Stomatal Size, Speed, and Responsiveness Impact on Photosynthesis and Water Use Efficiency

Tracy Lawson* and Michael R. Blatt

School of Biological Sciences, University of Essex, Colchester CO4 3SQ, United Kingdom (T.L.); and Laboratory of Plant Physiology and Biophysics, University of Glasgow, Glasgow G12 8QQ, United Kingdom (M.R.B.)

The control of gaseous exchange between the leaf and bulk atmosphere by stomata governs CO₂ uptake for photosynthesis and transpiration, determining plant productivity and water use efficiency. The balance between these two processes depends on stomatal responses to environmental and internal cues and the synchrony of stomatal behavior relative to mesophyll demands for CO₂. Here we examine the rapidity of stomatal responses with attention to their relationship to photosynthetic CO₂ uptake and the consequences for water use. We discuss the influence of anatomical characteristics on the velocity of changes in stomatal conductance and explore the potential for manipulating the physical as well as physiological characteristics of stomatal guard cells in order to accelerate stomatal responses to environmental and internal cues and the synchrony of stomatal behavior relative to mesophyll demands for CO₂.

In order for plants to function efficiently, they must balance gaseous exchange between inside and outside the leaf to maximize CO₂ uptake for photosynthetic carbon assimilation (A) and to minimize water loss through transpiration. Stomata are the “gatekeepers” responsible for all gaseous diffusion, and they adjust to both internal and external environmental stimuli governing CO₂ uptake and water loss. The pathway for CO₂ uptake from the bulk atmosphere to the site of fixation is determined by a series of diffusional resistances, which start with the layer of air immediately surrounding the leaf (the boundary layer). Stomatal pores provide a major resistance to flux from the atmosphere to the substomatal cavity within the leaf. Further resistance is encountered by CO₂ across the aequous and lipid boundaries into the mesophyll cell and chloroplasts (mesophyll resistance). Water leaving the leaf largely follows the same pathway in reverse, but without the mesophyll resistance component. Guard cells surround the stomatal pore. They increase or decrease in volume in response to external and internal stimuli, and the resulting changes in guard cell shape adjust stomatal aperture and thereby affect the flux of gases between the leaf internal environment and the bulk atmosphere. Stomatal behavior, therefore, controls the volume of CO₂ entering the intercellular air spaces of the leaf for photosynthesis. It also plays a key role in minimizing the amount of water lost. Transpiration, by virtue of the concentration differences, is an order of magnitude greater than CO₂ uptake, which is an inevitable consequence of free diffusion across this pathway. Although the cumulative area of stomatal pores only represents a small fraction of the leaf surface, typically less than 3%, some 98% of all CO₂ taken up and water lost passes through these pores. When fully open, they can mediate a rate of evaporation equivalent to one-half that of a wet surface of the same area (Willmer and Fricker, 1996).

Early experiments illustrated that photosynthetic rates were correlated with stomatal conductance (gs) when other factors were not limiting (Wong et al., 1979). Low gs limits assimilation rate by restricting CO₂ diffusion into the leaf, which, when integrated over the growing season, will influence the carbohydrate status of the leaf with consequences for crop yield. Stomata of well-watered plants are thought to reduce photosynthetic rates by about 20% in most C3 species and by less in C4 plants in the field (Farquhar and Sharkey, 1982; Jones, 1987). However, even this restriction has been shown to impact substantially on yield. For example, Fischer et al. (1998) demonstrated a close correlation between gs and yield in eight different wheat (Triticum aestivum) cultivars. Those studies highlighted the effects gs can have on crop yield, not only through reduced CO₂ diffusion but also through the impact on water loss and evaporative cooling of the leaf. Indeed, enhancing photosynthesis yields by only 2% to 3% is sufficient to substantially increase plant growth and biomass over the course of a growing season (Lefebvre et al., 2005; Zhu et al., 2007).
Stomata and their behavior profoundly affect the global fluxes of CO₂ and water, with an estimated $300 \times 10^{15}$ g of CO₂ and $35 \times 10^{18}$ g of water vapor passing through stomata of leaves every year (Hetherington and Woodward, 2003). Changes in stomatal behavior in response to changing climatic conditions are thought to impact on water levels and fluxes. For example, it is estimated that partial stomatal closure driven by increasing CO₂ concentration over the past two decades has led to increased CO₂ uptake and reduced evapotranspiration in temperate and boreal northern hemisphere forests (Keenan et al., 2013), with implications for continental runoff and freshwater availability associated with the global rise in CO₂ (Gedney et al., 2006). Concurrently, the increase in global water usage over the past 100 years and the expectation that this is set to double before 2030 (UNESCO, 2009) has put pressure on breeders and scientists to find new crop varieties, breeding traits, or potential targets for manipulation that would result in crop plants that are able to sustain yield with less water input. The fact that stomata are major players in plant water use and the entire global water cycle makes the functional and physical attributes of stomata potential targets for manipulation to improve carbon gain and plant productivity as well as global water fluxes.

There are several approaches for improving carbon gain and plant water use efficiency (WUE) that focus on stomata. It is possible to increase or decrease the gaseous conductance of the ensemble of stomata per unit of leaf area ($g_s$) through the manipulation of stomatal densities (Büssis et al., 2006). In addition, there is potential to alter the stomatal response or sensitivity to environmental signals through the manipulation of guard cell characteristics that affect stomatal mechanics (e.g. OPEN STOMATA [ost] mutants; Merlot et al., 2002). Such approaches have produced an array of mutant plants with altered characteristics and varying impacts on CO₂ uptake and transpiration, several of which we discuss in greater detail below. An intuitive measure of the efficacy of such manipulations is the WUE, commonly defined as the amount of carbon fixed in photosynthesis per unit of water transpired. In general, higher WUE values have been observed in plants with lower $g_s$, but these gains are usually achieved together with a reduction in $A$ and slower plant growth. Plants with higher $g_s$ have greater assimilation rates and grow faster under optimal conditions, but they generally exhibit lower WUE. An approach that has not been fully explored or considered in any depth is to select plants for differences in the kinetics of stomatal response or to manipulate stomatal kinetics in ways that improve the synchrony with mesophyll CO₂ demand (Lawson et al., 2010, 2012). To date, the majority of studies assessing the impact of stomatal behavior on photosynthetic carbon gain have focused on steady-state measurements of $g_s$ in relation to photosynthesis. These studies do not take account of the dynamic situation in the field. As we discuss below, a cursory analysis of stomatal synchrony with mesophyll CO₂ demand suggests that gains of 20% to 30% are theoretically possible.

Here, we address the question of the kinetics of the stomatal response to the naturally fluctuating environment, notably to fluctuations in light that are typical of the conditions experienced in the field. We focus on vascular seed plants, to which crop plants belong, and do not address seedless vascular plants, such as ferns. The characteristics of the latter, and hence the issues and challenges they present, are very different. In our minds, of paramount importance is whether there is potential for engineering guard cells of crop plants to manipulate the dynamic behavior of stomata so as to improve WUE without substantial cost in assimilation. Of course, in many circumstances, stomata are not the only factor to limit water flux through the plant (see other articles in this issue), but they are one of the most important “gatekeepers” and therefore, serve as a good starting point for such considerations. Thus, we explore the physical and functional attributes of stomata, their signaling, and the solute transport mechanisms that determine pore aperture as targets for potential manipulation of stomatal responses to changing environmental cues.

**INFLUENCE OF ANATOMY ON $g_s$**

In addition to stomatal sensitivity and responses to physiological drivers, $g_s$ is also dependent upon anatomical characteristics. Stomatal anatomical features define the maximum theoretical conductance (Dow et al., 2014a) and also influence the speed of response. Maximum $g_s$ is dictated by the size and density of stomata, which in turn can be influenced by the growth environment (Hetherington and Woodward, 2003; Franks and Farquhar, 2007). It is generally accepted that stomatal density is altered by atmospheric CO₂ concentration (Woodward, 1987; Gray et al., 2000), light (Gay and Hurd, 1975), and other environmental factors. More recent experimental evidence has demonstrated that stomatal density is negatively correlated with stomatal size (Hetherington and Woodward, 2003; Franks and Beerling, 2009). The interaction/correlation between stomatal size and density and the impact on stomatal function have received much attention, particularly with reference to the evolution of performance and plasticity in plants (Franks and Farquhar, 2007). The latest studies have also implied that physical attributes affect stomatal response times following environmental perturbations (Drake et al., 2013). Therefore, although it is possible to manipulate stomatal function, we must be fully aware of the interactions between stomatal size and numbers and the impact they can have on rapidity of stomatal movement.

**Stomatal Density**

Selecting crop plants with alterations in stomatal density to improve/reduce plant water use was first explored in the 1970s and 1980s (Jones, 1977, 1987) in various breeding programs with limited success. The original hypothesis was that increasing or decreasing stomatal numbers would, respectively, increase
or decrease $g_s$. However, several studies have demonstrated that this approach may be more complex than the simple argument outlined above. Arabidopsis (Arabidopsis thaliana) stomatal density and distribution (sdd1-1) mutants have a point mutation in a single gene that encodes a subtilisin-like Ser protease that results in plants with a 2.5-fold higher stomatal density compared with their wild-type counterparts (Berger and Altmann, 2000). The increase in stomatal density translates to an increase in $g_s$ and 30% greater photosynthetic rate under high-light conditions (Schlüter et al., 2003). Transgenic plants overexpressing SDD1 have a 40% reduction in stomatal densities compared with the wild type (Von Groll et al., 2002). In a comparative study, Büssis et al. (2006) showed no difference in $g_s$ or assimilation rate when measured under growth photosynthetically active photon flux density (PPFD) conditions (180 μmol m$^{-2}$ s$^{-1}$) between the overexpressing SDD1 plants, the sdd1-1 mutants, and wild-type controls. These findings indicated that increased stomatal aperture compensated for the lower stomatal density in the SDD1 plants while reduced aperture in the sdd1-1 plants offset the greater number of stomata. However, at high light intensities, low $g_s$ in the SDD1 overexpressors and restricted CO$_2$ diffusion limited A to 80% of the wild type (Büssis et al., 2006). These findings exemplify the role of both the physical and functional stomatal features in determining $g_s$ and that manipulation of physical attributes may be counterbalanced by modifications in function. However, more meaningfully, this work illustrates the importance of the surrounding environmental conditions on stomatal behavior and the significance of examining $g_s$ limitation on A at appropriate light and CO$_2$ concentrations. In the example above, no significant impacts on carbon gain were observed under growth PPFD; however, when photosynthesis was driven by greater PPFD, A was limited by CO$_2$ diffusion via reduced $g_s$. Such studies also provide an explanation for why breeding for increased WUE by altering stomatal density has so far been mostly unsuccessful (Jones, 1987). To date, there is only one report claiming a positive effect of stomatal density on $A$ in plants maintained in their growth conditions: Tanaka et al. (2013) used overexpression of STOMAGEN, a positive regulator of stomatal density, to produce plants with a 2- to 3-fold greater stomatal density than the wild type. A in these plants was increased by 30% due to greater CO$_2$ diffusion into the leaf rather than changes in photosynthetic carboxylation capacity (Tanaka et al., 2013). However, the resultant 30% increase in A was at the expense of transpiration, which was double that observed in the wild-type plants, resulting in a 50% decrease in WUE.

As stomatal density has been correlated with $g_s$ (Franks and Beerling, 2009), the rationale behind manipulating stomata anatomical characteristics to increase or decrease stomatal density may be considered relatively straightforward, helped by studies that have illustrated that manipulation of a single gene can alter stomatal patterning (Doherty-Adams et al., 2012). However, the manipulation of stomatal functional responses is clearly more complicated. Before such approaches are possible, we need a full understanding of the metabolic pathways (and underlying genetics) that form the basis of stomatal sensing, signaling, and response processes as well as a comprehension of physiological responses at the leaf level and at the cellular level. This understanding will need to include an appreciation for the hierarchical response of guard cells to internal and external signals and is likely to benefit from exploring the natural variation that exists in the magnitude of changes between individual stoma or groups of stomata within and between leaves (Lawson et al., 1998). Additionally, if such manipulations are directed at improving WUE, it is important to understand the coordination and synchronization of $g_s$ responses with mesophyll carbon assimilation (Lawson et al., 2012).

### Stomatal Patterning

Most research has focused on the physical behavioral aspects of stomata on gaseous diffusion, but investigations using density and patterning mutants have underpinned the physiological importance of stomatal patterning on CO$_2$ uptake and water loss. An interesting observation in the Büssis et al. (2006) study was the high-resolution chlorophyll fluorescence imaging that revealed two distinct areas of mesophyll in the SDD1 plants: one where mesophyll lay above stomata and the other where stomata were absent. Measurements of maximum and actual photosynthetic efficiency were identical to the wild type in areas with stomata; however, leaf areas without stomata showed lower maximum and actual photosynthetic efficiency, illustrating that stomatal patterning determined CO$_2$ concentration and photosynthesis across the leaf lamina and that lateral fluxes of gas could not compensate for reduced vertical diffusion as a result of reduced stomatal numbers (Büssis et al., 2006). This work agrees with reports that suggest that lateral fluxes can limit photosynthesis (Morison et al., 2005) but depend on species (Morison et al., 2007; Morison and Lawson, 2007) and leaf anatomy (Lawson and Morison, 2006).

There are many known density and patterning mutants (e.g. fama, sple, mute, and tmm) in which specific gene mutations have resulted in changes to cell division and differentiation and altered patterns of stomata and epidermal cells, resulting in stomatal pairing or clustering in which the “one-cell-spacing” rule is broken (for review, see Lau and Bergmann, 2012). The one-cell-spacing rule refers to the fact that stomata are separated from each other by a minimum of one cell, enabling efficient stomatal operation (Serna and Fenoll, 2000) and maintaining the efficiency of gas fluxes (Nadeau and Sack, 2002). This is exemplified in a recent study by Dow et al. (2014b), who revealed that high stomatal density (due to stomatal clustering) restricted CO$_2$ diffusion and lowered A. These findings were explained by the reduced functional capabilities of the guard cells from the reduced availability of ions required for driving membrane processes as well as the influence of turgor pressure for adjacent guard cells.
and the disruption of signaling processes due to the close proximity of the guard cells. This work reiterated the importance of the one-cell-spacing rule and indicated that optimal stomatal function depended to a large extent on the patterning of stomata. There is also a good understanding of environmental regulation (Lake et al., 2001; Bergmann, 2004; Casson and Gray, 2008; Casson and Hetherington, 2010) and what determines stomatal development as well as the underlying genetic pathways that lead to altered epidermal cell patterning (Bergmann and Sack, 2007; Casson and Hetherington, 2010). However, less is known about how environmental regulation influences stomatal density (e.g. CO₂ concentration) and even less about the impact of patterning on leaf transpiration.

Stomatal Anatomy

Doheny-Adams et al. (2012) investigated the impact of epidermal patterning factor (epf) mutants altered in density and patterning of stomata on gs, A, and WUE. Alterations in the expression of the different members of the EPF family affected stomatal and, in some cases, epidermal cell densities as well as the spacing of cells, indicating an impact on division and differentiation. In general, plants with no expression of EPF1 or EPF2, and double mutants of epf1 or epf2, showed increases in stomatal density; overexpressors showed reduced stomatal densities. It is noteworthy that the strong correlation between stomatal density and size was maintained within these plants: plants with lower stomatal densities also showed a greater mean stomatal size, whereas smaller stomata were found in leaves with greater stomatal densities. Interestingly, those plants with reduced density and larger stomata also showed reduced transpiration, greater growth rates, and a larger biomass (Doheny-Adams et al., 2012). The authors ascribed the improved growth rate to a combination of improved water status, higher metabolic temperatures, and lower metabolic costs associated with the development of guard cells. That growth rate was not enhanced in plants with greater stomatal densities indicates that resistance to CO₂ diffusion was not limiting in these conditions and that leaf water status dominated growth. It would be instructive to determine productivity in these plants when PPFD is increased and mesophyll demand for CO₂ is elevated or the plants are subjected to fluctuating light conditions. In contrast to the SDD1-expressing and ssl1 mutant plants with altered stomatal densities described above, the epf1 and epf2 mutants showed no compensatory behavior in stomatal aperture. It is important to remember that reduced gs often decreases transpiration at the expense of carbon gain. It is necessary, therefore, to assess both CO₂ uptake and water loss when considering strategies for improved WUE (McAusland et al., 2013). An excellent example of enhanced WUE driven by stomatal numbers are the glf1 mutants of Arabidopsis. GTL1 is a transcription factor that regulates trichome and stomatal development through its interaction with SDD1 expression (Breuer et al., 2009). Physiological analysis of these plants revealed no difference in photosynthetic rates over a range of light levels but reduced gs and transpiration (Yoo et al., 2009, 2010), illustrating that manipulating one gene related to stomatal development can “fine-tune” WUE.

Evidence from several studies has also suggested that smaller stomata respond faster than larger stomata, an observation that has been explained in the context of surface-to-volume ratios and the requirement for solute transport to drive movement (Hetherington and Woodward, 2003; Franks and Beerling, 2009; Drake et al., 2013). There are some notable exceptions to this relationship. The stomata of ferns are relatively large compared with many angiosperm species but nonetheless respond rapidly to changes in vapor pressure difference (VPD), which is the difference in water vapor pressure between inside the leaf and outside; however, their response to light is much slower than that of angiosperms (Brodribb and Holbrook, 2004; McAdam and Brodribb, 2012b, 2013). The differentiation in these responses between modern angiosperms and earlier vascular seedless plants most likely arises from the passive hydraulic characteristics of ferns (McAdam and Brodribb, 2012a, 2013). It is of interest that the size-speed relationship holds for the characteristics predicted of stomatal behavior in Vicia faba and Arabidopsis guard cells when modeled on the basis of quantitative information available for these two species (Chen et al., 2012c; Wang et al., 2012). In this case, the difference can be ascribed directly to the surface-to-volume ratios, an observation that is broadly consistent with the suggestions of Drake et al. (2013) and Hetherington and Woodward (2003). However, it should be borne in mind that stomatal opening is a physical-mechanical process and that, in order to open, guard cells must overcome the pressure exerted by the surrounding subsidiary cells. Franks and Farghali (2007) illustrated that stomatal opening in some species was only possible with a substantial reduction in subsidiary cell osmotic pressure and that rapid opening in wheat and other grasses, which have dumbbell-shaped guard cells and subsidiary cells, was due to complementary changes in turgor pressure between the guard cells and these surrounding subsidiary cells. Furthermore, as we note below, there can be substantial variations in the transport activities of guard cells, even within one species. So, direct comparisons of surface-to-volume ratios is likely to be uninformative as often as not.

SPEED OF THE STOMATAL RESPONSE

The opening and closing of stomata is driven by a number of external environmental and internal signaling cues (Blatt, 2000), and significant variation in sensitivity and responsiveness is known to exist among different species (Lawson et al., 2003, 2012; Lawson, 2009). In general, stomata open in response to light (with the exception of Crassulacean Acid Metabolism stomata), low CO₂ concentration, high temperatures, and low VPD, while closure is driven by low light or darkness, high CO₂, and high VPD (Outlaw, 2003). In the natural
environment, these factors exert compound effects on stomatal movements (Sharkey and Raschke, 1981; Zeiger and Zhu 1998; Talbott et al., 2003; Wang et al., 2008); therefore, stomata must respond to multiple signals in an integrated and sometimes hierarchical manner (Lawson et al., 2010). Short-term stomatal responses to changes in VPD (and to some extent temperature) are often considered to be related to the water status of the plant rather than the in situ photosynthetic carbon demand, while responses to CO2 concentration and irradiance are closely associated and correlated with mesophyll CO2 demand. 

Stomatal responses to changes in light, CO2 concentration, and VPD have been studied extensively in steady-state conditions (Jones, 1994; Lawson et al., 2010). However, such steady-state situations are rarely observed in nature (Jones, 1994) or in isolation (Lawson and Morison, 2004). Few studies have examined the dynamics of stomatal response and photosynthetic output in the face of environmental perturbations (Grantz and Zeiger, 1986; Knapp and Smith, 1987; Kirschbaum et al., 1988; Tinoco-Ojanguren and Pearcy, 1993; Barradas et al., 1994; Lawson et al., 2010; Wong et al., 2012; McAusland et al., 2013). The majority of these have concentrated on the impact of sun/shade flecks on carbon gain in understory plants, often without reference to stomata, and even fewer have looked at the impact on crop plants. Although grown in a monoculture, crops will also experience sun and shade flecks across the canopy from changes in cloud cover and sun angle to self-shading and shading from neighboring plants (Way and Pearcy, 2012), overlapping leaves, and wind-driven movements (Lawson et al., 2010). In the naturally fluctuating environment, stomata and photosynthesis respond continually to changing environmental cues, especially light and temperature. However, these responses are not always synchronized, as stomatal movements can be an order of magnitude slower than the more rapid photosynthetic responses to the same environmental stimuli (Pearcy, 1990; Lawson et al., 2010). For example, over the diel period, plants experience short-term fluctuations in light (sun/shade flecks) that drive the temporal and spatial dynamics of carbon gain and water loss (Fig. 1A). The temporal disconnect between gs and A means that under natural fluctuating environmental conditions, the coordination between carbon gain and water loss (and, therefore, WUE) is far from optimal (Fig. 1B) and can result in spatial heterogeneity over individual leaves (Lawson and Weyers, 1999) as well as throughout the crop canopy (Weyers et al., 1997). In C3 plants, photosynthetic rate can adjust in seconds to changes in irradiance (e.g., a sun fleck), but the lag in stomatal responses will constrain photosynthesis by limiting CO2 uptake (Tinoco-Ojanguren and Pearcy, 1993; Barradas et al., 1998; Lawson et al., 2010, 2012). The example in Figure 1 is of natural changes in irradiance, A, and gs in a bean (Phaseolus vulgaris) leaf over a 70-min period and clearly shows periods in which gs and A were uncoordinated, others in which the slower stomatal reopening limited A, and periods in which gs was greater than required for the A achievable under the available light. Intrinsic water use efficiency (WUEi, which used gs as an estimation of water loss rather than transpiration [A/gs]) over this period was between 0.03 and 0.11 μmol CO2 m⁻² s⁻¹/mmol water m⁻² s⁻¹ (Fig. 1B) and yielded a time-averaged value of 0.071. When recalculated assuming instantaneous responses and coordination of gs with A for the theoretical maximum WUE (determined from steady-state measurements of A and gs at different irradiances; Fig. 1B), this time-averaged value was improved by 22%, effectively accounting for the WUEi deficits illustrated by the shaded regions in Figure 1B. This simple experiment demonstrates the impact that the rapidity of stomatal responses to changing light environment has on carbon gain and water loss and illustrates the possible improvement in WUE if stomata respond rapidly (almost instantaneously) and in synchrony with mesophyll demands for CO2.

Pearcy and coworkers pioneered research on the impact of sun flecks on carbon gain and stomatal dynamics and dissected the photosynthetic response into several different phases, attributing the initial induction phase, periods of up to 10 min, to biochemical limitations (Barradas and Jones, 1996); induction was followed by a period dominated by stomatal limitation to the photosynthetic maximum; a third phase was identified in which gs remained high, effectively exceeding that needed for maximum assimilation rates under the given light conditions (Kirschbaum et al., 1988; Tinoco-Ojanguren and Pearcy, 1993) and, hence, out of synchrony with A (Lawson et al., 2010). Figure 2 shows an example of gs limiting photosynthesis, post induction, when irradiance was increased during a 2-min sun fleck. As the PPFD declined at the end of this period, gs continued to rise over several minutes, leading to a surplus in transpiration without the benefit of enhanced assimilation. If we consider the impact of this short increase in illumination on instantaneous WUE (Fig. 2, gray squares), it is clear that gs has less impact on the measurements of WUE after transition to the higher light intensity. However, it is also evident that assimilation was limited by gs during the sun fleck, with A rising by 0.6 μmol m⁻² s⁻¹, roughly 10% over the second half of the 2-min period, together with gs. Finally, Figure 2 illustrates the continued rise in gs following the sun fleck, leading to a significant decline in WUEi relative to the mean starting value.

The effects of shade flecks are less well studied (Lawson et al., 2010), but they yield dynamics similar to those observed during sun flecks. A decrease in light results in an immediate drop in A, to a new value imposed by the light level. gs rarely responds immediately, demonstrating a lag phase, before the start of closing. Although closing is often faster than the opening (Hetherington and Woodward, 2003), it can still take several tens of minutes to establish a new steady state in gs. Figure 3 illustrates dynamic responses in bean and V. faba subjected to 1-, 5-, and 15-min shade flecks. In agreement with previous observations, a decrease in light intensity of 1 to 2 min resulted in very little or no change in gs and a near-instant recovery in photosynthetic rate when light was restored.
For bean, longer shade flecks led to progressive declines in g,
and a slowing in the return of A to the initial value when light was restored (Fig. 3B). With a shade fleck of 15 min, the resulting drop in g, subsequently restricted the photosynthetic rate by some 35% in the first minutes after light was restored (T. Lawson and L. McAusland, unpublished data). The same light regimes had little effect on short-term g, in V. faba (Fig. 3, D–F). Consequently, these plants showed little evidence of CO2 limitation when irradiance was restored, but the much more...
sluggish response of the stomata and $g_s$ meant a proportionally higher rate of transpiration during the periods of reduced CO$_2$ demand. In short, the longer stomata take to close and reach a new $g_s$ value appropriate for the light level and $A$, the greater the surplus in transpiration and reduction in WUE (Fig. 1B).

Of course, the speed and magnitude of changes in $g_s$ in response to sun and shade flecks are species specific (Lawson et al., 2010) and may be dependent on differences in stomatal sensitivity or signaling mechanisms between species (Weyers et al., 1997; Mott and Peak, 2007; Mott, 2009). They will also depend on plant water status (Fig. 4), the history of stress (Pearcy and Way, 2012; Porcar-Castell and Palmroth, 2012; Wong et al., 2012; Zhang et al., 2012), leaf age (Urban et al., 2008), and the magnitude and duration of the change.

![Figure 3](image_url)
in irradiance (Lawson, 1997). As illustrated in Figure 3, *V. faba* showed little response to changes in irradiance. These plants were well watered and grown under moderate light intensities and temperatures of 18°C to 20°C to minimize stress. However, when the plants were challenged with the same 15-min shade fleck following 4 d without watering (Fig. 4), *g*ₙ was significantly lower compared with the well-watered plants (Fig. 3F) and showed an immediate and more rapid decrease during the shade fleck. Furthermore, *A* recovered in parallel with the relatively slow increase in *g*ₙ when the light was restored to its initial level, both *A* and *g*ₙ taking more than 30 min to recover (Fig. 4). Experiments of this kind illustrate the range of variations in speed and amplitude in *g*ₙ dynamics, and they suggest that stomatal size and density are often of secondary importance. Indeed, bean, which has smaller and more numerous stomata, here responded faster when well watered than well-watered *V. faba*, which has larger and fewer stomata, but when water stressed, *g*ₙ of *V. faba* responded in a manner similar to that of bean.

One feature of note is an apparent correlation between opening and closing rates. Vico et al. (2011) used 60 published gas-exchange data sets of the stomatal response to light variations to determine the impact of stomatal delays on photosynthesis and transpiration and its relationship to the energetic costs of stomatal movement. Unfortunately, the latter are compromised by the use of incorrect stoichiometries for the coupling of ATP to transport and by the assumption that stomatal closure is entirely passive, which it is not. However, among other outcomes, their studies show a general parallel in rates of stomatal movement across data sets: faster opening rates were generally associated with correspondingly faster closing rates. These findings agree with others (Hetherington and Woodward, 2003). Vialet-Chabrand et al. (2013) recently developed a new dynamic model to describe the diurnal time course of *g*ₙ using empirical parameters to accurately predict daily variation in WUEᵢ. This model uses non-linear methods to describe temporal changes in *g*ₙ driven by the fluctuating environment, and it underlines a coupling between rates of opening and closing.

Overall, from these various studies, we have learned a great deal about the manipulation of stomatal numbers and guard cell responses to environmental perturbations, but almost nothing is known about the mechanisms that control stomatal speed. However, research to date suggests that manipulating stomatal number and size is not necessarily the simplest or best approach, and the idea that manipulating the stomatal response to changing environmental conditions could provide a means to both improve the WUE of plants and, at the same time, increase the integrated photosynthetic carbon gain. Most important, they indicate that the rapidity of the stomatal response influences both daily carbon gain and WUE and that it is essential to consider the dynamics both of closing and opening together (Raschke, 1970; Kirschbaum et al., 1988; Knapp, 1993; Tinoco-Ojanguren and Pearcy, 1993; Cardon et al., 1994; Allen and Pearcy, 2000; Noe and Giersch, 2004; Pearcy and Way, 2012; Smith and Berry, 2013). With these points in mind, guard cell ion transport is an obvious target for exploration with the aim of altering stomatal dynamics and the rates of opening and closing.

**ION TRANSPORT, STOMATAL RESPONSE, AND WUE**

There is no question that stomatal movements of seed plants, including crop plants, arise from the transport, accumulation, and release of osmotically active solutes. A very large body of experimental evidence supports the collective role of ion transport across the plasma membrane and tonoplast in both stomatal opening and closing (Willmer and Fricker, 1996; Blatt, 2000; Chen et al., 2012c; Hills et al., 2012). The primary inorganic species transported are K⁺ and Cl⁻, which, with the organic anion malate⁻² (Mal) and Suc, comprise the bulk of solute that drives water flux and guard cell turgor (Willmer and Fricker, 1996; Roelfsema and Hedrich, 2005; McAinsh and Pittman, 2009). Because mature guard cells lack functional plasmodesmata (Wille and Lucas, 1984), these solutes must be transported across the plasma membrane. Much of this solute uptake must also be transported across the tonoplast. The guard cell vacuole, like that of most mature plant cells, makes up the bulk of the cell volume and, hence, plays a very important role as a “repository” for osmotically active solutes (Gao et al., 2005; MacRobbie, 2006; Chen et al., 2012c). Mal metabolism (notably its synthesis within the guard cell cytosol) makes a substantial contribution to the osmotic content of the guard cell, while Mal loss during stomatal closure occurs largely via efflux across the plasma membrane (Willmer and Fricker, 1996; Wang and Blatt, 2011). For this reason, questions of the extent and speed of stomatal
responses to environmental cues are intimately connected with characteristics of the guard cells, the most important being (1) the capacity for solute transport and exchange with the surroundings, and its relationship to the volume of the guard cells, and (2) the speed with which transport responds to environmental cues that determine stomatal movements.

Transport capacity is determined primarily by the density and activity of the various transport proteins at the membrane and is related secondarily to guard cell turgor and stomatal opening and closing by the surface-to-volume ratio of the cellular compartments. As noted before, guard cell size (and geometry) has been indicated to affect the speed of stomatal movements, with larger stomata often exhibiting slower responses. The importance of the size of the stomatal complex, as concluded from these studies, is predicated on the assumption that solute fluxes are largely uniform when normalized to the unit surface area; as a consequence, the time needed to adjust solute content within the cell volume is expected to decrease roughly in proportion with guard cell volume-to-surface ratio and, hence, the linear dimensions of the guard cell. However, quantitative data from which to compare solute fluxes are very limited (Willmer and Fricker, 1996) and depend on factors such as the history of water stress (Hiron and Wright, 1973; Figs. 3 and 4). The few studies that have examined specific transport activities directly, for example, of outward-rectifying K+ currents in intact guard cells of \textit{V. faba} tobacco (\textit{Nicotiana tabacum}), and Arabidopsis (Blatt, 1988b; Armstrong et al., 1995; Blatt and Gradmann, 1997; Blatt et al., 1999; Ache et al., 2000; Bauly et al., 2000; Johannson et al., 2006; Chen et al., 2012b; Eisenach et al., 2012, 2014), indicate substantial variations between species independent of surface area. These comparisons call in question the validity of assuming a priori that transport activity remains constant on a unit-surface-area basis.

There are a large number of studies reporting on the rates of net ion transport across the plasma membrane as the principle barrier to solute flux. Attention has often turned to changes in pH, as, arguably, the rates of H+ flux are a good measure of membrane energization by primary H+-ATPases (Marre, 1979; Shimazaki and Kondo, 1987; Goh et al., 1996; Kinoshita and Shimazaki, 1999; Merlot et al., 2007). By their nature, pH measurements report the net H+ flux rather than the true H+-ATPase activity and, therefore, are indirect, subject to the availability of other ions to balance charge during transport (Blatt and Clint, 1989; Clint and Blatt, 1989; Gradmann et al., 1993). This caveat aside, the rates of H+-ATPase activity recorded directly under voltage clamp (Blatt, 1987, 1988a; Lohse and Hedrich, 1992), and the corresponding fluxes of K+ and Cl (MacRobbie, 1981, 1983, 1984; Clint and Blatt, 1989), have generally proven consistent with the changes in solute content (MacRobbie and Lettau, 1979, 1980; Blatt, 2000; for a quantitative summary and comparison, see Hills et al., 2012).

Relating these data to the transport capacity of guard cells in mechanistic terms has proven much more complex, however. Difficulties arise in part because the predominant transport pathways for H+, K+, Cl−, and Mal are strongly dependent on membrane voltage over the physiological voltage range, especially at the plasma membrane. A second complication arises from the fundamental physical requirement for charge balance across all biological membranes. As a result, the flux of any one ionic species is necessarily connected to that of all others across the same membrane, unless this coupling is broken by introducing a “shunt” through the circuit of a voltage clamp (Blatt, 1991, 2004). Experiments in which membrane voltage has been brought under experimental control address the question of transport capacity directly and have proven quite revealing on several accounts. First and foremost, the dominant membrane transporters, including the K+ and Cl− channels, remain functional under most physiological conditions. This observation contrasts with a common misconception that transporters either activate or shut down fully in response to various stimuli. Their operation may be kinetically limited by substrate (ion) availability and especially by membrane voltage, but the activity of each transporter is generally evident under voltage clamp. For example, currents through the inward- and outward-rectifying K+ channels are present when voltage is clamped, both in the absence and presence of the water-stress hormone abscisic acid (ABA) that triggers stomatal closure (Blatt and Armstrong, 1993; Romano et al., 2000; Garcia-Mata et al., 2003). ABA affects the K+ carrying capacity of both channel populations; however, membrane depolarization is the overriding factor in driving K+ efflux and stomatal closure (Thiel et al., 1992; Romano et al., 2000). In other words, guard cells probably rarely, if ever, exist in a state in which the net flux of an osmotically active solute is zero. Instead, solute uptake and loss, and stomatal movements, arise through a dynamic balance in solute flux (Blatt and Armstrong, 1993; Gradmann et al., 1993; Chen et al., 2012c). A second point arises from the general finding that the ion transport underpinning stomatal movements reflects a small fraction only of the maximal capacity of the several transporters mediating these fluxes (Blatt et al., 1990; Thiel et al., 1992; Hamilton et al., 2000; Potosin and Schönknecht, 2007; DeAngeli et al., 2009). The activities of these transporters are kinetically limited by their inherent gating or other kinetic properties within the range of voltages typical for the plasma membrane and tonoplast. Two general conclusions may be drawn from these observations. First, the capacity for transport, especially through the several ion channels, is not inherently limiting for solute flux, although the balance of transport often is. Second, any attempt to manipulate solute accumulation and loss for accelerated stomatal movements must therefore address the balance between ionic fluxes at the plasma membrane and tonoplast, both individually and coordinately between membranes.

Information on the speed with which transport responds to environmental cues, especially to light and CO2, is similarly complicated by the connections between transport at each membrane. Again, deconstructing the sequence of events in mechanistic terms must draw on the voltage clamp to separate the individual transporter
Stomatal Impact on Water Use Efficiency

currents (Blatt, 2004) and is the focus of a number of recent reviews (Blatt, 2000; Hetherington and Brownlee, 2004; Blatt et al., 2007; Pandey et al., 2007; Melotto et al., 2008; Wang and Song, 2008; McAnish and Pittman, 2009; Hills et al., 2012). In general, however, stomatal movement arises as the cumulative sum of the net solute fluxes (i.e. of the rates of flux), especially for K+, Cl−, and Mal integrated over time. Therefore, the stomata aperture responds over substantially longer time scales, typically orders of magnitude greater than that needed for changes in transport activity and membrane voltage of the guard cell. For example, membrane depolarization in ABA stimulates K+ efflux within seconds through outward-rectifying K+ channels, in Arabidopsis the GORK K+ channel (Hosy et al., 2003; Suhita et al., 2004), and these K+ currents are enhanced during the subsequent 3 to 5 min as a consequence of a rise in cytosolic pH (Blatt and Armstrong, 1993; Grabov and Blatt, 1997). Stomatal aperture responds more slowly, typically with half-times of 10 to 20 min, reaching a new stable, (near) closed state after 45 to 60 min (Raschke et al., 1975; Roelfsema and Prins, 1995; Zhang et al., 2001). Thus, making a connection to the speed and efficacy of stomatal movements is necessarily indirect.

One promising way forward draws on quantitative systems modeling to relate transport and metabolic activities to stomatal movements. Wang et al. (2014a), in this issue, have undertaken such an approach using OnGuard models for V. faba and Arabidopsis (Chen et al., 2012a; Hills et al., 2012; Wang et al., 2012). OnGuard models incorporate all of our knowledge of molecular, biophysical, and kinetic characteristics of guard cell transport, Mal metabolism, and H+ and Ca2+ buffering, and they link this knowledge to stomatal aperture. These models have demonstrated true predictive power in uncovering previously unexpected and emergent behaviors of guard cells, several of which have been verified experimentally. OnGuard analysis of the Arabidopsis slac1 mutant exposed an unexpected connection between the Cl− channel and the plasma membrane K+ channels that was subsequently confirmed by experiments (Wang et al., 2012). This connection accounted fully for the effect of the slac1 mutant in slowing ion uptake and stomatal opening, even though the SLAC1 Cl− channel contributes directly only to solute loss and stomatal closure. Similarly, analysis of the Arabidopsis ost2 mutant, which affects H+-ATPase activity at the plasma membrane, has demonstrated an uncanny “communication” of transport between the plasma membrane and tonoplast (Blatt et al., 2013), predictions that now warrant investigation. Most importantly, the modeling underlines a commonality of kinetic limitations that arises from the sharing of substrates and membrane voltage across each membrane. These connections underpin the emergent characteristics identified so far.

So how might we approach the problem of improving water use by the plant without a cost in carbon gain? Different ion flux pathways are responsible for solute uptake during the opening and solute loss during the closing of stomata (Blatt, 2000; Roelfsema and Hedrich, 2005; Shimazaki et al., 2007), and at first glance, this separation should be a simplifying factor. However, a primary difficulty is that of transport interactions, such as those described above. Anticipating fully the consequences of such manipulations is generally beyond intuitive understanding and can only be addressed through quantitative modeling. Wang et al. (2014a) have explored systematically the most promising targets for genetic manipulation that will enhance the speed of stomatal opening and closing using the OnGuard platform. Their results indicate that, with few exceptions, modest changes to the populations of individual ion channels are largely ineffective. Only primary H+ transport, and those transporters affecting Ca2+ homeostasis directly, are predicted to have any substantial effects on stomatal movements. Surprisingly, the effects of manipulating almost all transporter populations were compound and, in part, at odds with the desired effects of accelerating opening and closing while maintaining the dynamic range of apertures. To the extent of our current knowledge, the results confirm past observations from a number of mutants, including the K+ channel gork (Hosy et al., 2003), the anion channel slac1 (Negi et al., 2008; Wang et al., 2012), the ost2 H+-ATPase mutant (Merlot et al., 2007), the clca H+-Cl− antiporter mutant (De Angelis et al., 2006), and the tpk1 K+ channel mutant (Gobert et al., 2007). They are supported, additionally, by the work of Wang et al. (2014b), who concurrent with this publication reported the effects of overexpressing the several K+ channels and the AHA2 H+-ATPase in Arabidopsis guard cells. Their results confirm the lack of effect on channel overexpression; they also demonstrate enhanced apertures, gK+, and A with H+-ATPase overexpression. However, these gains come with a corresponding reduction in WUE, suggesting that the acceleration in stomatal opening was not reflected in an increase in the rates of stomatal closure. Again, these findings confirm modeling predictions (Wang et al., 2014a) that interactions between transporters preempt most, if not all, of the intuitive solutions to accelerating stomatal movements. The most promising targets for manipulation to arise from these studies are the voltage-dependent characteristics of the two classes of K+ channels at the plasma membrane, rather than simple changes to the populations of these transporters. Wang et al. (2014a) note that a −18-mV shift in the gating of the outward-rectifying K+ channel (GORK in Arabidopsis) could be sufficient to accelerate closing by more than 30%, whereas doubling the number of these K+ channels counterintuitively slows the rate of closing in simulations. Other effective solutions may include manipulations of more than one transport pathway. In short, even at this simplified level, the problem of manipulating stomatal behavior throws up a surprising degree of complexity that is likely to challenge practical solutions to future efforts in “reverse engineering” of stomata.
ENGINEERING STOMATAL SIGNALING AND METABOLISM

Many of the challenges associated with manipulating ion transport in guard cells have analogs in efforts focused on their signaling, cell biology, and metabolism. Indeed, there are obvious functional overlaps, for example in the contributions of organic acid synthesis to the osmotic content of the cells. Thus, it is not surprising that altered Mal metabolism and transport will affect stomatal movements (Gruber et al., 2011). More still, a survey of the literature shows that the lessons learned from the systems biological approach of OnGuard about the (often counterintuitive) connections between transport and metabolism (Wang and Blatt, 2011; Wang et al., 2012) have direct bearing here, too. A thorough discussion of this literature is not possible here. Thus, we have selected a few of the more recent articles addressing leaf-level $g_\text{s}$ and $A$ that illustrate both the difficulties and the potential for manipulation of stomatal responses, especially in relation to stomatal kinetics. We stress that, to date, no single mutations of any structural gene products have surfaced that enhance stomatal function to improved WUE without a cost to carbon gain.

The overlaps with ion transport and the importance of stomatal dynamics are well illustrated by the vesicle-trafficking protein SYP121 in Arabidopsis. Eisenach et al. (2012) reported that the sup121 mutant impairs stomatal reopening following closing signals associated with elevated cytosolic free Ca$^{2+}$ concentration. The mutant mimicked the phenomenon of so-called “programmed closure,” previously ascribed to a “memory” of stress that leads stomata to open only slowly thereafter. The authors demonstrated the underlying mechanism that arises from the cycling of the KAT1 $K^+$ channel between the plasma membrane and endosomal membranes, thereby suppressing channel-mediated $K^+$ uptake by the guard cells and slowing stomatal reopening. Stomatal opening was slowed from a half-time of approximately 20 min to half-times of more than 60 min, sufficient to reduce long-term carbon assimilation and slow growth at low relative humidity. Slowed stomatal opening in the light has also been observed in the high leaf temperature1 mutant (Hashimoto et al., 2006), which exhibits alterations in responsiveness to CO$_2$, and thus may have important implications for synchronizing stomata with mesophyll-based photosynthesis. Finally, as noted above, Wang et al. (2012) analyzed the slac1 mutant that eliminates the predominant anion channel in Arabidopsis guard cells and greatly slows stomatal closure. They found that the mutation also slowed stomatal opening by altering the two $K^+$ channel currents. These observations were traced directly to changes in the baseline of cytosolic free Ca$^{2+}$ concentration and elevated cytosolic pH, the latter arising from the accumulation of Mal and suppression of its synthesis. In other words, mutation of a Cl$^-$ channel has unexpected and profound effects that extend well beyond Cl$^-$ transport, both to other ion channels and to organic acid metabolism.

Manipulation of photosynthetic carbon metabolism has likewise revealed some informative interactions between mesophyll and stomatal guard cells, although the conclusions are often seemingly contradictory. Transgenic plants with reduced photosynthesis showed that $g_\text{s}$ was largely unaffected (Quick et al., 1991; Stitt et al., 1991; Hudson et al., 1992; Evans et al., 1994; Price et al., 1998), thereby breaking the long-standing correlation between $A$ and $g_\text{s}$ that is usually conserved. More recent studies have similarly demonstrated that $g_\text{s}$ and $A$ can be uncoupled when carbon fixation via Rubisco (von Caemmerer et al., 2004) and electron transport (Baroli et al., 2008) are down-regulated. However, accelerated stomatal opening was observed when the regeneration capacity of ribulose 1,5-bisphosphate was suppressed in transgenic tobacco with reduced levels of Sedoheptulose-1,7-bisphosphatase (Lawson et al., 2008). Overexpression of maize (Zea mays) NAD-malic enzyme in tobacco resulted in plants with a decrease in $g_\text{s}$ but gains in biomass, suggesting that manipulation of both stomata and mesophyll processes could improve plant WUE (La Porte et al., 2002). Finally, Kelly et al. (2012) increased the expression of hexokinase in guard cells with the counterintuitive effect of decreasing $g_\text{s}$. Hexokinase drives starch breakdown through the phosphorylation of Glc, which then feeds into glycolysis, the tricarboxylic acid cycle, and malic acid synthesis. Analysis of these transgenics suggested a Suc feedback mechanism that coordinated stomatal behavior with mesophyll photosynthesis. Other, seemingly more indirect, manipulations can have unexpected consequences for stomata and WUE. Some examples include manipulations to SUC2 that increased guard cell invertase activity (Antunes et al., 2012). Aratújo et al. (2011) observed an elevated $g_\text{s}$ and a 25% increase in $A$ in tomato (Solanum lycopersicum) plants with altered succinate dehydrogenase levels. This response was only observed on ectopic overexpression with a 35S promoter, not when expression was restricted to the guard cells only. In short, these studies highlight the potential for manipulating metabolic processes, both of mesophyll and guard cells. However, they also underscore the need, in addressing complex behavior such as WUE, for quantitative systems approaches on a tissue-wide scale if we are to effectively target and exploit stomatal guard cell physiology.

CONCLUSION

Improving plant WUE and a plant’s ability to cope with reduced water availability is high on the scientific agenda. Stomata ultimately control 95% of all gaseous fluxes between the leaf and the environment. It follows that stomata represent an attractive target for manipulations aimed at reducing water loss. Given that stomata also regulate CO$_2$ access to the photosynthetic tissues of the leaf, the challenge will be to achieve this goal without compromising carbon gain (Lawson et al., 2012). Equally important, any manipulations to stomatal function, sensitivity, or responsiveness should not render the plants more vulnerable to environmental extremes or biotic
challenges. Progress to these ends is most likely to come now from combinations of physiological and molecular genetic methods together with those of quantitative systems analysis and, therefore, will benefit from additional information about the quantitative kinetics, especially of signal transduction.

Significant advances have been made in our understanding of the biochemical response pathways of guard cells and the underlying molecular biology and genetic basis that underpin these functional responses and anatomical characteristics. Recent advances in molecular biology tools and approaches to manipulate key biochemical processes or select for desirable traits mean that we can reconsider these earlier ideas and the manipulations of guard cell metabolism or specific stomatal traits as tangible targets, with real potential to deliver plants with improved WUE and yield. For example, transcriptional analysis of guard cells from epidermal fragments (Wang et al., 2012) or microdissection of individual guard cells (Gandotra et al., 2013), along with single-cell metabolic profiling (Burrell et al., 2007), have greatly assisted in our understanding of the metabolic signaling and response pathways within guard cells. The identification of highly specific guard cell promoters (Müller-Röber et al., 1994; Cominelli et al., 2005) and transcription factors (Cominelli et al., 2010; Yoo et al., 2010), the production of guard cell enhancer trap lines (Gardner et al., 2009), and the ability to induce transient expression in guard cells (Rusconi et al., 2013) open up new exciting potential approaches to not only fully understand signaling and transduction pathways in guard cells but also to provide us with the tools to manipulate guard cell metabolism in order to improve plant WUE. Such tools have greatly improved our understanding of the molecular networks that control guard cell perception of and response to internal and external environmental cues and have provided possible candidates for manipulation (Galbiati et al., 2008; Rusconi et al., 2013).

It is equally important that plant phenotyping approaches keep pace with the molecular and genetic technical developments described above to deliver rapid and practical screening approaches and tools to identify plants with faster stomata. MacAusland et al. (2013) recently used combined chlorophyll fluorescence and thermal imaging to develop the first imaging approach to screen plants for alterations in intrinsic WUE. This approach provides both spatial and temporal images of g and A as well as dynamic protocol capabilities. Using this technique, stomatal responsiveness and speed can be assessed simultaneously with rates of photosynthesis, which would be ideal for identifying plants with faster stomata with no impairment in carbon assimilation.

Received February 1, 2014; accepted February 25, 2014; published February 27, 2014.

LITERATURE CITED


MacRobbie EAC (2006) Osmotic effects on vacuolar ion release in guard cells. Proc Natl Acad Sci USA 103: 1135–1140


McAdam SA, Brodribb TJ (2012a) Fern and lycophyte guard cell do not respond to endogenous abscisic acid. Plant Cell 24: 1510–1521

McAdam SA, Brodribb TJ (2012b) Stomatal innovation and the rise of seed plants. Ecol Lett 15: 1–8


