Trends in Plant Science

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Feature Review

Engineering stomata for enhanced carbon capture and water-use efficiency

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Stomatal pores facilitate gaseous exchange between the inner air spaces of the leaf and the atmosphere. As gatekeepers that balance CO_2 entry for photosynthesis against transpirational water loss, they are a focal point for efforts to improve crop performance, especially in the efficiency of water use, within the changing global environment. Until recently, engineering strategies had focused on stomatal conductance in the steady state. These strategies are limited by the physical constraints of CO_2 and water exchange such that gains in water-use efficiency (WUE) commonly come at a cost in carbon assimilation. Attention to stomatal speed and responsiveness circumvents these constraints and offers alternatives to enhancing WUE that also promise increases in carbon assimilation in the field.

Highlights

The guard cells that surround each stoma regulate stomatal aperture and, hence, gaseous conductance, through osmotically driven water fluxes that commonly accompany ion transport and the metabolism of organic solutes.

Manipulating stomatal density at the leaf surface yields significant improvements in plant water use efficiency and drought resilience by reducing steady-state gaseous conductance, but also reduces net carbon fixation and can affect vegetative growth.

Engineering strategies that focus on the kinetics of stomatal opening and closing offer a means to circumvent the trade-off in the steady-state exchange of atmospheric CO_2 for water in the plant.

Both synthetic optogenetics and manipulations of the activity of ion channels native to guard cells demonstrate the gains in both water use efficiency and carbon fixation that are possible by engineering stomatal kinetics.

The stomatal context

Stomata are pores on the surface of leaves and other aerial parts of plants that form between specialised pairs of epidermal cells, the guard cells. Stomata circumvent the impermeable cuticle barrier of the plant surface to enable CO_2 entry to the air space within a leaf for photosynthesis in the mesophyll. Most stomata open in the light when the CO_2 demand of photosynthesis is high and close as the light and photosynthetic activity decline. The open stoma also provides a pathway for transpirational water loss from the saturated inner air space of the leaf to the atmosphere. Inevitably, diffusion of CO_2 into the leaf comes at the expense of water vapour diffusion from the leaf to the atmosphere. It is this exchange between transpiration and CO_2 entry to feed photosynthesis that can pose an existential challenge for survival of the plant. Guard cells therefore must balance the need for CO_2 in photosynthesis against the need to prevent drying of the leaf tissues by adjusting the pore aperture and, hence, stomatal conductance, $\mathbf{g_s}$ (see Glossary) in response to environmental as well as endogenous signals [1].

Not surprisingly, stomata connect the global water and carbon cycles and exert a major influence on both. Foliar transpiration has had a significant role in atmospheric modelling and weather prediction for more than a quarter of a century [2–5], and stomatal transpiration is at the centre of a crisis in water availability and crop production that is expected to unfold over the next 20–30 years. Globally, fresh water usage increased sixfold over the 20th century, twice as fast as the human population, and is expected to double again before 2030, driven mainly by agriculture [6]. Even in the UK, not generally considered a region of arid climate, irrigation has risen tenfold over the past 30 years and this trend is expected to continue [7].

Cowan and Farquhar [8] originally proposed that stomatal aperture is regulated, at least over shorter periods of time, to maintain an optimum in carbon gain against water lost. They introduced the concept of **WUE**, defined as the moles of carbon fixed by photosynthesis divided

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by the moles of water lost via transpiration, proposing that the cost in water associated with an aperture change affecting carbon assimilation

$$\lambda = (\partial A/\partial g_s)/(\partial E/\partial g_s) \quad [1]$$

should remain constant. In essence, the concept of WUE optimisation holds that the benefit of increasing the rate of photosynthetic carbon assimilation, ***A***, with a small increase in stomatal conductance, ∂g_s, should be offset by a proportional cost of increased water loss via transpiration, ***E***. Our understanding of why stomata behave the way they do has been dominated by this concept and its ability to explain empirical data across species and environments [9–15]. The simple elegance of the WUE concept rests with its ability to supersede other restrictive assumptions and its capacity to describe leaf and canopy g_s with photosynthesis [12,16,17].

Arguably, the WUE concept has also focused attention on g_s in the steady state [18]. Yet, for plants growing in the field, the steady state is the exception rather than the norm [19]. Stomata experience frequent, often rapid, changes in the environment, especially in light and temperature, and also in water availability and atmospheric humidity. For instance, stomata will close as clouds pass by and as the overhead canopy shadows the leaf, thereby avoiding water loss as light becomes limiting for photosynthesis and the demand for CO_2 declines. In many species, stomatal responsiveness also varies with the circadian period [20]. So, even when water is not limiting for the plant, changes in stomatal dynamics give rise to substantial differences in WUE over the diurnal cycle. It comes as no surprise, then, that WUE is rarely stable in the field.

Such behaviours underline the complexity of the challenges that face efforts to enhance carbon assimilation and WUE. They beg the question: if stomata are targets for bioengineering, then what features of stomata and their conductance are the most promising for study and, ultimately, for application? Here, we examine WUE and related measures as yardsticks for **stomatal efficiency (SE)**; we consider the benefits and limitations of efforts to engineer stomata through their static and dynamic characteristics; and, finally, we review progress to date and the promise for future efforts in bioengineering of stomata.

What defines stomatal efficiency?

In the simplest sense, SE reflects the capacity to regulate water loss via transpiration, and thereby to maintain the hydrated environment of the inner leaf air space, while ensuring an optimum of CO_2 influx to support photosynthetic carbon assimilation. Thus, SE incorporates the **instantaneous WUE**, hereafter referred to as WUE_i (= *A*/*E*), which is defined as the ratio of the momentary rates of assimilation and transpiration. It contrasts with time-integrated WUE that relates total water use and net biomass gain, typically calculated over the growth period or the lifetime of the plant. WUE subsumes metabolic investments in developmental and homeostatic processes of the plant implicitly [19–21], whereas SE extracts key elements of these factors explicitly to connect stomatal function in the short term with their longer-term effects on plant growth.

As a measure, WUE_i takes account of the environmental inputs of light, CO_2, and atmospheric humidity. For SE, however, there also needs to be a weighting for resilience to factors with direct impacts and/or dependencies on stomata and with consequences for plant growth. Among these factors, most obvious are temperature, water stress, and pathogen resistance. Where detail of the g_s dependence on a factor is known, an absolute weighting may be applied. For example, for temperature, the weighting

$$r^o_T = \partial g_s/\partial T \quad [2]$$

Glossary

δ_S: density of stomata across the leaf epidermal surface.
$[Ca^{2+}]_i$: cytosolic-free calcium ion concentration.
***A*:** assimilation rate of CO_2 as carbon fixed by photosynthesis.
A_s: cross-sectional (surface) area of the stomatal pore.
D_C: diffusion constant for CO_2 in air.
d_s: depth of the stomatal pore across the epidermis.
D_w: diffusion constant for water vapour in air.
***E*:** transpiration rate of water as vapour lost as evaporation from the leaf through stomata.
Emergent behaviour: a characteristic that arises through interactions between elements of a process that could not be foreseen from knowledge of the elements on their own, usually associated with nonlinearities in the properties of the individual elements.
Gas exchange: exchange of water vapour and of CO_2 between the atmosphere and the inner air space of the leaf.
g_s: conductance of the leaf (or ensemble of stomata) to gaseous diffusion [= $E/(100 - \%RH_{out})$].
Instantaneous (or momentary) WUE (WUE_i): commonly defined as the rate of CO_2 fixed by photosynthesis divided by the rate of water transpired (= *A*/*E*).
J_{CO2}: flux of CO_2.
J_{H2O}: flux of water vapour.
Membrane voltage: electrical potential difference across a membrane.
Optogenetics: study and application of biological tools that enable light-mediated control of cellular processes.
pC_i: partial pressure of CO_2 in the inner air space of the leaf.
pCO_2: partial pressure of CO_2 in the atmosphere.
pW: partial pressure of water vapour in the atmosphere.
pW_i: partial pressure of water vapour in the inner air space of the leaf.
r^o_T, r_T: absolute and relative weightings in stomatal efficiency for temperature.
r_P: relative weighting in stomatal efficiency for pathogen resistance.
r_W: relative weighting in stomatal efficiency for drought resistance.
Stomatal efficiency (SE): defined as the product of WUE_i and weighting factors for resilience to temperature, drought, and pathogen attack.
Vapour pressure difference (VPD): difference between the atmosphere and the inner air space of the leaf.

where $\mathbf{r^{o}_{T}}$ describes the absolute dependence of g_s on temperature, *T*. This dependence accounts for the quasi-linear response of g_s to a differential in temperature over the range of values typically experienced by the plant but may also be relevant to the breakdown in this relationship at temperature extremes [22,23].

In the absence of such detail, weightings are most easily addressed in relation to known standards, whether these be the wild-type in comparisons to mutant varieties or an established model in comparisons across species. For example, colonisation by penetrative bacterial pathogens [24,25] relative to stomata of the wild-type plant provides a semiquantitative weighting for pathogen resistance of a mutant, with the standard weighting for the wild-type (relative to itself) of unity. Similarly, the relative maintenance of leaf water potential in the face of defined soil hydraulic and atmospheric vapor pressures provides a measure of the capacity to withstand short-term water stress. Thus, in general we may consider

$$SE = WUE_i \cdot r_T \cdot r_W \cdot r_P \quad [3]$$

where the weightings $\mathbf{r_T}$, $\mathbf{r_W}$, and $\mathbf{r_P}$ replace the corresponding absolute weightings and account for the efficacy in responses to differentials in temperature, water stress, and pathogens, respectively, relative to standards in each case.

The physical properties of the leaf remain the obvious starting point when considering SE. Stomata operate within the framework of the laws of diffusion and mass action that delimit the primary characteristics of g_s (Box 1). Thus, factors affecting stomatal size and cross-sectional pore area, stomatal density, and the depth of the epidermal layer and, hence, of the stomatal pore, act

Water-use efficiency (WUE): commonly defined as the net CO_2 fixed by photosynthesis per water lost from the leaf via transpiration (= *A*/*E*) or the biomass accumulated divided by the total water used by the plant.

Box 1. What determines stomatal efficiency?

The fluxes of CO_2 and water vapour, $\mathbf{J_{CO2}}$ and $\mathbf{J_{H2O}}$, respectively, across the stomatal pore between the leaf and atmosphere can be represented by Equations I and II:

$$J_{CO2} = A_S D_C \delta_s (pCO_2 - pC_i)/d_s \quad [I]$$

and

$$J_{H2O} = A_S D_w \delta_s (pW_i - pW)/d_s \quad [II]$$

where $\mathbf{A_S}$ is the cross-sectional area of the stoma, $\mathbf{D_C}$ and $\mathbf{D_w}$ are the CO_2 and water vapour diffusion coefficients in air, $\boldsymbol{\delta_S}$ is the density of stomata distributed over the leaf surface, $\mathbf{d_S}$ is the depth of the stomatal pore, $\mathbf{pCO_2}$ and $\mathbf{pC_i}$ are the partial pressures of CO_2 in the atmosphere and substomatal cavity, respectively, and **pW** and $\mathbf{pW_i}$ are the corresponding partial pressures of water vapor, respectively. As a practical measure, stomatal conductance, g_s, is defined using Equation III

$$g_s = J_{H2O}/(pW_i - pW) = A_S D_w \delta_s / d_s \quad [III]$$

where pW_i is commonly assumed to be saturated in air.

In general, increasing d_s or decreasing the size of stomata (Figure IA) and, hence A_s, reduces J_{H2O}, g_s, and its dynamic range, and will have a proportional impact on J_{CO2}. Reducing guard cell size can benefit stomatal kinetics by increasing the surface:volume ratio. Increasing this ratio favours an accelerated exchange of solute and water for stomatal movements through its indirect impact on A_s dynamics. The dumbbell-shaped guard cells of grasses respond faster than the kidney-shaped guard cells of broad-leaved plants, in part because of the much smaller volumes and greater surface:volume ratios of the former [135]. Efforts to engineer stomatal performance have yet to focus on stomatal size. Nonetheless, a consequence of manipulating stomatal density is often to affect stomatal size, with both characteristics affecting g_s, WUE, and plant growth.

Manipulating guard cell metabolism and transport (Figure IB) affects A_s dynamics directly by altering the kinetics of solute metabolism, ion, and water transport needed to drive guard cell turgor and stomatal aperture changes [58,65].

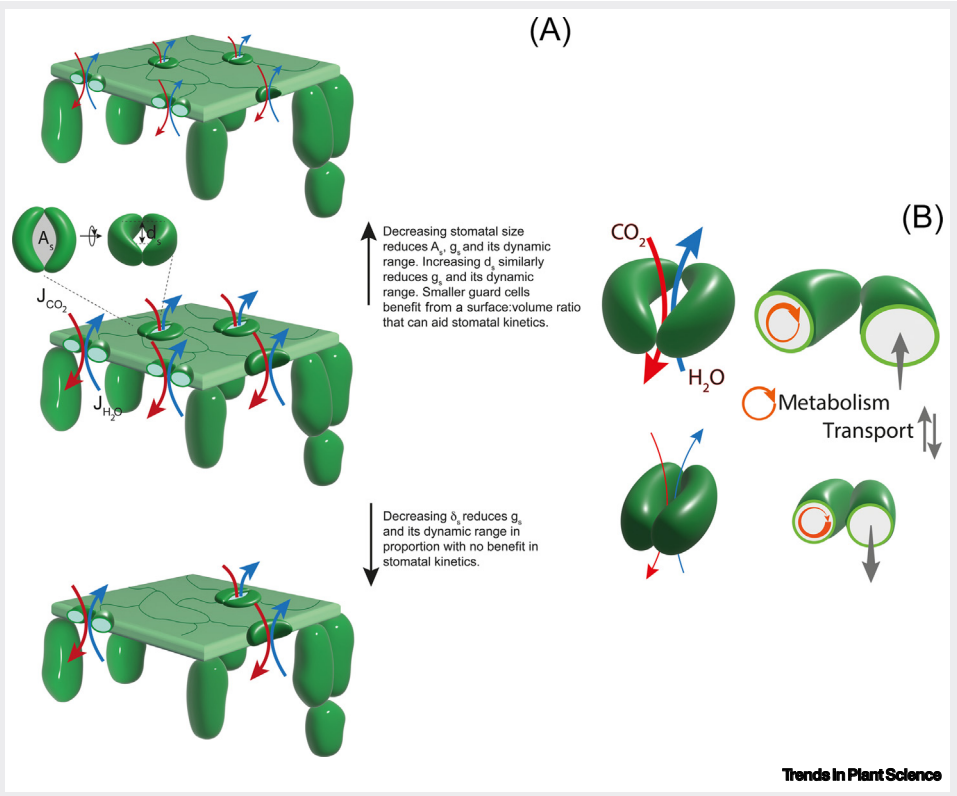

Figure I. Static and dynamic properties that determine stomatal efficiency.

as scalar parameters. These parameters define the upper limits for g_s. Indeed, evolutionary and species comparisons highlight the inter-relationships between stomatal size and density as key parameters affecting assimilation [26–28], although in a broader context the hydraulic relations of other tissue come into play [29]. In general, stomatal parameters affect WUE_i in both transpirational water loss and CO_2 influx, although the effects are most evident in transpiration. The bias to transpiration arises because of the absolute difference in partial pressures between water vapour and CO_2, the higher diffusion constant for water vapour compared with that for CO_2, and, most important, the nonlinear response of photosynthesis to CO_2 (Figure 1). So reducing g_s initially has a proportionally greater effect on transpiration compared with assimilation. A positive impact on r_W (>1.0) is a predictable consequence of reducing stomatal density [27,30], although this is likely balanced by a negative impact on r_T (<1.0). The consequence for r_P is less obvious.

Control of stomatal aperture alters the effective cross-sectional pore area and presents a second and major set of determinants behind g_s (Box 1). From the closed to the fully open state, stomatal movement in many species elevates transpiration by factors of five- to eightfold or more and, when closed, often includes significant contributions from water loss across the cuticle itself [31,32]. These characteristics establish the operational features of g_s. They determine stomatal agility in transiting between states – the kinetics of opening and closing – in response to fluctuations in the environment, notably in light, temperature, and humidity. Aperture control reflects the efficacy of the guard cell signalling pathways and, most important, the metabolic and transport mechanisms behind how stomata work.

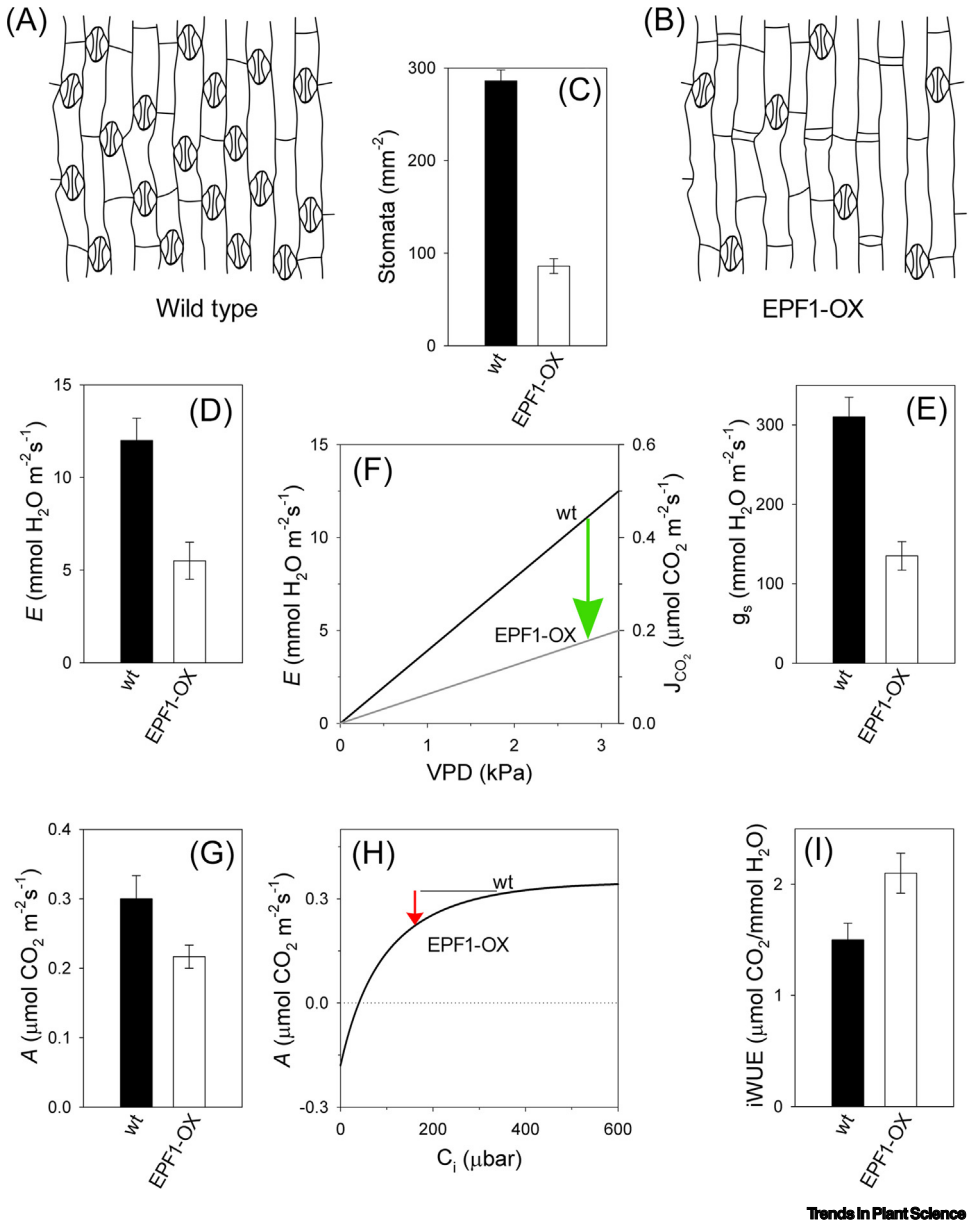

Figure 1. Stomatal density affects transpiration and water-use efficiency (WUE) but may have more modest impacts on assimilation. Overexpression of the epidermal patterning factor EPF1 (EPF1-OX) leads to a developmental reduction in the density of stomata on rice leaves. Data extracted from Mohammed *et al.* [49] show the pattern of stomata in wild-type (A) and EPF1-OX (B) lines leading to an approximate 2.6-fold reduction in stomatal density (C) and 2.5-fold reductions in transpiration *E* (D) and stomatal conductance g_s (E). The scalar reduction in *E* (green arrow) is a simple linear function of the vapour pressure difference and the corresponding decrease in the stomatal density δ_s (see Box 1) and infers a corresponding reduction in J_{CO2} (F). Carbon assimilation *A* (G) is less strongly affected (red arrow), at least in part because its relationship is strongly nonlinear over the range of likely partial pressures of CO_2 within the leaf airspace, pC_i (H), thereby allowing for a moderate gain in instantaneous WUE, WUE_i (I).

Both the static properties of stomata and our understanding of their mechanics offer opportunities to enhance stomatal efficiency with challenges and benefits as well as trade-offs. However, whereas the static properties of stomata inevitably affect both transpiration and CO_2 availability

for photosynthesis, strategies that arise from our understanding of stomatal dynamics hold the promise of circumventing the limitations arising from the exchange of water lost as CO_2 enters the leaf. In short, these strategies allow for gains in both WUE and assimilation.

Accessing the static properties of stomata

Stomata develop through a single asymmetric division within the epidermal cell layer, generating a guard mother cell that then divides evenly into two guard cells [33–36]. The lineage of cell divisions within the epidermis ensures an even distribution of stomata separated by at least one epidermal cell, the one-cell spacing rule that is evident in nearly every land plant. Mutations in the leucine-rich repeats (LRR) receptor gene *too many mouths* (*TMM*) disrupt this pattern by randomizing the plane of formative asymmetric divisions, and by permitting ectopic divisions in arabidopsis (*Arabidopsis thaliana*) [37]. Associated basic helix-loop-helix genes *SPCH*, *MUTE*, and *FAMA* have maintained their functions as regulators of stomatal differentiation across plant taxa [38–41].

Not surprisingly, stomatal development has provided many handles with which to manipulate SE. For example, signalling peptides encoded by epidermal patterning factor (EPF) genes negatively regulate stomatal development [42,43] and EPF overexpression has been shown repeatedly to reduce stomatal density and enhance drought tolerance across species [44–47]. Drought tolerance in these circumstances most likely arises because of the slowed transpiration that also reduces soil drying [48]. Decreased stomatal density by EPF overexpression thus enhances WUE [49] and reduces assimilation (Figure 1), although its effect on yield may be moderated [46] as a secondary consequence of metabolic adjustments. Conversely, increasing stomatal density with *EPF1* knockout enhances carbon assimilation and growth, but at a substantial cost in WUE_i [50] and, hence, in SE. These studies focused primarily on g_s in the steady state. Whether EPF expression affects stomatal kinetics is not known and is worth considering, because stomatal clustering is known to impair the speed of stomatal movements and WUE [51].

Anatomical features of the guard cell itself may provide other targets for engineering stomatal performance. Flexibility of the cell wall is important for guard cells to withstand turgor pressure cycling and may provide one such target [52,53]. Recent analysis suggested that thickening and pectic structures of the wall in polar regions of stomatal complexes are vital for effective stomatal movement [54]. Altering the pectic arabinan side chains in arabidopsis by overexpression of *ARABINAN DEFICIENT 1* (*ARAD1*) to increase cell wall flexibility enhanced carbon assimilation but with no impact on WUE [55]. By contrast, the mutant *FUSED OUTER CUTICULAR LEDGE 1 (foc1)*, which normally directs formation of the cuticular ledge around the pore, yielded stomata with pores fully occluded by a cuticular layer [31]. As expected, the *foc1* mutant reduced transpiration and enhanced drought tolerance relative to wild-type arabidopsis, thus enhancing r_w but at a cost in r_T. Its impact on assimilation was not reported, but the threefold reduction in g_s implies a substantial reduction in the rates of biomass gain. Thus, both *ARAD1* overexpression and the *foc1* mutant reinforce the limitations in SE that are likely to be achieved through static manipulations of stomata characteristics.

Targeting stomatal dynamics

Guard cells function independently of other leaf tissues and, even when isolated in epidermal peels, will respond to an array of extracellular signals, notably light and CO_2, to regulate stomatal aperture [56–58]. Changes in the stomatal aperture arise from osmotically driven water flux, commonly accompanying the metabolism of organic solutes and ion transport by the guard cells [19,58,59]. Transport across the guard cell plasma membrane connects with transport across the tonoplast through a complex network of controls to regulate the fluxes of the major osmotic solutes, principally K^+, Cl^-, and malate (Mal), as well as water. For transport especially, the

network of transport proteins driving guard cell turgor and stomatal aperture is exceptionally well defined [18,58] and marks the mechanics of the guard cells as promising targets for SE engineering.

Focusing on stomatal physiology widens the scope for engineering to include processes that affect stomatal kinetics as well as the dynamic ranges achieved between the closed and open states of the pore. There are substantial gains to be made here, especially if the speed of stomatal opening and closing can be increased. Enhancing stomatal responsiveness by accelerating stomatal opening and closing offers possibilities to improve WUE and carbon assimilation averaged over longer time periods (Box 2). Stomata close slowly compared with photosynthesis, a hysteresis in response that often leaves stomata lagging behind environmental changes by many minutes or even tens of minutes [19,20,60]. The result is that water is lost without a corresponding gain in carbon fixed. Similarly, slower stomatal opening frequently restricts CO_2 influx and fixation by photosynthesis when the leaf goes from shade to bright light [19]. In short, such delays undermine the relationship of Equation 1: the hysteresis in closing costs the plant in water loss and degrades WUE; the hysteresis in opening costs the plant in fixed carbon and, while it does not greatly affect WUE, it does reduce photosynthetic efficiency.

Identifying the most promising targets presents several challenges, nonetheless. First and foremost, ion transport across the major membranes of the cell is tied to the driving force of the **membrane voltage**, which is shared between the overwhelming majority of transporters at each membrane. Membrane voltage affects electrogenic transport directly and electroneutral transport indirectly, thus introducing an extraordinary degree of entanglement between transport activities [58,61,62]. Consequently, charge (ion) flux through any one transporter affects, and is affected by, all other transporters that move charge across the membrane.

A second challenge has its roots in the coordination of transport between the plasma membrane and tonoplast. Such coordination is vital for stomatal function, simply because the vacuole comprises some 80–90% of the volume of the guard cell and the major fraction of solute and water flux occurring during stomatal movements must therefore cross both membranes [1,58]. Transporters (Table 1) at these two membranes share a common pool of solutes within the cytosol, and transport of these solutes, including signalling molecules, such as Ca^{2+}, is affected by, and impact on, the activities at both membranes [63]. Furthermore, these solutes affect, and are affected by, metabolism [58,59].

Finally, to do work, cellular transport and metabolism must operate well away from thermodynamic limits. Guard cells are no exception and their physiology is dictated by the kinetic properties of each of the dominant pathways. In most cases, these kinetics are highly nonlinear, often with respect to multiple variables, including substrate concentrations and, for transport, membrane voltage, as well as regulatory inputs that engage ligand and other post-translational controls. These nonlinearities, and the entanglements noted above, give rise to **emergent behaviours** that often are counter-intuitive [58,63,64]. In short, selecting targets within the network of mechanisms operating in the guard cell is not straightforward and requires analysis of the system as a whole [18]. As a rough guide, however, such analyses have generally highlighted the importance of introducing new conductances and/or altering the biophysical regulatory properties rather than the densities of any one protein as the most promising targets [65–68]. To date, this guide holds true, as we note below.

Manipulating guard cell ion transport with optogenetics

The very idea of using **optogenetics** in a light-dependent, photosynthetic organism seems counterintuitive at first, but its application has proven remarkably successful. Optogenetic tools

Box 2. Stomatal kinetics influence both water use and carbon assimilation

Figure IA shows stomatal conductance, g_s (green line), as it lags behind photosynthetic carbon assimilation, *A* (red line), with light changes, such as when clouds pass overhead (shading above). Increases in light often lead to periods (stomatal limitation) in which *A* is limited by g_s (red shading). With decreasing light, *A* is commonly limited by the photon fluence rate (photon limitation) rather than by g_s, but because stomata close slowly there follow periods in which water is lost without a commensurate gain in photosynthesis (green shading). The resulting WUE_i (Figure IB) shows large decreases as light declines; increasing steps in light enhance WUE_i, at least transiently, but at a cost in carbon fixation (Figure IC). Broken lines throughout illustrate the potential gains in WUE_i and carbon assimilation that would be possible by speeding both stomatal opening and closing.

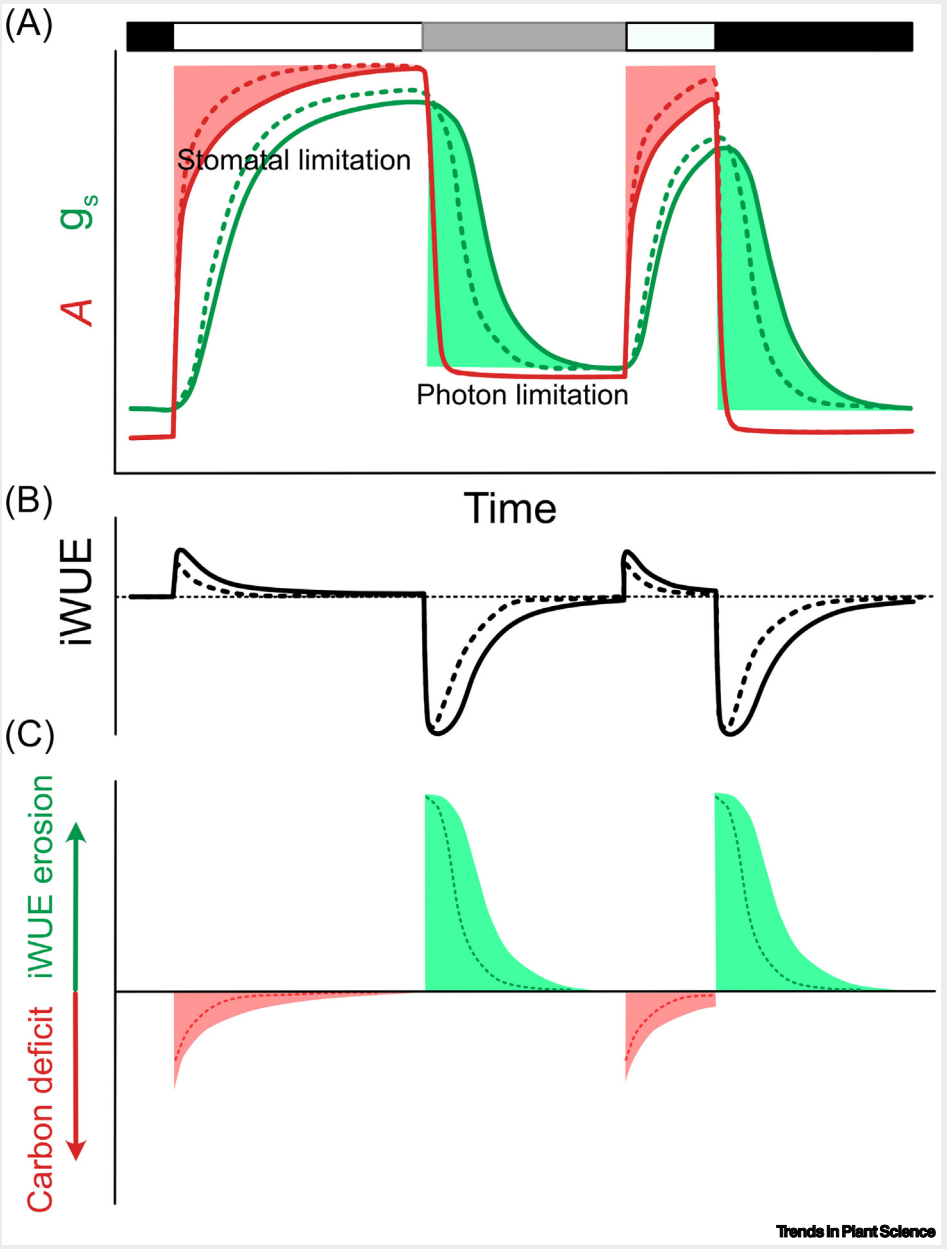

Figure I. Stomatal kinetics influence both water use and carbon assimilation.

Table 1. Major solute transporters of guard cells[a]

Transporter	Gene products[b]	Transported substrates	Function[c]
Plasma membrane			
H^+-ATPase	AHA1, AHA2, AHA5	H^+	Energization
Ca^{2+}-ATPase	ACA8, ACA10	Ca^{2+}, H^+	Ca^{2+} efflux
H^+/Ca^{2+} antiport	CAX11	Ca^{2+}, H^+	Ca^{2+} efflux
H^+-K^+ symport	KT1/KUP1/HAK5	K^+, H^+	K^+ influx, high affinity
H^+-anion symport	NPF7	$Cl^-(NO_3^-)$, H^+	Cl^- influx, high affinity
H^+-Mal symport	ABCB14	Mal^{2-}	Malate influx
H^+-sugar symport	SUC3, STP1, STP4	Sucrose, glucose	Sucrose influx
Ca^{2+} channel	Unknown	Ca^{2+}	Ca^{2+} influx
K^+ channel	KAT1, KAT2, AtKC1	K^+	K^+ influx
	GORK	K^+	K^+ efflux
$Cl^-(NO_3^-)$ channel	SLAC1, SLAH3	$Cl^-(NO_3^-)$	Anion efflux
Cl^-(Mal) channel	ALMT12	Cl^-, Mal^{2-}	Anion efflux, voltage oscillation
Aquaporin	PIP2;1, PIP2;2, PIP2;4	H_2O, ROS	Water flux
Tonoplast			
H^+-ATPase	VHA1	H^+	Energization
H^+-PPase	AVP1, AVP2	H^+	Energization
Ca^{2+}-ATPase	ACA4, ACA11	Ca^{2+}, H^+	Ca^{2+} efflux
$Cl^-(NO_3^-)$ channel	ALMT9	$Cl^-(NO_3^-)$	Anion influx
Mal channel	ALMT6	Mal^{2-}	Malate influx
H^+/Ca^{2+} antiport	CAX2, CAX3	Ca^{2+}, H^+	Ca^{2+} efflux
H^+/cation antiport	NHX1, NHX2	H^+, K^+	
H^+/Cl^- antiport	CLC1	$Cl^-(NO_3^-)$, H^+	$Cl^-(NO_3^-)$ sequestration
Ca^{2+} channel	Unknown	Ca^{2+}	Ca^{2+} influx
K^+ channel	TPK1	K^+	K^+ flux
	FV	K^+	K^+ flux
K^+/Ca^{2+} channel	TPC1	K^+, Ca^{2+}	Unknown
VCl	ALMT9	$Cl^-(NO_3^-)$	Anion flux
VMal	ALMT6	Mal	Anion flux
Aquaporin	TIP1	H_2O	Water flux

[a]For further details, see [18,58].
[b]Reference genes of *Arabidopsis thaliana*.
[c]Flux direction is relative to the cytosol.

are synthetically engineered, light-responsive proteins derived from photoreceptors and light-gated ion channels of plants, fungi, and bacteria, that can be targeted to tissues of interest and their activity controlled with high spatiotemporal resolution by light [69]. Until recently, they were used exclusively in mammalian cells for experimental control of gene expression, membrane voltage, protein–protein interactions, and signalling [70].

Following quantitative systems analyses favouring new ion conductances [65–68], Papanatsiou *et al.* [71] showed how optogenetics can be used in plants with real benefits for carbon fixation and WUE. This first application made use of the BLINK1 K^+ channel, a fusion of a blue light-sensitive phototropin photoswitch from *Avena sativa* and the Kcv K^+ channel from a *Chlorella*

virus [72]. Expressed in arabidopsis guard cells, the channel introduced a new, light-gated K^+ conductance across the plasma membrane, promoting K^+ flux and accelerating stomatal kinetics. Papanatsiou *et al.* [71] reported more than a twofold increase in biomass and WUE under fluctuating light both when plants were water replete and water stressed (Figure 2). Subsequent work (M. Blatt, unpublished) indicated that BLINK1 expression did not affect pathogen susceptibility. Thus, the work with BLINK1 provides an all-important demonstration that manipulating stomatal kinetics circumvents the trade-off in water lost for CO_2 gained that is otherwise intrinsic to g_s engineering, and it does so without concomitant impact on disease resistance. These studies thus show how real gains in SE are possible through alterations in stomatal speed.

Subsequent studies employed microbial channelrhodopsins that otherwise were hindered by the requirement for rhodopsin cofactor that normally is absent from angiosperm plants [70]. Coexpressing the genes for cofactor synthesis with channelrhodopsins has enabled new approaches to validating ion transport coordination in guard cells [73–75] that underpins stomatal function [18,58]. Among these, introducing a light-activated, anion-conducting channelrhodopsin reconfirmed the long-recognised role for anion efflux in stomatal closure [74]. Similarly, studies with the H^+-permeant channelrhodopsin 2 highlighted the well-known role of plasma membrane H^+-ATPases in generating the guard cell membrane voltage [75]. Without a doubt, these studies have established the utility of optogenetics in plant research; however, as synthetic constructs, they also face societal barriers to wider applications in agriculture.

Engineering native stomatal membrane transport

Efforts focused on transport native to the guard cell likewise bear out the importance of engineering that introduces new biophysical characteristics over transporter densities. Wang *et al.* [76] reported that two plasma membrane K^+ channels, AKT1 and KAT1, had no effect on g_s or WUE, even when overexpressed by three- to fivefold over the wild-type. While overexpressing the plasma membrane H^+-ATPase AHA2 enhanced stomatal opening and g_s in the steady state and promoted carbon assimilation [76], presumably by increasing the energetic driving force on transport, the gains came at a cost in WUE_i and, hence, in SE. These characteristics have since been translated successfully to fast-growing poplar (*Populus*), improving the growth of transgenic trees [77], but unsurprisingly with a similar cost in SE.

Much the same conclusions may be drawn from manipulations of the SLAC1 anion channel. Originally described in arabidopsis [78,79], SLAC1 is an important pathway for anion efflux and balances K^+ flux through the GORK K^+ channel for solute loss and stomatal closure [58,80]. Thus, in well-watered rice, as in arabidopsis, the *slac1* null mutant was found to slow stomatal closure, elevate steady-state g_s, and promote carbon assimilation, but at a cost in SE [81]. Stomatal opening is also greatly slowed in the *slac1* mutant, which similarly impacts SE and arises from metabolic and regulatory feedback on the K^+ channels mediating guard cell K^+ uptake [82]. Whether SLAC1 also inhibits the K^+ channels through direct binding [83] remains unclear without supporting evidence of K^+ channel protein levels *in vivo* and on heterologous expression in oocytes. However, the impact on SE of overexpressing SLAC1 is likely counterproductive.

By contrast, engineering strategies that address the biophysical regulatory properties of channels are proving more successful. Horaruang *et al.* [65] used quantitative systems modelling [66] and knowledge of the gating properties of the GORK K^+ channel to accelerate stomatal kinetics two- to threefold with similar gains in WUE and carbon assimilation under fluctuating light and water stress. GORK is the dominant outward-rectifying K^+ channel in arabidopsis guard cells and is the major pathway for K^+ efflux during stomatal closure [84–86]. Like its counterparts in other species [87–90], GORK gating is controlled by membrane voltage and is inhibited

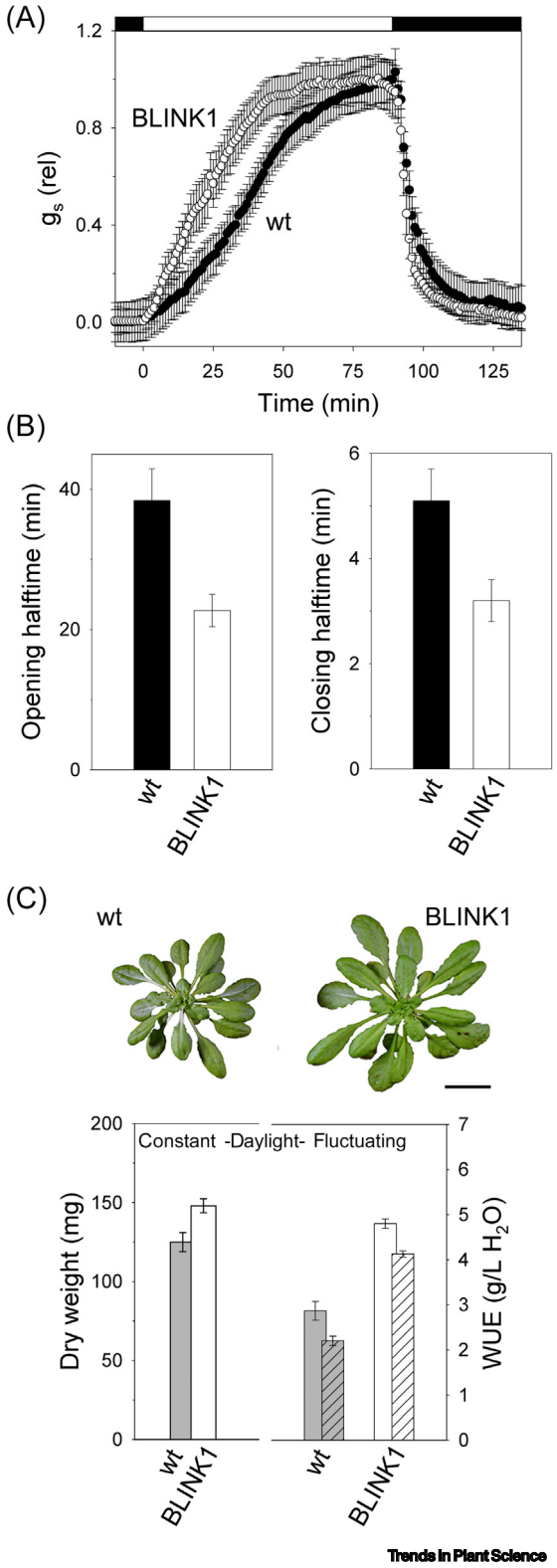

Figure 2. Accelerating stomatal kinetics with a synthetic, light-gated K^+ channel bypasses the limitations of steady-state CO_2–water exchange across the stomatal pore. Stomatal opening and closing (A) with light steps (above) for wild-type (wt) and BLINK1-expressing arabidopsis (*Arabidopsis thaliana*), here normalised to highlight the relative kinetics. Opening and closing halftimes (B) were calculated from these relaxations. Plants grown (C) under a 9:15 h, light:dark cycle, with constant daylight showed little difference in dry weight or water-use efficiency (WUE). When grown under the same total fluence with a varying daylight regime, both dry weight and WUE were reduced by almost 50% in wild-type plants but not in the BLINK1-expressing plants, either when well watered (open bars) or when water limited (hatched bars). Representative plants are shown above; scale bar: 5 cm. Data from [71].

by extracellular [K^+]. Horaruang *et al.* [65] engineered channels with an increased K_i (reduced affinity) for gating inhibition by K^+, thereby shifting voltage dependence of gating by –25 to –30 mV to promote GORK activity. In effect, these manipulations introduced an additional conductance for K^+ to accelerate solute efflux and stomatal closure. The modified channels also introduced a new conductance for K^+ influx, thereby enhancing stomatal opening kinetics and carbon assimilation (Figure 3). This success highlights channel gating over channel population as a target for SE engineering. As with BLINK1 engineering [71], it also demonstrates how stomatal kinetics can be used to circumvent the limitations of the trade-off between water loss and CO_2 uptake intrinsic to steady-state g_s. Given the structural conservation among GORK-like channels of angiosperms [65,91], it is likely that the same benefits will be realised through gene editing in several crop species.

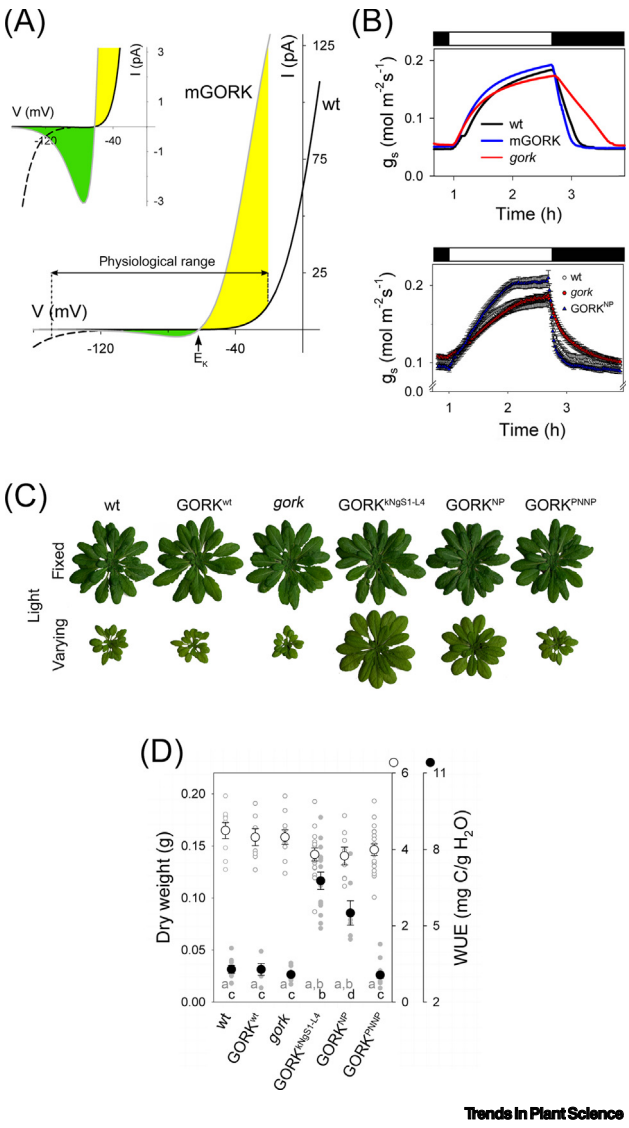

Figure 3. Relaxing the K^+ inhibition of GORK channel gating introduces a new K^+ conductance to accelerate its flux both in and out of the guard cell. Data extracted from [65] show (A) the modelled shift in mutant mGORK current that promotes both outward (yellow shading) and inward (green shading) K^+ flux depending on the steady-state membrane voltage, V, and the predicted and measured stomatal conductances g_s (B) on speeding of stomatal opening and closing with light steps (above). Highly significant gains were obtained under varying but not constant (fixed) daylight, in rosette size (C), and in dry weight and water-use efficiency (WUE) (D) of *gork*-null mutant plants complemented with the gating-shifted GORK$^{kNgS1-L4}$, and GORK^{NP} channels, but not in plants complemented with the GORK^{PNNP} mutant that showed a gating dependence on voltage equivalent to the wild-type (wt). Symbols in (D) are for constant (open symbols) and varying light (filled symbols) with data from individual transformants (small symbols) and mean±SE (large symbols).

Similar approaches targeting the gating properties of other channels that facilitate major osmotic solute fluxes have yet to be explored, but knowledge of channel gating and the tools required are already to hand. For example, mutations of the KAT1 voltage-sensor domain that stabilise the open channel are known to alter the voltage range for channel gating [92,93]. Single-residue substitutions displace the gating midpoint by +40 mV or more, equivalent to a reduction in gating free energy of almost 2 kcal mol^{-1} and sufficient to enhance by 20-fold the rate of K^+ uptake at voltages typical of guard cells. Less is known for potential modification of SLAC1 and its relatives. Nonetheless, critical cryo-electron microscopy structure data are now available for KAT1 [94], AKT1 [95,96], and SLAC1 [97,98] that should accelerate efforts in this direction.

To date, targeting of the guard cell tonoplast has proven more of a challenge, in part because of our comparatively poor understanding of transport at this membrane. Redundancies in function and the ability of several solutes to cross-substitute as osmotica also present challenges that will benefit from mechanistic modelling and analysis as guides to molecular engineering. The functional overlaps between organic and inorganic ions as osmotica may well explain why mutations affecting dicarboxylate transport have failed to show any effects on stomatal behaviour [99,100], despite its confirmed role in vacuolar malate transport [101].

Endomembrane transport, including that of the tonoplast, does present clear targets within the regulatory networks that operate in the guard cell. Not least, endomembrane Ca^{2+} sequestering and its release accounts for 90–95% of Ca^{2+} flux behind changes in cytosolic-free $[Ca^{2+}]$ ($\mathbf{[Ca^{2+}]_i}$), and it regulates some 70% of transporters known to operate at the guard cell plasma membrane and tonoplast [58,102]. How important is the control of endomembrane Ca^{2+} transport? The discovery of a 'carbon memory' of the guard cells underscores its significance. Jezek *et al.* [66] reported a short-term decline in stomatal responsiveness, as predicted through quantitative systems modelling, that slowed stomatal closure and re-opening following repeated steps in light and in the partial pressure of CO_2. The declines were shown to arise from the time needed to refill endomembrane Ca^{2+} stores following each closing stimulus, with the ensuing latency in responsiveness greatly extended by mutations of the major endomembrane Ca^{2+}-ATPases [103,104]. These findings beg the question whether stomatal responsiveness in the field might be enhanced by altering the regulatory controls on Ca^{2+} sequestration to reduce the latency period.

Finally, it is worth bearing in mind that solute transport of epidermal cells surrounding the guard cells contributes to stomatal kinetics, both as a reservoir for solutes and as part of a 'two-stage pump' mechanism [1,105], and could also provide targets for engineering stomatal performance. Behind the latter mechanism is substantial evidence for a shuttle of osmotic solutes, notably in grasses that incorporate highly specialized epidermal cells, so-called 'subsidiary cells', adjacent the guard cells [106]. These complexes incorporate two dumbbell-shaped guard cells that lend great mechanical advantage to the subsidiary cells [107–109]. However, epidermal cells also exert backpressure in stomata with kidney-shaped guard cells [1]. These epidermal cells are also likely to contribute to ion shuttling during stomatal movements [110,111] and a corresponding turgor 'exchange' [27,112–114].

So, are subsidiary cells important for stomatal kinetics? Certainly, ablating these cells in the model grass *Brachypodium* greatly slowed the rates of stomatal opening and closing [34]. However, to date, such studies offer few clues to the likely mechanics of epidermal cell transport or its regulation and, hence, to possible targets for engineering to accelerate stomatal kinetics. Among dicotyledonous plants, at least one K^+ channel, KC1, is known to contribute to K^+ transport in the surrounding epidermal cells: the arabidopsis *kc1* mutant was found to reduce K^+ accumulation in the epidermis, thereby lowering epidermal turgor and increasing stomatal aperture [115].

Furthermore, the regulation of K^+ and anion channel activities shows some variation from that of the guard cells, at least in the fast-responding grass stomatal complex. Determining the molecular identities of the main ion transport components in these specialised epidermal cells [116,117] may also prove an important step toward future engineering of stomata.

Metabolic engineering

Guard cell starch, sugar, and organic acid metabolism are tightly linked with membrane transport in regulating stomatal aperture [59,118]. They present a further dimension for potential engineering for stomatal performance and come with similar challenges (Figure 4). As with transport engineering, systems analysis suggests the most promising strategies are those that target kinetic and

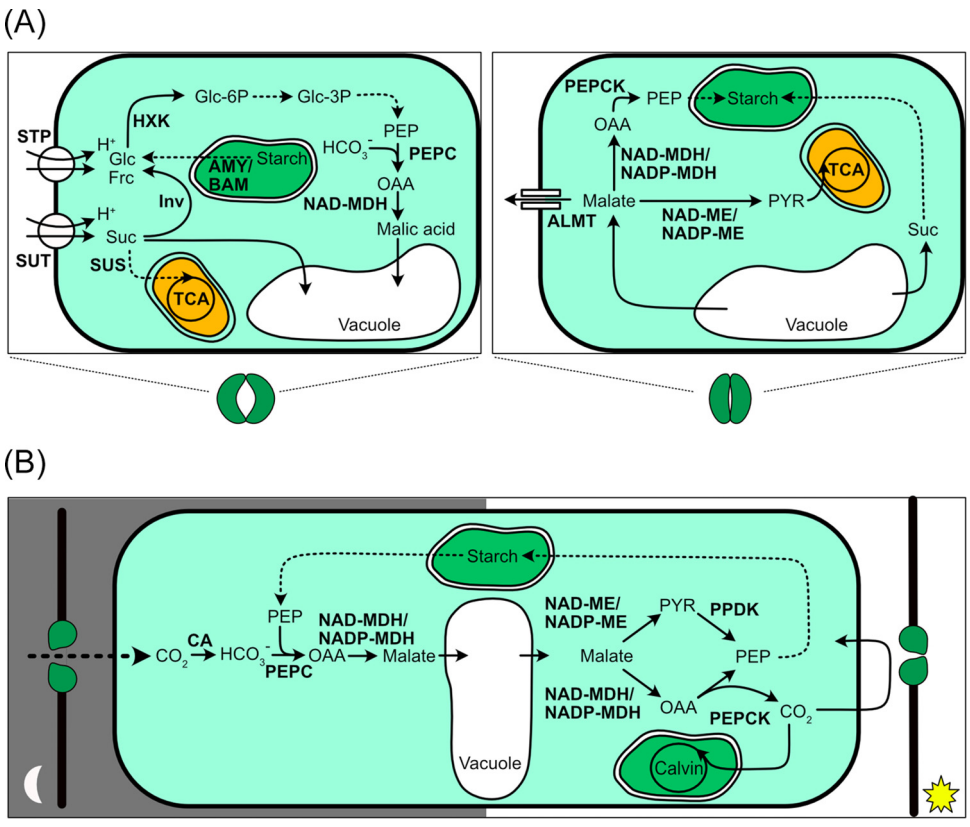

Figure 4. Potential targets for engineering of metabolic kinetics in guard cells. Major metabolic pathways (A) associated with organic osmotica in guard cells as targets for kinetic modification to accelerate stomatal opening (left) and stomatal closing (right). Of the enzymes highlighted in stomatal opening, H^+-coupled uptake of sugars is mediated by SUT and STP transporters; sucrose synthases (SUS) most likely feed into energy production; amylases (AMY and BAM) lead to glucose release from starch breakdown; hexokinases (HXK) and cytosolic invertase (INV) contribute to glucose metabolism; and phosphoenolpyruvate carboxylase (PEPC) and NAD-malate dehydrogenase (NAD-MDH) generate malic acid through phosphoenolpyruvate (PEP) and oxaloacetate (OAA). Enzymes highlighted in stomatal closing associate primarily with the removal of malate as an osmoticum, both by efflux through anion channels (ALMT) and by breakdown and conversion to starch through malate dehydrogenases (NAD-MDH and NADP-MDH) and phosphoenolpyruvate carboxykinase (PEPCK), and via the malic enzymes (NAD-ME and NADP-ME) through pyruvate (PYR) to the tricarboxylic acid cycle (TCA). Major elements of these malate metabolic pathways are repurposed in plants exhibiting crassulacean acid metabolism (CAM), that open the stomata at night and close them during the day (B). The mesophyll of CAM plants uses carbonic anhydrases (CA), PEPC, and malate dehydrogenases to fix CO_2 and store malate in the vacuole overnight; during the day, the mesophyll releases the malate and breaks it down to PEP, releasing CO_2 behind the closed stomata, thereby concentrating CO_2 for fixation by RuBisCO and the Calvin cycle.

regulatory properties rather than the densities of one or more metabolic enzymes [65–68]. To date, however, research has yet to address the question of metabolic kinetics and has focused principally on enzyme populations, either overexpressing or eliminating selected enzyme activities (Table 2). Thus, for example, overexpressing *Sucrose Synthase 3* (*SUS3*), encoding an enzyme catalysing sucrose synthesis from fructose, was found to increase flux through the tricarboxylic acid cycle and elevate steady-state g_s, thereby enhancing carbon assimilation and growth of water-replete plants but at a collateral cost in water loss [119] and, hence, SE. Conversely, antisense knockdown of *SUS3* reduced g_s and improved WUE, but at the expense of carbon assimilation, which resulted in slowed vegetative growth [120]. Guard cell-specific knockdown of *Sucrose Transporter 1* (*SUT1*) in engineered tobacco also decreased steady-state g_s and improved WUE, but with qualitatively similar reductions in assimilation and growth [121]. The same issues apply to monosaccharide metabolism, for example, in glucose release on starch breakdown by the gene products α-Amylase 3 (AMY3) and β-Amylase 1 (BAM1), which, in guard cells, facilitate stomatal opening at the beginning of the day [122]. Both starch and sucrose metabolism are

Table 2. Engineering guard cell metabolism[a]

Gene	Function	Manipulation	Plant	Phenotype	Refs
SUC2	Invertase	Overexpression	Potato	Steady-state g_s increase, reduced WUE	[111]
SUS3	Sucrose synthase	Antisense suppression	Potato	Steady-state g_s decrease, increased WUE	[111]
		Overexpression	Tobacco	Steady-state g_s increase, reduced WUE and drought resistance	[127]
HXK1	Hexokinase	Overexpression	Tomato	Steady-state g_s decrease, reduced growth	[128,129]
AMY3, *BAM1*	Amylase	T-DNA knockout	Arabidopsis	Steady-state g_s decrease, reduced growth	[113,130]
STP1, *STP4*	Sugar transporter	T-DNA knockout	Arabidopsis	Steady-state g_s decrease, reduced growth	[131]
SUT1	Sucrose transporter	Overexpression	Tobacco	Steady-state g_s decrease, increased WUE	[112]
PEPCK	Phosphoenolpyruvate carboxykinase	T-DNA knockout	Arabidopsis	Steady-state g_s increase, reduced WUE	[132]
NADP-ME	NADP-malic enzyme	Overexpression	Tobacco	Steady-state g_s decrease, increased WUE and drought resistance	[133]
				Steady-state g_s decrease, increased WUE and drought resistance, reduced growth	[13]
			Arabidopsis	Reduced growth	[135]
NAD-ME	NAD-malic enzyme	Overexpression	Arabidopsis	Steady-state g_s decrease, slowed growth	[122]
NADP-ME	NADP-malic enzyme	Overexpression	Arabidopsis	Steady-state g_s decrease, slowed growth	[122]
PPDK	Pyruvate phosphate dikinase	Overexpression	Arabidopsis	Steady-state g_s decrease, slowed growth	[122]
PEPC1	Phosphoenolpyruvate carboxylase	RNAi knockdown	Kalanchoe	Partial inversion of diurnal g_s cycle, reduced growth	[123]
		Overexpression	RNAi knockdown	Steady-state g_s increase, increased growth when well-watered	[122]
β-CA2	β-Carbonic anhydrase	Overexpression	RNAi knockdown	Steady-state g_s increase, increased growth when well-watered	[122]
NAD-MDH	NAD-malate dehydrogenase	Overexpression	RNAi knockdown	Steady-state g_s increase, increased growth when well-watered	[122]
PPCK1	Phosphoenolpyruvate carboxylase kinase	Overexpression	RNAi knockdown	Steady-state g_s increase, increased growth when well-watered	[122]

[a]Principal engineering efforts centred on guard cell sugar and organic acid metabolism.

potential targets for intrinsic kinetic alterations that, like manipulating K^+ channel gating [65], may prove an effective route to accelerating stomatal opening.

Apoplastic malate was initially proposed to regulate guard cell anion channel gating [123] through a voltage-dependent inhibition of the current, although the shift in activity has proven marginal compared with its regulation by $[Ca^{2+}]_i$ [124–126]. Nonetheless, guard cell malate transport affects stomatal closure kinetics, suggesting a role for its uptake as an osmotic solute from the apoplast [127], and malate metabolism has a substantial impact on stomatal function. Notably, overexpressing NAD-dependent and NADP-dependent malic enzymes (NAD-ME and NADP-ME), both contributing to malate decarboxylation (Figure 4 and Table 2), was found to reduce steady-state g_s, and to increase WUE and drought resistance, but at a cost to vegetative growth both in tobacco and arabidopsis [122,133–135].

Malate and its transport have additional roles in the guard cells of plants exhibiting crassulacean acid metabolism (CAM) [128]. The diurnal cycle of CAM stomata is reversed from that of other plants as an evolutionary adaptation to enhancing carbon assimilation and WUE (Figure 4): CAM stomata open at night for CO_2 uptake when temperatures are cool and the **vapour pressure difference (VPD)** driving transpiration is low. CO_2 is fixed and stored temporarily in the mesophyll vacuole as malic acid. During the day, this stored malate is hydrolysed to release CO_2 behind the closed stomata, thus avoiding water loss during daylight hours while concentrating CO_2 by 200- to 300-fold within the leaf to promote fixation by RuBisCO [129].

How organic acid metabolism integrates with CAM stomatal control is of particular interest, in part because engineering CAM-like characteristics offers substantial gains in SE [129] and because much could be learned from CAM models about how stomatal behaviour is coupled to mesophyll photosynthesis [128]. Several developments hold real promise for CAM engineering. Introducing cassettes of CAM-specific enzymes in arabidopsis has enhanced growth [130,131]. However, real challenges remain to understand CAM stomatal control. For example, in the model CAM species *Kalanchoe fedtschenkoi*, suppressing mesophyll *PEPC1* expression, the dominant PEP carboxylase that drives malate synthesis, highlighted the importance of metabolic communication with the guard cells [132], yet analysis of the guard cell anion channels has ruled out apoplastic malate as a direct intermediate [133].

Concluding remarks and outlook

More than two decades on since the seminal work that led to the drought-resistant Drysdale wheat with reduced stomatal density [134], approaches in stomatal engineering have greatly expanded in scope and have demonstrated real benefits in SE, promoting both WUE and assimilation. Although several of the most recent developments have yet to be taken to crop field trials, they promise substantial gains without obvious costs, for example, in disease resistance. These developments come on the back of an ongoing revolution in the molecular tools available for precision engineering of gene structure, transcriptional regulation, and physiological controls at the cellular level [39,47,70]. They offer new ways to address and analyse the mechanics of stomata, WUE, and their connections with plant growth and biomass yield (see Outstanding questions).

Most important, our perception of stomatal engineering has matured substantially. Research building on knowledge of stomatal development has generated crops with a range of stomatal densities over the surface of leaves [44,46]. Nonetheless, evidence from these studies, as from previous breeding efforts, indicates that the gains in WUE are carbon neutral at best and may come at a cost in plant biomass, yield, or growth rate. These costs are inevitably tied to the

Outstanding questions

Are there circumstances in which the static characteristics of stomatal conductance might enhance carbon flux for photosynthesis as well as WUE? If so, what are the possible biological targets for such engineering?

What are the predominant elements in guard cell membrane transport that influence the speed of stomatal opening and stomatal closing? Are there synergies between these elements that could be deployed to further enhance stomatal kinetics?

Could engineering the substrate affinities of metabolic enzymes in guard cells be used to accelerate stomatal kinetics, much as has been achieved through manipulations of gating inhibition of the GORK K^+ channel?

Are there substantial differences in factors affecting stomatal kinetics that are evident between species and, if so, how might these be accommodated through differential bioengineering?

trade-off between transpiration and CO_2 entry across the leaf epidermis. Such constraints do not apply across the temporal dynamics of the environment and the hysteresis in stomatal movements typical of plants in the field: we need only look beyond the static properties of stomatal conductance to find strategies that enhance WUE and assimilation against the background of environmental fluctuations that are common in the field. Simply put, approaches that focus on stomatal kinetics show how it is possible to circumvent the physical constraints of steady-state **gas exchange**.

To date, research centred on the kinetic behaviour of stomata has focused on the mechanics of ion transport, primarily that of the plasma membrane. These studies indicate real gains in SE, both in reduced transpiration and in enhanced assimilation, that arise from the simple expedient of promoting K^+ flux [65,71,91]. A common theme to these studies is their targeting of the kinetic properties of transport through changes in voltage sensitivity and substrate inhibition, effectively altering the regulatory energetics for transport. The benefits of this focus, rather than one addressing the steady-state rates of transport through changes in transporter populations, are amply supported by quantitative modelling that has also yielded insights into how kinetic manipulations may be achieved.

Indeed, mechanistic modelling [18] suggests a number of other potential targets that include, but are not limited to, ion transport. Among these targets, addressing the latency, or 'carbon memory', of stomata in response to fluctuating light and CO_2 within the leaf [66] is likely to have even greater impact on SE. Equally promising, we suspect, will be efforts toward addressing metabolism, especially of starch, sugar, and organic acids, through its kinetic regulation. There is a wealth of data across species, described in many thousands of research papers over the past half-century, that is sure to help refine efforts and offer additional strategies for stomatal engineering. We need only keep in mind the lessons of stomatal kinetics in engineering efforts going forward.

Acknowledgments

We acknowledge support of grants BB/N01832X/1, BB/L019025/1, BB/T006153/1, and BB/T013508/1 from the UK Biotechnology and Biological Sciences Research Council. Our thanks also go to colleagues who contributed to some of the research summarised in this review and to many informal discussions over the past decade around the development of the OnGuard modelling platform and the studies leading to the advances in BLINK1 optogenetics and GORK channel gating. Among these colleagues we thank especially Tracy Lawson, Maria Papanatsiou, Anna Amtmann, John Christie, Graham Farquhar, and Tom Buckley.

Declaration of interests

No interests are declared.

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