Metabolomics for understanding stomatal movements

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Abstract Stomata control the exchange of CO_2 and water in land plants. For this reason, plants evolved to quickly respond the surrounding environment and endogenous cues in order to maintain their photosynthetic rates, but avoiding excessive water loss. Although guard cell has been used as model for characterization of signaling pathways, mainly regarding abscisic acid (ABA) response, several important questions about its functioning remain elusive. Recently, transcriptomics, proteomics, and metabolomics studies carried out using guard cells as model have contributed substantially for our understanding on how guard cells sense and respond to relative air humidity, CO_2, ABA, and sucrose. Comparatively, proteomics and metabolomics platforms need substantial improvement to increase the number of analytes detected. However, with the introduction of metabolomics-based technologies, several studies have been published increasing our knowledge on guard cell function. Here, we review these new exciting findings as well as discuss the importance of using these new data to improve prediction when modelling stomatal behavior.

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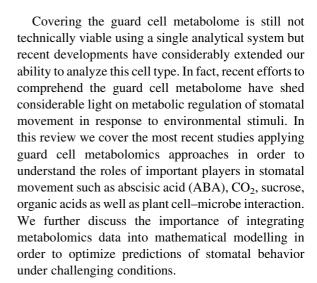Viçosa, Minas Gerais 36570-900, Brazil

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1 Introduction

Stomata, microscopic pores surrounded by two specialized cells—the guard cells -in the leaf epidermis, control the exchange of carbon dioxide (CO_2) and water in land plants. Therefore, they are the main gate for two essential process in plants; photosynthesis and transpiration. For this reason, plants need to adapt quickly and efficiently the environment and endogenous cues in order to gain sufficient CO_2 to maintain their photosynthetic rates and avoid excessive water loss and thus prevent dehydration (Lawson et al. 2014; Schroeder et al. 2001).

Guard cells have been extensively studied for over a century, and considerable efforts have been made to better understand their structure, development, physiology, and metabolism (Bergmann and Sack 2007; Daloso et al. 2017; Dow and Bergmann 2014; Kim et al. 2010; Mott 2009; Santelia and Lawson 2016). These advances have rendered the guard cell as one of

the best studied plant cell models for membrane transport, signaling, and homeostasis (Blatt 2000; Engineer et al. 2016; Hetherington and Brownlee 2004; Hills et al. 2012; Israelsson et al. 2006).

Stomatal pores are structurally very simple, but the surrounding guard cells are one of the most specialized cell in land plants. They are morphologically distinguished from other epidermal cells and possess a complex signal transduction network, tightly regulated membrane ion system, and specialized metabolic pathways which modulate guard cell turgor and subsequently promote opening and closure of stomata (Santelia and Lawson 2016). Generally, the stomatal pores open in response to increases in guard cell volume driven by decreases in the water potential of the guard cell as result of the osmolytes accumulation and subsequently water influx. Conversely, during stomatal closure, efflux of osmolytes from guard cells is required with associated increase in guard cell water potential and in turn efflux of water (Assmann and Wang 2001). Potassium (K^+) and chloride (Cl^-) act as the main inorganic ions, while malate^{2-} and sucrose (Suc) are considered as the main organic osmolytes during stomatal movements (Kollist et al. 2014; Roelfsema and Hedrich 2005; Vavasseur and Raghavendra 2005).

Most of the knowledge we have regarding stomatal movements came from the discovery and characterization of several components of the protein transporting system in guard cells (Eisenach and De Angeli 2017; Jezek and Blatt 2017). However, lately guard cell metabolism has been revisited and we have recently witnessed great efforts in assessing the importance of a range of metabolites in the regulation of stomatal function in elegant biochemical and physiological studies. The knowledge from these studies has increased our understanding on how guard cells regulate their primary metabolism through organic acid, sucrose, starch, and lipid biosynthetic and degradative pathways in order to control stomatal movements. Although we discuss some of these studies in the following sections the focus of this review is to give an update on how metabolomics approaches have been applied in guard cells studies rather than providing an overview of the regulation of guard cell metabolism for which several recent reviews document the most important recent findings (Daloso et al. 2017; Lima et al. 2018; Santelia and Lawson 2016).

Covering the guard cell metabolome is still not technically viable using a single analytical system but recent developments have considerably extended our ability to analyze this cell type. In fact, recent efforts to comprehend the guard cell metabolome have shed considerable light on metabolic regulation of stomatal movement in response to environmental stimuli. In this review we cover the most recent studies applying guard cell metabolomics approaches in order to understand the roles of important players in stomatal movement such as abscisic acid (ABA), CO_2, sucrose, organic acids as well as plant cell–microbe interaction. We further discuss the importance of integrating metabolomics data into mathematical modelling in order to optimize predictions of stomatal behavior under challenging conditions.

2 Metabolomics studies in guard cells

While earlier studies on guard cells focused on changes in accumulation of sugars, organic acids, and K^+ in guard cell (Amoedo et al. 1996; Lu et al. 1995; Talbott and Zeiger 1993, 1996), the recent introduction of new high throughput technologies has reignited guard cell metabolism research. Metabolomics approaches have been applied to analyze changes in guard cell metabolism, being highly useful in providing a broader understanding of guard cell function. However, despite the improvements in guard cell separation methods, all the studies involving guard cell metabolome still face the bottleneck of high quality and rapid cell isolation, in order to disturb as few as possible guard cell primary metabolism. In the last 5 years several studies were published, in which metabolomics approaches were used for answering several unsolved questions on guard cell metabolism, below we review these exciting new findings. The platforms used in each study are summarized in Table 1.

2.1 Metabolic signatures for ABA response

Guard cells are well established as a model cell type for deciphering ABA signaling in plants. The regulatory role of ABA signaling pathway on stomatal regulation has being recently improved by using metabolomics tools. For instance, the metabolic response to ABA treatment was recently followed in

Table 1 Technologies applied for metabolomics studies in guard cell

Platform used	Remarks	References
LC–MS	Guard cell protoplasts from Columbia-0 and *gpa1* Arabidopsis plants subjected in response to ABA	Jin et al. (2013)
GC–MS/MS and LC–MRM–MS	Mesophyll and guard cells from *B. napus* subjected in response to $NaHCO_3$	Misra et al. (2015b)
GC–TOF–MS	Feeding with $NaH^{13}CO_3$ in guard cell-enriched epidermal fragments (EF) from tobacco	Daloso et al. (2015)
GC–TOF–MS	Feeding with $NaH^{13}CO_3$ in EF from transgenic tobacco plants	Daloso et al. (2016b)
GC–MS/MS, LC–MRM–MS, and LC–TOF–MS	EF from Arabidopsis under 800 ppm CO_2	Geng et al. (2016)
GC–TOF–MS	EF from *tdt* Arabidopsis mutant, apoplastic leaflet fluid, and non-aqueous fractionation samples	Medeiros et al. (2017)
GC–MS/MS and LC–TOF–MS	Guard cell protoplasts from *B. napus* in response to ABA	Zhu and Assmann (2017)
GC–TOF–MS	Validation of modeling flux-based predictions using mesophyll and guard cell protoplasts fed with $NaH^{13}CO_3$	Robaina-Estevez et al. (2017)
GC–TOF–MS	Feeding with [U-^{13}C]-sucrose in EF from Arabidopsis	Medeiros et al. (2018b)
LC–MRM–MS	EF incubated with bacteria (Pst DC3000) and labeled with ^{13}C and ^{15}N isotopes	Pang et al. (2018)

LC liquid chromatography, *MS* mass spectrometry, *GC* gas chromatography, *MRM* multiple reaction monitoring, *TOF* time of flight

a time-course experiment utilizing liquid chromatography–multiple reaction monitoring–mass spectrometry (LC–MRM–MS) in order to perform targeted analysis of 85 signaling-related metabolites in Arabidopsis guard cell protoplasts (Jin et al. 2013). Arabidopsis wild-type plants and *gpa1* mutants displaying ABA-hyposensitive stomata were evaluated. This targeted metabolomics study revealed, as would perhaps be anticipated, coordinated regulation of signaling metabolites even in unrelated pathways. For instance, hormone related metabolites were more responsive to ABA treatment in wild type than in *gpa1* guard cells. Additionally, the majority of hormones also displayed different ABA responses in guard cell metabolite profiles when compared with those in the mesophyll cell metabolome, suggesting that ABA is most likely acts upstream of the other hormones (Jin et al. 2013).

More recently, a non-targeted metabolomics study utilizing a combination of gas chromatography–mass spectrometry (GC–MS/MS) and liquid chromatography–time of flight–mass spectrometry (LC–TOF–MS) was applied to identify the metabolic signatures in response to ABA in *B. napus* guard cell protoplasts
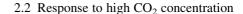
(Zhu and Assmann 2017). Non-targeted metabolomics was used as a complementary approach to targeted metabolomics—providing not only mass/charge ratios but also the mass spectra of all detectable precursor molecules. The platform combination resulted in a metabolite profile of 390 non-redundant metabolites, of which 77 were ABA responsive. On performing an enrichment analysis of the metabolic changes flavone and flavonol biosynthesis and metabolism of amino sugar, nucleotide, sugar, sucrose, and starch were highly affected by ABA (Zhu and Assmann 2017).

Recently, primary metabolism in guard cells has received increased attention mainly regarding the roles of organic acids, sucrose (discussed below) and starch metabolism (we suggest the read of Santelia and Lawson (2016), Santelia and Lunn (2017), and Daloso et al. (2017)). On the other hand, secondary metabolism of guard cells is poorly understood. New insights have, however, been published showing the connection between flavonol biosynthesis and ABA signaling (Watkins et al. 2017, 2014). Flavonol accumulation was observed in guard cells of Arabidopsis, but not in surrounding pavement cells (Watkins et al. 2014). Accordingly, CHALCONE SYNTHASE, a flavonol

biosynthetic enzyme, was demonstrated to be expressed in guard cells, but not pavement cells. Interestingly, null mutation for the gene encoding CHALCONE SYNTHASE increased the levels of reactive oxygen species (ROS) in the guard cells. Further, guard cells of mutant plants showed faster ABA-induced closure compared to wild type, suggesting that flavonols may reduce the ABA-dependent ROS burst that drives stomatal closing (Watkins et al. 2014). Curiously, flavonols levels were positively regulated in guard cells by ethylene treatment in the wild type, but not in an ethylene-insensitive mutant, suggesting an ethylene-regulated mechanism of flavonols controlling ROS burst under ABA signaling (Watkins et al. 2014). Similarly, it was also demonstrated that in tomato (*Solanum lycopersicum*), ABA-induced ROS accumulation is followed by stomatal closure (Watkins et al. 2017). Ethylene treatment of wild-type tomato plants increased flavonol accumulation in guard cells; however, no flavonol increases were observed in Neverripe (Nr) plants, an ethylene receptor deficient mutant. Taken together, these results strongly suggest the roles of flavonols as important players during the ABA-dependent ROS production for the regulation of stomatal opening. Intriguingly, the link between flavonols and stomatal functioning was also observed in an independent recent study investigating metabolic components of water stress in a Mediterranean vineyard (Gago et al. 2017).

Returning to the hormone response it is clear that hormones are good targets to investigate guard cell metabolomics. Indeed, considerable attention has been paid to ABA, but the crosstalk between ABA and other hormones can potentially also affect stomatal movements. For instance, brassinosteroid and jasmonate can act concurrently with ABA promoting senescence and programmed cell death (Hossain et al. 2011; Zhang et al. 2009). Other hormones such as cytokinin, auxin, salicylic acid and ethylene have been showed to act antagonistically to ABA under water deficit conditions (Chen et al. 2013; Ha et al. 2012; Meguro and Sato 2014; Tanaka et al. 2005). Therefore, we believe that metabolomics tools for specific cells can be useful in order to evaluate the metabolic changes provoked by the hormone signaling network and improving methods for single cell hormone measurements will likely greatly increase our ability to understand and model hormonal aspects of the control of stomatal movements.

2.2 Response to high CO_2 concentration

High ambient CO_2 concentrations mediate closure of stomatal pores in plants and conversely low ambient CO_2 concentration triggers the opening of stomatal pores. Within the context of the raising atmospheric CO_2 concentration to understand how guard cells adjust their physiology and metabolism in response to CO_2 is a key step for the development of plants better adapted to the shifting climate condition (Geng et al. 2017). Indeed, although the whole network of CO_2 response is not fully clear, recent studies have addressed the molecular and cellular mechanisms mediating CO_2 regulation of stomatal movements (Engineer et al. 2016; Zhang et al. 2018). For instance, changes in the metabolite profile in response to increases in CO_2 concentration was investigated over time. For this propose mesophyll and guard cells protoplasts from *B. napus* were fed with sodium bicarbonate ($NaHCO_3$) (Misra et al. 2015b). In this study, targeted metabolite profiling in untreated and NaHCO3 treated mesophyll and guard cell was performed using LC–MRM–MS and GC–MS/MS based technologies, in order to identify 268 metabolites related to both, primary and secondary metabolism. Interestingly, opposing metabolic responses were observed between the two cell types, in that while mesophyll cells presented increases in amino acid, phenylpropanoid, redox metabolite, auxin and cytokinin contents all of which were decreased in treated guard cells. The outcome from both cell type metabolite profile revealed that 26 and 27 metabolites in mesophyll and guard cells were exclusively identified, respectively. Regarding primary metabolism, constant increase of malate was observed across the experiment in $NaHCO_3$ treated guard cells. Additionally, in these cells increases in fructose, glucose, and mannose were observed at the early time-points (Misra et al. 2015b).

In another study by using three different approaches; GC–MS/MS, LC–MRM–MS, and LC–TOF–MS it was able to successfully annotate 358 metabolites (Geng et al. 2016). In this study the metabolic responses in guard cell-enriched epidermal fragments of *B. napus* were evaluated in response to elevated CO_2 (800 ppm) in a time-course experiment (over 1 h). Interestingly, these authors reported changes in primary metabolites such as sugars, fatty acids, amino acids and nucleotides under elevated CO_2

treatment. Additionally, ROS production increased and stomatal aperture decreased over the time. Curiously, following the previous findings in metabolic guard cell response to ABA (Zhu and Assmann 2017), alterations in flavonoid, organic acid, sugar, fatty acid, phenylpropanoid and amino acid metabolic pathways indicated changes in both primary and specialized metabolic pathways in guard cells. However, the novelty presented by this work was that after 10 min under elevated CO_2 treatment the jasmonic acid biosynthesis pathway was significantly induced (Geng et al. 2016).

2.3 Understanding the roles of Suc in guard cells

For a long time sucrose was believed to exclusively play an osmolytic role in stomatal opening (Daloso et al. 2016a). However, by using metabolomics tools recent findings have not only challenged this claim but also proposed an energetic role of sucrose during light-induced stomatal opening (for a complete overview about sucrose roles during stomatal movements please read Daloso et al. (2016a) and Lima et al. (2018)). The section below compiles interesting reports focused on the application of metabolomics tools to solve the open questions about guard cell metabolism.

The metabolic changes in guard cell-enriched epidermal fragments from tobacco (*Nicotiana tabacum* L.) during light-induced stomatal opening were evaluated alongside with isotope kinetic labelling experiment by feeding the guard cell-enriched epidermal fragments with NaH^{13}CO$_3$ under light conditions in order to understand the distribution of photosynthetic fluxes in guard cells (Daloso et al. 2015). By using a GC–TOF–MS platform it was observed that concomitant with the decrease in sucrose, there were increases in the level and ^{13}C enrichment of the TCA cycle-related metabolites. This report was the first evidence supporting the hypothesis that sucrose is catabolized within guard cells in order to provide carbon skeletons for organic acid production. Another important finding from this work was the qualitative demonstration that CO_2 fixation in tobacco guard cells can be catalyzed both by ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPc) (Daloso et al. 2015).

A follow-up study was performed using transgenic tobacco plants specifically overexpressing *SUCROSE SYNTHASE 3* (*SUS3*) in guard cells (Daloso et al. 2016b). A kinetic isotope labeling experiment following the metabolic fate of NaH^{13}CO$_3$ during the dark-to-light transition was performed using guard cell-enriched epidermal fragments which were analyzed via GC–TOF–MS. Minor changes were observed in the metabolite profile of whole leaves, whereas increased fructose and decreased organic acids levels were observed in the transgenic plants. In good agreement with the results from the previous study, increased labeling of the TCA cycle-related metabolites was observed, suggesting that breakdown of sucrose is a mechanism providing carbon skeletons during stomatal opening (Daloso et al. 2016b).

Despite the above-discussed evidence, the direct proof that sucrose acts as an energetic substrate during the dark-to-light transition was still missing until earlier this year when the work using a combination of stomatal aperture assays and kinetic [U-^{13}C]-sucrose isotope labelling experiments was published (Medeiros et al. 2018b). This work aimed to directly follow the fate of the carbons released from sucrose breakdown during light-induced stomatal opening. In this study GC–TOF–MS was used revealing rapid and high ^{13}C enrichment in fructose and glucose following dark-to-light transition. Clear ^{13}C enrichment in glycolysis and TCA cycle intermediates as well as glutamate and glutamine was also observed indicating activation of these pathways during light-induced stomatal opening. These new findings allowed a reevaluation of the current models concerning the role of sucrose in stomatal opening. It is now clear that during stomatal opening sucrose is degraded providing carbon skeletons for glutamine biosynthesis, however, the physiological reason behind this is, as yet, far from being understood (Lima et al. 2018; Medeiros et al. 2018b).

2.4 Metabolic changes due to altered organic acids transport in guard cell

Malate and fumarate have been highlighted as important players during stomatal movements. Given their importance in controlling stomatal movement, channels and transporters were recently identified and functionally characterized to be involved with organic acid transport at both plasma membrane and tonoplast of guard cells (Eisenach and De Angeli 2017; Martinoia 2018). In this context metabolomics approaches

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have been used in order to evaluate changes in the metabolism of both leaves and guard cells (Medeiros et al. 2016, 2017).

QUAC1 (Quick activating Anion Channel 1), also known as ALMT12, is a member of ALMT (Aluminum activated malate transporter) family, was identified as an ABA-dependent anion-selective channel at the guard cell plasma membrane responsible for malate efflux during stomatal closure in a voltage-dependent manner (Meyer et al. 2010). Loss-of-function of QUAC1 resulted in an impaired kinetic of stomatal closure in response to dark and high CO_2 levels as well as reduced ABA-induced stomatal closure coupled with significant changes in organic acid accumulation as well as increases in both stomatal and mesophyll conductance (Medeiros et al. 2016). By using the GC–TOF–MS system 40 metabolites related to primary metabolism were successfully annotated, showing relatively few changes in the metabolite profile of mutant leaves. Interestingly, looking at the organic acids levels, it was observed that maleic acid, malate, fumarate, succinate increased in the knockout lines. These results indicate that changes in organic acids homeostasis in guard cell can affect the whole leaf metabolism highlighting the coordination between mesophyll and guard cell metabolism.

A vacuolar malate transporter (tonoplast Dicarboxylate Transporter—tDT) was also recently characterized in Arabidopsis. tDT was considered the main malate transport system at the tonoplast and required for the proper accumulation of malate in Arabidopsis leaves (Emmerlich et al. 2003; Hurth et al. 2005). tDT was only recently functionally characterized (Frei et al. 2018). Highly purified tDT protein showed transport activity as malate and citrate 1:1 in an antiport mode, also accepting fumarate and succinate as substrates (Frei et al. 2018). However, the functional role of tDT in guard cells remained unclear until recently when the metabolic impact of the tDT absence was studied (Medeiros et al. 2017). This study also used GC–TOF–MS technology to assess metabolic changes in leaves and guard cell-enriched epidermal fragments of wild type and *tdt* knockout lines. This study showed that manipulating vacuolar organic acid transport by suppressing tDT greatly impacts mitochondrial metabolism but has minor effects on stomatal and photosynthetic capacity. Interestingly, by comparing the metabolite profiles of leaves and guard cell-enriched epidermal

fragments, intermediates of the tricarboxylic acid cycle were altered differently in the two tissues. For instance, lower malate and fumarate accumulation was observed in leaves but not in epidermal fragments (Medeiros et al. 2017). These findings confirmed the previous hypothesis of functional redundancy for organic acids transport at the tonoplast in guard cells (Meyer et al. 2011).

Recently the report of a new ALMT member (ALMT4) demonstrated it to be a phosphorylation-dependent anion channel, releasing malate from the vacuole during stomatal closure in response to ABA (Eisenach et al. 2017). *almt4* knockout mutants displayed normal stomatal opening, but by contrast to other organic acids transporters at the tonoplast, *almt4* plants presented impaired stomatal closure in response to ABA, but not to darkness (Eisenach et al. 2017). Altogether these exciting findings suggest that independent mechanisms may be activated under specific conditions and individual organic acid transporters are regulated independently in a stimulus-dependent manner at the tonoplast (Medeiros et al. 2018a).

2.5 The guard cell metabolome during plant cell–microorganism interaction

The interaction between plants and microorganisms is known to triggers signaling cascade and metabolic responses. However, the main issue of analyzing changes in the plant metabolic profile during the interaction plant cell–microorganism is avoiding contamination with metabolites from the coexisting microorganism (Pang et al. 2018). A very recent work addressed this issue by implementing an effective stable isotope labeling approach coupled to LC–MRM–MS measurements (Pang et al. 2018). The plant bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 was incubated in isotope-labeled medium (Celtone complete medium with > 98% ^{13}C and > 98% ^{15}N). This procedure was used in order to differentiate plant metabolites from bacterial metabolites. This study suggested that bacterial infection may affect stomatal movement by reprograming the guard cell signaling network and primary metabolism. It was observed that at 30 min after bacterial infection the most affected pathways were related to amino acid metabolism. Whereas at 180 min, aminoacyl-tRNA biosynthesis, the tricarboxylic acid (TCA) cycle, and

carbon fixation as well as the biosynthesis of phenylalanine, tyrosine, and tryptophan were dramatically affected.

In summary, metabolite profiling has aided the identification of several novel interactions between the TCA cycle, photosynthesis and stomatal function as well as guard cell responses to both biotic and abiotic stresses. Due to the vast complexity of the metabolic and molecular regulatory pathways underlying guard cell function they remain not fully understood. However, the improvements of new technologies toward metabolomics approaches will help us to better understand stomatal responses. Furthermore, the usage of metabolic data into mathematical models can optimize predictions of stomatal behavior in a changing environment. Studies in this direction have been performed and are discussed in the following section.

3 Metabolomics data for improving stomatal behavior predictions

Transcriptomics, proteomics, and metabolomics studies carried out using guard cells as model have contributed substantially for our understanding on how plant cells sense and respond to environmental and endogenous signals such as relative air humidity, CO_2, ABA, and sucrose (Medeiros et al. 2015). All of the knowledge gained from these studies are useful for the establishment of systems biology approaches. Transcriptomic data has advantage over other omics platforms for the use in mathematical models given the higher coverage of transcriptomic techniques (Alseekh and Fernie 2018). Comparatively, proteomics and metabolomics platforms need substantial improvement to increase the number of analytes detected. This is an important issue for the creation of genomic scale metabolic models, in which several reactions of the metabolic network are included in the model (Hyduke et al. 2013). However, it is noteworthy that gene expression does not always correlate to protein expression/activity and subsequently to metabolic changes (Fernie and Stitt 2012; Lehmann et al. 2009). In this scenario, metabolomics approaches provide vital information by which to understand the structure and the regulation of metabolic networks as well as to enhance the predictive power of metabolic models.

Despite the fact that mass spectrometry-based metabolomics platforms have been widely used in plant biology, these platforms have only been recently used to investigate guard cell metabolism (Medeiros et al. 2015; Misra et al. 2015a). The establishment of a reproducible and reliable analytical platform is crucial for the performance of -omics studies, which can subsequently aid in the generation of mathematical models. Some models for leaf metabolism of C3, C4, and CAM plants have been published (Arnold and Nikoloski 2014; Cheung et al. 2014, 2015; Chew et al. 2014; Masakapalli et al. 2010, 2013; Pfau et al. 2018; Seaver et al. 2015; Shameer et al. 2018; Sweetlove et al. 2013; Wang et al. 2012). Recently the AraCORE model created for leaf primary metabolism (Arnold and Nikoloski 2014) has been adapted to model guard cell metabolism (Robaina-Estevez et al. 2017). Large-scale omics data have been used to constrain different models and thus more faithfully predict metabolic fluxes through metabolic networks (Nikoloski et al. 2015; Orth et al. 2010). This is part of a systemic characterization of plants, in which the data generated is used either to create or to improve mathematical models, following a "design–build–test–learn" cycle (Sweetlove et al. 2017).

Existing models and their combinations have clearly contributed to the predictive modelling and refinement of the relationship between stomatal conductance and the players regulating stomatal function. Unfortunately, limited success in clarifying how changes in stomatal movements, by consequence water loss and CO_2 gain, are affected by the interplay of environment, cellular levels, as well as the coordination between mesophyll and guard cells. The OnGuard platform is one of the first modelling approaches created specifically for guard cells (Hills et al. 2012). Although the possibility to analyze guard cell signaling pathways using the theory of biological networks was postulated much earlier (Hetherington and Woodward 2003). OnGuard is a software built based on the knowledge of transport and accumulation of important molecules for guard cell osmoregulation such as K^+, Cl^-, Ca^{+2}, malate, and sucrose. Simulations of the transport of these molecules through both plasma and tonoplast membranes of guard cells can be now performed by OnGuard (Chen et al. 2012; Hills et al. 2012; Wang et al. 2014). Despite its great contribution to understand important process that regulates stomatal movements, OnGuard software is

not able to predict metabolic alterations in guard cells. This is partially due to the relative scarcity of guard cell metabolomics data. However, given that recent metabolomics approaches (reviewed above) being adapted to study guard cells, this hurdle is no longer valid.

LC–MS and GC–MS-based metabolomics studies have provided important information regarding the guard cell response to ABA, bicarbonate, and sucrose (Daloso et al. 2015; Jin et al. 2013; Medeiros et al. 2018b; Misra et al. 2015b; Zhu and Assmann 2017). Furthermore, the establishment of a methodology to perform ^{13}C-feeding experiments with the use of labelled substrates resolve a long debate regarding the role of sucrose for guard cell regulation and the mode of CO_2 fixation found in guard cells (Daloso et al. 2015; Medeiros et al. 2018b). RubisCO-mediated CO_2 fixation has long been thought to be absent in guard cells (Outlaw and Manchester 1979). However, the ^{13}C distribution found in guard cells subjected to light indicate a clear CO_2 fixation mediated by both RubisCO and PEPc (Daloso et al. 2015). The results of this study were substantially different from those observed in the Arabidopsis rosette which is largely composed of mesophyll cells (Szecowka et al. 2013). Taking this into account, a systems biology study compared the $NaH^{13}CO_3$ distribution in mesophyll and guard cell protoplasts by combining flux predictions using a genome scale metabolic model and the ^{13}C distribution measured in both cells via GC–TOF–MS (Robaina-Estevez et al. 2017). The modelling strategy of this work was based in previous published transcriptome data (Bates et al. 2012; Pandey et al. 2010) that were integrated into the AraCORE model (Arnold and Nikoloski 2014). As output, the new model, namely AraCOREred, provides cell-specific metabolic predictions of 455 reactions which comprise 374 primary metabolites of mesophyll and guard cells (Robaina-Estevez et al. 2017). Interestingly, the results of this work confirmed that guard cells have higher anaplerotic CO_2 fixation by PEPc than mesophyll cells as well as regarding the source of sucrose and malate within guard cells.

The use of such genome scale, constraint-based metabolic models is important to understand the regulation of plant metabolism (Sweetlove et al. 2017). Mainly because the model can predict metabolic changes at subcellular level under different conditions, given that the input of signals and the output of the model can be manipulated according to the aim of the study (Medeiros et al. 2015; Orth et al. 2010). The AraCOREred model indicates that transcriptome data can be used to predict cell-specific metabolic changes. This model can then be used in the future to predict guard cell metabolic responses to ABA and sucrose, given the availability of transcriptome data under ABA and sucrose treatment (Bates et al. 2012; Bauer et al. 2013; Leonhardt et al. 2004; Pandey et al. 2010). However, it is important to highlight that AraCOREred model was created assuming the guard cell metabolism at steady states. Due to the dynamic nature of the guard cell metabolism, the current challenge is to model guard cells under non-steady state condition, which would seem to be more physiologically relevant given the high plasticity of guard cell metabolism under varying conditions. Modelling strategies such as dynamic flux balance analysis seems to be great option to fulfil this gap. The establishment of such non-steady state modelling, while challenging, may increase the reliability of predictions by the current available models for guard cell functioning.

4 Concluding remarks

Over the past 5 years the application of metabolomics approaches in order to better understand guard cell metabolism and the mechanisms underlying the stomatal responses to environmental and endogenous cues has increased. Metabolite profiling of isolated or enriched-guard cells extracts has aided the identification of several novel interactions between the TCA cycle, photosynthesis, and stomatal function. Furthermore, guard cell metabolomics studies have fulfilled some important unsolved questions regarding the roles of sucrose and organic acids transport and metabolism during stomatal movements as well as the guard cell responses to water deficit, high CO_2 concentrations, and plant defense against pathogens.

Alongside other omics technologies, metabolomics studies of the highly specialized guard cell have proven to be a powerful tool for understanding the hormonal and metabolic regulation of stomatal movements. This is particularly important since plant growth, development, and yield depend greatly of the fine control of plant–environment gas exchanges. Given the complexity of the interconnected networks

governing stomatal movements under varying conditions, we argue that metabolomics approaches are highly valuable. An emerging technology that may greatly aid in this vein is metabolite imaging (Bjarnholt et al. 2014), which may soon be able to aid our understanding of metabolite movements between the mesophyll and guard cells. Ongoing systems biology approaches, combining data from different omics technologies will aid in elucidating the mechanisms underlying stomatal control and furthermore could ultimately unravel targets for modulation of stomatal responses to environment and towards the design of new strategies for engineering better plant performance in the field.

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