazaki et al., 1982). Early studies provided little evidence of Calvin cycle activity in guard cells and demonstrated that CO_2 is incorporated as malate (K Raschke & Dittrich, 1977). Subsequent experiments demonstrated that guard cell chloroplasts lacked ribulose-1,5-bisphosphate carboxylase (RuBPC) and ribulose-5-phosphate kinase (Ru5PK) activity (Outlaw & Manchester, 1979) and other key enzymes (Outlaw & Manchester, 1979; Schnabl, 1981) for the photosynthetic carbon reduction pathway. These findings concluded that there was insignificant Rubisco activity, confirming the conclusion of Hampp, Outlaw, and Tarczynski (1982) that photo-reduction of CO_2 by guard cells was absent. Despite these findings of lack of carbon fixation in the guard cell chloroplasts, studies in the last two-three decades have shown that photosynthetic carbon fixation takes place in the guard cells. For instance, studies have shown that guard cells contain Rubisco (Madhavan & Smith, 1982; Zemel & Gepstein, 1985) and several other key Calvin cycle enzymes (Gotow, 1998; Shimazaki & Zeiger, 1985). Chlorophyll fluorescence measurements in guard cells of intact leaves (Lawson et al 2001; 2002) and epidermal peels have shown distinct features associated with Calvin cycle activity (Melis & Zeiger, 1982). The demonstration of photorespiration and CO_2 fixation in single guard cells by chlorophyll fluorescence kinetics measurement (Cardon & Berry, 1992), the dichlorophenyl dimethyl urea (DCMU) sensitivity of sucrose accumulation in guard cells from sonicated epidermal peels incubated under red light (Matthew Poffenroth, David B Green, & Gary Tallman, 1992) indicated the ability of the guard cell chloroplasts to fix carbon. Zeiger et al, (2002) also detected significant Calvin cycle activity and demonstrated that it was osmotically important without the breakdown of starch (Talbott & Zeiger, 1993; Tallman & Zeiger, 1988) for stomatal function. Carbon dioxide uptake into 3-PGA and ribulose 1,5-bisphosphate along with evidence for guard cell production of sucrose during red light-induced stomatal opening in *V. faba*, where no starch breakdown was observed and sugar import was ruled out as a result of the use of epidermal peels (K.

Gotow, S. Taylor, & E. Zeiger, 1988; M. Poffenroth, D. B. Green, & G. Tallman, 1992; Talbott & Zeiger, 1993; Tallman & Zeiger, 1988). In the last few decades, more evidence for guard cells carbon reduction has been published. For instance, tobacco plants with reduced levels of Rubisco had been found with substantially low photosynthetic capacity and with stomatal responses to light and changing [CO_2] similar to those of the wild type (I. Baroli, G. D. Price, M. R. Badger, & S. von Caemmerer, 2008; Susanne von Caemmerer et al., 2004).

All the above have demonstrated that guard cell photosynthesis exists and either supplies the energy for the proton pumps and/or produces solutes that contributes to guard cell osmoregulation of stomatal behaviour, however more further studies using recent advances in molecular technology, such as cell-specific promoters are highly needed to fully elucidate the role of guard cell photosynthesis in stomatal function (Lawson, 2009; Lawson and Blatt, 2014).

The overall aim of the work was to determine the influence and presence of mesophyll on stomatal function and behaviour in *Vicia faba*. The approach used epidermal peels from plant material either in isolation or grafted onto mesophyll from the same plant and subjected these to changes in light intensity and CO_2 to assess stomatal response. Infra-red gas analysis was used to examine the effect of irradiance and CO_2 concentrations on mesophyll photosynthesis as well.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of broad bean *Vicia faba L*. (long pod) from the University of Essex, UK were planted in pots containing commercial potting soil (seed and modular compost plus sand, (Levington F2, Fisons, Ipswich, UK). The compost contained the following nutrient composition N:144; P:73; K:239. Plants of *V. faba* were grown in a controlled growth chamber (F1-totron PG660, Sanyo Gallenkamp Plc) at 64% humidity and temperature 23-24°C under photoperiod of 12hrs PPFD (380-400 µm of light) using halogen quartz iodide lamps (Powerstar HQ1-TS 250 W/NDL, Osram, Munich) and 12 hrs of darkness at 19°C for 1-5 weeks and watered 2-3 times weekly. Two growth chambers were used for growing the plants. One chamber was programmed for the lights to come on at 9 am and switched off at 9 pm. Using both these chambers enable plant material to be selected from the complete diel period.

Peeling method of plant material and incubation medium

Fully expanded leaves were excised from the plant and placed on a glass slide and cut with a razor blade into lamina strips. A tab was made on the lamina by cutting through the upper epidermis without damaging the lower epidermis. The leaf section was turned over and the epidermal strips were peeled manually from the lower surface (abaxial) using the tab of the leaf according to the method of Weyers and Travis, 1981. For measurements on isolated epidermal peels, the peels were placed directly in an incubation buffer. The incubation medium of 50mM KCL+10mM PIPES-KOH (pH 6.8) was prepared freshly each day (Weyers & Meidner, 1990). For epidermal-mesophyll experiments, peels were removed from the mesophylls and placed back onto mesophyll with the epidermis removed from a leaf of the same species. Control leaf segments were prepared from intact detached leaves cut into 1 x 1 cm square and the abaxial side viewed directly. All plant materials were kept hydrated with incubation medium and inside the chamber.

Experimental setup of the novel epidermal-mesophyll transfer

The sample chamber consisted of two aluminium blocks mounted on a purpose built microscope stage. An aperture on the top of the chamber was allowed entry of the long-distance objective (x40, Leica Ltd). A condom with the tip removed was mounted around the circumference of the aperture and attached to the objective to gas seal the chamber. Gas conditions in the chamber were controlled using a setup that consisted of two large cylinders (CO_2-free air) and (10% CO_2 air) passed through a mass flow controller (EL-flow, Bronkhorst high tech, New Market UK) used to provide air containing the selected CO_2 concentration at a flow rate of 200 ml min^{-1}. Licor (L1-820, Licor Biosciences, Lincoln, Nebraska) was used to monitor the concentrations of CO_2 inside the incubation chamber as well. Chamber temperature was controlled by a circulated cooler that maintained the temperature at 23°C. The plant material was placed in the chamber and subjected to different light intensity of either 0 µmol $m^{-2}s^{-1}$ and 400 µmol $m^{-2}s^{-1}$ and CO_2 concentration was maintained at either (120 µmol mol^{-1} and 650 µmol

mol^{-1}). The protocol used were as follows : light intensity of PPFD (400 µmol m^{-2} s^{-1}) + [CO_2] (120 µmol mol^{-1}) were first applied for a period of 1 hour with continuous monitoring and recording of stomatal sizes (after 5-10 mins) after which it was changed to darkness + [CO_2] of 120 µmol mol^{-1} for another 1 hour, and then returned back to the initial conditions of PPFD (400 µmol m^{-2} s^{-1}) + (120 µmol $mol^{-1)}$ for an hour. Again light intensity of PPFD (400 µmol m^{-2} s^{-1}) + high [CO_2] of 650 µmol mol^{-1} were applied for another hour then finally, PPFD (400 µmol m^{-2} s^{-1}) + [CO_2] of 120 µmol mol^{-1}were applied for the last hour.

Stomatal apertures were measured using a (Leica DMRX Leitz 567030 Wetzler Germany) with a long focal objective lens (50X). The epidermal peels were kept from drying out by placing a tube connected to a syringe and inserted into the chamber and buffer dropped into the system at a rate of 1 drop per min. Digitalised photo images of stomata were obtained using image-analysis hardware and software (Bresser Microcam 5.0MP GmbH & Co Gutenberger Rhede-Germany) connected to a computer where the measurements were recorded every 5-10 min over the 5-7 h measuring period.

Gas exchange experiments

Attached leaves from whole plants of *Vicia faba* were used to measure carbon assimilation (*A*) and stomatal conductance *(g_s)* in response to change in light and CO_2 concentrations following a similar protocol used for the epidermal peel transfer experiments. Measurement were made using a portable gas exchange system (CIRAS 2, PP systems, Hitchin, Hertsfordshire,UK) and recorded every 1 min for 5 h using the same protocol as the one in the experimental set up.

Statistical Analysis

The data are shown as means of ± SE of five independent experiments and differences between means were analysed using the student t-Test: sample for means.

RESULTS

Diel stomatal responses in isolated epidermis and whole leaves

Diel stomatal behaviour were assessed in both isolated epidermal peels incubated in buffer and whole attached plant leaves using gas exchange in the C3 plant (*Vicia faba*).

Diel stoma behaviour in C3 plant

The diel stomatal response in C3 epidermal peels isolated from the mesophyll were determined by measuring the aperture of individual stoma (taken from new peel at each measurement over the course of a day. The stomatal response in *Vicia faba* over the diel period (Fig.1.) displayed a typical and expected pattern of stomatal behaviour, consistent with previous observations in many C3 plants. Stomata opened in the light during the diurnal period and closed in the dark. Stomatal aperture increased through the diurnal light period. When the light was switch off at the end of the diel period stomatal aperture reduced rapidly reaching a minimum value of 7.6 µm almost immediately which was generally maintained through the nocturnal period albeit with some fluctuation in the aperture.

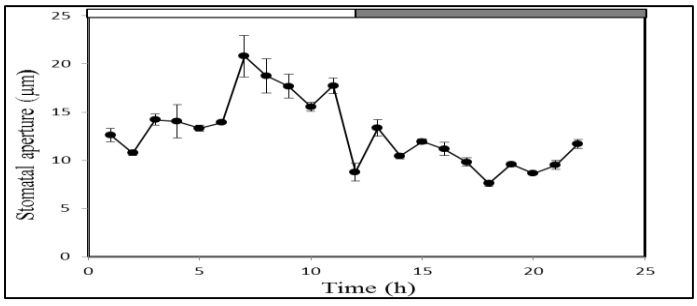

Figure 1. Diel stomatal aperture in *Vicia faba.*

Measurements were taken from individual stoma of a leaf segment of biological replicates. The leaf sections placed in a specially designed gas chamber to control the conditions during measurements. Chamber conditions maintained at [CO_2] of 400 µmol mol^{-1} and photosynthetic photon flux density (PPFD) of 400 $µmol^{-2}s^{-1}$. Stomatal

measurement were taken every hour. White and black boxes represent light and dark respectively. Values are means of five replicates (± SE).

Diel whole leaf gas exchange in C3 plant

The diel whole leaf gas exchange measurements enabled both CO_2 assimilation rate (*A*) and stomatal conductance (g_s) to be determined in *Vicia faba* (C3). In *Vicia faba* (C3) (Fig. 2), photosynthetic carbon fixation rate (Fig. 2a) increased during the diurnal period reaching values of *Ca*. 8 and 12 µmol m^{-2} s^{-1} in the reverse and standard illumination cabinet respectively. In the dark/reverse cabinet, this rate was maintained for the majority of the diurnal period, however *A* in the light/standard illumination cabinet (Fig. 2c) initially was greater than that of the dark/reverse growth cabinet and decreased half way through the diurnal period dropping to a value similar to the dark/reverse cabinet around 8 µmol m^{-2} s^{-1}. Stomatal conductance follows a similar pattern, increasing the light to a maximum value of about 300-400 mmol m^{-2} s^{-1} at the start of the light period before gradually decreasing throughout the diurnal period (Fig b &d). However, in the dark, as expected no photosynthetic activity took place and stomatal conductance remained relatively low with an average nigh-time stomatal conductance of 40 mmol m^{-2} s^{-1}. Similar patterns of g_s and *A* were observed in plant grown in either the standard or reverse light cabinets illustrating that the different cabinet did not affect the growth of the plants or the diel pattern of gas exchange which also showed the coordination between *A* and g_s.

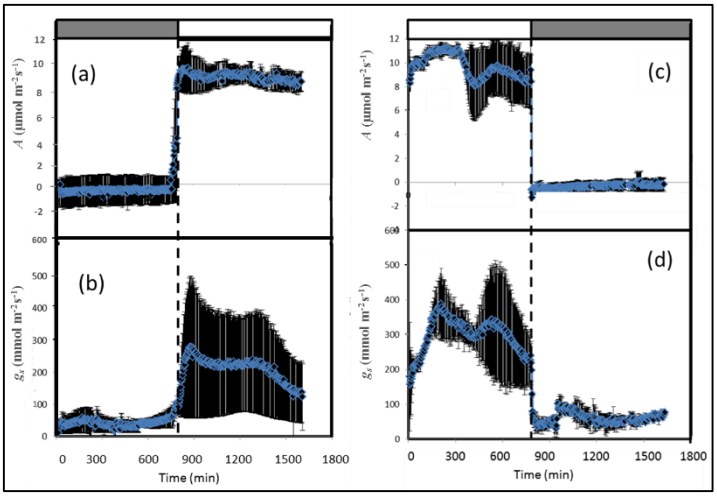

Figure 2. Diel whole leaf gas exchange measurements of *Vicia Faba*

(a) Photosynthetic carbon assimilation rates and **(b)** stomatal conductance in reverse cabinet and **(c)** Photosynthetic carbon assimilation rates and **(d)** stomatal conductance in standard cabinet of *Vicia faba* plants. The chamber conditions maintained [CO_2] of 400 µmol mol^{-1} and photosynthetic photon flux density (PPFD) of 400 $\mu mol^{-2}s^{-1}$. White boxes represent light (400 µmol $m^{-2}s^{-1}$) while grey boxes represent darkness. Dotted lines separate light and dark period. Data are means of five replicates (± SE).

Functional stomatal responses to changes in light and [CO_2] in isolated epidermal peel, epidermal-mesophyll transfer, detached and whole leaf gas exchange

Although guard cells can photosynthesize on their own, the accumulation of mesophyll-derived metabolites act as signals which contribute to the regulation of stomatal movement (Daloso et al., 2017; J. Lee & Bowling, 1993; Keith A Mott et al., 2013; Keith A Mott et al., 2008). It has long been hypothesized that the breakdown of starch, sucrose and lipids is an important mechanism during stomatal opening, which may aid in the production of ATP through glycolysis. Accumulation of osmolytes such as sugars and malate have been suggested to acts as signaling components that connect mesophyll photosynthesis with stomata or guard cell behaviour (K. Gotow et al., 1988; M. Poffenroth et al., 1992; S. Wong et al., 1979).

Funtional stoma response in epidermal peel, epidermal-mesophyll transfer and detached leaf in Vicia faba,

*S*tomatal function in responses to light and [CO_2] were examined in epidermal peels in which the influence of the mesophyll has been removed and in material in which peels have been grafted onto mesophyll tissue from the same plant species (Fig 3.)

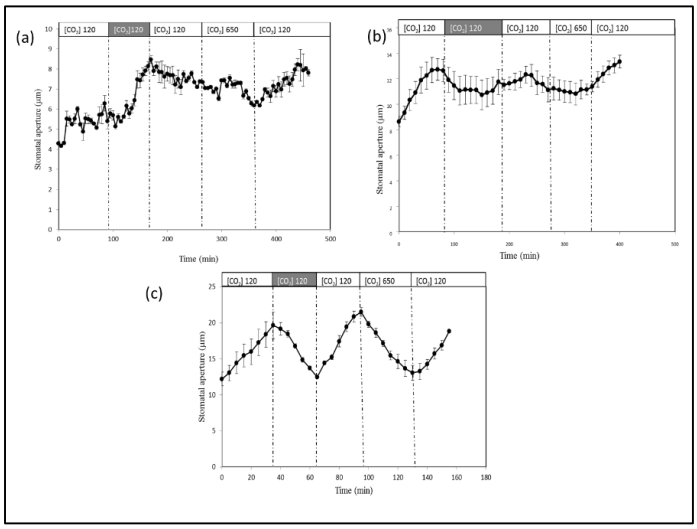

Figure 3. Stomatal response to change in PPFD and CO_2 concentration in C3

(a) Epidermal peel of *Vicia faba;* **(b)** epidermal-mesophyll experiment of *Vicia faba* strip grafted on *vicia faba* mesophyll and (**c**) whole detached leaf of *Vicia faba*. Stomata responded significantly to all conditions as indicated in the graph **(c).** The chamber conditions were maintained at the following: light/ [CO_2] of 120 µmol mol $^{-1}$: Dark/[CO_2] of 120 µmol mol $^{-1}$ light/ [CO_2] of 120 µmol mol $^{-1}$: light/[CO_2] of 650 µmol mol^{-1} and light/ [CO_2] of 120 µmol mol^{-1}. White and dark boxes represent light (400 µmol $m^{-2}s^{-1}$) and dark respectively. Dotted lines represent change from one light level/[CO_2] to another. Data are means of five replicates (± SE).

Fig. 3.a shows the result of an isolated epidermal peel of *Vicia faba.* Stoma opened with 400 µmol $m^{-2}s^{-1}$ light and a [CO_2] of 120 µmol mol^{-1} reaching a maximum aperture of about 9.0µm. When light was turned off aperture decreased significantly to 3.9 µm by the end of the dark period. Stomatal aperture was restored to about 6. 0 µm when light turn back on. When [CO_2] was increased a small decrease in aperture was observed which increased when CO_2 was returned to the low concentration of 120 µmol mol^{-1}.

The stomatal behaviour in a detached leaf of *Vicia faba* (Fig.3.c,) clearly demonstrates large stomatal responses to all the conditions of light and darkness, low and high CO_2 concentrations. Stomata within two and a half hours demonstrated the pattern shown in Fig.3c due to the rapid response to change in light and CO_2 concentrations (opening and closing respectively). Within 30-35 min, stomata reached a significant aperture of 19.6µm and responded to darkness by reducing significantly aperture to 12.4µm. When the light was resumed, stomata opened reaching an aperture of 21.4 µm after 90 min. When [CO_2] was increased to 650 µmol mol^{-1} stomatal aperture decreased to 13.0 µm. Changing [CO_2] back to the original level of 120 µmol mol^{-1} resulted in a rise in aperture significantly to 18.8 µm. Fig. 4 shows the stomatal movement during the detached leaf experiment.

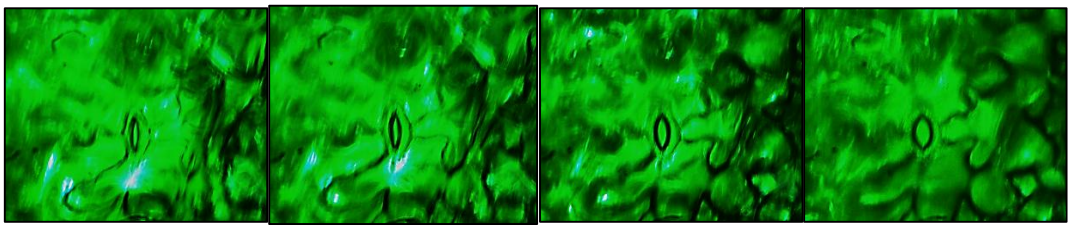

Figure 4. Influence of mesophyll to individual stoma opening.

Slides of increasing stoma opening in the detached leaf of *Vicia Faba* after every 5 mins (refer to Fig 3c above) in controlled environment chamber. Condition were maintained at PPFD of 400 µmol $m^{-2}s^{-1}$ and [CO_2] of 120 µmol mol^{-1}.

Stomatal and photosynthetic responses in Vicia faba plants in response to changes in light and CO_2 concentrations

Functional whole leaf plant gas exchange analysis was performed on the same plants used above in the epidermal peel-mesophyll experiment in order to assess if g_s responded in a similar manner as individual stoma. Carbon assimilation (*A*) in the light/standard cabinet (Fig. 3.9a) where plants were subjected to 12 h of dark prior to the gas exchange measurements ranged between 3.5-3.9 µmol CO_2 $m^{-2}s^{-1}$ (highest value) at [CO_2] of 120 µmol mol^{-1} $m^{-2}s^{-1}$ and in the light while the lowest value was around -1.8 µmol CO_2 $m^{-2}s^{-1}$ in the dark. However, when [CO_2] was increased to 650 µmol mol^{-1} $m^{-2}s^{-1}$, as expected *A* increased, reaching about 16.6 µmol CO_2 $m^{-2}s^{-1}$.

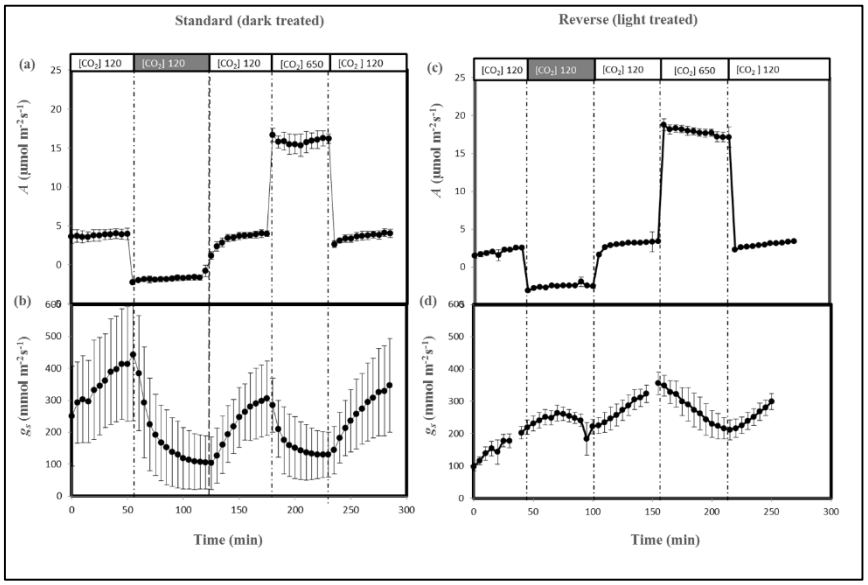

Figure 5. Gas exchange measurements in *Vicia faba*

(a) Photosynthetic carbon assimilation rates and (b) stomatal conductance of *Vicia faba* subjected to 12 hr darkness prior to experiment from the standard cabinet while (c) carbon assimilation and (d) stomatal conductance subjected to 12 hr light from the reverse cabinet in response to changes in Photon flux density (PPFD) and [CO_2] as indicated. The chamber conditions were maintained at light/ [CO_2] of 120 µmol mol^{-1}: Dark/[CO_2] of 120 µmol mol^{-1}: light/ [CO_2] of 120 µmol mol^{-1}: light/[CO_2] of 650 µmol mol^{-1} and light/ [CO_2] of 120 µmol mol^{-1}. White and dark boxes represent light (400 µmol $m^{-2}s^{-1}$) and dark respectively. Dotted lines represent change from one light level/[CO_2] to another. Data are means of five replicates (± SE).

Similarly, in the dark/reverse cabinet (Fig. 5 c&d) where plants were subjected to 12 h of light prior to the gas exchange measurements. *A* range between 2.5-3.3 µmol (highest value) at [CO_2] of 120 µmol mol^{-1} $m^{-2}s^{-1}$ and in the light while lowest value was around to -2.4 µmol CO_2 $m^{-2}s^{-1}$ in the dark. Increasing [CO_2] to 650 µmol mol^{-1} increased *A* to

18.7 µmol $m^{-2}s^{-}$ (Fig 5c). Stomatal conductance (g_s) in both cabinets responded very similar and mirrored the pattern of *A*. Although the pattern in behaviour are similar, there are situation where it is obvious that g_s responded to changes in light intensity and [CO_2] more slowly or with lags that resulted in a non-coordinated response. For example the increase in g_s in the first phase (initial 50 min at the

beginning) of the response in Fig. 5 b, whilst *A* remains stable (Fig. 5 a), had reached its steady state, g_s was still increasing showing that the diel correlation between *A* and g_s can be perturbed by modulation of the length of light or dark phases as experienced in the short term (Hennessey & Field, 1991). It is also interesting to note that g_s continued to increase even while in the dark (Fig. 5d).

Discussion

The coordination between stomatal conductance and mesophyll photosynthesis is important to help maximise WUE and plant productivity (Lawson, Simkin, Kelly, & Granot, 2014b). However, what is not clear is the mechanism(s) that co-ordinate g_s and *A* and the influence of the mesophyll on stomatal behaviour in this co-ordination. To date it has been assumed that the concentration of CO_2 inside the leaf (C_i) is maintained at a constant ratio between the concentration of CO_2 inside the leaf and that of the surrounding atmosphere C_i:C_a ratio; (Mott et al., 1988) and this drives a co-ordinated response of g_s with *A*. However, several studies have suggested that stomatal responses to C_i are too small to account for the large changes in g_s that have been observed in response to light (Farquhar & Raschke, 1978; Farquhar & Sharkey, 1982; Klaus Raschke, 1975; Sharkey & Raschke, 1981). Additionally several studies have reported stomatal response to changing light intensity even when C_i is held constant (Lawson, Lefebvre, Baker, Morison, & Raines, 2008; Messinger et al., 2006; Wang & Song, 2008). Further evidence against a C_i mechanism that co-ordinates g_s with *A* is from studies that have examined stomatal behaviour in transgenic plants with reduced levels of photosynthesis and shown stomatal responses to changing light despite high C_i values (Irene Baroli, G Dean Price, Murray R Badger, & Susanne von Caemmerer, 2008; Lawson et al., 2008; Susanne von Caemmerer et al., 2004). Take together all of these studies suggest that C_i cannot be the only signal that co-ordinates g_s with *A*. Several reports have suggested a mesophyll driven signal is responsible for the co-ordination between *A* and g_s. Chloroplastic ATP, NADPH and RuBP have all been proposed to be the possible candidates for the mesophyll signal (J. Lee & Bowling, 1992; Tominaga, Kinoshita, & Shimazaki, 2001; S. Wong et al., 1979; Zeiger & Zhu, 1998). Other possibilities include malate and sugars transported from the mesophyll which is highlighted later in this section.

In this study, a comparison between stomatal responses to changing light intensity and [CO_2] were examined in epidermal peels of C3 (*Vicia faba*) which opens in the light and closes stomata in the dark. As it is well-established that circadian rhythms influence stomatal behaviour (Dodd et al., 2005) diurnal measurements of stomatal aperture from epidermal peels (Fig. 1 and gas exchange measurements of g_s (Fig. 2) showed that there were diel patterns of behaviour in stomatal behaviour. However, stomatal aperture changed little during the initial 6h of the diurnal period in *Vicia faba* which means that there would be little influence on circadian driven changes in the stomatal aperture in the subsequent epidermal mesophyll transfer experiments. The similar diel patterns of behaviour between peels and intact plants confirm that the protocol used for isolating and measuring the epidermal peels was appropriate and stomata responded similar to intact leaves.

Differences in stomatal responses to various environmental stimuli (including light intensity and CO_2 concentration) in epidermal strips and intact leaf have been reported previously in literature (J.-S. Lee & Bowling, 1995; Travis & Mansfield, 1979; CM Willmer & Dittrich, 1974). For instance Schwartz et al. (1988) and Travis and Mansfield (1979) demonstrated that although stomata in epidermal peels were able to open in response to light intensity and low [CO_2] the change in stomatal aperture was much less than that observed in intact leaves. However, others have argued that stomata in isolated epidermal peels do not respond to light and [CO_2] in the guard cell (Takashi Fujita et al., 2013; J. Lee & Bowling, 1992; Keith A Mott et al., 2008), suggesting an important role of the mesophyll in stomatal response. In response to changes in light intensity and [CO_2], stomata in epidermal peels of *Vicia faba* (Fig.3a), responded, albeit with a lesser magnitude of change and reduced sensitivity, as expected and similar to the intact leaf (Fig. 3.c), agreeing with reports that stomata respond and function when in isolation, (Meidner & Mansfield,

1968; Outlaw et al., 1981; Webb, McAinsh, Mansfield, & Hetherington, 1996; Colin Willmer & Fricker, 1996), but showed different responses than in intact leaves and the magnitude and/or speed of the stomatal responses are not the same as when the mesophyll is present (Schwartz et al. (1988) Travis and Mansfield (1979). Wang et al., (2014) found lower guard cell ATP levels in isolated epidermis compared with intact leaves, which they accredited to the weaker stomatal opening response to white light in the epidermis. Their results provide evidence that both guard cell chloroplasts and mesophyll contribute to the ATP source for H^+ extrusion by guard cells for osmoregulation.

The dampened stomatal response to changing [CO_2] in the epidermal peels in *Vicia* compared with the intact leaves points to a role of C_i in these responses and signaling pathways (Mott 1988).

The measurements (Fig 3.c) on the detached whole leaf clearly demonstrated an excellent responses between g_s and A as well as rapid responses unlike those recorded on the isolated epidermis or the peels grafted onto mesophyll (Fig. 3b). These finding indicate that stomata are influenced by the underlying mesophyll, however this could simply be due to mesophyll photosynthetic consumption of CO_2 altering C_i leading to stomatal opening and closing in response to changes in light and [CO_2] or it could be that sucrose produced in the meosphyll plays a key role in stomatal osmoregulation (Kelly et al., 2013) synchronising stomata with mesophyll. It is also worthy to mention here that the response in the intact detached leaf aside from being very rapid in response to light and CO_2 changes, stomata failed to respond to either light nor [CO_2] at a certain point in the experiment (result not shown) which could possibly be due to an effort to balance or coordinate *A* and *gs* in order to check excess transpiration. This could probably be (in addition to C_i) due to accumulation of sucrose in the walls of the guard cells that were not translocated to other parts of the plant (since the plant was a detached one) hence the shutdown of the stomatal walls. This is an interesting finding in this study which seemed to be in line with the many hypothesised theories of a mesophyll-derived signal(s). Recalling again, several studies have argued that changes in C_i are often too small to account for the large changes that occurs in stomatal aperture which has even been demonstrated by stomatal responses to PPFD even when C_i is held constant (Lawson et al., 2008; Messinger et al., 2006; Wang et al., 2008). S. von Caemmerer and Griffiths (2009) has also demonstrated that high intercellular CO_2 is not the sole cause for stomatal closure during phase lll of CAM. Apoplastic sucrose has also been proposed to co-ordinate mesophyll photosynthesis with stomatal behaviour (Kang, Outlaw, Fiore, & Riddle, 2007). Lu, Outlaw Jr, Smith, and Freed (1997) implied the presence of multiple sucrose pools in mesophyll cells which are a localized mesophyll-apoplast region that exchanges with phloem and stomata, and mesophyll-derived sucrose in guard-cell walls which excess of it is able to diminish stomatal opening. Fujita et al, (2013) also suggested that stomata close as a result of high apoplastic sucrose concentration when mesophyll sucrose efflux exceeds translocation. When photosynthesis is high and sucrose production, exceed the capacity of the phloem to translocate the sucrose, the apoplast sucrose content increases and travels in the apoplast to the guard cells were the sucrose acts as an osmotic and reduces stomatal aperture (Kang et al., 2007) see review by Lawson et al, (2014).

Fig 5 illustrate stomatal and photosynthetic responses to the step changes in light intensity and [CO_2] in *Vicia* grown in either the light/standard cabinet or the dark/reverse cabinet. The identical photosynthetic responses in *Vicia* illustrate that the different 12h pre-treatment did affect the potential photosynthetic rates. However what was interesting was the fact that stomatal conductance was clearly co-ordinated with *A* in the plants grown in the light standard cabinet (with some indication of slow stomatal response limiting assimilation rates (Lawson & Blatt, 2014). Although g_s and A were also well co-ordinated in the dark/reverse cabinet grown plants in the end period of the measurements, g_s increase in with light but unexpectedly continued to increase even when the light was turned off suggesting that g_s could respond to darkness as well.

These data strongly suggest that the mesophyll photosynthetic behaviour plays a key role in stomatal behaviour (as seen in the epidermal peel and intact leaf) but indicates a C_i driven responses. More metabolite signal like the sugar can be evaluated in their response to stomatal responses. In conclusion, the main findings from this study are summarised as follows;

***C*i driven signal:** This was illustrated by the dampened stomatal responses to changes in [CO_2] and light in the isolated epidermal peels in *Vicia faba* compared to the detached whole leaf experiment in *Vicia* faba plants. Plants have demonstrated faster responses of g_s to *A* as a result of CO_2 consumption for mesophyll photosynthesis unlike those of the isolated epidermis or the peels grafted onto mesophyll which again points to the role of the C_i mediating *A* and g_s. These finding indicate that stomata are influenced by the underlying mesophyll to changes in light and [CO_2].

The role of apoplastic sucrose has also been proposed to co-ordinate mesophyll photosynthesis with stomatal behaviour (Kang et al., 2007) which can further be investigated.

Wang et al., (2014) found that stomata in the epidermal peels function to a lesser degree than when the mesophyll is present and proposed that the amounts of ATP required for the proton pumps for guard cell osmoregulation were lower in isolated epidermis which resulted in a decreased stomatal opening in response to white light, suggesting that both guard cell chloroplasts and mesophyll contributed to the required ATP for osmoregulation.

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