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Guard Cell Metabolism and Stomatal Function

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Keywords

stomata, guard cells, osmoregulation, photosynthesis, subsidiary cells, mesophyll

Abstract

The control of gaseous exchange between the leaf and external atmosphere is governed by stomatal conductance (g_s); therefore, stomata play a critical role in photosynthesis and transpiration and overall plant productivity. Stomatal conductance is determined by both anatomical features and behavioral characteristics. Here we review some of the osmoregulatory pathways in guard cell metabolism, genes and signals that determine stomatal function and patterning, and the recent work that explores coordination between g_s and carbon assimilation (A) and the influence of spatial distribution of functional stomata on underlying mesophyll anatomy. We also evaluate the current literature on mesophyll-driven signals that may coordinate stomatal behavior with mesophyll carbon assimilation and explore stomatal kinetics as a possible target to improve A and water use efficiency. By understanding these processes, we can start to provide insight into manipulation of these regulatory pathways to improve stomatal behavior and identify novel unexploited targets for altering stomatal behavior and improving crop plant productivity.

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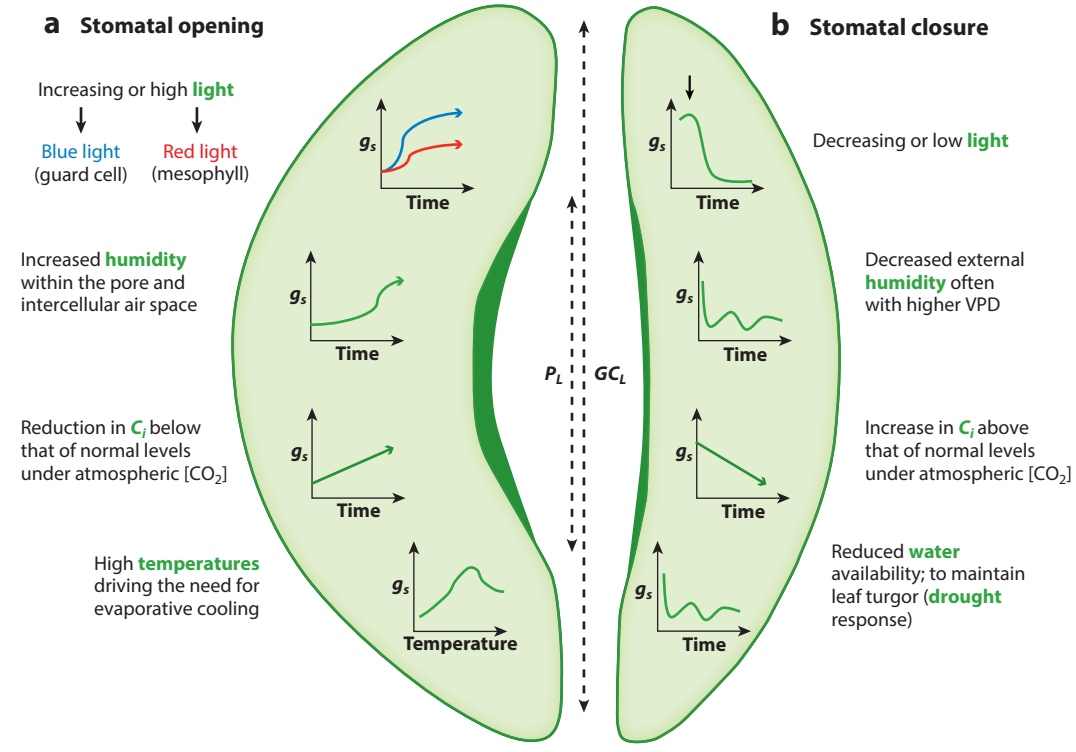
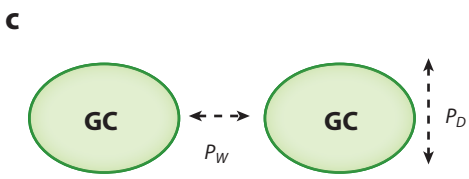
INTRODUCTION

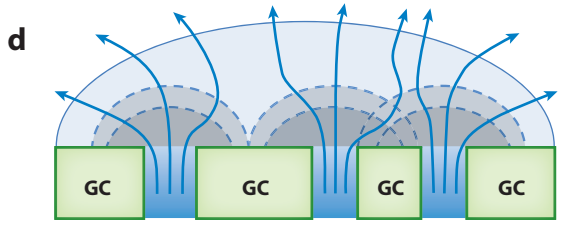
Stomata are small pores on the aerial parts of most plants that first appeared in the fossil record about 400 million years ago (48) and that, along with the development of the leaf cuticle, were instrumental in plants colonizing the terrestrial environment. The leaf cuticle is often considered impermeable to water and CO_2, and therefore almost all water lost through transpiration and CO_2 absorbed for photosynthesis pass through stomatal pores (34, 84). That said, a recent study has suggested that water lost through cuticular conductance has potentially been underestimated and can represent up to 18% of total water loss, with implications for estimations of transpiration and internal CO_2 concentration (C_i) (20, 67). Stomata typically occupy 0.3–5% of the leaf surface, and 95% of all gas exchange between the leaf and surrounding atmosphere takes place through these pores; therefore, stomatal behavior has major implications for global carbon and hydrological cycles (86).

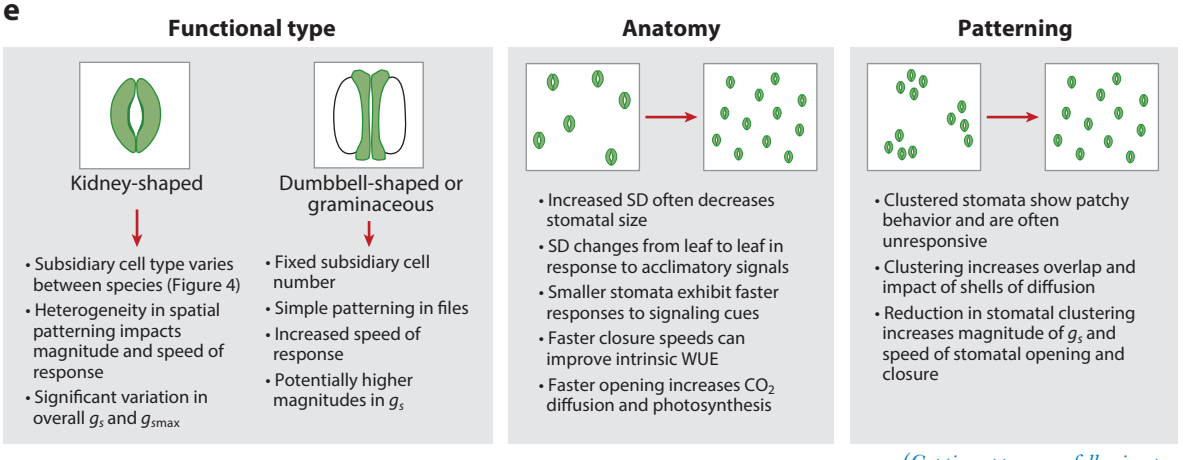
Internal CO_2 concentration (C_i): the concentration of CO_2 in the intercellular airspaces

Stomata adjust aperture in response to both environmental and internal cues (see **Figure 1**). In general, stomata in C_3 and C_4 plants open under conditions of high or increasing light intensity, low C_i, and low evaporative demand, while closure is driven by low or decreasing light intensity and high C_i and under conditions of high evaporative demand or low soil water availability (see, e.g., 104, 149, 197, 212). Stomata also respond to endogenous signals including phytohormones, hydrogen peroxide, nitric oxide, and other mesophyll-derived metabolites. In addition to facilitating CO_2 uptake for photosynthesis, stomata play a key role in transpiration that not only influences









(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Impact of stomatal characteristics on response to environmental cues. (*a*,*b*) Key environmental factors contributing to stomatal opening and closure, including but not limited to light, humidity, CO_2 concentration, and temperature. (*a*–*c*) Stomatal dimensions contributing to the calculation of g_{smax}, including pore depth (P_D), pore width (P_W), pore length (P_L), and guard cell length (GC_L). (*d*) Shells of diffusion: semicircular pattern of gaseous flux from the stomatal pore, overlapping shells (with increased stomatal density or clustered patterning) reduce and alter flux (*blue arrows*) into and out of the pore. (*e*) Key anatomical features of stomata and the impact these have on behavior, g_s, and stomatal response kinetics. Abbreviations: g_s, stomatal conductance; C_i, internal CO_2 concentration; GC, guard cell; SD, stomatal density; VPD, vapor pressure deficit; WUE, water use efficiency.

nutrient uptake (132) but is also essential for plant water status and translocation of photosynthate (2), as well as evaporative cooling and the maintenance of optimal leaf temperature for metabolic processes (140, 194).

The regulation of aperture balances CO_2 uptake for photosynthesis (A) with water loss through stomata (g_s), influencing photosynthetic rates and intrinsic water use efficiency (iWUE = A/g_s). As such, stomatal anatomy, kinetics, and biochemistry represent a mostly unexploited target for manipulating photosynthetic carbon gain and plant water use (94, 107). With unpredictable rainfall patterns and the growing pressure to increase crop productivity, stomata are central to efforts in improving photosynthesis and water use and are unexploited targets for manipulation to improve both processes and the balance between these. Here we provide an overview of various aspects of stomatal anatomy, behavior, and biochemistry that could provide novel targets for incorporation into future breeding programs. In the next sections we discuss both anatomical features and biochemical processes that influence stomatal function.

Photosynthetic CO_2 assimilation (A): the rate of CO_2 assimilation per unit leaf area

Intrinsic water use efficiency (iWUE): the ratio of CO_2 uptake (A) relative to water vapor lost through stomata (g_s)

Subsidiary cell: cell adjacent to the guard cells that is morphologically distinct from the surrounding epidermal cells

Stomatal complex: guard cells together with surrounding subsidiary cells that form a functional unit

Stomatal conductance (g_s): the capacity of stomata to allow CO_2 and water flux between the leaf and atmosphere, measured as a molar flux per unit area

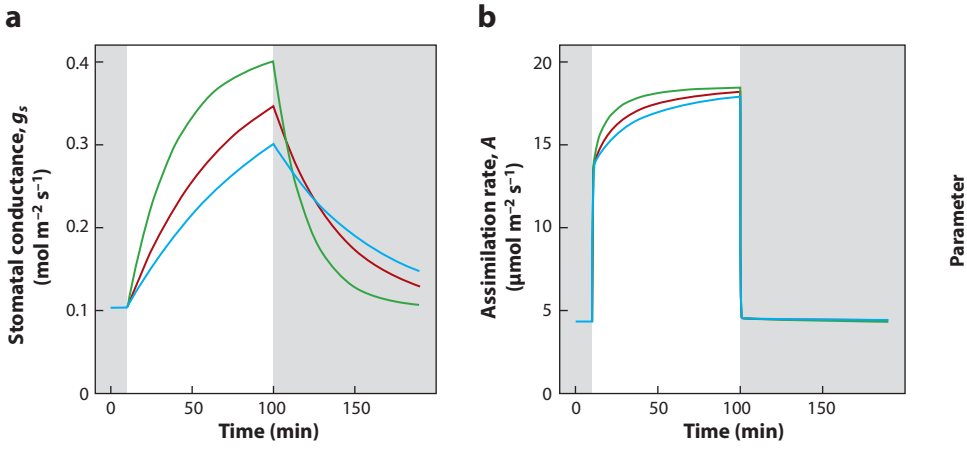
Stomatal density (SD): the number of stomatal pores per unit leaf area

STOMATAL CONDUCTANCE IS DETERMINED BY ANATOMY AND BEHAVIOR

Stomata are formed from two guard cells that surround the stomatal pore, and in many species the guard cells are surrounded by subsidiary cells that are morphologically distinct from the adjacent epidermal cells. Together these are known as the stomatal complex. The presence, number, and shape of subsidiary cells in a stomatal complex are species-specific, and these cells have been shown to play a major role in stomatal function (60, 161), which is discussed in greater detail below. Guard cells can be characterized on the basis of two distinct shapes: graminaceous, or dumbbell-shaped, and kidney-shaped (73). Dumbbell-shaped guard cells are typically found in grasses (including the majority of our major crop species) and other monocots (73). Kidney-shaped guard cells first appeared roughly 400 million years ago (47) and are found in the majority of the eudicots as well as some monocots (e.g., *Commelina*; 93). The diffusion of gases into and out of the leaf depends for the most part upon stomatal conductance (g_s), which is a measure of the maximum capacity for gaseous diffusion (of water) and is determined by both the density of stomata and aperture of the pores (96, 98, 210) (**Figure 1**). The physical pore dimensions and density of stomata determine the maximum stomatal conductance (g_{smax}). g_{smax} can be calculated from the following equation, which assumes an elliptical stomatal shape (58, 60, 156) for kidney-shaped guard cells (**Figure 1**).

$$g_{smax} = \frac{\frac{Dw}{v} SD.pa_{max}}{P_D + \frac{\pi}{2}\sqrt{pa_{max}/\pi}},$$

where Dw is diffusivity of water vapor in air at 25°C (0.0000249 $m^2\ s^{-1}$) and v is molar volume of air (0.0245 $m^3\ mol^{-1}$); both are constants. SD is stomatal density (stomata m^{-2} leaf area), pa_{max} is maximum stomatal pore area (m^2), and P_D is stomatal pore depth (m). However, in most plant species g_{smax} is rarely achieved in the field (98, 131) and therefore if stomatal aperture is measured



Parameter	Rapidity: fast	slow	middle
G_{min}	0.01	0.01	0.01
G_{max}	0.5	0.5	0.5
k_i	30	90	60
k_d	20	60	40
Vc_{max}	90	90	90
J_{max}	130	130	130
g_m	0.4	0.4	0.4

Figure 2

The influence of the speed of stomatal opening and closing on the response of (*a*) stomatal conductance (g_s) and (*b*) assimilation rate (A) to a step increase in light. Theoretical temporal response for the impact of step change in light from 100 μmol m^{-2} s^{-1} PPFD (*gray*) to 1,000 μmol m^{-2-1} (*white*). Using the model of Vialet-Chabrand et al. (198), the temporal response of g_s using a time constant (k), starting g_s (G_{min}), and a predicted steady-state g_s target at 1,000 μmol m^{-2} s^{-1} PPFD (G_{max}) is described. k_i represents the speed of opening and k_d closure. The impact of fast (*green*), slow (*blue*) and middle (*red*) values of k_i and k_d were used to assess the impact on g_s and A. Assimilation rate was determined using the Farquhar, von Caemmerer, and Berry (53) model, using fixed values of the maximum carboxylation rate of Rubisco (Vc_{max}), maximum rate of electron transport (J_{max}), and mesophyll conductance (g_m). Unpublished data of Vialet-Chabrand & Lawson. Abbreviation: PPFD, photosynthetic photon flux density.

directly and substituted for maximum pore area, operational g_s can be determined using the same equation. There is a negative relationship between *SD* and size (58), and density appears to override size or aperture when determining g_{smax} (130, 131). Because g_s is determined by both, these are obvious targets for manipulation to tune stomatal behavior. However, as stomatal behavior is influenced by continually changing environmental conditions, g_s is dynamic and therefore an obvious target for manipulating g_s is the speed of stomatal responses to changing environmental conditions (94, 104). It could be predicted that plants with more rapid stomatal kinetics would facilitate greater CO_2 uptake and avoid unnecessary water loss and therefore have a higher intrinsic water use efficiency (*i*WUE). **Figure 2** illustrates the impact of the speed of stomatal response on g_s following a step increase in photosynthetic photon flux density (PPFD) and the influence this has on CO_2 diffusion and photosynthetic carbon assimilation. Stomatal anatomy also influences stomatal function, with smaller stomata generally considered to have faster kinetics (45). Although this relationship appears to hold true for closely related species, it may not over a wide range of different species (50, 103, 128). However, what is clear is that stomata with dumbbell-shaped guard cells open and close much faster than stomata with kidney-shaped guard cells (60, 73, 128), which is due to the greater guard cell membrane surface area to volume ratio (60, 165). Interestingly, McAusland et al. (128) reported faster stomatal responses in C_4 species compared with C_3 species suggesting the importance of biochemical mechanisms as well as anatomical features (**Figure 1**).

ANATOMICAL TARGETS FOR ALTERING g_s

Alterations in density of stomata (and often with a concurrent change in stomatal size) have been an important adaptive strategy for plants in response to changing environmental signals (60) and have been key in the evolution of plants and plant acclimation to new environmental conditions.

Shells of diffusion: semicircular pattern of gaseous flux from the stomatal pore

For example, it is well established that elevated [CO_2] decreases stomatal density in the majority of but not all species (e.g., 73, 215) and that reduced water availability initiates a signal from mature leaves to developing leaves to reduce stomatal density (175). Many of the genes involved in the developmental pathway of stomata are well established, and genetic modification (GM) approaches have successfully demonstrated that reduction of stomatal density through manipulation can improve water use efficiency (WUE) at least in C_3 plants without impacting yield (27, 41, 77) as well as reducing entry of bacteria and other pathogens (46). On the other hand, increasing stomatal density through GM methods has facilitated greater CO_2 diffusion and therefore higher photosynthetic rates, which has translated into larger plants (although this was at the expense of WUE) (190). There is, however, a limit to how many stomata can be present per unit leaf area/surface, due to the cost associated with developing and operating more stomata relative to the benefits of greater g_s (40). Furthermore, high stomatal density can have a detrimental effect on stomatal functioning (40). Dow et al. (43) examined the importance of appropriate stomatal patterning and spacing using high–stomatal density mutants that also exhibited clustered stomata and demonstrated reduced g_{smax} diffusion and lower assimilation rates. Three possible mechanisms were proposed for the reduction in aperture and g_s observed in these mutants. The first is insufficient supply and movement of ions due to reduced contact with surrounding subsidiary cells. This mechanism is supported by a reduction in K^+ accumulation in guard cell cluster mutants *too many mouths*, although this was independent of any flux from the subsidiary cells (154). Secondly, the higher competitive turgor pressure exerted by surrounding guard cells would be greater than that of surrounding subsidiary cells; and finally, the development of surrounding guard cells could interrupt signaling pathways resulting in altered stomatal function (43). Additionally, as water escapes from the pore, stomata shells of diffusion are created that extend like a semicircle over the stomatal pore (**Figures 1** and **3**). Closely grouped pores would have overlapping diffusion shells (**Figures 1*d*** and **3**), which would decrease the concentration gradients and therefore the rate of gaseous diffusion (40, 43, 111, 212).

Additionally, other mechanisms such as hydraulic constraints have been shown to impact stomatal function (see **Figure 3**). For example, if hydraulic capacity is insufficient to supply water to groups of stomata, guard cell turgor pressure would be lower than that required to open the pore (40, 43), as it is well established that there is a strong correlation between leaf water supply and stomatal conductance (e.g., 22). Therefore, although manipulating stomatal density is relatively straightforward, and increasing density can lead to greater photosynthetic rates, further consideration of the underlying anatomy and hydraulic supply is needed, and it may be worth considering manipulation of the genetic constraints on mesophyll cell division and vein differentiation. Furthermore, we should contemplate how mesophyll cell structure, vein density, and stomatal anatomy are intimately connected if we are to attempt to create leaves/plants with optimal performance.

APPROPRIATE STOMATAL PATTERNING IS ESSENTIAL IN ENSURING AN OPTIMAL PATHWAY FOR CO_2 DIFFUSION

The arrangement of stomata over a leaf lamina is not random and obeys the one-cell spacing rule that states that for efficient functioning, stomata must be separated from each other by at least one cell to ensure optimal gaseous fluxes (145). The spatial distribution of stomata across the leaf is not uniform (210) and differs between and within leaves (106, 159, 180) due to both cell expansion and cell differentiation (35, 95), although this has received much less attention than clustering or functional analyses of stomata (111). Appropriate spatial arrangement of stomata across the leaf lamina relative to the underlying airspaces is fundamental in ensuring an optimal pathway for the

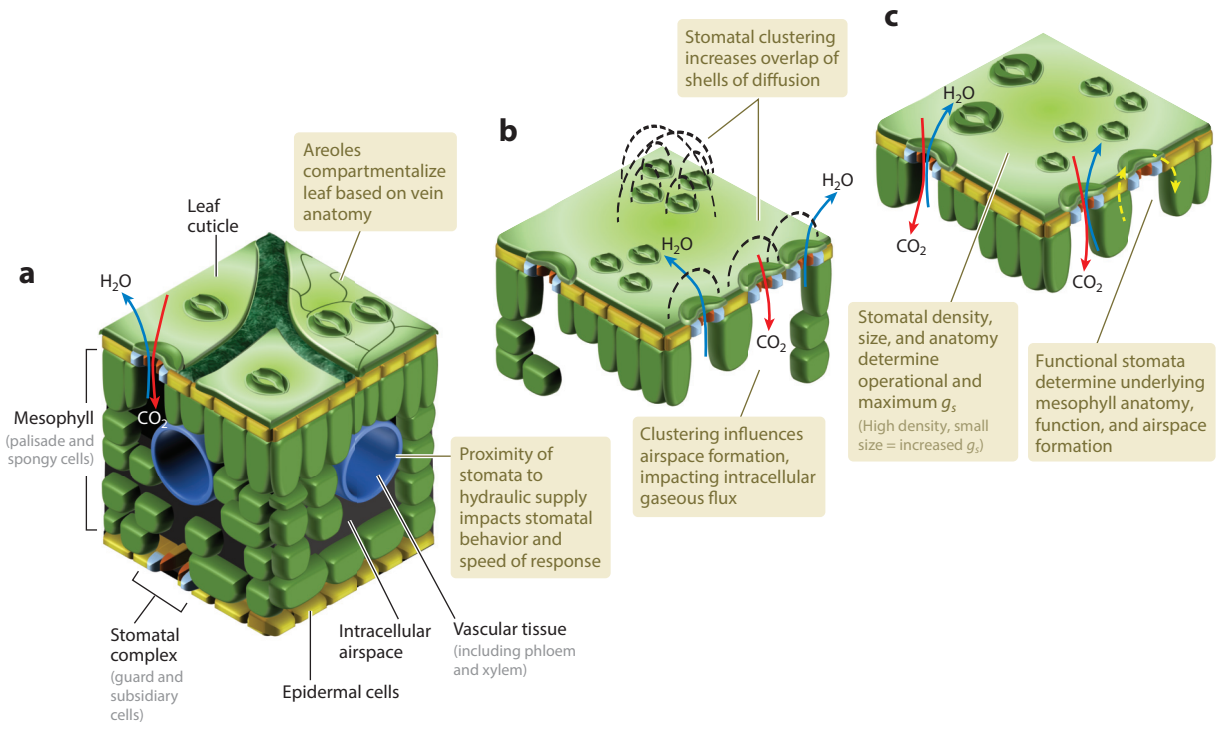


Figure 3

Schematic illustration of the impact of changes in stomatal density and patterning on mesophyll airspace, hydraulic supply, and shells of diffusion. (*a*) Example of cell structure and key components inside the leaf. (*b*,*c*) Impact of stomatal density and spacing on mesophyll airspace formation, shells of diffusion, and gaseous flux. (*b*) Stomatal clustering increases patchy stomatal behavior and increases the impact of shells of diffusion (*dashed lines*) on gaseous flux in and out of the stomatal pore. (*c*) Presence of functional stomata (*yellow dashed arrows*) and higher stomatal densities increases mesophyll airspace formation.

supply of CO_2 (**Figure 3**), to meet mesophyll demands for photosynthesis (106). Recent work demonstrated that manipulating mesophyll cell density and airspace patterning can increase leaf photosynthetic capacity (112); however, such changes need to consider the impact of spatial patterning of stomata on vertical gas diffusion. Furthermore, it has been shown that the arrangement of stomata and mesophyll cells can influence lateral gaseous flux and therefore CO_2 assimilation (e.g., 141). It has been suggested that some mesophyll signals are involved in the coordination of stomatal patterning relative to the mesophyll, including the mesophyll-driven Stomagen, which is a member of the EPFL family (EPLF 9) of cysteine-rich secretory proteins (43, 183). Stomagen is expressed in mesophyll tissues and acts in a dose-dependent manner to positively regulate stomatal density, demonstrating that photosynthetic tissue optimizes function by regulating stomatal density for optimal CO_2 uptake (183). However, assimilation rate and the number of stomata are not necessarily always correlated, as numerous studies on transgenic plants with increased rates of carbon fixation showed no change in density (e.g., 11, 97, 203), demonstrating that the link between photosynthetic demand and stomatal anatomy is complex and dynamic.

Stomatal pattern may also be important with regard to "patchy stomatal behavior," in which groups of stomata (within areoles) have much smaller pore apertures than neighboring areoles, compartmentalizing gaseous fluxes across the leaf lamina (142) (**Figure 3**). Patchy stomatal behavior is particularly prevalent under water-limited conditions, leading to reduced CO_2

Patchy stomatal behavior: groups of stomata within areoles that have a significantly different aperture to those in an adjacent areole

diffusion (98), which previously led to the incorrect conclusion that water stress directly impacted photosynthesis rather than via stomatal conductance, due to the incorrect calculation of C_i in gas exchange measurements (44, 96, 210). However, in amphistomatous leaves, Mott et al. (142) showed that stomatal responses on the upper and lower surface were not always coordinated, which raised questions regarding the signals that link mesophyll demands for CO_2 and g_s (see below). Lundgren et al. (120) have recently demonstrated that mesophyll airspace formation and patterning are linked to stomatal function and therefore provide a novel target for future improvements in photosynthesis and water use. However, what is not clear is what the optimal balance between mesophyll cell and stomatal density is, given that more stomata would initiate additional air spaces that would require extra hydraulic supply, all of which would be at the expense of main photosynthesizing cells (**Figure 3**). Further work is therefore needed to determine the exact coordination of these tissues and cells, if we are to exploit these as targets for enhanced productivity.

HYDRAULIC CAPACITY AND SUPPLY INFLUENCE STOMATAL FUNCTION

In order for stomata to open to supply sufficient CO_2 for mesophyll demands, a continuous supply of water is required to replace that lost through transpiration supplied by the vein network (55) (**Figure 3**). Increasing vein density and reticulation have occurred through natural selection as increases in hydraulic supply have been closely correlated with higher photosynthetic rates (21–23, 220). However, increased vein density also has a cost associated with it due to the space that would otherwise be occupied by mesophyll cells (as discussed above) (31, 55). Therefore, for optimal plant performance coordination between the density of stomata and veins exists, although most studies examining this relationship have focused on the development of these relationships over evolutionary time (29, 55, 131). Fiorin et al. (55) investigated the spatial arrangement of stomata and veins in a range of different species (mostly trees and shrubs) and demonstrated that stomata and veins are spatially coordinated and that the distance between these limits stomatal numbers, due to hydraulic supply (22). They reported uniformity in spatial patterning within leaves, suggesting that optimal spatial organization of veins and stomata enables a constant mesophyll hydraulic resistance throughout the leaf. Additionally, these authors reported a uniformity of spatial patterns of stomatal density with measurements taken at a number of different positions across the leaf lamina. However, several studies have demonstrated significant heterogeneity in stomatal density over the leaf surface (180) that influenced photosynthetic rates (105, 210). The majority of leaves examined in the study by Fiorin et al. (55) would be defined as heterobaric with looping vein contours providing areoles and compartmentalizing the gaseous exchange in the leaf (106), and therefore it would be interesting to examine homobaric leaves to see if similar conclusions could be drawn. This further emphasizes the need to understand spatial variation in anatomical features if we are to understand the whole leaf–level requirement to optimize stomata function with photosynthetic demands and water use.

THE IMPORTANCE OF SUBSIDIARY CELLS IN STOMATAL FUNCTION

In order for stomata to open, the guard cells must swell and overcome the back-pressure of the surrounding subsidiary and epidermal cells, which also exert a turgor pressure in the opposite direction to stomatal opening. Franks & Farquhar (60) examined the mechanical characteristics of four species with diverse evolutionary histories and differences in guard cell morphology. They demonstrated that it would be nearly impossible for the species with dumbbell-shaped guard cells (*Triticum aestivum*) to achieve the g_s measurements recorded without a substantial decrease in subsidiary cell turgor pressure and a reduction in the mechanical advantage (60). These authors

proposed osmotic and turgor pressure seesaws between guard and subsidiary cells during stomatal opening and closing. The evolution of dumbbell-shaped guard cells in graminaceous plants as well as the movement of solutes between subsidiary and guard cells to facilitate rapid stomatal movement and high g_s were proposed to underpin the success of grasses (60). The importance of subsidiary cells for stomatal function in grasses was confirmed by Raissig et al. (161), who identified a transcription factor required for the formation of subsidiary cells in *Brachypodium distachyon*. Removal of this transcription factor resulted in plants without subsidiary cells and stomata that were less responsive and had smaller apertures. This work clearly demonstrates the importance of the surrounding subsidiary cells in stomatal behavioral responses and the contribution they make to overall plant performance through enhanced tuning of stomata to facilitate increased carbon assimilation and WUE (161).

Osmoregulation: flux of ions, solutes, and water that regulate osmotic pressure and water potential

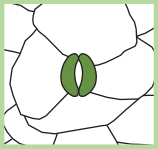
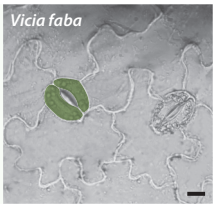
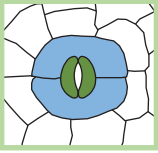
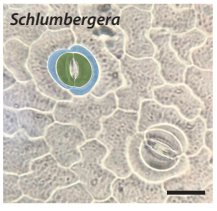
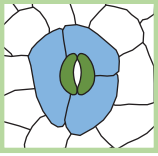
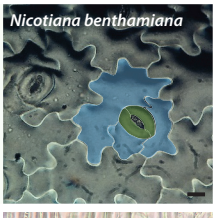
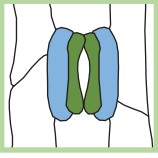
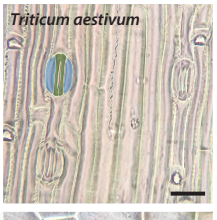
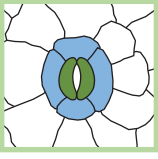
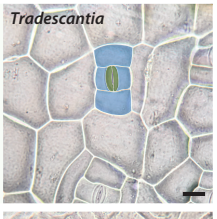
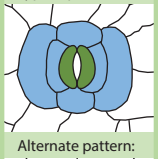
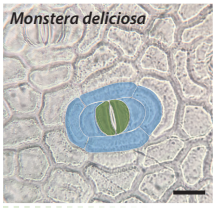
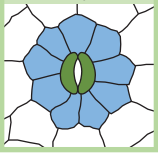
Although there have been several early studies that described the different morphologies of subsidiary cells (see **Figure 4**), few have focused on elucidating the role of these cells in stomatal function. The classical definition of subsidiary cells is the following: cells that directly surround guard cells and are differentiated from other epidermal cells in dimension, structure, or form. Those that are not differentiated from epidermal cells are considered neighboring cells (139). However, many reports in the literature apply the term to all cells that border guard cells, irrespective of whether they are morphologically distinct from the remaining epidermal cells. Here, we provide an example of some of the vast number of stomatal complexes described [following the classic terminology of Metcalfe & Chalk (139)]. **Figure 4** shows examples of distinct stomatal complexes that represent some of the most commonly observed, including anomocytic (which occurs in the Ranunculaceae), anisocytic [which occurs in the Cruciferae and includes *Arabidopsis* (176), potato, and tobacco], and graminaceous (Metcalfe later classified the monocots as tetracytic, characterized by two lateral and two dorsal guard cells, e.g., Poaceae, which includes many crop species such as wheat and barley), as well as some of the most obscure morphotypes, e.g., actinomytic, which is found in *Commelina* spp. What is intriguing is the diversity of subsidiary cell types and shapes, which together with the two guard cell types make up the complex, particularly given the current revived interest in subsidiary cells and their potential importance in stomatal function and responsiveness (see above). The vast array of anatomical types raises the question of potential differences in the importance of subsidiary cells in guard cell osmoregulation in different species, as well as the possible spatial variation in channels associated with the transfer of solutes/ions between the two cell types. These differences may influence the speed or sensitivity with which guard cells respond to changing environmental cues and could represent an unexploited target for manipulation.

SUBSIDIARY CELLS ARE INVOLVED IN OSMOREGULATION

Early studies in the 1970s demonstrated the transfer of solutes from the subsidiary cells to the guard cells, and the importance of these solute stores for guard cell osmoregulation was realized (e.g., 157, 164, 181, 213). More recently, K^+ channels in the plasma membrane of subsidiary cells were reported, and it was demonstrated that they were inversely polarized to guard cells to enable the antiparallel K^+ fluxes between these two cell types during stomatal movement (123). Büchsenschütz et al. (24) examined expression patterns of members of the shaker family of K^+ channels and demonstrated overlapping and differential expression between subsidiary and guard cells, indicating the interaction between these two cell types in maize. This work agrees with that of Pallaghy (152), who, using isolated epidermal strips of maize, showed that stomata were still able to open, even when K^+ or Cl^- was excluded from the incubation medium, while species such as *Vicia faba* that lack subsidiary cells could not open. These findings support the idea that

Figure 4

Examples of stomatal complex types composed of guard cells (*green*) and surrounding subsidiary cells. In all illustrations and photographs, guard cells are shaded dark green and subsidiary cells are shaded blue. Included are anatomical descriptions and example families. Complex types and nomenclature follow information from References 139 and 196. Scale bars represent 20 μm.

Type of stomatal complex	Description	Example families	Examples
Anomocytic (irregular celled) 	Guard cells surrounded by cells no different from epidermal cells in size and shape, and may lack true subsidiary cell function. Metcalfe & Chalk (1950) reported these occur in 142 families. Sometimes called ranunculaceous.	Chenopodiaceae Cucurbitaceae Apocynaceae Boraginaceae	*Vicia faba* 
Diacytic/Paracytic (two celled) 	Guard cells surrounded by two perpendicular (diacytic) or parallel (paracytic) subsidiary cells. Illustration is diacytic, example given is paracytic.	Caryophyllaceae Acanthaceae Cactaceae	*Schlumbergera* 
Anisocytic (unequal celled) 	Guard cells surrounded by three unequally sized subsidiary cells, one of which is distinctly smaller than the other two. Metcalfe & Chalk (1950) reported these occur in 37 families.	Crassulaceae Solanaceae Brassicaceae	*Nicotiana benthamiana* 
Graminaceous (grass-like) 	Dumbbell-shaped guard cells surrounded by subsidiary cells that lie parallel to the long axis. Metcalfe & Chalk (1950) described different types.	Cyperaceae Poaceae	*Triticum aestivum* 
Tetracytic (four celled) 	Guard cells are surrounded by four subsidiary cells—two lateral and two polar. Found mostly in monocots but can occur in dicots such as *Tilia*.	Poaceae Asclepiadaceae Asphodelaceae Commelinaceae	*Tradescantia* 
Actinocytic (star-celled) Typical pattern: 	Typical pattern for *Commelina communis* (monocotyledon with kidney-shaped guard cells). A great deal of early stomatal work was conducted on this species due to the ease with which the epidermis peels.	Typical pattern: Ebenaceae Commelinaceae Araceae	*Monstera deliciosa* 
Alternate pattern: 	Guard cells surrounded by four or more subsidiary cells, elongated radially to the stomata.	Alternate pattern: Araceae Musaceae	Guard cell Subsidiary cell Epidermal cell

the subsidiary cells provide a reservoir of ions for stomatal function. Recent studies have shown drought-induced accumulation of the signaling messenger H_2O_2 in subsidiary cells was important for stomatal closure (216), further demonstrating their significance in stomatal signaling mechanisms. A recent report has established that a glucose transporter found in the subsidiary cells of maize is the missing link in the feedback regulation of stomatal movement and photosynthesis (204). In this study Wang et al. (204) showed that mutations in *closed stomata1* (*cst1*), which encodes for a subsidiary cell glucose transporter, had reduced g_s and A, which resulted in carbon starvation and an early senescence phenotype. It is clear from current and previous literature that subsidiary cells play a significant role in stomatal function and signaling mechanisms. The subsidiary cell components of the stomatal complex represent an obvious and currently unexploited target for improving stomatal function and kinetics, but before such targets can be utilized, more information is needed regarding their anatomy, biochemistry, and membrane transport channels, as well as their relationship with guard cell behavior.

GUARD CELL OSMOREGULATION

Despite decades of research on stomatal responses to environmental cues, the signaling mechanisms and osmoregulatory pathways involved are not all fully understood, although there have been major advancements over the last two decades (66, 171). The role of the mesophyll and the mechanisms that coordinate mesophyll activity with guard cell response are also a current topic of debate. Furthermore, it is still not clear whether the guard cells perceive changes in these cues (e.g., $[CO_2]$ and PPFD) or respond as a result of a signal from the mesophyll (10). Modifications in guard cell shape are the result of accumulation or loss of solutes, which alters water potential triggering water to move in or out of the guard cell, increasing or decreasing turgor pressure. The large plasticity in guard cell function demonstrates the difficulty in fully elucidating the specific mechanisms involved in stomatal behavior and responses to specific cues.

OVERVIEW OF OSMOREGULATORY PATHWAY

Early reports that investigated osmoregulation in guard cells supported the concept that the main osmolyte necessary for stomatal opening was sucrose, produced from the breakdown of starch in stomatal guard cells. The starch-sugar hypothesis (116) was the established theory for osmoregulation well into the 1960s, when uncertainties about the correlation between starch content and stomatal aperture led to exploration of further mechanisms. It was eventually replaced by the K^+-malate theory (78, 162), which correlated stomatal opening with K^+ uptake and the counterions malate and/or Cl^- (3, 147, 173). This theory was acknowledged as the main osmoregulatory pathway and replaced the starch-sugar hypothesis. It was not until MacRobbie & Lettau (122) suggested K^+ and its counterion malate could not provide all the osmolytes required to support stomatal apertures measured in *Commelina communis* that the original starch-sugar hypothesis was revisited, with further reports supporting this theory (118, 158, 186, 188). Later, Talbott & Zeiger (187) provided evidence for both osmoregulatory pathways operating in guard cells, but at different times of the day. K^+ was proposed to be important for stomatal opening early in the day, and sucrose later in the diel period to maintain stomatal aperture (4, 174, 187).

ION TRANSPORT AND STOMATAL FUNCTION

There have been many studies that recognize that stomatal opening and closing occur from the collective role of ion transport across the plasma membrane and tonoplast (15, 32, 33, 74, 82, 207, 212). The main solutes transported across the plasma membrane are the inorganic K^+ and Cl^-

ions and the organic anion malate as well as sucrose, which also make up the bulk of solute that drives the changes in water flux and guard cell turgor associated with stomatal movement (127, 169). As mature guard cells lack functional plasmodesmata (211), these solutes must be transported across the plasma membrane. A considerable amount of this solute taken up by the guard cell must be transported across the tonoplast; the guard cell vacuole makes up the majority of the cell volume, playing an important role as a store for these solutes (33, 63, 121). Malate metabolism and synthesis make a considerable contribution to the osmotic content of the guard cell, while the loss of malate during the process of stomatal closure occurs via efflux across the plasma membrane (206). Over the years, the validity of assuming a priori that transport activity remains constant on a unit-surface-area basis has been called into question, with several studies indicating substantial variation between species, for example, of outward-rectifying K^+ currents in intact guard cells of *Arabidopsis*, *Vicia faba*, and *Nicotiana tabacum* (1, 14, 18, 33, 49, 83, 207). Analyzing, in mechanistic terms, the sequence of events that occur during ion and solute transport, in order to separate the individual transporter currents, is the focus of a number of reviews, and we would refer the reader accordingly (15, 17, 72, 74, 81, 127, 137, 153, 205).

THE ROLE OF STARCH IN STOMATAL FUNCTION

Starch has long been implicated in stomatal function and shown to be at higher levels in the guard cells of *V. faba* when stomata were closed (151) and correlated with volume changes during opening (172). However, until recently, it was still a matter of debate whether starch is present in *Arabidopsis* guard cells at the end of the night or, indeed, whether it is required for stomatal opening (37, 92, 182), to the point that starch metabolism in guard cells was considered to differ among species (101). It has, however, recently been shown that starch degradation within the first 30 min of (blue) illumination is correlated with a corresponding increase in stomatal aperture (75), confirming earlier work by Tallman & Zeiger (189) showing that starch breakdown in guard cells was under the direct control of the blue light signaling pathway (see below). However, it is still not entirely clear whether starch breakdown provides carbon skeletons for malate synthesis or produces sucrose that is used either as an osmoticum or a respiratory energy source to provide ATP, or a combination of both. Although earlier work on the *Arabidopsis* starchless mutants did exhibit reduced rates of stomatal opening under blue light (92) and supported a role for PEPc activity in the formation of malate (135), Horrer et al. (75) also demonstrated a distinct set of hydrolytic enzymes (BAM1 and AMY1) involved in starch degradation specific in guard cells that are not used in other leaf tissues. Furthermore, in β-amylase (*bam1*) mutants, reduced water uptake and limited cell wall extension associated with stomatal closure from impaired guard cell starch breakdown led to improved drought tolerance (160). This highlights that blocking starch degradation results in elevated starch levels within the guard cell, impacting stomatal osmoregulation, functional response, and even biomass production (75, 160, 195).

STARCH BIOSYNTHESIS IN GUARD CELLS

Horrer et al. (75) showed that starch biosynthesis in guard cells begins 1 to 3 h after light is turned on, when stomata have fully opened, and proceeds slowly through the diurnal periods and into the night. These authors demonstrated that red light promotes efficient starch synthesis in guard cells, whereas blue light promotes starch degradation, although both stimuli open stomata. It is conceivable that red light–induced CO_2 fixation provides the precursors needed for starch synthesis. Furthermore, a recent study suggested that starch biosynthesis in guard cells, but not in mesophyll cells, is involved in high-CO_2-induced stomatal closure (8). These authors compared stomatal

responses to changes in [CO_2] in *Arabidopsis* mutants that were starch deficient in either all plant tissues (*ADGase*) or just the mesophyll while retaining starch accumulation in the guard cell (*pgi1*) and demonstrated that *ADGase* but not *pgi1* exhibited impaired CO_2-induced stomatal closure. This suggests that starch biosynthesis in the guard cell functions as part of CO_2-induced stomatal closure, whereas in the mesophyll tissues it does not. Starch in the guard cell can be synthesized from CO_2 fixed via the Calvin cycle or from sugars and/or organic acids that have accumulated early in the day (synthesized by the guard cells themselves or imported from neighboring mesophyll cells). The relative contributions of these mechanisms to the pool of accumulated starch, as well as their timing, remain a matter of debate and may in fact differ among species and acclamatory states (94, 125, 170). Guard cells can produce malate by the carboxylation of PEP using CO_2, as demonstrated by experiments with isolated epidermis exposed to $^{14}CO_2$ (37, 163, 213). Furthermore, hexose phosphate needed for guard cell starch accumulation can also be derived from the metabolism of sucrose stored in the vacuole or from sugars imported directly from the apoplast (38, 39, 115, 171). It is possible that sucrose, or its degradation products, is metabolized to hexose phosphate, which may then move into the chloroplast to be converted to starch. If so, hexose phosphate transport would reduce the phosphate concentration in the chloroplast and provide carbon for starch synthesis in the form of ADP-glucose (ADPGlc). These findings suggest that hexose phosphate could represent a target for direct manipulation of guard cell metabolism and function and/or the signaling pathways linking mesophyll carbon assimilation and stomatal behavior.

ROLE OF SUCROSE IN STOMATAL FUNCTION

Sucrose accumulation in guard cells is possible via three main paths: produced from starch breakdown in the guard cells (116); produced by photosynthesis in mesophyll cells and translocated via the apoplast to the guard cells (158, 186, 187, 189); and produced from guard cell photosynthetic carbon metabolism. It would seem, however, that photosynthesis and starch breakdown within the guard cell may only provide limited amounts of sucrose, and therefore sucrose transported from the apoplast represents the greatest source of guard cell sucrose (166, 191, 197), which is taken up into the guard cell symplast (85, 117, 118, 150). This occurs through the plasma membrane directly as sucrose via the sucrose-H^+ symporter and/or hexose-H^+ symporters following cleavage of sucrose by cell wall invertase. It has further been suggested that sugars themselves do not act as guard cell osmolytes but are instead degraded by invertase and sucrose synthase producing hexose monomers that are sensed by hexokinase, and result in stomatal closure via a mechanism mediated by abscisic acid (ABA) produced in the guard cells (66, 89). However, it is not entirely clear whether sucrose is degraded by apoplastic invertases and hexose enters the guard cells through hexose transporters, or whether it is imported through sucrose transporters and broken down directly in the guard cells themselves (114). Constitutive overexpression of hexokinase (HXK) in tomato and *Arabidopsis* enhanced sugar sensing and reduced stomatal conductance, transpiration rate (87, 89), photosynthesis, and growth (36, 80, 87). Furthermore, overexpression of HXK in guard cells using a guard cell–specific promotor (88) also resulted in reduced stomatal apertures and transpiration rates (89, 119), demonstrating that guard cell sucrose metabolism could represent a target for manipulation for crop improvements. Subsequently, recent studies have examined the response of stomata to different concentrations of externally added sucrose (133) and the HXK substrates glucose, fructose, and mannose (70, 113). While low concentrations had little or no effect, higher concentrations of sucrose and the HXK substrates resulted in stomatal closure, further supporting the idea that sucrose accumulation in the guard cells and hexokinase activity stimulate stomatal closure. Guard cell transcriptomic analyses have shown that the major sugar transporters across

the plasma membrane are the sucrose-H^+ transporters SUC1 and SUC3 and the monosaccharide-H^+ transporters STP1 and STP4, with suggestions that sucrose is converted to monosaccharide in the guard cell apoplast (12, 13, 182). It has been suggested that these cotransporters exhibit high levels of expression at times when guard cells accumulate high amounts of sugars, such as midday (182, 187), and therefore analysis of these transporters and their impact on stomatal function is needed (see recent reviews 170, 171). It has been suggested that if the effect of sucrose in guard cells is solely related to a role as an osmolyte, plants with lower accumulation of guard cell sucrose should have smaller stomatal apertures and lower levels of g_s. However, Daloso et al. (38) recently highlighted the importance of the breakdown of sucrose in the guard cell, via overexpressing sucrose synthase (SuSy), which converts sucrose to fructose and glucose, with a guard cell–specific promotor. These authors found that the transgenic lines displayed markedly higher stomatal conductance, photosynthetic rates, and overall biomass, which were attributed to an increase in sucrose degradation capacity (6, 38). Furthermore, Daloso and colleagues have demonstrated that the manipulation of guard cell–specific sucrose transporters, such as SUT1, can improve WUE and the interplay between carbohydrate metabolism and K^+ accumulation (5). The same authors have suggested that the function of sucrose is primarily energetic (38) and that breakdown provides substrates for glycolysis and the tricarboxylic acid (TCA) cycle (133). Furthermore, Lima et al. (115) used a modeling approach to propose that a futile cycle (involving sucrose synthase, invertase, hexokinase, phosphoglucomutase, and UDP-glucose pyrophosphorylase, which are highly expressed in guard cells) would circulate C around sucrose making it available for glycolysis when required, although this has yet to be confirmed experimentally. With evidence such as this, it is easy to assume that sucrose accumulation and breakdown both play roles in the opening and closing behavior of stomata. Based on the literature there is evidence that sucrose may act as an osmoticum at certain times of the day and/or under particular conditions, provides energy through glycolysis and the TCA cycle, provides carbon skeletons for malate formation, and is a signal coordinating photosynthesis and stomatal behavior.

ROLE OF GUARD CELL CHLOROPLASTS IN STOMATAL FUNCTION

The role of guard cell photosynthesis and its contribution to stomatal function have been debated for several decades (93). Guard cell electron transport can be moderated by [CO_2] (136), suggesting that Calvin cycle activity acts as a major sink for electrons (99) and sucrose production is osmotically important for stomatal opening (186, 189, 218). On the other hand, several studies argue against a role of guard cell photosynthesis, suggesting that levels of Rubisco and chlorophyll in guard cells are too low to produce any significant contribution to osmoregulation (e.g., 148), with only a 2% contribution calculated by Reckmann et al. (166). Stomata in transgenic tobacco plants with reduced photosynthetic capacity, in both guard and mesophyll cells, responded to light and changing [CO_2] similar to wild type (11, 97, 203), suggesting that photosynthesis (in either cell type) is not essential for stomatal function. However, a recent study by Azoulay-Shemer et al. (9) has renewed interest in the role of the guard cell chloroplasts and guard cell photosynthesis in stomatal function. Transgenic *Arabidopsis* plants with degraded chlorophyll in their guard cells, and therefore impaired photosynthesis, exhibited a deflated, thin phenotype, suggesting that photosynthesis in guard cells is critical for guard cell turgor. Interestingly, these plants showed typical wild-type responses to [CO_2] and ABA, indicating that guard cell photosynthesis is not involved in these responses (9). Furthermore, Wang et al. (207) found that, in an *Arabidopsis crumpled leaf* mutant (*cr1*), stomatal aperture in plants lacking guard cell chloroplasts (*cr1*—no chl) was 40% to 50% smaller than that of the wild type, possibly due to reduced ATP levels. These studies provide evidence that guard cell chloroplasts and mesophyll contribute to ATP (37) for H^+ extrusion and

play an essential role in light-induced stomatal opening (207); however, further work is required to fully elucidate the role of chloroplasts in guard cells. Recently, Robaina-Estévez et al. (168) suggested that CO_2 diffusion (as in mesophyll cells) does not support the Calvin cycle in guard cells, but CO_2 was provided by the decarboxylation of cytosolic malate by a plastidial malate dehydrogenase after malate had been imported into guard cell chloroplasts by a dicarboxylate transporter. It is clear from recent studies that guard cell chloroplasts do contribute to stomatal behavior, through either the supply of energy for proton pumps or the production of osmotica, or as some type of signaling component, although the extent of this contribution remains to be determined. Further studies using advances in molecular technology, such as cell-specific promoters and the ability to perform single-cell metabolomics, may allow us to better elucidate the role of guard cell photosynthesis in stomatal function.

SPEED OF STOMATAL RESPONSES

Questions regarding the magnitude and speed of stomatal responses to environmental stimuli are closely associated with characteristics of the guard cells, including capacity for the transport of solutes in relation to guard cell volume and the speed with which this transport responds to environmental cues (94). The density and activity of transport proteins across the plasma membrane determine transport capacity and are related to guard cell turgor, which in turn governs stomatal opening and closing (45, 58, 73). Guard cell characteristics, such as size, geometry, and in some cases density, have been shown to affect the speed of stomatal movements and the associated magnitude of g_s, with larger stomata often in conjunction with lower densities exhibiting slower responses (51, 60, 94). The importance of the size of the stomatal complex is based upon the assumption that the flux of solutes is essentially uniform when normalized to the surface area. Therefore, the time needed to adjust solute content within the cell volume would decrease approximately in proportion with guard cell volume to surface area ratio and the dimensions (length, width, depth) of the guard cell (73, 96, 165). The speed with which transport of solutes responds to environmental cues, especially to light and CO_2, is determined by the connections between transporters at each membrane (94). Stomatal aperture responds to signaling cues over substantial time scales, often far greater than that needed for changes in guard cell transport activity and membrane voltage (101, 207). As an example, depolarization of the membrane by ABA stimulates K^+ transport within seconds through outward-rectifying K^+ channels, such as the GORK K^+ channel in *Arabidopsis* (76, 184); a rise in cytosolic pH then enhances the currents of K^+ during the next 3 to 5 min (16, 65). Guard cell movement, stomatal aperture, and therefore g_s respond more slowly, typically with opening times varying between 10 min and several hours, depending on guard cell type, plant species, and acclimatory state (45, 126, 128, 202). Thus, the correlation between the speed of stomatal response and the speed of solute flux and accumulation is not necessarily direct. Drawing on quantitative systems modeling in order to relate ion transport with guard cell metabolic processes, Blatt and colleagues developed OnGuard models for *V. faba* and *Arabidopsis* (19, 33, 74, 199, 207, 209). OnGuard models integrate the molecular, biophysical, and kinetic characteristics of guard cells, including ion transport, malate metabolism, and H^+ and Ca^{2+} buffering, and attempt to connect this information to stomatal kinetics and behavior. These OnGuard models have demonstrated a high degree of predictive power and have highlighted previously unknown and in some cases unexpected ion channel behavior in guard cell movement (see 81, 94), for example, the role of K^+ channel activity in guard cell responses to vapor pressure deficit (208). This is a useful resource for establishing targets for potential manipulation of stomatal dynamics, including but not limited to ion transport, sucrose metabolism, and starch biosynthesis. It is also important to consider how

manipulation of such processes may connect to stomatal anatomy and patterning, with several recent studies, for example, highlighting the potential of altering stomatal density and kinetics for increased carbon gain, drought tolerance, and biomass accumulation (27, 59, 71, 77, 103, 155).

COORDINATION BETWEEN MESOPHYLL AND STOMATAL RESPONSE

Although g_s is closely linked with mesophyll demands for photosynthesis and a strong correlation between A and g_s has been demonstrated many times (e.g., 25, 52, 124, 214), stomatal responses to changing environmental cues can be an order of magnitude slower than photosynthetic responses (94, 104). Therefore, short-term changes to environmental cues under dynamic fluctuating environments can lead to a temporary disconnect between A and g_s (91, 104, 105, 125, 126, 128, 192, 200, 201). Low g_s or slow stomatal responses can limit A by restricting CO_2 diffusion, which when integrated over the entire growing season can reduce biomass and yield. It has been reported that stomatal resistance to CO_2 diffusion can limit photosynthetic rates by 20% in well-watered C_3 species, although this is less in C_4 plants (52, 84), while high g_s can lead to unnecessary water loss that can make the plant more vulnerable to water stress or cavitation (depending on the species). Reduced stomatal aperture can also impact evaporative cooling and maintenance of optimal leaf temperatures, which can have a further knock on effect on A and yield (56, 57). Therefore, manipulation of stomatal behavior and/or the mechanisms that correlate A and g_s could provide potential targets for manipulating CO_2 assimilation in the field; however, in order to do this more information on the mechanisms and signaling pathways that coordinate A and g_s is required, as well as an understanding of the hierarchy of stomatal responses.

Both A and g_s respond to light, which is one of the most dynamic environmental signals and plays a key part in the coordination of A and g_s. Stomatal response to light is dependent on two distinct signaling mechanisms, termed the red and blue light responses (7, 169, 178, 217) (**Figure 5**). The red light or mesophyll response occurs at high fluence rates and saturates at similar intensities as photosynthesis and is often considered to be the link between A and g_s, as it can be eliminated with the application of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (138, 146, 177, 193). For many years the concentration of CO_2 inside the leaf (C_i) was believed to coordinate the response of g_s with A. However, several studies have demonstrated a stomatal red light response even when C_i is held constant (97, 138), supporting the idea of a specific mesophyll signal (see below). The stomatal blue light response is specific and saturated at light levels too low to drive net carbon assimilation, and therefore not related to mesophyll photosynthesis (**Figure 5**). On a quantum basis the stomatal blue light response is 20× more effective than red light at driving stomatal opening (90, 177, 178). It is thought to facilitate responses to rapid changes in light (such as sunflecks; 7, 79), and is responsible for dawn opening of stomata (when the spectrum is enriched with blue light) to maximize photosynthetic rate early in the day (187, 188). Although it is well known that some species do not have a stomatal blue light response (42), little it is known about the diversity in the magnitude or rapidity of these responses. This is especially important when considering the impact stomatal behavior in response to blue light might have on carbon uptake and water use in major crop species. The fact that stomata open with blue light even when photosynthesis is already saturated with red light (178) means that g_s can be higher than required to achieve maximum CO_2 uptake for photosynthesis, and therefore *i*WUE is greatly reduced. Reducing stomatal sensitivity to blue light has the potential to optimize crop resource use, thereby maintaining photosynthetic rates while using water more efficiently in certain environments. Decreasing water loss would enable sustained photosynthetic rates through the

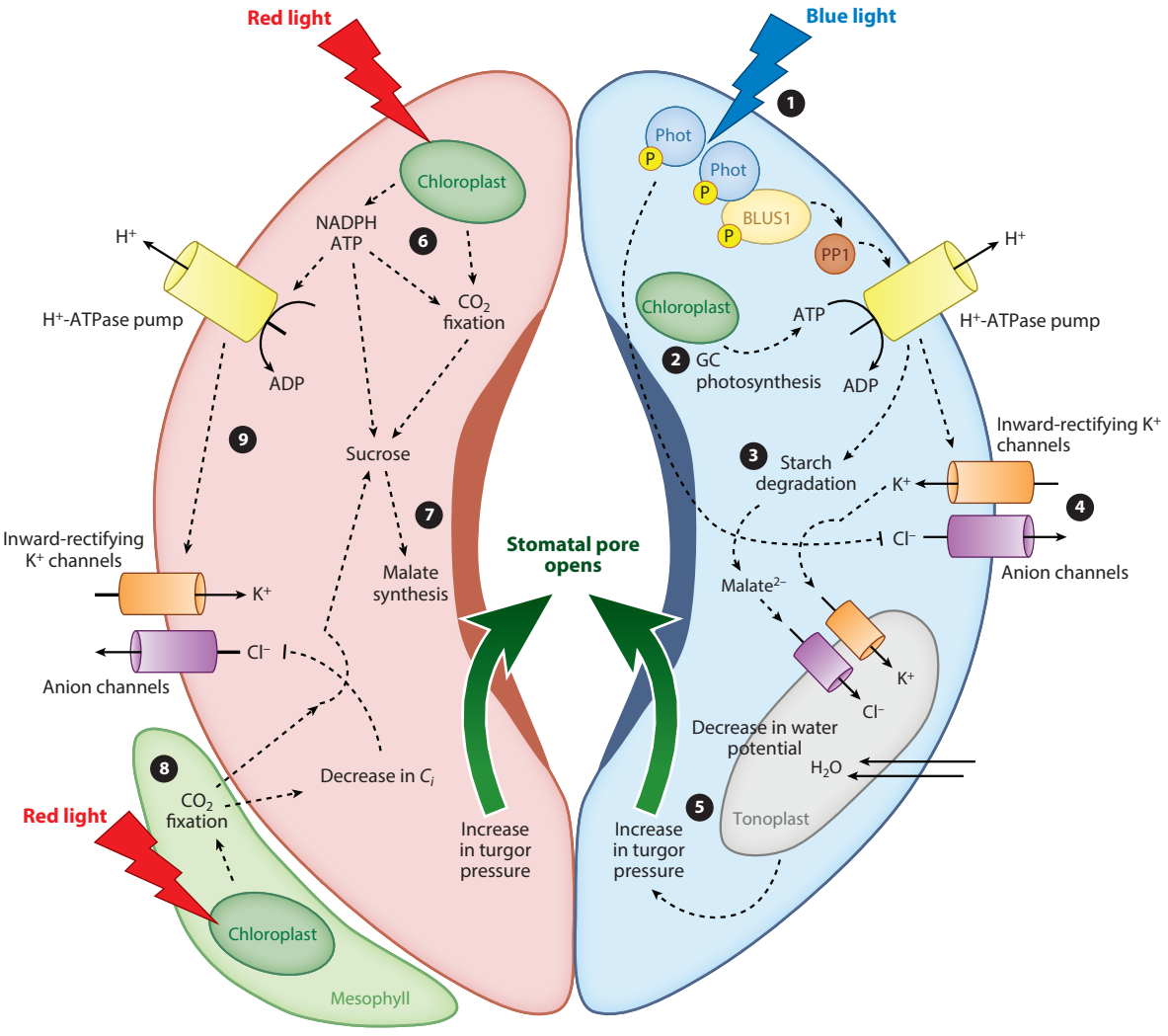


Figure 5

Simplified schematic of red and blue light signaling pathways in stomatal guard cell and mesophyll tissues. Osmoregulation steps involved in (①–⑤) blue light– and (⑥–⑨) red light–driven stomatal opening. (①) Phototropin activation by blue light phosphorylates BLUS1 kinase leading to activation of H^+-ATPase protein pumps. (②) GC chloroplast photosynthesis provides ATP for activation of H^+-ATPase pumps. (③) Starch degradation in the GC leads to malate^{2-} synthesis. (④) Activation of inward-rectifying K^+ channels along with deactivation of Cl^- anion channels leads to K^+ and Cl^- accumulation in the GC. (⑤) Transport of K^+, Cl^- and malate^{2-} into the vacuole decreases GC water potential and increases turgor pressure as water enters the GC vacuole from the subsidiary cell. The GCs swell, opening the stomatal pore. (⑥) Production of ATP and NADPH via GC photosynthesis leads to (⑦) malate synthesis and accumulation via CO_2 fixation and sucrose production. (⑧) Alternatively, photosynthesis in the mesophyll cell causes a reduction in C_i inside the GC, deactivating Cl^- anion channels. (⑨) Inward-rectifying K^+ channels and H^+-ATPase protein pumps are activated via GC photosynthesis. Abbreviations: BLUS1, blue light signaling1; C_i, internal CO_2 concentration; GC, guard cell; PP1, protein phosphatase 1. For further reference, see 38, 75, 79, 93 and 185.

grain-filling period when water becomes limiting, thus enhancing photosynthetic potential and overall grain yield (30). Of additional note is that nocturnal stomatal conductance has been reported for a number of different species (28), which results in significant water loss for no carbon gain. The magnitude of nighttime conductance is species specific and depends upon the surrounding environmental conditions. Although the purpose and mechanisms behind this are not fully understood, it has been postulated that nocturnal g_s aids in nutrient and mineral uptake (129) or supports removal of excess CO_2 (134) and ensures sufficient O_2 for respiration and growth during the night. However, a recent reanalysis of published data sets on nocturnal g_s by Resco de Dios et al. (167) did not support any of the above benefits but indicated that nocturnal conductance was positively correlated with relative growth rates and agreed with the suggestion that circadian-driven nocturnal conductance enhances predawn g_s, priming stomata for photosynthesis early in the light period.

SIGNALS THAT COORDINATE MESOPHYLL AND GUARD CELL BEHAVIOR

Several reports have questioned C_i as the main driver that coordinates A and g_s. For example, it was reported that under red light g_s increased even when C_i was held constant (97, 138, 205). Furthermore, in transgenic plants in which manipulation of key enzymes in the Calvin cycle led to reduced photosynthetic rates, stomata opened in response to PPFD, irrespective of higher C_i values observed in these mutants (11, 97, 203). These findings agree with earlier studies that suggested that stomatal responses to C_i are too small to account for the changes observed in response to light (52, 162, 177). These findings led to von Caemmerer et al. (203) postulating that stomata respond to external atmospheric [CO_2] (C_a) rather than C_i. The breakdown in the relationship between A and g_s in these transgenic plants raised further questions regarding the mechanism that links these two processes, with recent reports suggesting that an as yet unidentified signal produced by the mesophyll is sensed by the guard cells triggering a response. Early work by Lee & Bowling (108, 109) suggested an aqueous metabolic signal, with possible candidates including ATP, NADPH, and RuBP (54, 193, 214, 219), as well as malate and sugar (68, 69, 110). More recent research proposed a gaseous vapor phase ion signal (143, 144, 179), with alternative suggestions such as guard cell photosynthesis (93, 98, 100). However, Fujita et al. (61) tested several hypotheses using a combination of different pore-sized cellophane and polyethylene films, between the epidermis and mesophyll, with only gaseous substances able to pass through the polyethylene, and both gaseous and aqueous solutions through the cellophane. They concluded that the signal must be aqueous based (see 101, 102), which agrees with previous research (108).

Sucrose metabolism in the mesophyll has also been proposed to play a role in the longer-term coordination of A and g_s over the diurnal period, where sucrose produced in the mesophyll during periods of high photosynthesis and in excess of that loaded into the phloem is carried toward the guard cells via the apoplast, where an extracellular osmotic effect causes stomatal closure (85, 117, 149). Additionally, meta-analysis of multiple species revealed a negative correlation between photosynthetic rate and stomatal conductance with leaf sugar content (62), highlighting that sucrose concentration does at least partially regulate the trade-off between stomatal water loss and carbon assimilation (170). However, this mechanism could only occur over longer time scales and could be responsible for the decrease in g_s and A observed over the diurnal period (125, 126, 200), as periods of high photosynthesis are generally not correlated with low g_s or stomatal closure (101). Another potential mechanism for coordinating A and g_s is the redox state of chloroplastic quinone A (Q_A) (26). Q_A is the primary electron acceptor downstream of photosystem II (PSII), and its oxidized state reflects the balance between excitation energy at PSII and Calvin cycle activity. Reports

Table 1 Examples of studies on transgenic plants that displayed markedly different stomatal and/or photosynthetic behavior, through manipulation of key guard cell–specific and constitutive/mesophyll genes associated with guard cell behavior

Manipulation/ Identification	Description	Outcome/Impact	Future/Targets	Reference(s)
Hydrolytic enzymes (BAM1 and AMY1)	Involved in guard cell–specific starch degradation	Stomatal closure and reduced water uptake, due to impaired starch breakdown	Targets for improved WUE and drought tolerance	75, 160
SUT1	Downregulation of guard cell–specific sucrose transporter SUT1	Improved WUE; revealed the interplay between carbohydrate metabolism and K^+ accumulation in regulating stomatal opening	Target for improving WUE and carbon gain	5
Sucrose synthase (SuSy)	Overexpression of SuSy increases sucrose degradation capacity	Increased g_s, photosynthetic capacity, and total biomass	Target for improving photosynthetic capacity through increases in g_s, leading to improved crop performance	6, 38
Hexokinase (HXK)	Overexpression of HXK in guard cells enhanced sugar sensing	Reduced stomatal aperture and transpiration rates	Guard cell sucrose metabolism as a potential target for improving crop performance	87, 119
ADGase + *pgi1*	Starch deficient in all plant tissues (*ADGase*) or retaining accumulation in guard cells only (*pgi1*)	Starch biosynthesis in the guard cells functions as part of CO_2-induced stomatal closure	Target for improving WUE via altered stomatal closing dynamics	8
Degraded chlorophyll	Degraded chlorophyll in guard cells to investigate role of guard cell photosynthesis in stomatal function	Thin phenotype with deflated chlorophyll-less guard cells that were continuously closed	Highlights importance of guard cell photosynthesis for maintaining turgor, and for energization of guard cell function	9
crumpled leaf mutant (*cr1*)	Plants lacking guard cell chloroplasts	Stomatal aperture 40–50% lower than that of wild type due to reduced ATP production	Highlights importance of guard cell chloroplasts in providing energy for stomatal function	207
Mesophyll density	Relationship between cell spacing, patterning, and intercellular airspace	Increased mesophyll cell density leads to increased photosynthetic capacity	Engineer improved photosynthesis	112
Mesophyll airspace formation	Mesophyll porosity is modulated by functional stomata	Mesophyll airspace formation is linked to stomatal function in monocots and eudicots	Target mesophyll and stomatal coordination for improved WUE	120
closed stomata1 (*cst1*)	Encodes a subsidiary cell glucose transporter found to be important in the feedback regulation of stomatal movement and photosynthesis	Reduced g_s and A, leading to carbon starvation and early senescence	Target for coordination of A and g_s to improve feedback regulation	204

(*Continued*)

Table 1 (*Continued*)

Manipulation/ Identification	Description	Outcome/Impact	Future/Targets	Reference(s)
Blue light signaling1 (BLUS1)	Manipulation of BLUS1, which mediates a primary step in phototropin signaling of blue light in guard cells	Removes stomatal response to blue light reducing g_s and therefore A	Target for reducing g_s to improve WUE and crop performance	185
Photosystem II subunit S (PsbS)	Altered expression of PsbS and genes related to NPQ at PSII, to examine impact on redox state of Q_A	Q_A strongly correlated with g_s in plants with increased PsbS, leading to 25% increase in WUE	Target for improved WUE and potentially A	64
OnGuard modeling	Modeling of ion transport, sucrose metabolism, and starch biosynthesis to establish stomatal behavior	Shows relationship between ion channels and stomatal function, to establish targets for future manipulation	Target specific factors to improve WUE and photosynthesis depending on plant-specific requirements	82, 155, 208

Abbreviations: g_s, stomatal conductance; NPQ, non-photochemical quenching; PSII, photosystem II; Q_A, chloroplastic quinone A; WUE, water use efficiency.

have suggested that a more reduced Q_A pool corresponds to increased g_s (26, 64). The importance of the redox state of the plastoquinone pool was demonstrated by Głowacka et al. (64) in transgenic tobacco plants with altered expression of the PSII subunit S (PsbS) and expression of genes related to the xanthophyll cycle that are involved in nonphotochemical quenching at PSII. PsbS alters the rate of excitation absorbed by the antenna complex of PSII while NPQ protects the PSII apparatus, and both influence reduction of Q_A. In a range of plants with varying expression levels, Q_A was strongly correlated with g_s, and a 25% saving in WUE was found in plants with increased levels of PsbS (64).

CONCLUDING REMARKS

The key aspects of guard cell metabolism have long been known, although the interplay between cell type (guard, mesophyll, subsidiary) and function is still largely unidentified. Within the last decade, there has been a significant push toward understanding the mechanisms involved in guard cell movement and response to environmental and signaling cues, in an attempt to highlight previously unexploited novel targets for potential crop improvement. Manipulation of key stomatal characteristics, such as density, has demonstrated the potential for employing genetic techniques to future-proof crops to growing climate change (**Table 1**). However, the impact of these manipulations on stomatal function along with the complexity and hierarchy of these responses have been largely overlooked. Similarly, the osmoregulatory aspects of the links between A and g_s have also often been ignored, and despite decades of research there are still major gaps in our knowledge of the mechanisms synchronizing these two factors. Evaluating stomatal function in conjunction with subsidiary cell osmoregulation, hydraulic supply, and underlying mesophyll anatomy is vital to advancing our holistic and mechanistic understanding of the impact of stomatal behavior in response to changing environmental conditions. Here we highlight that with a greater understanding of the coordination between stomatal function, guard cell metabolism, and mesophyll photosynthesis, we can start to unlock new mechanisms and novel targets for refining stomatal response to improve crop performance.

SUMMARY POINTS

1. Anatomy is fundamental in determining stomatal conductance (g_s), photosynthesis (A), and water use efficiency (WUE). Studies have shown anatomy to be a key target for manipulation to improve crop performance.
2. Spatial patterning of stomata, hydraulic supply, and underlying mesophyll airspace formation greatly impact stomatal function and CO_2 diffusion, providing evidence that stomatal distribution plays a fundamental role in maximizing gas exchange and plant performance.
3. The role of subsidiary cell type and function has largely been overlooked; however, recent findings demonstrate the importance of these complex features and the potential of these cells as novel targets for manipulation to improve stomatal response.
4. Future work should focus on producing new transgenic and nontransgenic lines with alterations in key genes involved in osmoregulation, to improve stomatal response in the face of changing environmental interactions.
5. There have recently been advancements in understanding the importance of sucrose and starch metabolism in guard cells, as well as their role as signaling components for coordinating A with g_s.
6. The speed of stomatal response represents an unexploited target to remove diffusional constraints on mesophyll demands for CO_2 to improve plant performance and resource use.
7. The role of guard cell chloroplasts and photosynthesis in terms of stomatal function is still largely unknown, and further work is required to elucidate their importance in stomatal behavior and dynamic response.
8. Temperatures and atmospheric CO_2 concentrations have risen and will continue to rise, creating opportunities for enhancing WUE and carbon gain through the coordination of photosynthesis and stomatal behavior, potentially future-proofing crops to climate change.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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