Systems dynamic modelling of the stomatal guard cell predicts emergent behaviours in transport, signalling and volume control

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Running Head: OnGuard diurnal modelling of stomatal guard cells
ABSTRACT
The dynamics of stomatal movements and their consequences for photosynthesis and transpirational water loss have long been incorporated into mathematical models, but none have been developed from the ‘bottom-up’ that are widely applicable in predicting stomatal behaviour at a cellular level. We previously established a systems dynamic model incorporating explicitly the wealth of biophysical and kinetic knowledge available for guard cell transport, signalling and homeostasis. Here we describe the behaviour of the model in response to experimentally-documented changes in primary pump activities and malate synthesis imposed over a diurnal cycle. We show that the model successfully recapitulates the cyclic variations in H⁺, K⁺, Cl⁻ and malate concentrations in the cytosol and vacuole known for guard cells. It also yields a number of unexpected and counterintuitive outputs. Among these, we report a diurnal elevation in cytosolic-free Ca^{2+} concentration and an exchange of vacuolar Cl⁻ with malate, both of which find substantiation in the literature but had previously been suggested to require additional and complex levels of regulation. These findings highlight the true predictive power of the OnGuard model in providing a framework for systems analysis of stomatal guard cells, and they demonstrate the utility of the OnGuard software and HoTSig library in exploring fundamental problems in cellular physiology and homeostasis.

Keywords: Systems biology software / membrane ion transport kinetics / cytosolic-free Ca^{2+} concentration/ pH, cytosolic and vacuolar / Cl⁻ - malate exchange / stomatal guard cell

In-text footnotes:
1A complete list of abbreviations will be found in Supplemental Appendix 5 of the companion article.
INTRODUCTION

The guard cells, which surround stomatal pores in the epidermis of plant leaves, regulate the pore aperture to balance the often conflicting demands for CO₂ in photosynthesis with the need to conserve water by the plant. Stomatal transpiration accounts for much of the nearly 70% of global water usage associated with agriculture and has a profound impact on the water and carbon cycles of the world (Gedney et al. 2006; UNESCO 2009). Recent studies have associated increases in continental water run-off with the rise in available CO₂ and decreases in stomatal transpiration (Gedney et al. 2006) and have suggested that stomatal behaviour skews the impact of greenhouse gasses on fresh water resources (Betts et al. 2007). The past half century has generated a vast wealth of knowledge for guard cell transport, signalling and homeostasis, resolving the properties of all of the major transporters and many of the signalling pathways that control them (Blatt 2000a; Schroeder et al. 2001; Blatt et al. 2007; Wang and Song 2008; McAinsh and Pittman 2009). Even so, resolving many aspects of stomatal dynamics remains a challenge. These studies have yet to yield any detail about how the entire network of transporters works as a unit to modulate solute flux and regulate stomatal aperture. Quantitative systems analysis offers one approach to this problem that is now much needed. Efforts to model stomatal function to date generally have been driven by a ‘top-down’ approach: the mechanics of stomatal movements are subsumed within a few empirical parameters of linear hydraulic pathways and conductances (Farquhar and Wong 1984; Ball 1987; Williams et al. 1996; Eamus and Shanahan 2002; West et al. 2005). These models have proven useful at the plant and community levels; but they have not incorporated the essential detail in order to support an understanding of the molecular and cellular mechanics that drive stomatal movements.

In the previous article (Hills et al. 2012) we introduced a computational approach to developing a dynamic model of the stomatal guard cell based on the HoTSig library and OnGuard software. We resolved an OnGuard model that takes account of all of the fundamental properties for transporters at the plasma membrane and tonoplast, the salient features of osmolite metabolism, and key homeostatic and dynamic signalling characteristics that have been described in the literature. The model successfully integrated a number of the steady-state characteristics of guard cells, recapitulating the patterns in guard cell response to the extracellular variables of KCl and CaCl₂ concentrations and to extracellular pH. Here we explore the capacity of the model to reproduce diurnal oscillations in guard cell membrane
transport and malate metabolism, and its consequences for the dynamics of guard cell volume, turgor pressure and stomatal aperture. We demonstrate the true predictive power of the OnGuard model in generating a number of unexpected and counterintuitive outputs. Among these, the model yields counterintuitive changes in cytosolic-free $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) and a daily exchange of Cl$^-$ with malate that are well-documented in the literature, but have been suggested to require additional and complex levels of regulation. These behaviours are accounted for entirely by the known kinetic features of the transporters encoded in the model. Thus, the results demonstrate the predictive power of the OnGuard model as a framework from which to test the basic tenets of the stomatal behaviour and to explore the interactions of transport and metabolism in the guard cell system.
THE DIURNAL MODEL

By nature guard cells define a closed cellular system within the surrounding apoplastic volume of the leaf tissue (Wille and Lucas 1984). Transport of K\(^+\) and Cl\(^-\), both major contributors to the osmotic content of guard cells, and of H\(^+\) and Ca\(^{2+}\) must take place across the plasma membrane and coordinate with the metabolism of the predominant, osmotically-active organic compounds, notably of sucrose and malate. A number of excellent reviews summarise the very large body of experimental data that pertain to transport, metabolism and their regulation in guard cells (Blatt and Thiel 1993; Willmer and Fricker 1996; MacRobbie 1997; Thiel and Wolf 1997; Blatt 2000b; Schroeder et al. 2001; Hetherington 2001; Outlaw 2003; Dreyer et al. 2004; Blatt et al. 2007; Pandey et al. 2007). Details of the kinetic parameters incorporated for each of the transporters and for malate and sucrose metabolism are provided in the preceding article (Hills et al. 2012). We draw attention here to a few key conceptual points that bear on dynamic behaviour of the guard cell.

First and foremost, electrophysiological and radiotracer flux studies (Willmer and Fricker 1996; MacRobbie 1997; Blatt 2000b; Schroeder et al. 2001; Dreyer et al. 2004) have shown that the dominant membrane transporters are active under most physiological conditions, although their operation may be kinetically limited by substrate (ion) availability and/or membrane voltage. This observation contrasts with a common misconception that transporters either activate or shut down fully in response to various stimuli and, thus, may be modelled kinetically as simple ‘on-off’ or Boolean processes (Li et al. 2006). For example, currents through both inward- (I_{K,in}) and outward-rectifying (I_{K,out}) K\(^+\) channels are clearly evident under voltage clamp both in the absence and presence of the water-stress hormone abscisic acid (ABA) that triggers stomatal closure (Blatt and Armstrong 1993; Romano et al. 2000; Garcia-Mata et al. 2003). ABA reduces the activity of I_{K,in} and promotes that of I_{K,out} through qualitative changes in gating, although membrane depolarisation is an overriding factor in determining the K\(^+\) flux (Thiel et al. 1992; Romano et al. 2000). Thus, one conclusion to be drawn from these observations is that guard cells are unlikely to be found in a state in which the net flux of an osmotically-active solute is zero. Instead, the observations suggest that guard cells transit between situations of osmotic solute uptake and loss, to achieve by ‘time-averaged’ approximation a dynamic range of (quasi-)steady-state of solute contents and, consequently, of stomatal apertures.
This conclusion resonates with a second set of observations that guard cell membrane voltage can oscillate with periods from a few tens of seconds to many minutes (Thiel et al. 1992; Gradmann et al. 1993; Grabov and Blatt 1998) between two quasi-stable states, a depolarised state associated with K⁺ and Cl⁻ flux, and a hyperpolarised state which can be shown to drive K⁺ uptake (Blatt and Clint 1989; Clint and Blatt 1989; Blatt and Thiel 1993; Blatt 2000b). Such oscillations have been observed to occur spontaneously; they can be induced by changes in external solute (e.g. KCl) concentration as well as hormone treatments, including ABA and auxin (Thiel et al. 1992; Blatt and Armstrong 1993; Blatt and Thiel 1994; Sokolovski and Blatt 2007); and they have been associated with substantial changes in [Ca²⁺], (Grabov and Blatt 1998; Blatt 2000b). These characteristics lead to an expectation of considerable temporal dynamism in membrane transport and its regulation in guard cells.

Finally, a third point that helped inform our approach to modelling arises from the general finding that the ion fluxes facilitating stomatal movements reflect a small fraction only of the potential activities of several transporters, notably of the predominant voltage-activated K⁺ channels and Ca²⁺ channel at the plasma membrane [cf. Thiel, et al. (Blatt et al. 1990b; 1992; Hamilton et al. 2000)], the CLC H⁺-Cl⁻ antiporter, the SV (TPC) and FV cation channels, and the VCL and VMAL (ALMT) anion channels at the tonoplast [cf. Pottosin and Schoenknecht (2007) and DeAngeli, et al. (2009)]. In general, the activities of these transporters are kinetically limited by their inherent gating properties within the range of voltages typically found across the plasma membrane and tonoplast. Thus, the channel activities recorded under voltage clamp at the voltage extremes do not represent the typical current amplitudes in vivo, even if they are useful as measures of the biophysical and regulatory properties associated with the currents.

The Reference Cycle

Formulating a dynamic model of cellular homeostasis normally begins with the definition of a Reference State representing the resting physiological condition of the system under study. However, when the model describes periodic cellular responses, it is convenient to circumscribe the associated parameters within a “Reference Cycle”. Previously (Hills et al. 2012), we introduced paired Reference States corresponding to the guard cell of the closed and open stomata and reflecting the pattern of nocturnal closure and diurnal aperture found in most plant species (Willmer and Fricker 1996). We envisaged the Reference Cycle as a
logical extension of these paired states, with the nocturnal closed state equating with our earlier Closed Reference State (Hills et al. 2012) in which the guard cells retained a baseline of osmotic load and a minimum of ion flux across the tonoplast and plasma membrane. To define the diurnal cycle, the turnover rates of all primary pumps (H⁺-ATPases, Ca²⁺-ATPases, H⁺-PPase) across tonoplast and plasma membrane were assigned values in the nocturnal (closed) state of 5, 10 or 20 percent of their maximum output consistent with experimental estimates of the known light-stimulated activities [see Kinoshita, et al. (2001), Goh, et al. (1995; 1996), Assmann, et al. (1985), Gotow, et al. (1985) and Shimazaki, et al. (2007)]. The rates of sucrose and malate synthesis were set to zero in the dark.

Transition to the light was introduced by incorporating a hyperbolic dependence on light for these components. For simplicity we made use of a common L½ value of 50 μmol m⁻² s⁻¹ for the fluence dependence of all of the light-dependent processes and we simulated the light cycle as a 6-h ramp from 0 to 2200 μmol m⁻² s⁻¹, a further 6-h ramp down to 0 μmol m⁻² s⁻¹, and a 12 h dark period. All other model parameters were kept constant in any one simulation. As described in the following paragraphs, one consequence of using a simple ramp cycle was to delay the final steady-state achieved in daytime outputs until late in the daylight period. A steady-state in these outputs was achieved much earlier in the daylight period when a gaussian-shaped light regime was used (not shown, available to the user in OnGuard). We chose nonetheless to use the ramp cycle here because it yields simple hyperbolic functions in the activation/deactivation of the pumps and metabolism in relation to elapsed time and, as a result, it is much easier to appreciate the interactions between transporters. The parameter ensemble for the osmotic composition of the guard cell, buffering constants for H⁺ and Ca²⁺, the relevant transporters, sucrose and malate metabolism and their kinetic descriptors are those described in the preceding article (Hills et al. 2012) and are based on published data for Vicia. This final model was resolved through fine adjustments to the ensemble, introduced between simulations. The process was constrained throughout by experimental data, including electrophysiological data on single channels and pumps in isolated guard cells, data from measurements of stomatal apertures and calculated turgor pressures in populations of stomata, and from metabolic studies on malate and sucrose metabolism (Dittrich and Raschke 1977; Talbott and Zeiger 1993; Willmer and Fricker 1996; Hills et al. 2012). Tabulations of sample adjustments and effects on simulation outputs are summarised in Figs. 4 and 5 of the preceding article (Hills et al. 2012).
RESULTS

Diurnal changes in macroscopic outputs

In vivo (Raschke 1979; MacRobbie 1987; Willmer and Fricker 1996), the diurnal cycle is associated with a progressive accumulation of K⁺ with Cl⁻, and subsequently with malate, leading to an increase in turgor pressure and stomatal aperture in the first half of the day; only later do lengthening of the spectral wavelength and other factors lead to a decline in H⁺-ATPase and other transport activities. Malate synthesis and accumulation delays the decline in osmotic load, often effectively exchanging with Cl⁻ during the daylight period. Daylight events are thus accompanied by a large solute and water fluxes in and out of guard cells, mostly between vacuole and apoplast passing through the cytosolic compartment of the guard cells, and by an accelerated metabolic activity to generate and degrade additional osmolites, all with a highly regulated time course. Figures 1-7 summarise the principle outputs of simulation with the OnGuard model and its capacity to reconstruct these, and related characteristics of the guard cell diurnal cycle.

As anticipated, modulating the activities of the primary ion pumps, sucrose and malic acid synthesis resulted in a cycle of diurnal stomatal opening and nocturnal closure. As shown in Fig. 1, stomatal apertures varied over a physiological range between roughly 4 μm and 13 μm and were paralleled by physiologically reasonable changes in guard cell volume, turgor and vacuolar volume percent [cf. Willmer and Fricker (1996)], the latter defined as the percentage of the cell volume occupied by the vacuole. Stomatal closure at the end of the day was followed by a small and gradual rise in aperture and the associated macroscopic outputs, in effect anticipating the start of the next day much as has been observed in vivo (Gorton et al. 1993; Meidner and Willmer 1993). In the simulation conditions – 10 mM KCl and 1 mM CaCl₂, pH 6.5, typical of many studies with epidermal peels – the onset of daylight was associated with hyperpolarisation of the plasma membrane to voltages near -130 mV and the dark period was accompanied by depolarisation of the plasma membrane to voltages near the equilibrium voltage for K⁺, consistent with the diurnal cycle in energetic outputs of the ATP-driven pumps [see (Raschke 1979; Spanswick 1981; Blatt 1987b; McClure et al. 1989; Blatt and Clint 1989; Clint and Blatt 1989; Kinoshita et al. 1995) and below]. The tonoplast showed much smaller diurnal variations and an inverse pattern, with voltages of -20 mV in daylight and near -50 mV at night, indicating a dominance of secondary conductances especially during the daylight hours (Goldsmith and Goldsmith 1978; Gobert et al. 2007).
Transition to the dark was marked by a period of voltage excursions, or action potentials, at the plasma membrane, and was followed at first by a period during which the membrane rose to voltages near -40 mV. We return to these observations later.

**Osmotic contents and flux of $K^+$**

Figure 2 shows that stomatal opening was accompanied by increases in $K^+$ concentration in the cytosol and in the vacuole, these increases following monophasic kinetics, and decreased again in the first hours of dark during stomatal closure, consistent with experimental measurements [see Hills et al. (2012)]. The $K^+$ concentrations in both compartments gradually rose again later at night in parallel with stomatal aperture (Fig. 1). In the cytosol, the $K^+$ concentration varied between approximately 110 mM in the closed state and 210 mM in the open state; in the vacuole, $K^+$ concentrations ranged between approximately 20 and 120 mM (Fig. 2A). Analysis of the total $K^+$ flux (Fig. 2B) showed that the larger proportion of $K^+$ influx across the plasma membrane was shunted across the tonoplast to the vacuole during the day and this pattern reversed in the first hours of dark, as expected to accommodate the large changes in vacuolar volume (Fig. 1). [Note that here, and in all subsequent analyses, we have defined a positive flux as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.] At the plasma membrane (Fig. 2C), $K^+$ influx was dominated by $I_{K,in}$ in the first half of the day, this flux relaxing to approximately 20% of its maximum, roughly equivalent to that through the $H^+-K^+$ symport in the second half of the day. Closure was marked by the predominance of $K^+$ efflux through $I_{K,out}$, which relaxed to a near-zero value before the second half of the night. At the tonoplast (Fig. 2D), $K^+$ flux in both directions was mediated largely by the TPK channel with approximately 10% passing through the FV channel, consistent with experimental evidence for the importance of TPK1 for $K^+$ homeostasis (Gobert et al. 2007). Less than 1% of the $K^+$ flux passed through the TPC channel (not shown), consistent with evidence that in *Arabidopsis* the *tpc1* mutant has little effect on tissue $K^+$ contents (Peiter et al. 2005). These fluxes largely mirrored the diurnal pattern of those at the plasma membrane, with the exception that both influx and efflux were observed, primarily through the TPK channel, later during stomatal closure as the channel provided the major flux pathway for charge balance across the tonoplast (see Figs. 3, 4 and 6).
Sucrose and malic acid metabolism, and the malate osmoticum

Malate is known to be a major contributor to the osmotic contents of the guard cell, notably in the vacuole, and may accumulate to concentrations in excess of 150 mM during the day in some circumstances (Van Kirk and Raschke 1978a; Outlaw 1990; Talbott and Zeiger 1993; Willmer and Fricker 1996). For modelling purposes, we neglected sucrose transport, instead using Mal as a proxy for sucrose as an osmoticum (Hills et al. 2012), consistent with the predominance of Mal in many circumstances (Willmer and Fricker 1996). Thus, with sucrose synthesis activated in the OnGuard model in the daylight, malic acid production was engaged and then relaxed to a near-steady-state rate of production near 12 amol s⁻¹, sufficient to accumulate over 40 mM h⁻¹ in the cytosol (Fig. 3A); in the first 2-3 h of dark, a fraction of this Mal was metabolised to sucrose and broken down as it was recovered from vacuolar stores [see below, and (Van Kirk and Raschke 1978a; Outlaw 1990; Talbott and Zeiger 1993)]. During the day, the bulk of Mal production was diverted by transport of Mal²⁻ across the tonoplast, leading to a rise in total vacuolar Mal concentration from approximately 20 mM in the closed state to over 100 mM in the open state; in parallel, Mal in the cytosol ranged from 0.7 to values near 25 mM, respectively (Fig. 3B,C), much as has been estimated from experimental data [see Hills et al. (2012)]. Mal accumulation during the day was accompanied by a small increase in export across the plasma membrane, culminating in a substantial efflux from the vacuole and cytosol during closure in the first hours of dark (Fig. 3C-E). Again, these characteristics match well the experimentally-documented evidence for Mal flux (Allaway 1973; Van Kirk and Raschke 1977; Van Kirk and Raschke 1978b; Gotow et al. 1985; Tarczynski and Outlaw 1990). At the plasma membrane, the OnGuard model predicted Mal efflux to be mediated largely by the SLAC-type anion channel during daylight and through the R- (ALMT-) type anion channel during stomatal closure in the first hours of dark (Fig. 3C), much as has been deduced from experimental data (Hedrich et al. 1990; Schmidt and Schroeder 1994; Dietrich and Hedrich 1998; Vahisalu et al. 2008; Negi et al. 2008). Mal flux in both directions across the tonoplast was dominated by the VMAL (ALMT) anion channel, as previously postulated (Meyer et al. 2011), the flux thus driven by the balance of electrochemical driving forces across the tonoplast and plasma membrane, and the rapid decline in cytosolic Mal early during stomatal closure (see Fig. 3B).
Osmotic contributions from Cl

Both cytosolic and vacuolar Cl\(^-\) concentrations exhibited more complex, biphasic responses to the diurnal cycle in the OnGuard model (Fig. 4A), consistent with experimental observation (Raschke and Schnabl 1978; Talbott and Zeiger 1993; Talbott and Zeiger 1996). In the cytosol, the Cl\(^-\) concentration rose during the first hours of daylight to a maximum near 15 mM, declining thereafter to below 10 mM at the end of the day. This pattern was repeated in reverse during the night: the cytosolic Cl\(^-\) concentration falling in the first 2 h of dark, thereafter rising from a minimum of 2 mM to 6 mM at the end of the dark period. Vacuolar Cl\(^-\) concentration, by contrast, decreased monotonically from 45 mM to less than 20 mM during daylight, declining further to near 15 mM in the first 2-3 h of dark, before rising again in parallel with the cytosolic Cl\(^-\) concentration. The dark rise in both vacuolar and cytosolic Cl\(^-\) concentrations was suppressed when the external Cl\(^-\) concentration was reduced from the standard 12 mM (10 mM KCl + 1 mM CaCl\(_2\)) and the decline in cytosolic and vacuolar Cl\(^-\) concentrations during the second half of the daylight period was absent at external Cl\(^-\) concentrations of 22 and 32 mM (not shown).

Analysis of the Cl\(^-\) flux (Fig. 4B) showed a net influx across the plasma membrane that gradually declined during the dark period, and a persistent but smaller efflux throughout much of the day. The first 2-3 h of dark were marked by excursions in Cl\(^-\) flux between efflux and influx across the plasma membrane. Across the tonoplast, the inverse pattern was observed: a small Cl\(^-\) influx occurred throughout the daylight, the first 2-3 h of dark were marked by large excursions in Cl\(^-\) influx, and during the remainder of the dark Cl\(^-\) was transported outward (into the vacuole). Thus, stomatal opening was accompanied by a net flux of Cl\(^-\) from the vacuole to the apoplast; closure at the start of the dark period was marked by much larger fluxes of Cl\(^-\) from the vacuole to the cytosol and export across the plasma membrane; and this pattern reversed after the first 2-3 h of dark. The rise in cytosolic Cl\(^-\) concentration during the first hours of the day arose from the rapid Cl\(^-\) influx across the tonoplast and a slower rise in the rate of Cl\(^-\) export across the plasma membrane.

At the plasma membrane (Fig. 4C), Cl\(^-\) influx was mediated by the H\(^+\)-Cl\(^-\) symport throughout the daylight period and to a lesser extend during the night. The decline and reversal in net flux arose as the consequence of Cl\(^-\) efflux through the SLAC channel during the day and the first 2-3 h of the dark period. Cl\(^-\) export was augmented by efflux through the R- (ALMT-) type anion channel in the first 2-3 h of dark. By contrast, Cl\(^-\) flux at the
tonoplast was dominated throughout the diurnal cycle by the VCL channel, the CLC H⁺-Cl⁻ antiport making a lesser contribution to Cl⁻ accumulation in the vacuole throughout the 24 h cycle (Fig. 4D). As a consequence, reversal of the next flux of Cl⁻ across the tonoplast was driven by changes in the electrochemical driving force for Cl⁻ and a passive shift in VCL flux, which followed the decline in Cl⁻ concentration in the cytosol and changes in vacuolar membrane voltage (Figs. 1 and 4A). One conclusion to be drawn from this analysis is that net fluxes of Cl⁻ and Mal²⁻ are not linked. It is certainly the case that cytosolic Mal affects both the major anion channels at the plasma membrane, but the effect is a moderate suppression in channel activity (Wang and Blatt 2011) which could be expected to enhance Cl⁻ retention rather than enhancing its efflux. Thus, the OnGuard model successfully reproduced the apparent daytime exchange between Cl⁻ and Mal (Van Kirk and Raschke 1977; Raschke and Schnabl 1978), but without a requirement for direct control of Mal on Cl⁻ flux as previously postulated (Hedrich and Marten 1993).

_Cytosolic pH, the H⁺-ATPase and H⁺-coupled transport_

Direct measurements in guard cells (Irving et al. 1992; Blatt and Armstrong 1993; Thiel et al. 1993; Armstrong et al. 1995; Grabov and Blatt 1997; Zhang et al. 2001) have indicated resting pHi values near 7.5-7.7, with one notable exception (Thiel et al. 1993), and vacuolar pH generally has been estimated to situate between pH 4.5 and 6 [see Hills, et al. (2012)]. Figure 5A shows that the OnGuard model faithfully reproduced the characteristics anticipated for the pH, both in the cytosol (pHi) and vacuole (pHv). Values for pHi remaining close to 7.6-7.7 throughout much of the day, dropping to 7.5 during the night and rising again above 7.6 before the start of the subsequent daylight period. This daily cycle was punctuated by an acid-going transition in the first 1-2 h of daylight and a similar period at the end of the day when the model showed a 0.1-unit pHi rise to a value near 7.8. Details of the diurnal changes in pHi in the guard cell remain to be explored in vivo. However, a 0.1-0.3 unit rise in pHi is known to occur during the first 10-30 min of stomatal closure evoked by ABA (Irving et al. 1992; Blatt and Armstrong 1993; Zhang et al. 2001), so it is significant that a similar rise in pHc coincided with the initial and very rapid fall in stomatal aperture (compare Figs. 1 and 5).

Direct measurements of pHc indicate substantial static buffering, as incorporated in the OnGuard model (Hills et al. 2012), and a capacity for dynamic pHc control (Grabov and
Blatt 1997). However, static buffering will accommodate finite changes in H⁺ concentration and cannot account for long-term H⁺ loads such as imposed by metabolism. At 1-3 pmol h⁻¹ (guard cell)⁻¹, maximum rates of malic acid synthesis alone in Vicia guard cells are sufficient to generate a H⁺ load near 0.5 M h⁻¹ (Gotow et al. 1985; Outlaw 1990; Tarczynski and Outlaw 1990). Thus, H⁺ elimination from the guard cell is essential for pHₖ homeostasis. As expected, the OnGuard model predicted the vast bulk of the H⁺ production associated with daytime Mal synthesis (Fig. 3A) to be exported via the plasma membrane H⁺-ATPase, with roughly 20% transported to the vacuole (Fig. 5B). The model returned a net H⁺ efflux that reached a maximum close to 30 amol s⁻¹ in the first 2 h of the day and settled to a rate near 20 amol s⁻¹ in the second half of the daylight period before declining to near zero, consistent with the diurnal pattern of H⁺ flux in vivo (Willmer and Fricker 1996). A substantially higher H⁺ flux was estimated through the H⁺-ATPase than was generated as net H⁺ export, much as can be concluded from its activity in vivo (Blatt 1987a; Blatt 1988; Blatt and Clint 1989; Fricker and Willmer 1990; Lohse and Hedrich 1992), such that 30-40% of the H⁺ export via the H⁺-ATPase was balanced by H⁺ entry coupled with Cl⁻ uptake and, to a lesser extent, with K⁺ uptake (Fig. 5C). One prediction to come from the OnGuard analysis, therefore, is that pHₖ homeostasis is likely to be affected by the absence of inorganic ions available for symport with H⁺, and this conclusion accords with analogous considerations in several plant cells (Smith 1973; Sanders et al. 1989; Guern et al. 1991) and in fungi (Blatt and Slayman 1987).

Vacuolar pH, H⁺ and malate transport

Malic acid comprises the major pH buffer in the vacuole and its accumulation is associated with acidification of the vacuolar contents (Van Kirk and Raschke 1978a; Outlaw 1990; Talbott and Zeiger 1993; Willmer and Fricker 1996). In the OnGuard model these diurnal changes resulted in pHₖ oscillating between 4.6 units during the day and around 5.0-5.4 units during the second half of the night (Fig. 5A). All evidence indicates that the organic acid is transported as the fully deprotonated (Mal₂⁻) form – with the VMAL channel as the primary pathway for tonoplast Mal₂⁻ flux (see Fig. 3D and reviewed by Hills, et al. (2012) – thereby implying that a component of charge balance is achieved with parallel H⁺ transport via the tonoplast VH⁺-ATPase and H⁺-PPase. In the OnGuard model (Fig. 5D), H⁺ transport to the vacuole was roughly divided between the VH⁺-ATPase and H⁺-PPase, as estimated
from experimental data in several plant species and cell types (Rea and Poole 1993; Martinoia et al. 2007). The model predicted H+ re-entry to the cytosol to be dominated by its exchange with Ca2+ mediated by the CAX H+-Ca2+ antiport, primarily during stomatal closure in the first 2-3 h of dark; H+ exchange with Cl− was indicated to contribute a smaller fraction to H+ return from the vacuole, at least within the context of the extracellular parameters of the simulation (above). In total, however, the H+ flux represents a small fraction of the total H+ load estimated for the guard cell: a comparison of fluxes and ions accumulated in the simulation indicates that net H+ flux through the combined pathways of the VH+-ATPase and H+-PPase contributed approximately 30% to vacuolar charge balance with the remainder assumed by K+ flux (see Fig. 2).

One underlying assumption in these estimations is of the diurnal variations in energy-dependent H+ transport. To validate this assumption, we turned to Arabidopsis, carrying out quantitative PCR for the VHA-a2, VHA-a3, A and C subunits of the VH+-ATPase on mRNA isolated at 4 h time intervals from leaves; additionally, we isolated microsomal membranes from the same leaf samples to assay for Concanamycin A-sensitive ATPase activity (Palmgren 1990; Brux et al. 2008). The results of these experiments (Supplementary Figs. S1 and S2), demonstrated that both the transcription and enzymatic activity of the VH+-ATPase pass through a diurnal cycle, with a maxima during mid-day and a minimum close to 10% of this value during the nocturnal period. Furthermore, analysis of the model outputs showed that transport of the major osmotica and changes in pHv followed the pattern consistent with experimental observation (above). Thus, our prediction of a diurnal cycle in VH+-ATPase activity as built into the model are borne out, and the results gave us confidence to assume a comparable cycle of activity for the H+-PPase.

**Diurnal variations in cytosolic-free [Ca2+]**

Figure 6A shows that stomatal opening was accompanied by an increase in total [Ca2+] in the vacuole, rising from a mean value near 15 mM in the dark to 25 mM at the end of the daylight period; total [Ca2+] in the cytosol remained between 0.2 and 0.4 mM throughout much of the daylight period. Free [Ca2+] in the cytosol ([Ca2+]i) rose from a resting value near 180 nM in the dark to a quasi-steady-state close to 300 nM in the second half of the daylight period (Fig. 6B). In the final hour of daylight and the first 2-3 h of dark, the OnGuard model generated a series of voltage and [Ca2+] excursions that culminated with
stomatal aperture relaxing to a minimum closed value and recovery of dark levels in total
\([Ca^{2+}]\) and \([Ca^{2+}]_i\). These characteristics are broadly consistent with a number of previous
observations: they mirror the action potential-like oscillations in membrane voltage and
\([Ca^{2+}]\), elevations that have been associated with stomatal closure (Gilroy et al. 1991; Thiel
et al. 1992; Irving et al. 1992; McAinsh et al. 1992; Blatt and Armstrong 1993; Gradmann
et al. 1993; Grabov and Blatt 1998; Staxen et al. 1999), and they recapitulate diurnal
variations in \([Ca^{2+}]\), with resting values elevated in the daytime relative to the night (Dodd et
al. 2005; Dodd et al. 2006).

Detailed analysis of the OnGuard output (Fig. 6C) showed a net \(Ca^{2+}\) influx across the
plasma membrane and export to the vacuole throughout the daylight period, although the bulk
of this flux occurred in the first 8 h of the day. The flux direction was reversed during the first
2-3 h of dark, albeit with excursions in tonoplast flux to positive (outward, directed to the
vacuole) values, before both membrane fluxes relaxed to near-zero values. At the plasma
membrane (Fig. 6D), this \(Ca^{2+}\) flux was dominated by the inward-rectifying \(Ca^{2+}\) channel
during daylight hours and by the \(Ca^{2+}\)-ATPase in the first hours of dark. Much of \(Ca^{2+}\)
exported to the vacuole (Fig. 6E) by the vacuolar \(Ca^{2+}\)-ATPase was returned through the
TonVCa \(Ca^{2+}\) channel during daylight hours. Activity of the CAX \(H^+\)-\(Ca^{2+}\) antiport was
evident in the first 2-3 h of dark, and all three transporters underwent a series of excursions,
albeit with \(Ca^{2+}\) return via the \(Ca^{2+}\) channel dominating, before the component \(Ca^{2+}\) fluxes
relaxed to near-zero values in the second half of the dark period. Comparison of the flux
amplitudes predicted by the OnGuard model for the plasma membrane and tonoplast shows
that vacuolar \(Ca^{2+}\) transport dominated by at least one order of magnitude throughout the
diurnal cycle. This prediction is generally in accord with the major roles for endomembrane
sequestration and release in \(Ca^{2+}\) homeostasis (Sanders et al. 2002; Blatt et al. 2007;
McAinsh and Pittman 2009) and with direct evidence in guard cells for its importance in
potentiating and shaping \([Ca^{2+}]_i\), signals (Gilroy et al. 1991; McAinsh et al. 1991; Grabov
and Blatt 1997; Grabov and Blatt 1998; Grabov and Blatt 1999; Garcia-Mata et al. 2003).

Of the model outputs, the diurnal variation predicted for resting \([Ca^{2+}]_i\) is
provocatively counterintuitive. The OnGuard model incorporated a 5-fold increase in the
activities of the \(Ca^{2+}\)-ATPases during daylight hours at both the plasma membrane and the
tonoplast. Yet resting \([Ca^{2+}]_i\), rose substantially in the light, despite the enhanced \(Ca^{2+}\) export
from the cytosol. How can we explain such behaviour? The OnGuard model outputs yield a
simple, if unexpected answer. Figure 7A illustrates the underlying characteristics for Ca\(^{2+}\) transport at the plasma membrane, cross-referenced to membrane voltage and Ca\(^{2+}\) flux (Fig. 7C,D), with data captured from screenshots at time points throughout the diurnal cycle [curves for other transporters are not shown for clarity see also Hills, et al. (2012)]. The IV curves for the Ca\(^{2+}\) channel and Ca\(^{2+}\)-ATPase shown here encapsulate the kinetic descriptors for each of these transporters as a function of the membrane voltage, and they correspond to the component IV curves that would be recorded under voltage clamp. Comparing the currents at the resting (free-running) membrane voltages, indicated by the vertical, dashed lines, it is evident that the balance of Ca\(^{2+}\) transport led to a net Ca\(^{2+}\) influx into the cytosol early in the day, a gradual decay in this flux towards the end of the day, and a greater (and oscillating) Ca\(^{2+}\) efflux early in the night before the net Ca\(^{2+}\) flux relaxed back to a value near zero. Thus, at the plasma membrane, stimulating the pumps and the resulting membrane hyperpolarisation during the day affects directly the kinetic limits for Ca\(^{2+}\) export by the Ca\(^{2+}\)-ATPase, suppressing its ability to export Ca\(^{2+}\) and at the same time promoting Ca\(^{2+}\) entry through the hyperpolarisation-activated Ca\(^{2+}\) channels.

Analysis of current at the tonoplast (Fig. 7B) shows a complementary pattern of Ca\(^{2+}\) flux. In this case, enhancing Ca\(^{2+}\)-ATPase activity and the small membrane depolarisation during the day can be seen to have promoted Ca\(^{2+}\) efflux to the vacuole sufficient to keep pace with Ca\(^{2+}\) entry from the apoplast. Additionally, as [Ca\(^{2+}\)]\(_i\) rises during the day it enhanced Ca\(^{2+}\) release, which largely balanced efflux via the tonoplast Ca\(^{2+}\)-ATPase. Finally, the decline in pump activities at the end of the day left the predominant flux for Ca\(^{2+}\) directed inward across the tonoplast over the same time period that depolarisation of the plasma membrane favoured Ca\(^{2+}\) export through the Ca\(^{2+}\)-ATPase (Fig. 7C,D). Thus, stimulating the ATP- and pyrophosphate-dependent pumps in the OnGuard model, including the Ca\(^{2+}\)-ATPases at the plasma membrane and tonoplast, translates to a net influx of Ca\(^{2+}\) from the apoplast, through the cytosol, to the vacuole during the day; this net flux reverses as the stomata closed during the early hours of the night. In short, the counterintuitive changes in [Ca\(^{2+}\)]\(_i\) during the day resulted from membrane hyperpolarisation and consequent alterations in kinetic restrictions on the Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) channel at the plasma membrane; these changes were paralleled by a progressive decline in the capacity for Ca\(^{2+}\) sequestration. It is worth noting, too, that the predicted variations in Ca\(^{2+}\) flux and [Ca\(^{2+}\)]\(_i\) were achieved without encoding any extrinsic feedback such as has been suggested to affect the durnal capacity for
endomembrane Ca\(^{2+}\) release (Dodd et al. 2007). Of course, the simulation does not preclude this or other feedback regulation, but it demonstrates the ability of the model to encapsulate otherwise unexpected biological phenomena within the emergent properties of a relatively simple system. As a corollary, it also suggests more subtle roles for control mechanisms such as post-translational regulation affecting the capacity for Ca\(^{2+}\) release.

**Predicting endomembrane Ca\(^{2+}\) flux and \([Ca^{2+}]_i\), oscillations**

One requirement for the OnGuard model was our inclusion of a self-limiting Ca\(^{2+}\) flux pathway for Ca\(^{2+}\) release from the vacuole. Such pathways are essential for evoked Ca\(^{2+}\) release and, like the mammalian IP\(_3\)- and ryanodine-receptor channels of animals (Bezprozvanny et al. 1991; Hille 2001), must incorporate an element of self-inhibition to prevent prolonged [Ca\(^{2+}\)], elevation and exhaustion of the Ca\(^{2+}\) store. Ca\(^{2+}\) channels that are activated by Ca\(^{2+}\) and ligands such as inositol-trisphosphate (IP\(_3\)), cyclic ADP-ribose and nitric oxide are known or have been demonstrated indirectly to reside in the endomembranes and vacuoles of plant cells, including those of *Vicia* guard cells (Alexandre et al. 1990; Leckie et al. 1998; Garcia-Mata et al. 2003). With the exception of the plasma membrane Ca\(^{2+}\) channel of guard cells (Hamilton et al. 2000), self-inhibition has not been demonstrated in plants per se; nevertheless, it is implicit in evoked [Ca\(^{2+}\)], oscillations such as those observed in *Vicia* guard cells (McAinsh et al. 1995; Grabov and Blatt 1998; Staxen et al. 1999; Garcia-Mata et al. 2003; Sokolovski et al. 2005). The SV (TPC1) channel defines a Ca\(^{2+}\)-permeable pathway in the guard cell tonoplast with gating characteristics well-constrained experimentally [cf. Peiter, et al. (2005), Dacz-Narloch, et al. (2011), and Schulze-Lessdorf and Hedrich (Schulzlessdorf and Hedrich 1995)], but this channel is unsuited to the task of Ca\(^{2+}\) release using any of the range of gating and permeation parameters that are reported in the literature [see Hills, et al. (2012)]. Without self-limitation, trials of the SV channel in the OnGuard model either failed to release Ca\(^{2+}\) and to drive stomatal closure, or yielded prolonged elevations, lasting tens of minutes to hours, with [Ca\(^{2+}\)], pinned to values in excess of 50-100 μM until the vacuolar Ca\(^{2+}\) content dropped below 3% of its mean daytime value. Such behaviour is not consistent with the physiology of the guard cell.

To overcome this difficulty we included in the model (Hills et al. 2012) a Ca\(^{2+}\) channel (TonVCa) with [Ca\(^{2+}\)], activation analogous to the IP\(_3\)-receptor channels of the Beta vacuole (Alexandre et al. 1990). We added to this channel self-inhibition with time-
dependent inactivation and reactivation above and below fixed \([Ca^{2+}]_i\) thresholds, respectively, similar to the slow inactivation and latency of the animal IP3-receptor channels (Bezprozvanny et al. 1991). Finally, gating of this hypothetical \(Ca^{2+}\) channel was assigned a \([Ca^{2+}]_i\)-sensitivity to its voltage dependence in order to avoid depleting the endomembrane \(Ca^{2+}\) store. These characteristics remain undocumented, and therefore should be viewed as ‘placeholders’ predicting the characteristics of one or more endomembrane \(Ca^{2+}\) release pathways. They may represent features of a tonoplast channel, but need not be restricted to this membrane; in vivo intracellular \(Ca^{2+}\) release depends on other compartments including the endoplasmic reticulum (Clapham 1995; Bootman et al. 1997; Hamilton et al. 2000; Navazio et al. 2001; Garcia-Mata et al. 2003; Sokolovski et al. 2008). In short, the TonVCa characteristics are a predictive summation of the dynamic outputs essential for the \([Ca^{2+}]_i\)_ physiology of the guard cell, rather than the literal descriptors for a \(Ca^{2+}\) channel yet to be identified.

These caveats aside, the TonVCa channel successfully constrained \([Ca^{2+}]_i\) excursions to maxima near 1-2 \(\mu M\), and it resulted in simulated \([Ca^{2+}]_i\) oscillations with periods of 5-10 s to 10-20 min, characteristics qualitatively and quantitatively similar to experimental measurements in vivo (Gradmann et al. 1993; Blatt 2000b; Blatt et al. 2007)(McAinsh and Pittman 2009). Figure 8 shows the logged output from the train of \([Ca^{2+}]_i\)_ and voltage excursions simulated by the OnGuard model at the end of the daylight period (see also Fig. 7) and associated IV curves taken from screenshots at the timepoints indicated. Also plotted are the associated fluxes of \(Ca^{2+}\) through the \(Ca^{2+}\) channels and \(Ca^{2+}\)-ATPases at the two membranes and the change in stomatal aperture. Analysis of the flux and voltage over time indicated within each cycle a clear sequence of events that may be summarised as follows. (1) Initially, the negative membrane voltage drives \(Ca^{2+}\) influx through \(Ca^{2+}\) channels at the plasma membrane, raising \([Ca^{2+}]_i\) (timepoint a). (2) This rise in \([Ca^{2+}]_i\)_ promotes TonVCa channel activity and \(Ca^{2+}\) influx across the tonoplast. (3) A threshold is reached near the \(K_{Ca}\) for TonVCa channel activation, beyond which the positive feedback of \([Ca^{2+}]_i\) leads to self-activation of the TonVCa channel (timepoint b). (4) Elevated \([Ca^{2+}]_i\)_ stimulates SLAC and R-(ALMT-) type channels and suppresses the H+-ATPase at the plasma membrane, consistent with the \(K_{Ca}\) for each transporter [see Hills, et al. (2012)]; initially the effect is to generate an ‘N’ shape and two quasi-stable voltages (two inflections of positive slope with the voltage axis) in the total plasma membrane IV curve before the membrane collapses to the more...
positive of these two voltages (timepoint c). (5) Elevated \([\text{Ca}^{2+}]\), enhances the activities of the \(\text{Ca}^{2+}\)-ATPases at both membranes and, with depolarisation, the kinetic restriction on the plasma membrane \(\text{Ca}^{2+}\)-ATPase is relieved. (6) With \([\text{Ca}^{2+}]\), above its inactivation threshold, the TonVCa channel passes slowly into inactive latency, thereby shifting the flux balance towards \(\text{Ca}^{2+}\) export and a gradual recovery in \([\text{Ca}^{2+}]\), (timepoint d). (7) As \([\text{Ca}^{2+}]\), falls, the activities of the SLAC and R- (ALMT-) type anion channels decline and the \(\text{H}^+\)-ATPase recovers until the plasma membrane hyperpolarises again. (8) Finally, the TonVCa channel escapes latency as \([\text{Ca}^{2+}]\), falls below its minimum threshold.

The parallels to neuromuscular action potentials are striking (Jack et al. 1983; Hille 2001), notably the underlying feedback between membrane voltage, \(\text{Ca}^{2+}\) entry and \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release, \([\text{Ca}^{2+}]\), and membrane voltage recovery. In this case, however, each depolarising cycle at the plasma membrane and tonoplast promoted \(\text{K}^+\) and anion (\(\text{Cl}^-\) and \(\text{Mal}^{2-}\)) flux from the vacuole, through the cytosol, and out across the plasma membrane, resulting in a loss in turgor pressure and volume. As a consequence, the OnGuard model yielded an oscillatory decline in stomatal aperture, each step-like fall in aperture arising from the loss in osmotic solutes and water during the periods of depolarisation (Fig. 8A). This prediction accords with experimental observations of stomatal closure following a \(\text{Ca}^{2+}\) stimulus (Wang et al. 2006), and it may also relate to oscillations in transpiration long known to occur in leaves of several plant species (Lang et al. 1969; Cowan 1972; Farquhar and Cowan 1974; Cardon et al. 1994).

Other features of the oscillations generated by OnGuard model are equally noteworthy. We highlight three of these here. First, the model predicts that the initial rise in \([\text{Ca}^{2+}]\), is linked to hyperpolarisation of the plasma membrane, much as was postulated a decade ago from experimental data for the \(\text{Ca}^{2+}\) channels and \([\text{Ca}^{2+}]\), recordings (Grabov and Blatt 1998; Hamilton et al. 2000; Blatt 2000b; Hamilton et al. 2001). These earlier studies showed that \(\text{Ca}^{2+}\) channel activity is strongly dependent on membrane voltage and promoted by membrane hyperpolarisation, thus favouring \([\text{Ca}^{2+}]\), elevation; they also indicated slower, \(\text{Ca}^{2+}\)-sequestering and export activities that must underpin the recovery phase of the \([\text{Ca}^{2+}]\), and voltage. Two conclusions to be drawn from the experimental data, and from the simulations above, are that the rise in \([\text{Ca}^{2+}]\), is strongly dependent on membrane voltage and that, once elevated, membrane voltage is dependent on \([\text{Ca}^{2+}]\), recovery. In other words, \([\text{Ca}^{2+}]\), and plasma membrane voltage form two major arms of a cyclic control loop that
regulates plasma membrane transport. The simulations also predict that the recovery phase, but not the initial transient depolarisation and \([\text{Ca}^{2+}]\), elevation phase, is dependent on \(\text{Ca}^{2+}\) export at the plasma membrane and especially at the tonoplast. Thus, suppressing or eliminating these activities – either of the \(\text{Ca}^{2+}\)-ATPases or of CAX \(\text{Ca}^{2+}\)-H\(^{+}\) antiport activity via block of vacuolar H\(^{+}\) transport – is predicted to slow the recovery. Some experimental evidence supports this prediction: notably, mutations affecting the vacuolar H\(^{+}\)-ATPase and tonoplast energisation greatly prolong \([\text{Ca}^{2+}]\), elevations (Allen et al. 2000). Finally, a third conclusion to arise from the simulations is the fundamental importance of H\(^{+}\)-ATPase inhibition at the plasma membrane by elevated \([\text{Ca}^{2+}]\). Trials with the H\(^{+}\)-ATPase uncoupled from \([\text{Ca}^{2+}]\), – that is, either independent of \([\text{Ca}^{2+}]\), or lacking an appreciable inhibition with \([\text{Ca}^{2+}]\), elevations above 300-400 nM – favoured prolonged non-physiological increases in steady-state \([\text{Ca}^{2+}]\). Analysis of the corresponding IV curves showed the behaviour to arise from insufficient inward current to drive the voltage positive and bias the membrane for \(\text{Ca}^{2+}\) efflux. In general, the H\(^{+}\)-ATPase held the membrane voltage sufficiently negative that the \(\text{Ca}^{2+}\)-ATPase was kinetically restricted, unable to keep pace with the \(\text{Ca}^{2+}\) influx passing through the \(\text{Ca}^{2+}\) channels at the plasma membrane. Thus the model predicted a progressive accumulation of \(\text{Ca}^{2+}\) in the vacuole and a pronounced elevation of \([\text{Ca}^{2+}]\).
DISCUSSION

Stomatal dynamics have long been incorporated into models, notably to predict gas exchange characteristics at the level of the plant community (Ball 1987; Williams et al. 1996; Eamus and Shanahan 2002). These models subsume the cellular mechanics of the guard cell within a few empirical parameters of linear hydraulic pathways and conductances. However, no model of stomata behaviour to date has taken full advantage of the wealth of knowledge available at the cellular level for guard cell transport, signalling and homeostasis. The complexity of the guard cell transport in itself precludes any quantitative description of how guard cells regulate stomatal aperture, let alone a clear understanding of the emergent properties of the guard cell system as a whole. To address this gap in understanding, we have taken a computational approach to dynamic modelling of the guard cell. The OnGuard software and model described in the preceding article (Hills et al. 2012) and elaborated above incorporate all of the fundamental properties for transporters at the plasma membrane and tonoplast, and the salient features of osmolite metabolism. Here we demonstrate its capacity for true predictive power in generating a number of counterintuitive outputs in the emergent behaviour of the system, using the diurnal cycle as a testbed. Many of these outputs find support in experimental data already extant in the literature, and we provide additional validation in support of our starting parameters for primary transport activity from an analysis of gene and protein expression for the tonoplast H+-ATPase. Of particular note, we show that the OnGuard model faithfully reproduces the diurnal variations in K⁺, Cl⁻ and Mal flux and content, in cytosolic and vacuolar pH, and in cytosolic-free Ca²⁺ concentration ([Ca²⁺]i) that have been reported in the literature. Furthermore, it generates action potential-like variations in membrane voltage and in [Ca²⁺], similar to spontaneous oscillations observed experimentally and posulated to play a role in control of stomatal aperture, and it predicts these oscillations to facilitate changes in the osmotic solute content of the guard cell. Significantly, the OnGuard model arrives at each of these predictions without ad hoc assumptions of signalling pathways to connect the various transport and metabolic activities. Other model outputs will now fuel substantive research projects in their own right and are beyond the scope of the present study. In short, the OnGuard software establishes a framework for the systems biological analysis of stomatal guard cells, and it sets out a flexible modelling environment that should find application in explorations of similar physiological and related problems in the future.
Guard cell systems modelling

Of the few models developed, bottom-up from knowledge of guard cells, the only recent effort (Li et al. 2006) surprisingly made deliberate use of Boolean network analysis, thus ignoring the large body of detailed kinetic information available. This choice was not an obvious one. The difficulty with a Boolean approach is its inability to encompass the kinetic dimension essential to dynamic modelling of physiological processes. The power of such network analysis is as a tool to study systems for which there is little quantitative information and a large number of components and possible connections between them. Its most common application is to identify critical components – for example, to answer the question whether signal transmission might be blocked by eliminating one component or its connection to another – in order to ‘map’ the major nodes and causal pathways of a network. However, the simplicity of Boolean models, in which components and their links can only be ‘on’ or ‘off’, precludes any useful application in studies that aim to explore emergent behaviours arising from dynamic interactions within a well-defined network. Of course, it is possible to apply pseudo-temporal characteristics to Boolean models, but the outputs are inevitably disconnected from any meaningful comparison with physiology. That the Boolean guard cell model (Li et al. 2006) yielded an output which had been demonstrated experimentally more than a decade earlier (Blatt and Armstrong 1993), but went without mention, does little to support the claim to its predictive power.

Some past success was achieved in recapitulating guard cell voltage oscillations based on a computational approach and provided an impetus for the work we present here. Gradmann, et al. (1993) incorporated the predominant K⁺ and Cl⁻ channels, H⁺-ATPase and H⁺-Cl⁻ symport activities to demonstrate the minimum of transporters necessary to simulate the oscillations in voltage observed to arise spontaneously in guard cells. This modelling effort was limited in several respects. Among others, it did not include components necessary for Ca²⁺ transport, or parameters for the sensitivities of the various transporters to [Ca²⁺], and pH. The latter omissions are especially important, because pH and [Ca²⁺], are known to exert control on a number of these transport processes. Furthermore, it failed to satisfy the basic requirements for a stable Reference State or the equivalent – lacking a sufficient complement of pathways to balance the modelled ion fluxes, a minimum that might be dictated by our Reference State analysis – and thus incorporated substantial predictive indetermination.
Nonetheless, the Gradmann model was successful in demonstrating voltage oscillations similar to those observed in vivo, and it led to the proposal of a time-averaging mechanism controlling K\(^+\) and anion fluxes across the plasma membrane, elements of which are evident in the OnGuard model (see Figs. 7 and 8). Analogous results were achieved in an application to ion transport in complex tissues of the plant root (Shabala et al. 2006), although similar limitations and lack of indetermination undermine its predictive utility.

**Predictive power of the OnGuard model**

The few instances in which mathematical modelling has been applied to cellular homeostasis with sufficient rigour have been remarkably successful both in reproducing known cellular physiology and in predicting unexpected behaviours. For example, dynamic models of cellular homeostasis (Lew et al. 1979) correctly predicted a transient cell shrinkage and protracted fall in epithelial short-circuit current following ouabain inhibition of the Na\(^+\)/K\(^+\)-ATPase. Both predictions were counterintuitive but experimentally confirmed (MacKnight et al. 1975a; MacKnight et al. 1975b) and, at the time, appeared to contradict the general validity of Ussing’s now widely-accepted, two-barrier description of mammalian epithelia (Ussing 1982). The OnGuard model demonstrates a similar, if not a greater, degree of success in integrating knowledge of stomatal physiology and generating novel insights. Validation of the model using a set of basic parameters within a simple, diurnal regime gave a remarkably close approximation to the guard cell behaviour in vivo and its variation with the diurnal cycle; physiologically sensible outputs were obtained for guard cell turgor pressure, volume, partial volumes of the cytosol and vacuole, stomatal aperture, and values for total osmotic solute content as well as compartmental ion and metabolite concentrations, cytosolic and vacuolar pH, plasma membrane and tonoplast voltage (Figs 1-5).

In addition, the OnGuard model generated a surprising wealth of predictions, many of which we have highlighted above. Among these, the model yielded an apparent exchange of Cl\(^-\) with Mal, and counterintuitive changes in [Ca\(^{2+}\)], and pH over the diurnal cycle, each of which finds direct support in independent experimental data. It is especially significant that these outputs were achieved without additional feedback controls, such as those suggested to affect the diurnal capacity for endomembrane Ca\(^{2+}\) release (Dodd et al. 2007). Thus, these simulations demonstrate the capacity of the OnGuard model to encapsulate otherwise unexpected biological phenomenology within the emergent properties of the known network.
of intrinsic kinetic parameters of the guard cell. Other predictions that find support in experimental literature (Blatt 2000b; Schroeder et al. 2001; Blatt et al. 2007; McAinsh and Pittman 2009) arose from simulated trains of short-term oscillations in [Ca\(^{2+}\)]\(_i\), that took place over periods of seconds to minutes. These demonstrated (i) a coupling to plasma membrane voltage during the early phase of [Ca\(^{2+}\)]\(_i\), elevation and, conversely, the dependence of membrane voltage on [Ca\(^{2+}\)], recovery, (ii) the central role for anion channels in driving the transition between [Ca\(^{2+}\)], elevation and its recovery, and (iii) the fundamental importance of H\(^+\)-ATPase inhibition at the plasma membrane by elevated [Ca\(^{2+}\)]\(_i\) to engage the recovery phase of the oscillatory cycle (see Figs. 6-8). Significantly, all of these predictions arise from the basic Reference Cycle that we implemented as a starting point for exploratory simulations. We have yet to extend our studies to the effects, for example, of environmental and hormonal challenges or of single transporter mutations and genetic ablations, and therefore anticipate many more fundamental insights to come of this work in the future.

**Limitations of the OnGuard model**

These successes notwithstanding, the OnGuard model is subject to a number of restrictions that will need revisiting as our modelling efforts advance. Of these, the inclusion of additional intracellular compartments with different dynamic Ca\(^{2+}\) and H\(^+\) buffering (sequestration) capacities and K\(_d\) values is likely to prove important in fine-tuning signalling events and, for example, could allow for the mid-day depression in stomatal aperture previously documented in several species (Dodge et al. 1992; Hirasawa and Hsiao 1999). We have refrained from adding compartments other than the vacuole at present, because there remains insufficient experimental data to adequately constrain the parameters without predictive indetermination. Similarly we have yet to introduce mechanisms that accommodate protein (de-)phosphorylation cascades or changes in redox status (Wang and Song 2008; Kim et al. 2010). These, and similar omissions do not mean that such processes are unimportant, but rather that specific kinetic detail has yet to become available that would justify their explicit inclusion. Subsuming their effects within the functioning of the individual target processes offers an alternative, effectively ‘black-boxing’ the underlying mechanism(s) within the phenomenological behaviour of the targets (Endy and Brent 2001). This approach is useful, too, in reducing complexity and computational burden without a loss in predictive power. There are many examples of the successful use of black-box
phenomenology, including the Hodgkin-Huxley equations used to represent the operation of the Na\(^+\) and K\(^+\) channels responsible for neuronal action potentials; the Hodgkin-Huxley equations explained the fundamental physiological processes of channel gating long before the underlying molecular mechanisms were elucidated (Hille 2001). We have devised mathematical black boxes that effectively subsumed ensembles of passive conductances across the plasma membrane of *Neurospora* (Blatt and Slayman 1987), *Chara* (Blatt et al. 1990a) and *Vicia* guard cells (Blatt 1987a), and the same approach has been used in the past (Ferreira and Lew 1976; Raftos et al. 1999), and in the present OnGuard model (Hills et al. 2012), to accommodate cytoplasmic proton and divalent cation buffering reactions.

In conclusion, we have built on the concept of a Reference Cycle, using the diurnal cycle of activities known for the primary, ATP-dependent pumps, sucrose and malate metabolism to generate a number of quantitative, and often counterintuitive, predictions emergent in the behaviour of the stomatal guard cell. Significantly, the OnGuard model yielded each prediction from a single, common framework of model parameters and without ad hoc assumptions of signalling pathways to connect the various transport and metabolic activities. Thus, we are able to demonstrate the capacity of the OnGuard software to generate a model with true predictive power for the stomatal guard cell.
MATERIAL AND METHODS

OnGuard modelling

The OnGuard software (www.psrg.org.uk) was driven through a diurnal 12:12 h light:dark cycle as described in the text. Light intensity was elevated in a linear ramp from zero at the start of the cycle to 2200 μmol m$^{-2}$s$^{-1}$ at 6 h into the cycle, and then was reduced in the same manner to zero at 12 h into the cycle. All light-sensitive processes – the transport ATPases and PPase, and sucrose synthesis – wereassigned fluence dependencies with a $K_{1/2}$ of 50 μmol m$^{-2}$s$^{-1}$ with minimum (dark) and maximum (light) activities as described previously (Hills et al. 2012). All other model parameters were fixed. Parameter values will be found in Supplementary Appendix 6 of the previous article (Hills et al. 2012) and are available with the OnGuard software (www.psrg.org.uk).
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Figures:

Fig. 1. Macrosopic outputs from the OnGuard model resolved over the diurnal Reference Cycle (see text) with the standard environmental parameters of 10 mM KCl, 1 mM CaCl₂ and pH 6.5. Data represent a 5-d window of the stable Reference Cycle (12 h light:12 h dark, indicated by bars above). OnGuard model parameters are those described in the preceding article (Hills et al. 2012). Shown are (A) plasma membrane and tonoplast voltage, (B) stomatal aperture, turgor pressure and total guard cell volume, and (C) the percentage of cell volume occupied by the vacuole.

Fig. 2. K⁺ contents and analysis of K⁺ fluxes at the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown are (A) cytosolic and vacuolar [K⁺], (B) the net K⁺ flux across the plasma membrane and tonoplast, (C) the K⁺ flux through the K⁺-permeable transporters at the plasma membrane, comprising the two K⁺ channels and the H⁺-K⁺ symporter, and (D) the K⁺ flux through the K⁺ permeable transporters at the tonoplast, comprising the TPK and FV channels. K⁺ flux through the TPC channel accounted for less than one percent of either of the other channel fluxes, and has therefore been omitted for purposes of clarity. Note that positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.

Fig. 3. Sucrose and malic acid synthesis, total malate (Mal) contents and analysis of Mal²⁻ fluxes at the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown are (A) the rates of sucrose (Suc) and Mal synthesis and metabolism, (B) total cytosolic and vacuolar [Mal], (C) the net flux of Mal²⁻ across the plasma membrane and tonoplast, (D) the Mal²⁻ flux through the Mal²⁻-permeable transporters at the plasma membrane, comprising the SLAC and R-(ALMT-) type anion channels, and (E) the Mal²⁻ flux through the Mal²⁻ permeable transporters at the tonoplast, comprising the VMAL and VCL channels. Note that positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.
Fig. 4. Chloride contents and analysis of Cl\(^-\) fluxes at the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown are (A) total cytosolic and vacuolar [Cl\(^-\)], (B) the net flux of Cl\(^-\) across the plasma membrane and tonoplast, (C) the flux of Cl\(^-\) through the Cl\(^-\) -permeable transporters at the plasma membrane, comprising the SLAC and R- (ALMT-) type anion channels and H\(^+\)-Cl\(^-\) symporter, and (D) the flux of Cl\(^-\) through the Cl\(^-\) - permeable transporters at the tonoplast, comprising the VCL channel and CLC H\(^+\)-Cl\(^-\) antiporter. Note the difference in scales between (C) and (D). Note that positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.

Fig. 5. Cytosolic and vacuolar pH, and analysis of H\(^+\) fluxes across the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown are (A) cytosolic and vacuolar pH, pH\(_c\) and pH\(_v\) respectively, (B) the net H\(^+\) flux across the plasma membrane and tonoplast, (C) the H\(^+\) flux through the H\(^+\)-permeable transporters at the plasma membrane, comprising the H\(^+\)-ATPase, and the H\(^+\)-K\(^+\) and H\(^+\)-Cl\(^-\) symporters, and (D) the H\(^+\) flux through the H\(^+\) permeable transporters at the tonoplast, comprising the VH\(^+\)-ATPase, VH\(^+\)-PPase, the CLC H\(^+\)-Cl\(^-\) antiporter and the CAX H\(^+\)-Ca\(^{2+}\) antiporter. Note that positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.

Fig. 6. Total cytosolic and vacuolar [Ca\(^{2+}\)], cytosolic-free [Ca\(^{2+}\)], and analysis of Ca\(^{2+}\) fluxes across the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown are (A) the total cytosolic and vacuolar [Ca\(^{2+}\)], (B) cytosolic-free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]), (C) the net flux of Ca\(^{2+}\) across the plasma membrane and tonoplast, (D) the Ca\(^{2+}\) flux through the Ca\(^{2+}\)-permeable transporters at the plasma membrane, comprising the hyperpolarisation-activated Ca\(^{2+}\) channel and the Ca\(^{2+}\)-ATPase, and (E) the flux of Ca\(^{2+}\) through the Ca\(^{2+}\)-permeable transporters at the tonoplast, comprising the Ca\(^{2+}\)-ATPase, the CAX H\(^+-\)Ca\(^{2+}\) antiporter, and the TonVCa Ca\(^{2+}\) channel. Note the difference in scales between (D) and (E). Note that
positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.

Fig. 7. Cytosolic-free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)), and analysis of the energetics for Ca\(^{2+}\) flux across the plasma membrane and tonoplast resolved over the diurnal Reference Cycle as described in Fig. 1 (12 h light:12 h dark, indicated by bars above). Shown in (A) and (F) are the current-voltage curves for the dominant Ca\(^{2+}\) transporters at the plasma membrane and tonoplast, respectively. Total membrane current is indicated in each case by the dotted line; the free-running membrane voltage is defined by the point at which this line crosses the voltage axis. Also summarised are (B) the plasma membrane and tonoplast voltages, (C) the [Ca\(^{2+}\)]\(_i\), and the Ca\(^{2+}\) channel and Ca\(^{2+}\)-ATPase fluxes at the (D) plasma membrane and (E) tonoplast. Current-voltage curves are cross-referenced to time points in (B)-(E) by number. Time point 2 is characterised by plasma membrane hyperpolarisation, which favours Ca\(^{2+}\) influx through the Ca\(^{2+}\) channels at the plasma membrane (A); time point 3 is characterised by plasma membrane depolarisation, which favours Ca\(^{2+}\) efflux through the plasma membrane Ca\(^{2+}\)-ATPase. Note the difference in scales between (D) and (E), which underlines the predominance of Ca\(^{2+}\) circulation between the cytosol and the endomembrane store of the vacuole. Note that positive flux is defined as movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.

Fig. 8. Cytosolic-free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)), and analysis of the Ca\(^{2+}\) flux across the plasma membrane and tonoplast during voltage and [Ca\(^{2+}\)] excursions at the end of the daylight period. Shown are (A) the plasma membrane and tonoplast voltages, and the stomatal aperture, (B) the Ca\(^{2+}\) influx through the Ca\(^{2+}\) channels at the plasma membrane and tonoplast, (C) the Ca\(^{2+}\) efflux through the plasma membrane and tonoplast Ca\(^{2+}\) ATPases, and (D) the [Ca\(^{2+}\)]\(_i\). Current-voltage curves for these Ca\(^{2+}\) transporters at the plasma membrane and tonoplast are shown in (E) and (F), respectively. Total membrane current is indicated in each case by the dotted line; the free-running membrane voltage is defined by the point at which this line crosses the voltage axis. Current-voltage curves are cross-referenced to time points in (A)-(D) by number. As in Fig. 7, time point 1 is characterised by plasma membrane hyperpolarisation, which favours Ca\(^{2+}\) influx through the Ca\(^{2+}\) channels at the plasma membrane; time point 3 is characterised by plasma membrane depolarisation, which favours Ca\(^{2+}\) efflux through the plasma membrane Ca\(^{2+}\)-ATPase. Note the Ca\(^{2+}\) influx at the plasma membrane is replaced by Ca\(^{2+}\) influx across the tonoplast shortly before plasma membrane depolarisation (B); thereafter, the characteristics of the [Ca\(^{2+}\)]\(_i\) rise are determined by Ca\(^{2+}\) flux from the tonoplast and, as the tonoplast Ca\(^{2+}\) channels inactivate, by [Ca\(^{2+}\)], recovery mediated Ca\(^{2+}\)-ATPases at the two membranes (C). Note that positive flux is defined as
movement of the ionic species (not the charge) out of the cytosol, either across the plasma membrane or the tonoplast.
Fig. 1
Fig. 2
Fig. 6
Fig. 8