

Opinion

Utilizing systems biology to unravel stomatal function and the hierarchies underpinning its control

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ABSTRACT

Stomata control the concomitant exchange of CO₂ and transpiration in land plants. While a constant supply of CO₂ is need to maintain the rate of photosynthesis, the accompanying water losses must be tightly regulated to prevent dehydration and undesired metabolic changes. The factors affecting stomatal movement are directly coupled with the cellular networks of guard cells. Although the guard cell has been used as a model for characterization of signaling pathways, several important questions about its functioning remain elusive. Current modeling approaches describe the stomatal conductance in terms of relatively few easy-to-measure variables being unsuitable for *in silico* design of genetic manipulation strategies. Here, we argue that a system biology approach, combining modeling and high-throughput experiments, may be used to elucidate the mechanisms underlying stomata control and to determine targets for modulation of stomatal responses to environment. In support of our opinion, we review studies demonstrating how high-throughput approaches have provided a systems-view of guard cells. Finally, we emphasize the opportunities and challenges of genome-scale modeling and large-scale data integration for *in silico* manipulation of guard cell functions to improve crop yields, particularly under stress conditions which are of pertinence both to climate change and water use efficiency.

INTRODUCTION

Stomata are microscopic pores formed by pairs of specialized epidermal guard cells, which control the essential exchange of CO₂ with the environment concomitantly with transpiration in land plants. Although plants are in constant need of sufficient quantities of CO₂ to maintain their photosynthetic rates, the water losses associated with the CO₂ entry must also be strictly regulated to prevent dehydration and the subsequent unfavourable changes of metabolic state that this invokes. Furthermore, the influx of CO₂ and the absorption of minerals depend highly on the availability of water, which is usually a major limiting factor for the development and growth of terrestrial plants. For these reasons, most terrestrial

plants are coated with a water-impermeable cuticle layer that prevents evaporation and water loss (Bessire *et al.* 2007; Schreiber 2010; Buschhaus & Jetter 2011). Accordingly, the majority of water lost to transpiration, and CO₂ absorption for that matter, occurs through stomatal pores rendering the stomata as the main control point, which regulates the flow of gases between plants and atmosphere (Schroeder *et al.* 2001).

Changes in guard cell metabolism can regulate the magnitude of the pore size in response to a range of environmental and endogenous plant stimulus (Kim *et al.* 2010). This regulation allows plants to occupy habitats with fluctuating environmental conditions. Therefore, it has been postulated that stomata must be important contributors to speciation and evolutionary adaptation (Hetherington & Woodward 2003). Moreover, stomatal behaviour and stomatal density are of fundamental importance not only for individual plant performance, but also for crop productivity and the terrestrial CO₂ and water cycles (Buckley & Mott 2013; Schroeder *et al.* 2013).

Stomatal pores open in response to an increase in guard cell volume driven by influx of water, which in turn is the result of a decrease in the water potential of the guard cell. Conversely, during stomatal closure, an efflux of osmolytes from guard cells is necessary with an associated increase in guard cell water potential, leading to efflux of water (Schroeder *et al.* 2001; Kim *et al.* 2010; Araújo *et al.* 2011a,c). Therefore, water potential is one of the key determinants of stomatal movements that is opening and closing.

In addition to the control of water loss, stomatal movements directly affect CO₂ assimilation in response to environmental cues by integrating a variety of stimuli, such as: light, vapour pressure deficit (VPD), abscisic acid (ABA) and CO₂ concentration (Blatt 2000; Hetherington & Woodward 2003; Shimazaki *et al.* 2007; Kim *et al.* 2010; Hubbard *et al.* 2012). Although guard cells have often been used as a model system in which to discover ABA receptors (Liu *et al.* 2007; Pandey *et al.* 2009; Park *et al.* 2009) and for the *in vitro* characterization of the ABA signalling pathway (Fujii *et al.* 2009), several important questions about guard cell functioning, particularly under suboptimal conditions, remain elusive.

For over a century, guard cells have been extensively studied, and considerable efforts have been made to better

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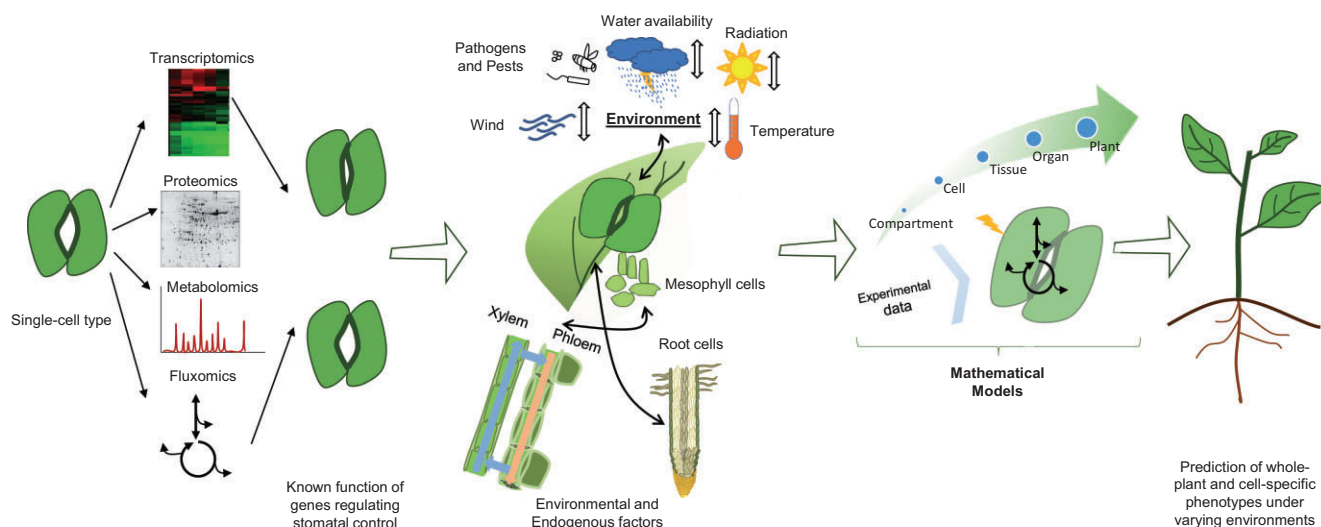


Figure 1. Schematic representation showing how systems analyses and modelling can help the knowledge of molecular hierarchy undergoing stomatal movements and in predictions about phenotypes. The system biology approaches (transcriptomics, proteomics, metabolomics, fluxomics and others) may aid the understanding of unknown questions still unclear about stomatal movements coupling with experimental data from interactions between guard cell – environmental and guard cell – endogenous signals from different cell types that, together with the feedback loops those interactions determine the integrated and coordinated plant behaviour. These data can be grouped in mathematical models to stomatal response and associated with reverse engineering will allow to provide biotechnological targets for generation of improved crop yields and to predict the phenotype under varying environments.

understand their structure, development and physiology (Bessey 1898; Bergmann & Sack 2007; Mott 2009; Berry *et al.* 2010; Casson & Hetherington 2010; Kim *et al.* 2010; Dow & Bergmann 2014). In addition, the relative ease of the isolation of the guard cells, which gate the stomatal pore, has facilitated experimental interrogation contributing to the increase in the understanding of these cells relative to other plant cell types (Hetherington & Brownlee 2004; Israelsson *et al.* 2006; Kim *et al.* 2010). These advances have rendered the guard cell as one of the best studied plant cell models for membrane transport, signalling and homeostasis (Blatt 2000; Roelfsema & Hedrich 2010; Hills *et al.* 2012).

Thorough understanding of stomatal behaviour and its regulation represents an important step towards obtaining plants with better water-use efficiency, which can be defined as the carbon fixed per unit of water (Lawson & Blatt 2014; Lawson *et al.* 2014; Way *et al.* 2014). Here, our first aim was to focus on high-throughput studies that have recently made considerable progress in elucidating the molecular and genetic bases underlying stomatal function and movement. However, the complexity of interactions involved in regulatory processes ranging from metabolite transport to the cell-autonomous metabolic and buffering activities of guard cells remains the main barrier to understand the regulatory networks governing stomatal movement (Blatt *et al.* 2013). In this context, we suggest that a systems biology approach offers itself as a comprehensive way to aid in elucidation of the mechanisms governing stomatal function and the molecular hierarchies underpinning the stomata control. Furthermore and given that systems biology approaches necessitate data-driven modelling to integrate datasets capturing various read-outs of the cell as well as to generate and

validate (or refute) hypotheses, they can also ultimately be used to predict biotechnological targets for the manipulation of stomatal behaviour. To this end, our second aim was to outline the steps that are prerequisites for coupling reverse engineering of stomatal responses with *in silico* design and modelling in order to direct genetic manipulation for improved crop yield and water-use efficiency (Fig. 1). We additionally suggest an alternative approach to those commonly used to illuminate stomatal behaviour and in doing so hope to bridge the gap between ecophysiological and molecular views of this important cell-to-environment interaction.

Why apply the systems biology approach to study stomatal function?

Over the last 20 years we have witnessed an increasing interest in the understanding of the molecular mechanisms by which guard cells sense and respond to stimuli such as ABA, plant water status, light, CO₂, temperature and humidity. As a consequence, guard cells have become an important model for studies of membrane transport and cell signalling (Assmann 2010). Advances in our understanding over this time period are mostly due to the identification and characterization of mutants deficient in stomatal closing and opening coupled with the currently challenging, but all the same feasible, isolation and screening of large populations of guard cells, employed in various high-throughput technologies (Table 1).

Several seminal studies and numerous reviews (Tallman 2004; Shimazaki *et al.* 2007; Kim *et al.* 2010; Meyer *et al.* 2010; Araújo *et al.* 2011a; Song *et al.* 2014), point out that stomatal

Table 1. Description of *Arabidopsis* mutants plants which have stomatal dysfunction and were identified and/or characterized by omics approaches

Gene(s) AGI code	Mutant name	Functional description	Phenotype	Characterization	Technology*	References
AT1G72770	<i>ap2C-HA-1</i>	Encodes the protein phosphatase 2C; regulates the activation of the Snf1-related kinase OST1 by ABA	ABA-hypersensitive regulation of stomatal closing ^a	Transcriptome	Microarray	Leonhardt <i>et al.</i> 2004
AT4G34460.1	<i>agb1</i>	Encodes the heterotrimeric G-protein β subunit	ABA-hypersensitive	Transcriptome	Microarray	Pandey <i>et al.</i> 2010
AT2G26300.1	<i>gpa1</i>	Encodes the heterotrimeric G-protein α subunit	hypersensitive to ABA inhibition of stomatal opening	Transcriptome	Microarray	Pandey <i>et al.</i> 2010
AT4G34460.1; AT2G26300.1	<i>agb1 gpa1 (double mutant)</i>	Encodes the heterotrimeric G-protein α and β subunits	ABA-hypersensitive	Transcriptome	Microarray	Pandey <i>et al.</i> 2010
AT4G17770.1	At4g17770	Encodes an enzyme putatively involved in trehalose biosynthesis.	Stomatal conductance decreased without change stomatal density	Transcriptome	Microarray	Bates <i>et al.</i> 2012
AT1G65370.1	At1g65370	Encodes a TRAF-like family protein	Stomatal conductance decreased without change stomatal density	Transcriptome	Microarray	Bates <i>et al.</i> 2012
AT1G15440	At1g15440	Encodes a nucleolar protein that is a ribosome biogenesis co-factor.	Stomatal conductance decreased without change stomatal density	Transcriptome	Microarray	Bates <i>et al.</i> 2012
AT1G16540.1	<i>aba3-1</i>	Encodes molybdenum cofactor sulfurase. Involved in the conversion of ABA-aldehyde to ABA	Guard cell-autonomous ABA synthesis is sufficient for stomatal closure in response to low relative humidity	Transcriptome	Microarray	Bauer <i>et al.</i> 2013
AT5G65590.1	<i>scap1</i>	Encodes a plant-specific DoF-type transcription factor (TF). It regulates stomatal guard cell maturation and functions as a key TF regulating the final stages of guard cell differentiation	Impaired CO ₂ -induced stomatal closing and light-induced stomatal opening	Transcriptome	Microarray	Negi <i>et al.</i> 2013
AT5G26000.1	<i>tgg1</i>	Member of glycoside hydrolase family 1. Encodes one of two known functional myrosinase enzymes in <i>Arabidopsis</i> . The enzyme catalyses the hydrolysis of glucosinolates into compounds that are toxic to various microbes and herbivores.	Hypersensitive to ABA inhibition of guard cell inward K ⁺ channels and stomatal opening	Proteome	Identification from broad and narrow pH range 2D gels, and 2D liquid chromatography matrix-assisted laser desorption/ionization multidimensional protein identification	Zhao <i>et al.</i> 2008
AT2G26300.1	<i>gpa1-4</i>	Encodes the heterotrimeric G-protein α subunit	Hypersensitive to ABA inhibition of light-induced stomatal opening	Proteome	iTRAQ	Zhu <i>et al.</i> 2010
AT4G33950.1; AT3G50500.2; AT5G66880.1	<i>srk2dei (triple mutant)</i>	The three genes encode ABA-activated protein kinases, members of SNF1-related protein kinases (SnRK2)	ABA-insensitive showing severe wilting phenotype because of fully opened stomata	Proteome	Phosphoproteomic analyses; Nano-LC-MS/MS	Umezawa <i>et al.</i> 2013
AT2G26300.1	<i>gpa1</i>	Encodes the heterotrimeric G-protein α subunit	Hypersensitive to ABA inhibition of stomatal opening	Metabolome	LC-MS/MS with (MRM)	Jin <i>et al.</i> 2013

*Abbreviations for the technologies used.

iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring.

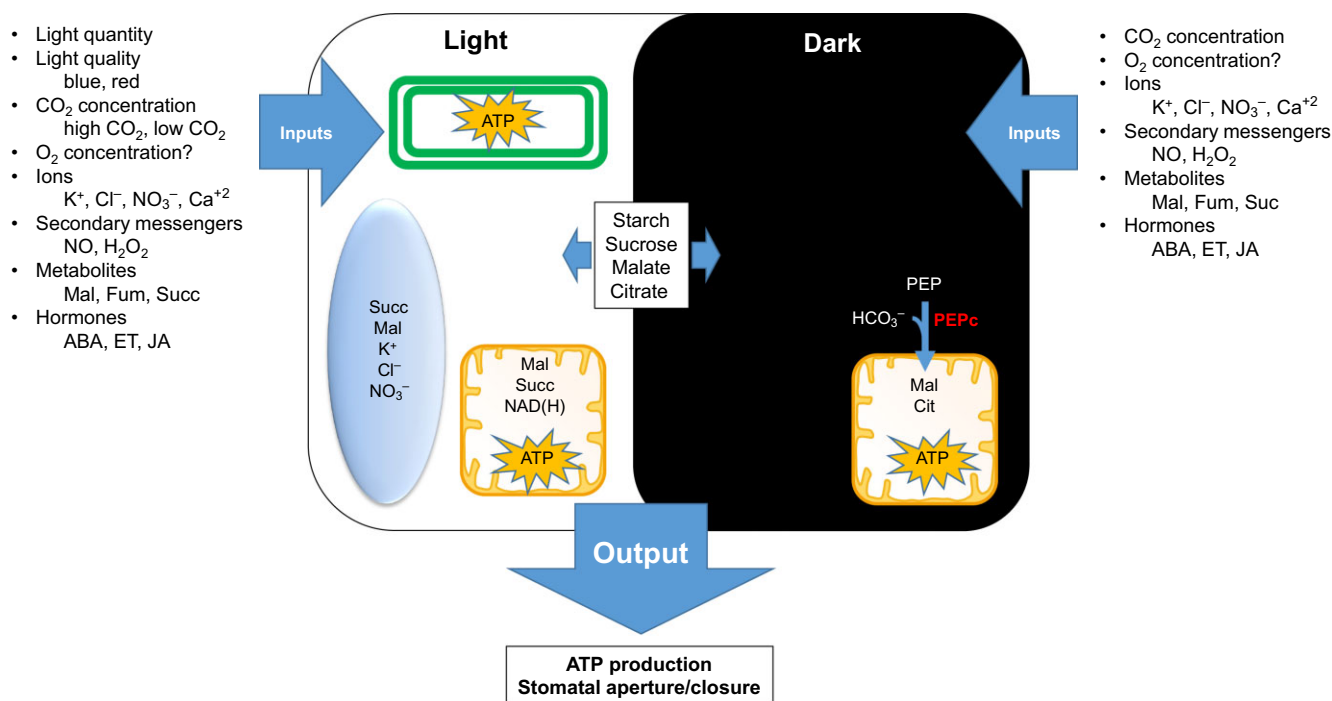


Figure 2. The main regulators of guard cell movements. Schematic representation of guard cell metabolism of C3 plants in both light and dark phases. For guard cell modelling it is important to consider the particularities of guard cell metabolism, which are represented in the figure by the number of possible inputs (environment signals, ions, secondary messengers, metabolites, hormones) and the high compartmentalization of the metabolism, which involves transport and accumulation of ions and metabolites in the apoplast and organelles (plastids, mitochondria and vacuole). It is also important to consider that guard cells have a metabolism similar to C4 plants and not C3 plants, represented in the figure through the activity of the enzyme phosphoenolpyruvate carboxylase (PEPc) in the dark phase. Beyond ATP production, which is commonly used as output, guard cells have a specific output that has substantial physiological importance – stomatal aperture or closure. Abbreviations: ABA, abscisic acid; Ca⁺², calcium; Cl⁻, chloride; Cit, Citrate; CO₂, Carbon dioxide; ET, ethylen, Fum, fumarate; HCO₃⁻, Bicarbonate; H₂O₂, hydrogen peroxide; JA, jasmonic acid; K⁺, potassium; Mal, malate; NO₃⁻, nitrate; NO, nitric oxide; O₂, Oxygen; Succ, Succinate.

movement depends on many exogenous variables (Fig. 2) that affect the internal networks on multiple levels of cellular organization ranging from aspects of transcriptional regulatory networks, signalling and metabolism. For this reason, system perturbation by the application of a series of independent environmental cues (or their combinations), would make it increasingly difficult to distinguish between their primary and secondary effects. This complexity is further exacerbated in instances wherein these stress perturbations are not simply applied to wild-type tissues, but also to genetic variants such as mutants or transgenic lines. Moreover, the differential coverage of cellular components by high-throughput technologies currently renders data interpretation rather difficult (Ferne & Stitt 2012). It should be mentioned that these incoherencies are not only due to technical issues, but also related to the complexity and structure of metabolic networks (Araújo *et al.* 2012).

To this end, data-driven integrative modelling will likely prove highly useful for both *in silico* investigation of the effects of environmental changes and reverse engineering of the networks underlying guard cell functions. Moreover, using the outcome of the system-level simulations to plan validation experiments will be highly instructive and will likely result in several iterative cycles of experimentation and

modelling. Such an approach may alleviate some of the problems encountered by guard cell experimentalists most acutely perhaps is reducing the number of experiments needed, which given that difficulties in obtaining the required number of guard cells often represents the bottleneck in the experimental studies of guard cells.

Finally, the ultimate goal of the systems biology approach is to understand the system with the aim of manipulating its function towards a desired outcome. Therefore, systems approach, carefully integrating high-throughput data with (possibly) large-scale models of metabolism and regulation/signalling can further contribute to identifying novel determinants on a molecular level as well as to plan intervention strategies (discussed in detail later in the section ‘Opportunities and challenges in systems modelling of stomatal function’).

Application of systems biology approaches to stomata

The differential analysis of multiple cell types and organs is one approach to dissect regulatory signalling networks and metabolic pathways underlying complex molecular functions, and has been deemed crucial for the development of systems

biology (Ideker & Krogan 2012). Such comparative analyses require the use of high-throughput phenotyping technologies that permit the generation of large volumes of data. These data can, in turn, be used to either generate or test hypotheses with respect to the genes and their products (i.e. proteins) as well as to downstream metabolites, which exhibit differential behaviour across tissues or cells under different environmental conditions. A major bottleneck and challenge in plant cell-specific studies, however, is in obtaining different cell types in high enough quantity and quality for downstream analysis (Misra *et al.* 2014). Nevertheless, several elegant studies involving the parallel usage of different high-throughput technologies have employed guard cells. Most of these studies, mainly performed in *Arabidopsis*, focused on the identification of molecular components of the ABA signal cascade. We highlight these studies and the knowledge advances, which they provided in the first part of this paper and subsequently offer a perspective on the modelling of guard cell function. Finally, we will present what in our opinion are the major outstanding questions in understanding guard cell function.

Transcriptomics studies

As a first step towards a deeper understanding of guard cell functioning, we provide a summary of publicly available transcriptomic data from guard cell studies. It is important to mention that most of studies available to date have been used to analyse the response to ABA and the ABA signalling pathway. Accordingly, most of the data available was generated in *Arabidopsis* mutant plants (Table 1). Given that the response of plants to environmental cues, particularly stresses, often involves the coordinated induction or repression of gene expression, transcriptomics studies provide investigation of one critical facet of the molecular response of guard cells.

Oligomer-based DNA microarray was used in earlier studies to profile gene expression in *Arabidopsis* guard cells, facilitating the identification of ~1300 genes, which were expressed in guard cells (Leonhardt *et al.* 2004). On comparison of guard cell expression profiles with those from mesophyll cells, 64 transcripts were identified to be preferentially expressed in guard cells. Importantly, the reliability of this approach was further confirmed by quantitative RT-PCR. Analysis of the expression profile of guard cells had additionally revealed the ABA-responsive genes that govern the behaviour of guard cell ABA-signalling components (Leonhardt *et al.* 2004).

In a more recent study, guard cell transcriptome data were used to identify novel modes of (heterotrimeric) G-proteins action (Pandey *et al.* 2010). G-proteins comprise three subunits, alpha ($G\alpha$), beta ($G\beta$) and gamma ($G\gamma$), and are important transmembrane components of signal transduction (Temple & Jones 2007; Kumar *et al.* 2014). Given that G-proteins can mediate a wide range of essential signalling pathways in eukaryotes this protein family might be involved in aspects of guard cell signalling (see discussion on 'Proteomics studies' later). By analysing microarray

data from guard cells and excised leaves of G-protein subunit mutants of *Arabidopsis*, with and without ABA treatment, the classical mechanisms of G-protein signalling was confirmed and a novel route of G-protein signalling, in which $G\beta$ subunit regulates gene expression independently of $G\alpha$ subunit, was discovered. Notably, it was also observed that G-protein mode of transcriptional regulation differs between guard cells and leaves, supporting a system-specificity of G-protein signalling. These observations clearly improve resolution of the molecular network of ABA-responsive genes and could plausibly be used as new inputs in modelling approaches that predict stomatal movements in addition to those involving solute transport across membranes.

Another recent study involving ABA regulation of gene expression in guard cells have identified a unique *cis*-acting motif, GTCGG, associated with ABA-induction of gene expression specifically in guard cells (Wang *et al.* 2011). In addition, approximately 300 genes displaying ABA-specific regulation were uncovered in guard cells, including genes known to encode ion transporters associated with stomatal opening (e.g. the H^+ -ATPase *OPEN STOMATA 2 – OST2*, the *POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 and 2 – KAT1* and *KAT2*, the *SUGAR TRANSPORTER 1 – STP1*, the endosomal Na^+/H^+ antiporter *CATION/H + EXCHANGER 20 – CHX20*, and the *NITRATE TRANSPORTER 1 – NRT1*). These genes were down-regulated upon ABA treatment, suggesting that this regulation may be an important aspect of mid- to long-term inhibition of stomatal opening by ABA as well as ensuring the maintenance of stomatal closure during stress situations such as drought. Moreover, these results provided evidence for cross-talk at the transcriptional level between ABA and another hormonal inhibitor of stomatal opening, namely, jasmonate (Wang *et al.* 2011).

Microarray analysis was additionally performed on guard cells from leaves of *Arabidopsis* treated with sucrose to test the hypothesis that photosynthesis-derived sugar acts as a signal in guard cells, which allows adjustment of their stomatal opening (Bates *et al.* 2012). This study revealed that the expression of 440 genes changed in response to sucrose in guard cells including genes involved in several important cellular functions such as photosynthesis and transport of sugars, water, amino acids and ions. Remarkably, investigation of T-DNA insertion lines for 50 genes highly responsive to sucrose revealed that 12 genes, which were not previously known to function in guard cells, are important in leaf conductance control, water-use efficiency and/or stomata development. Of these, three are of particular interest given that they showed similar effects in nearly all test of stomatal function without a change in stomatal density: a trehalose phosphate synthase *TPS5* (At4g17770), a TRAF domain-containing protein (At1g65370), and a WD repeat-containing protein (At1g15440) (Bates *et al.* 2012). Collectively, these results demonstrate the power of large-scale gene expression studies as an effective tool in revealing candidate genes with potential for practical application in field-related experiments.

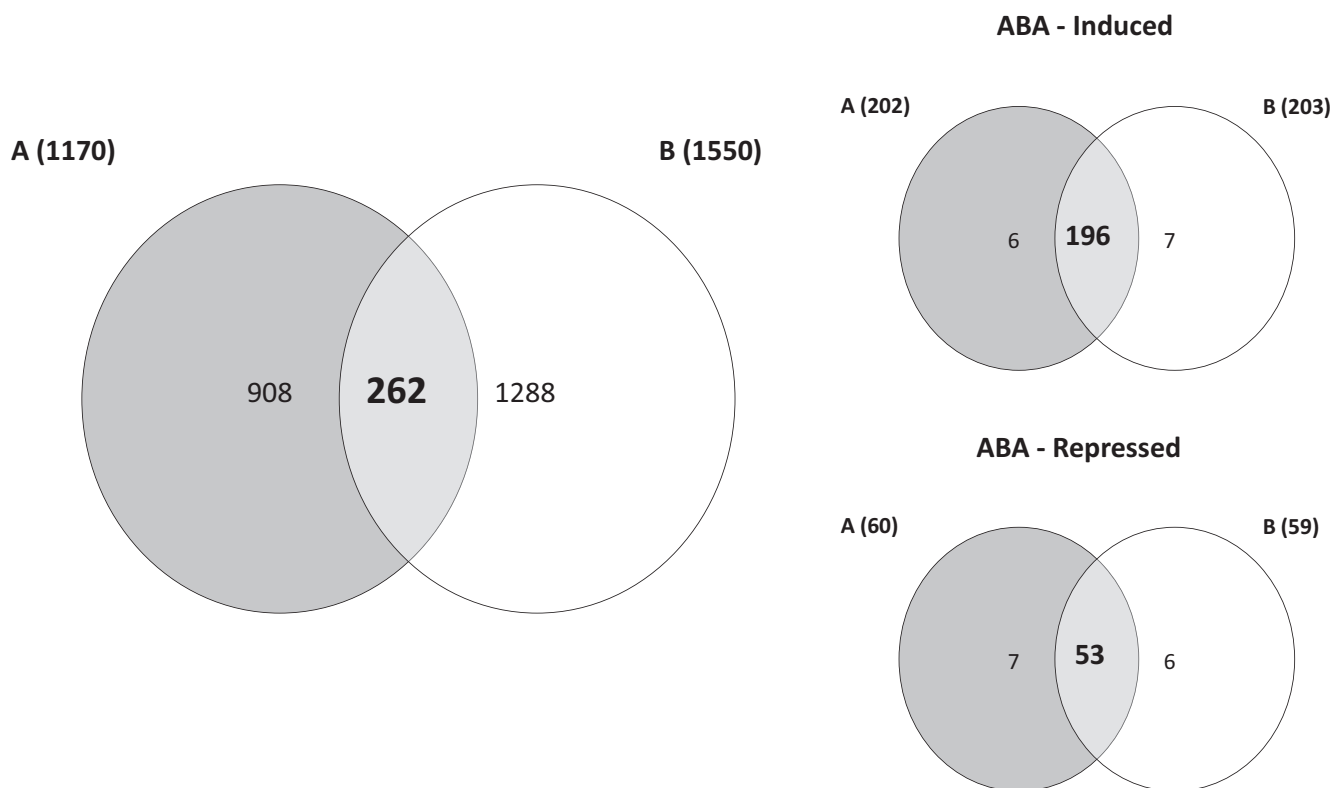


Figure 3. Venn diagram showing the transcriptome overlap of studies on ABA response in guard cells. (a) and (b) correspond to Wang *et al.* (2011) and Bauer *et al.* (2013), respectively. Notably, we found 262 genes that were responsive to ABA treatment in both studies. Additionally, we overlapped the genes that were ABA-induced (196) or repressed (53) from the list of the 262 genes previously found. The data used are available in the Supporting Information Table S1. The Venn diagram was constructed in the Pangloss Venn diagram generator (<http://www.pangloss.com/seidel/Protocols/venn4.cgi>).

In a similar approach, the expression profiles of *Arabidopsis* plants under dry air conditions, characterized by long-term stomatal closure, revealed 588 differentially regulated genes (Bauer *et al.* 2013). Given that transport is an essential activity of guard cells, it is not surprising that genes associated with this function were found to be highly expressed in guard cells (e.g. *KAT1*, *KAT2*, *GATED OUTWARDLY-RECTIFYING K⁺ CHANNEL – GORK*, *SLOW ANION CHANNEL-ASSOCIATED 1 – SLAC1*, *SLAC1 HOMOLOGUE 3 – SLAH3*, and *QUICK-ACTIVATING ANION CHANNEL 1 – QUAC1*). It is worth mentioning that when treated with exogenous ABA, 1550 genes were sensitive to ABA of which 1080 were up-regulated and 470 were down-regulated (Bauer *et al.* 2013).

Most recently, Negi and collaborators isolated an *Arabidopsis* mutant, stomatal carpenter 1 (*scap1*) that develops irregularly shaped guard cells lacking normal stomatal function, and showing defects in CO₂-induced stomatal closing and light-induced stomatal opening from an M2 population of ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis* plants (Negi *et al.* 2013). *SCAP1* was identified to encode a plant-specific DNA binding with one finger (Dof) transcription factor, and its role in guard cell gene expression was investigated by microarray experiments.

These experiments identified 1540 genes that are expressed in guard cells, but not in mesophyll cells. The *scap1* mutation resulted in decreased expression of genes for several factors directly involved in stomatal opening and closure, namely, *GORK* (Hosy *et al.* 2003), *PYL2*, a regulatory component of ABA receptor 2 (Park *et al.* 2009) and *MYB60*, an essential transcriptional regulator for guard cell movements (Cominelli *et al.* 2005). Thus, *SCAP1* regulates not only stomatal guard cell maturation, but also stomatal functions through its direct involvement in the final stages of guard cell differentiation.

By focusing on well-known ABA-responsive genes, we here utilize a Venn diagram to visualize the overlap of the findings from these most recent guard cell studies employing transcriptome analyses (Wang *et al.* 2011; Bauer *et al.* 2013). Notably, we found 262 genes that are responsive to ABA treatment in both experiments (Fig. 3 and Supporting Information Table S1). These genes could thus be strong candidates for being involved in the functional response to changes in ABA levels as well as representing potential targets for future experimental work to further dissect the ABA-response network.

Notably, transcriptomes as assessed by microarrays have produced an unprecedented amount of data regarding transcript identity and levels in plant systems and also in guard

cells network and cascades that have been elegantly elucidated. Additionally, although next-generation DNA sequencing technologies are driving increasingly rapid, affordable and high resolution analyses of plant transcriptomes through sequencing of their associated cDNA (complementary DNA) populations; an analytical platform commonly referred to as RNA-sequencing (RNA-seq) (Martin *et al.* 2013), not so much has been performed in guard cells thus far. Remarkably RNA-sequencing is currently well-established as a versatile platform with applications in an ever-growing number of fields of plant biology research, including naturally guard cell studies. Collectively, there is plenty of reason to believe that guard cell microarrays and transcriptome sequences can provide a valuable resource for plant breeding and research helping in the identification of novel insights into common plant responses to several environmental stress and offer candidate genes or markers that can be used to guide future efforts attempting to breed tolerant plants.

Proteomics studies

Proteomics analyses complement transcriptomics-based studies and can provide better understanding of the interplay between cellular levels since proteins are generally functionally involved either in regulatory, signalling or metabolic processes. At the proteomic level, comparative analysis of purified guard cells and mesophyll cells from *Brassica napus* has first been performed using the isobaric tags for relative and absolute quantification (iTRAQ) technology alongside two-dimensional (2D) liquid chromatography mass spectrometry (Zhu *et al.* 2009). This strategy identified around 1450 non-redundant proteins in both guard cells and mesophyll cells. Interestingly, 427 of these proteins were quantified, and 74 of them were found to be enriched in guard cells. As would perhaps be anticipated, proteins involved in energy metabolism, transport, transcription, cell structure, and signalling were preferentially expressed in guard cells, providing further support to the idea that guard cells can, at least partially, function autonomously (Schroeder *et al.* 2001; Hashimoto *et al.* 2006; Young *et al.* 2006; Sirichandra *et al.* 2009).

In another study related to ABA signalling and stomatal movement, more than 1700 unique proteins from *Arabidopsis* guard cell protoplasts were identified using three complementary proteomic methods (Zhao *et al.* 2008). This extensive single-cell proteomics approach identified not only proteins whose encoding genes were not previously identified in transcriptome analyses of guard cells, but also signalling proteins, of which two were suggested to be related to guard cell function. For instance, THIOGLUCOSIDE GLUCOHYDROLASE1 (TGG1), a myrosinase, catalyses the production of toxic isothiocyanates from glucosinolates. This protein was more abundant in the guard cell proteome, and the *tgg1* mutants were hyposensitive to ABA inhibition of guard cell inward K⁺ channels and stomatal opening. As such, this study revealed that the glucosinolate-myrosinase system, previously identified as a defense against biotic

invaders, is also required for key ABA responses of guard cells of *Arabidopsis* (Zhao *et al.* 2008). That said it is important to note that glucosinolates are mainly present in the family Brassicaceae (Fahey *et al.* 2001), and as such this is very much a specialized rather than a general mechanism of guard cell regulation.

A further component of the *Arabidopsis* ABA-responsive network underpinning stomatal movement was also identified by using iTRAQ technology. In this study guard cell proteins whose abundance was affected by ABA supply and/or mutation in *G PROTEIN ALPHA SUBUNIT 1* (*GPA1*) in *Arabidopsis thaliana* were investigated. Accordingly, this elegant study has demonstrated that the G-protein α subunit, GPA1, is implicated in ABA-regulated guard cell movement, confirming thus a previous transcriptome analysis (Pandey *et al.* 2010). It is worthy to mention that the abundance of two proteins in Col-0 and six proteins in *gpa1-4*, a null mutant lacking the $G\alpha$ subunit, was affected by ABA treatment in guard cells, whereas 18 guard cell proteins were quantitatively affected by mutation in *GPA1*. Remarkably, these are proteins clearly involved in photosynthesis as they participate in the Calvin–Benson cycle, photosynthetic light reactions, ATP synthases and chloroplastic electron transfer. It is also worth mentioning that a further five proteins whose abundance increased in *gpa1-4* guard cells are members of the so-called ‘response to stress’ family including proteins involved in oxidative stress (Zhao *et al.* 2010). Altogether, the results of these studies indicated that GPA1 not only inhibits guard cell photosynthesis, but also promotes the availability of reactive oxygen species (ROS) in guard cells.

Analysis of ABA responsive proteins in *B. napus* guard cells has also been performed, with a total of 431 unique proteins being unequivocally identified, of which 66 increased and 38 decreased (Zhu *et al.* 2010). The proteins that were more highly abundant following ABA treatment included proteins involved in stress and defence while proteins with functions in spermidine synthesis, rhamnose biosynthesis, jasmonic acid biosynthesis, fatty acid beta-oxidation, purine metabolism, alkaloid biosynthesis and protein synthesis decreased. Surprisingly, some of the metabolic pathways that were suppressed are additionally important in plant stress and defence (Zhu *et al.* 2010). Furthermore, given that many of these proteins have not previously been reported as either being ABA responsive or being involved in stomatal movement, it suggests that further novel players involved in guard cell ABA signalling may yet remain to be identified.

At a more mechanistic level, a recent phosphoproteomic approach identified proteins that were phosphorylated following ABA treatment or under dehydration stress in *Arabidopsis* wild-type plants, but not in mutants lacking all three kinases of the SUCROSE NON FERMENTING 1 (SNF1)-RELATED PROTEIN KINASE 2s (SnRK2s) family (Umezawa *et al.* 2013), which are central components of ABA signalling pathways. The number of differentially phosphorylated peptides was greater in *srk2dei* plants treated with ABA than in the ones subjected to dehydration, suggesting that SnRK2 was mainly involved in ABA signal-

ling rather than dehydration. SnRK2 also promotes the ABA-induced activation of the Mitogen-activated protein kinases (MAPKs) AtMPK1 and AtMPK2, AREB1 (ABA-responsive element binding protein 1), and stimulate ABA-responsive gene expression. Furthermore, SnRK2-SUBSTRATE 1 (SNS1), a previously unknown protein, was phosphorylated *in vivo* following isolation and functional characterization of knockout mutants of SNS1 it was revealed that SNS1 is a negative regulator of ABA signalling at the post-germination stage in *Arabidopsis*.

As discussed earlier, proteomic approaches have recently expanded our view of the protein landscape required for and during stomatal movements and thus contributed significantly in advancing our mechanistic understanding of ABA signalling controlling guard cell movements. However, there are still large gaps in the knowledge concerning the changes of the proteome during adaptation to environmental stress conditions that lead to changes in stomatal aperture. With the application of proteomic methods, particularly 2D-gel and gel-free, LC/MS-based methods, insights into the composition and dynamic changes of the guard cell proteome could be obtained. It is reasonable to assume that a thorough understanding of guard cell response to abiotic stress at the molecular level is a prerequisite for its effective management. Notwithstanding, the molecular mechanism involved in guard cell movements is seemingly complex and requires information at the post-genomic level to understand it effectively. In this regard, the ever-growing field of proteomics is also being effectively employed for investigating guard cell responses in land plants. The development of proteomics techniques allowing increased proteome coverage and quantitative measurements of proteins will be particularly instrumental to characterize proteomes and their modulation during guard cell movements. Despite important advances, plant proteome analysis, including those of model plant species, remain constrained by limitations inherent to proteomics techniques and data interpretation. Here we have, accordingly, highlighted the prominent approaches and achievements of proteomics with model plant *Arabidopsis* and have not discussed the current limitations of plant proteomics, which has been expertly reviewed elsewhere (Lilley & Dupree 2007; Abril *et al.* 2011; Nakagami *et al.* 2012; Vanderschuren *et al.* 2013). We anticipate future directions that could advance the contribution of plant proteomics to crop improvement and additionally indicates that proteomic approaches could generate a huge amount of data, and therefore adequate advancements in computational and mathematical tools are a must to be achieved for effective analysis. It is important to note, however, that the integration of genomic-scale information to address complex genetics and physiological questions still remains challenging.

Metabolomics studies

Last, but by no means not least, it is plausible that metabolomics of guard cells will ultimately become a highly powerful approach in dissecting the functioning of guard cells. Although no single analytical system is ever likely to

cover the whole metabolome technological developments have considerably extended our ability to analyse complex biological systems, facilitating the simultaneous detection of different compound classes with diverse chemical properties (Osorio *et al.* 2012). Notably, the development of new technologies might ameliorate the problem of limited metabolite coverage. It is important to mention, however, that given the level of physical and chemical complexity of properties of metabolites coupled with other factors, such as the dynamic measurement range that is required, make the development of a single analytical platform that will provide a comprehensive analysis of the cellular metabolome a formidable challenge (Fernie *et al.* 2004; Misra *et al.* 2014), rendering metabolomics more complicated than other post-genomic analysis. To date, metabolomic analyses in guard cells are still incipient compared with transcriptomic and proteomic. Although the focus of metabolomics has largely been to elucidate the flow of mass in the substrate–product conversions, metabolomics may also be used as a tool for the elucidation of the information flow in cellular signalling networks, as we illustrate later.

Using liquid chromatography–multiple reaction monitoring mass spectrometry for targeted analysis of 85 signalling-related metabolites in *Arabidopsis* guard cell protoplasts over a time course of ABA treatment, it was possible to dissect signalling aspects of the guard cell in response to ABA (Jin *et al.* 2013). For this purpose, *Arabidopsis* wild-type plants (Col-0) and *gpa1* mutants, which have ABA-hyposensitive stomata (Zhao *et al.* 2010), were evaluated. The metabolomes of these plants revealed coordinated regulation of signalling metabolites in unrelated biochemical pathways. Interestingly, hormone metabolites were responsive to ABA, with generally greater responsiveness in Col-0 than in *gpa1*. Thus, for instance, indole-3-acetic acid (IAA) levels were decreased by ABA in wild-type guard cells, but not in *gpa1* guard cells. Additionally, the majority of hormones also displayed different ABA responses in guard cell metabolite profiles when compared with the one observed in mesophyll cell metabolomes, suggesting that ABA is most likely an upstream actor to regulate other hormones (Jin *et al.* 2013).

Indeed, it seems clear that hormones are good targets to investigate guard cell metabolomics and although great attention has been paid to ABA, there is a crosstalk between ABA and other hormones that can also affect stomatal movements. For instance, brassinosteroid and jasmonate can act concurrently with ABA promoting senescence and programmed cell death (Zhang *et al.* 2009; Hossain *et al.* 2011). On the other hand, other hormones such as cytokinin, auxin, salicylic acid and ethylene can act antagonistically to ABA in response to water deficit (Tanaka *et al.* 2005; Pinheiro & Chaves 2011; Ha *et al.* 2012; Chen *et al.* 2013; Meguro & Sato 2014).

The increased photosynthesis and whole-plant biomass observed in tomato plants where antisense inhibition of the iron–sulphur subunit of the succinate dehydrogenase (SDH) was performed seems to be related to an organic acid-mediated effect on stomatal aperture (Araújo *et al.* 2011c). These results are diametrically opposite to those obtained for

antisense inhibition of fumarase, where decreased photosynthesis and biomass were observed (Nunes-Nesi *et al.* 2007). Furthermore, measurement of apoplastic organic acid levels in SDH and fumarase antisense plants, revealed a negative correlation between the levels of fumarate and stomatal conductance, although the influence of fumarate appears to be weaker than that of malate (Araújo *et al.* 2011c). It is highly interesting that similar results were obtained by inhibiting two different complex II subunits, the iron–sulphur subunit in tomato (Araújo *et al.* 2011c) and the flavoprotein in *Arabidopsis* (Fuentes *et al.* 2011). Taken together, the results of these work provided compelling evidence to support that modulation of malate and fumarate concentration can strongly impact stomatal movements. Perhaps more importantly, these results also provide strong support of the theory that guard cells are not autonomously regulated (Mott 2009) and that the mesophyll harbours significant control over guard cell movements (Lee *et al.* 2008b; Fernie & Martinoia 2009; Mott 2009; Araújo *et al.* 2011b). Collectively, these results also indicate that mesophyll-derived signals (e.g. malate and fumarate), which play an important role in regulating stomatal movements (Hedrich *et al.* 1994; Araújo *et al.* 2011c), may be candidate signalling molecules. Importantly, further identification of mesophyll-derived signal molecules will provide essential information about CO₂-signalling mechanisms including guard cell receptors that sense CO₂ signals from the mesophyll. In summary, metabolite profiling has aided in the identification of novel interactions between the tricarboxylic acid (TCA) cycle and photosynthesis and stomatal function. Nevertheless, the metabolic and molecular regulatory hierarchy underlying this highly specialized cell type is as yet not fully understood and therefore further work is clearly required in order to establish the regulatory mechanisms involved in such responses.

Overall, while post-genomic technologies have already aided our understanding of guard cell metabolism at a systems level through additions to the 'parts list' of the metabolic network, they are poised to become more useful still through the discovery of new interactions and the provision of the quantitative data required for the construction of predictive models of metabolism. It should be mentioned that, alongside other studies, metabolic studies of this highly specialized cell type has demonstrate to be useful in increasing our understanding of the hormonal crosstalk and metabolic regulation of stomatal movement that is important for plant growth, development, yield and response to environmental factors. It is no doubt that the further development and combination of many analytical techniques (Fig. 1) will additionally allow a fuller description of the metabolic network in a guard cell context.

Opportunities and challenges in systems modelling of stomatal function

Classical modelling approaches focus on expressing the stomatal conductance (g_s) as a function of other external and internal factors. The goal of this section is not to offer an

overview of the existing modelling approaches; the reader is instructed to consult previous comprehensive reviews (Damour *et al.* 2010; Buckley & Mott 2013). These reviews have already categorized the existing models into three groups, largely based on the methodology used for their establishment, namely: purely data-based (e.g. developed by the boundary-line method; Jarvis 1976), mechanistic (e.g. based on the consideration of hydraulic properties and biophysical properties of guard cells) (Cowan 1972), and optimization-based (i.e. based on the idea that plants use the limiting resources in the most optimal way towards fulfilling a task) (Cowan & Farquhar 1977).

The above mentioned experimental evidence has clearly indicated that understanding of stomatal function has to directly consider the gradient in spatio-temporal scales, starting from modelling of single guard cell over fast changes in environmental determinants to theoretical investigations of the interactions between guard and mesophyll cells via various feedback loops and their coupling to data read-out to modelling of canopy over developmental and environmental gradients. The particular modelling question would determine the type of modelling strategy that has to be used; for instance, dynamic changes of well-characterized (sub)systems over short time intervals can be readily undertaken through kinetic modelling, whereas analysis of typical long-term or steady-state behaviour as well as integration of data from multiple cellular components is more readily tackled by means of large-scale stoichiometric models which often do not require kinetic parameterization (see following paragraphs for detailed discussion). The vast time scales of cellular processes that occur at different levels of cellular organization, ranging from metabolism (as fast) to protein synthesis and gene regulation (as slower) will also delineate the applicability of quasi-steady-state assumption as well as the lumping of various components as it is usually performed in modelling studies (Famili & Palsson 2003; Jamshidi & Palsson 2008).

The existing model categories and their combinations have clearly contributed to the predictive modelling and refinement of the relationship between g_s and its determinants. They have, however, had limited success in illuminating how the changes in stomatal movement, and through it g_s , are affected by the interplay of the environment, the various cellular organizational levels (e.g. transcripts, proteins, and metabolites), and the coordination between different cell types (e.g. guard and mesophyll cells). Therefore, despite efforts made to link environmental and endogenous factors to stomatal movements in mathematical models, these modelling approaches have not been used to determine well-planned *in silico* genetic manipulation strategies to investigate means by which to alter conductance-dependent traits and do not integrate data from multiple environments with the purpose of more accurate parameterization. Furthermore, these modelling approaches need to be continuously improved and augmented by inspecting not only the fit of the model predictions under various environmental factors but also from genetic manipulation strategies altering conductance-dependent traits. In fact, our current view is that

to develop an integrated understanding of stomatal function, the modelling approaches must couple the several processes across all levels of stomatal movements. Accordingly, it seems reasonable to assume that a single model of stomatal function must encompass all of those surrounding processes, including naturally the interactions between guard cells and surrounding tissues and their coupling with the environment (Lawson & Blatt 2014). Finally, the importance of these models will become prominent due to their ultimate test from investigating and validating genetic manipulation strategies from seemingly unrelated aspects of cellular subsystems, from metabolism down to gene regulation.

These challenges could, in part, be addressed by kinetic stomatal models of smaller scale, for which the kinetic form of the reaction rates (e.g. Michaelis–Menten) together with its numerous parameters, may be more facile to obtain. The sole prominent example of this direction is the kinetic-based modelling approach proposed initially (Hills *et al.* 2012) and more thoroughly validated later (Chen *et al.* 2012; Wang *et al.* 2012; Blatt *et al.* 2013). This kinetic model is implemented within the OnGuard software package (Available at <http://psrg.org.uk/guard-cell-modelling/>), a computational platform incorporating properties of transporters at the plasma membrane and tonoplast, features of osmolyte metabolism, as well as the characteristics such as the cytosolic pH and Ca^{2+} buffering capacity (Lawson & Blatt 2014). We would like to point out that the current implementation includes data-driven parameterization of the model as well as investigation of the effects of parameter changes on the quantitative predictions. Thus, the existing *in silico* studies based on OnGuard have demonstrated the capacity of this framework to provide a multitude of quantitative predictions which can now readily be used for goodness-of-fit testing with data from independent experiments and for improved calibration of the model. While the currently implemented model has aided the quantitative understanding of stomata behaviour as a function of few metabolic reactions, related to proxies for osmolytes, transport and signalling, its role in design of metabolic engineering strategies, because of the number of considered genes, enzymes and metabolites, remains to be experimentally demonstrated. Therefore, cell-specific manipulation of metabolic pathways and their effects on stomatal functions seem challenging to investigate in the framework of OnGuard.

Genome-scale metabolic modelling offers the opportunity to overcome the aforementioned problems of the existing modelling approaches. This is due to the fact that genome-scale metabolic models consider the entirety of the characterized biochemical reactions in a particular organism (Bordbar *et al.* 2014). Therefore, they readily facilitate a direct *in silico* inspection of the effects of environmental changes or reaction manipulations (e.g. knock-out mutants, silencing of expression or overexpression strategies) (Lewis *et al.* 2012) on particular composite traits such as biomass or reaction fluxes. Moreover, methodological advances have allowed direct integration of transcriptomics and proteomics datasets resulting in more physiologically relevant predictions of metabolic states of specific cell types under different

conditions (Sweetlove & Ratcliffe 2011; Töpfer *et al.* 2013, 2014). Most importantly, recent studies have focused on using the data generated by these high-throughput technologies to arrive at context-specific metabolic networks, where a context stands either for a particular genotype, organ and cell type (e.g. guard cell), or for different environmental conditions (Estévez & Nikoloski 2014). Such models will have to be general enough to simulate different environments, which challenges the usage of condition-independent biomass reaction (Feist & Palsson 2010) (see critique in Töpfer *et al.* 2013 for plants) or different tasks such as ATP production (Fig. 2) usually assumed to be optimized for modelling using flux balance analysis (FBA) (Boyle *et al.* 2009; Raman & Chandra 2009), which is reasonable to apply for guard cell modelling given the importance of the ATP turnover in guard cells (Suetsugu *et al.* 2014; Wang *et al.* 2014a,b).

In parallel with modelling advances for C3 model plants, *Arabidopsis*, (de Oliveira Dal'Molin *et al.* 2010a; Arnold & Nikoloski 2013, 2014; and references therein), there have been developments and refinement of genome-scale metabolic models for C4 model plants (e.g. maize). The latter are more involved as they capture the interplay between two cell types, bundle sheath and mesophyll, via transport of different metabolites through the plasmodesmata (de Oliveira Dal'Molin *et al.* 2010b; Saha *et al.* 2011; Simons *et al.* 2014). Therefore, they can provide the impetus for modelling the communication between guard and mesophyll cells to simulate leaf growth. Such an approach would also provide the necessary comparison between C3 and C4 species with respect to the behaviour of such multicellular systems in terms of different properties, including efficiency of resource usage and patterns of (re)allocation upon different changes, giving this line of research an evolutionary perspective (Mallmann *et al.* 2014).

However, this approach also has numerous challenges because of the nature of the model and the type of reactions considered. First, as prominent constraint-based methods to analyse genome-scale models, FBA and its derivatives largely assume steady-state behaviour, although stomatal movement is a dynamic process. Therefore, it is necessary to develop novel methods to allow investigations of dynamical aspects of large-scale models, particularly as the existing solutions, such as dynamic FBA, can only be applied to models of small to moderate size because of the large computational demands of the employ formulation (Kleessen & Nikoloski 2012; Kleessen *et al.* 2012). Second, these genome-scale metabolic models mostly neglect regulatory and signalling cascade, which can modulate the behaviour of metabolism, although recent method developments have rendered the integration of transcriptional regulatory cascades possible in *Escherichia coli* and *Micobacterium tuberculosis* (Chandrasekaran & Price 2010, 2013) and signalling cascades in a small network for *Saccharomyces cerevisiae* (Lee *et al.* 2008a). Moreover, as the behaviour of signalling pathways usually depends on the concentration of the ligand (e.g. metabolite, which modulates the transduction of the signal to the other cellular levels), the genome-scale models will have

to move from flux-centric to concentration-centric in order to allow the association of flux distributions with the concentrations of the considered metabolites (Töpfer *et al.* 2015). Resolving these challenges in parallel with the development of large-scale models of guard cell metabolism will most likely pave the way for truly predictive modelling (and validation), which will certainly have far-reaching applications.

Finally, aside from the issues related to simulation of the dynamics process, one has to consider the effect of spatial scales. To this end, the existing models of g_s for single leaves have been scaled up to the level of canopy, often performed following the big-leaf top-down approach (Baldocchi *et al.* 1991). To this end, there is the alternative of determining the canopy g_s as the sum of the conductance of individual leaves (see Blonquist *et al.* 2009) or following the Penman–Monteith equation (Monteith 1981; Furon *et al.* 2007). The former could be used to combine multiple large-scale models of illuminated and shaded leaves, with the idea of expanding the applicability of the large-scale models on the level of canopy, a much desired extension of the applicability of constraint-based approaches.

CONCLUDING REMARKS

Prompted by the discovery that genetic manipulation of guard cells is a promising strategy to improve plant growth, water-use efficiency and drought tolerance (Lebaudy *et al.* 2008; Antunes *et al.* 2012; De Angeli *et al.* 2013; Schroeder *et al.* 2013; Wang *et al.* 2014b), recent years have seen the accumulation of experimental evidence concerning different aspects of stomatal behaviour. This is largely due to elegant studies of molecular physiology of guard cells based on application of high-throughput technologies ranging from transcriptomics and proteomics to metabolomics. Our opinion is that the gathered large-scale datasets provide the basis for the creation of dynamic genome-scale metabolic models of guard cells. We posit that their coupling to models of other cell types will allow us to predict targets for genetic manipulation to improve crop yield, particularly under sub-optimal conditions.

Given the complexity of the interconnected networks governing stomatal movements under extremely variable internal and external conditions, we argue that a systems approach, which simultaneously uses different high-throughput technologies to gather data and then integrates them within large-scale models of regulation, signalling and metabolism is a viable strategy towards the rational design of intervention strategies. Such an approach is greatly facilitated by recent advances in context-specific models of plant metabolism and methods for integration of metabolomics data in flux-oriented approaches.

The grand challenge for this research field is to gain a fuller understanding as to how the regulation of stomatal movement occurs and how it may have evolved because of the interplay between genotypes and changing environ-

ments. Use of mathematical approaches to enhance our understanding of the interaction of the various molecular components of the cell (and their dynamic changes underlying this process) and how they are adjusted following environmental changes, can be evaluated in large-scale models in a relatively facile manner. While we are aware that achieving this goal is a vastly challenging task for any cell type we feel that the accessibility of guard cells and the relatively easy enrichment procedures involved in their isolation render them a valuable cell type not only for investigating this specific interaction between a plant cell and its environment as well as between different tightly coupled cell types, but also for looking at the specialization of plant cell function in general.

ACKNOWLEDGMENTS

This work was supported by funding from the Max Planck Society (to A.R.F., Z.N., W.L.A.) and the National Council for Scientific and Technological Development (CNPq-Brazil, Grant 483525/2012-0 to W.L.A.). Scholarships granted by CNPq-Brazil and FAPEMIG to D.B.M. and D.M.D., and research fellowships granted by CNPq-Brazil to W.L.A. are also gratefully acknowledged.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received 25 October 2014; received in revised form 20 January 2015; accepted for publication 27 January 2015

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Transcriptomic data from studies on ABA response in guard cells used for a Venn diagram analysis.