Starch Metabolism in Guard Cells and its Impact on Stomatal Function

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SUMMARY

The appearance of stomata over 400 million years ago represents a major evolutionary invention, which enabled plants to conquer land. Stomata are turgor-driven valves enclosed by a pair of highly specialized guard cells allowing the exchange of carbon dioxide, oxygen and water between the leaf interior and the environment. The reversible changes in guard cell turgor result from the uptake and release of a variety of osmotically active solutes. Much of the guard cell research conducted over the past 80 years focused on transmembrane ion transport. Although its importance for stomatal movement regulation is inarguably, adjustment of the stomatal pore size requires a complex network of interactions between ion transport, metabolism and solute partitioning. Carbon metabolism has been implicated with stomatal aperture control ever since von Mohl (1856) and Lloyd (1908) observed the presence of starch granules in guard cells. However, the lack of suitable experimental techniques imposed by the microscopic size of guard cells has long hindered the investigation of metabolic rearrangements during stomatal movements.

We only recently resolved the diurnal temporal dynamics of starch turnover in guard cells. Substantial amounts of stomatal starch are present throughout the majority of day and night. After dawn, this starch gets transiently fully hydrolyzed by the concerted action of the glucan hydrolases α-amylase 3 (AMY3) and β-amylase 1 (BAM1) accelerating stomatal opening. This process is directly triggered by the blue-light-dependent activation of the plasma membrane H^+-ATPase, which activity links membrane ion transport to guard cell carbon metabolism. However, one big unresolved question was how guard cell starch degradation integrates with light-induced ion transport processes in the control of stomatal opening kinetics.

In Chapter I of this thesis, we demonstrate that guard cell starch degradation does not directly affect the capacity for the transport of H^+, K^+ and Cl^- ions across the plasma membrane, suggesting that starch degradation does not directly affect the ability for ion transport. Moreover, we examined a long-lasting hypothesis that malate accumulates upon guard cell starch mobilization. Using newly developed enzymatic quantification assays, we revealed that the major end product of blue light-induced starch breakdown in the guard cells of Arabidopsis thaliana is glucose and not malate. The rapid generation of glucose from starch is thus essential for fast stomatal opening and contributes to the coordination between photosynthesis and transpiration. Although guard cell starch plays this essential role during stomatal opening, starch synthesis in guard cells is poorly understood. In Chapter I, we further demonstrate that
the capacity of guard cells for autonomous CO₂ fixation is limited and starch synthesis largely depends on the supply of mesophyll-derived substrates.

Along this line of evidence, the two proton-coupled hexose carriers Sugar Transport Proteins 1 and 4 (STPs) were identified as the major plasma membrane-localized monosaccharide transporters in guard cells in Chapter II. Using a large set of physiological and biochemical techniques, along with phenotyping technology, allowed us to show that their combined activity is required for glucose uptake to guard cells at dawn, delivering carbon substrates for starch synthesis and light-induced stomatal opening.

In Chapter III, in a review under revision for the "New Phytologist", we provide a critical summary of the latest research about guard cell carbon metabolism and identify remaining knowledge gaps.

Chapter IV reveals that in addition to the STPs, three members (1, 4 and 5) of the hexose facilitators from the Sugars Will Eventually be Exported (SWEET) family are highly expressed in guard cells and supply them with sugar precursors for starch synthesis. We further provide evidence that guard cell starch acts as a sink for osmolytes during high CO₂-induced stomatal closure. We identified the three responsible vacuolar exporters involved in this process: the Aluminum-activated Malate Transporter 4 (ALMT4), the Early Response to Dehydration Like 6 (ERDL6) and the Sucrose-H⁺ symporter SUC4.

Chapter V deals with the enzymatic pathway(s) of guard cell starch synthesis as guard cells show traits of both auto- and heterotrophic tissues. We demonstrate that the enzymes of the classical leaf pathway of starch synthesis play also a significant role in guard cells. Concomitant with hexose uptake to guard cells for starch synthesis, we identified the Glucose-6-phosphate/Phosphate Translocator 1 (GPT1), which catalyzes glucose uptake into chloroplasts, as an essential player of starch accumulation in guard cells.

Given the largely heterotrophic nature of guard cells, in Chapter VI, we conducted research in collaboration with the lab of Dr. Boon-Leong Lim (University of Hong Kong) to uncover how guard cell chloroplasts obtain energy for metabolic processes. We used genetically encoded ATP and NADPH biosensors and demonstrated that guard cell chloroplasts surprisingly import cytosolic ATP via the Nucleotide Transporter 1 (NTT1), which is used among other processes for the formation of starch.
Finally, in Chapter VII, we collaborated with the lab of Prof. Anne Borland (University of New Castle, UK) to peel back the layer and investigate differences in the proteomes of mesophyll and epidermis cells in *Kalanchoë fedtschenkoi*, revealing substantial differences in guard cell starch metabolism among C3 and CAM species.

Collectively, the findings of this thesis represent a major advance in our knowledge about the molecular mechanisms linking guard cell carbon metabolism and stomatal movement control. The presented data highlight the tight connection between carbohydrate metabolism in mesophyll and guard cells, revealing glucose as an essential metabolite coupling the energetic and metabolic status of these two cell types. Finally, we significantly enhance the awareness of energy production and fluxes within guard cells, paving the way for future research.
ZUSAMMENFASSUNG


Im ersten Kapitel zeigen wir, dass der Stärkeabbau keinen direkten Einfluss auf den Transport von H⁺, K⁺ und Cl⁻-Ionen an der Plasmamembran hat. Dies lässt darauf schliessen, dass die Stärke nicht primär zur


Im dritten Kapitel fassen wir kritisch die neusten Erkenntnisse über den Kohlenstoffmetabolismus der Schliesszellen zusammen und zeigen die derzeitigen ungeklärten Fragen in diesem Bereich der Forschung auf.

Im vierten Kapitel beschreiben wir drei weitere Transporter (1, 4 und 5) der Sugars Will Eventually be Exported (SWEET) Proteinfamilie, die für die Bereitstellung von Kohlenhydraten für die Stärkesynthese in den Schliesszellen zuständig sind. Wir konnten zeigen, dass unter hohen CO₂ Konzentrationen der Stärkeaufbau vakuoläre Osmolyte aus dem System entzieht und somit zur Schliessbewegung der Stomata beiträgt. Die drei Exportproteine Aluminum-activated Malate Transporter 4 (ALMT4), Early Response to Dehydration Like 6 (ERDL6) und Saccharose-H⁺-Symporter SUC4 des Tonoplasten sind dabei entscheidend.

Da Schliesszellen überwiegend heterotroph sind, begannen wir ausserdem nach der Energiequelle für metabolische Prozesse in den Chloroplasten zu suchen. Dafür kollaborierten wir mit Dr. Boon-Leong Lim (Universität Hong Kong). Im sechsten Kapitel haben wir mithilfe von genetisch kodierten Biosensoren den Nucleotide Transporter 1 (NTT1) identifizieren können, welcher cytosolisches ATP in die Schliesszellchloroplasten transportiert. Dieses Nukleotid wird unter anderem für die Synthese von Stärke genutzt.

Im siebten Kapitel kollaborierten wir mit Prof. Anne Borland (Universität Newcastle, UK) um die Mesophyll- und Epidermisproteome in Kalanchoe fedtschenkoi zu untersuchen und diese mit C3-Pflanzen zu vergleichen. Dadurch konnten wir gravierende Unterschiede in den Schliesszellmetabolismen von C3- und CAM-Pflanzen feststellen.

Zusammenfassend tragen die Ergebnisse dieser Dissertation wesentlich zum jetzigen Wissen über die molekularen Mechanismen der Schliesszellenbewegung bei, welche eng mit dem Kohlenstoffmetabolismus verbunden sind. Wir haben gezeigt, dass die Glukose als essenzieller Metabolit Mesophyll- und Schliesszellenstoffwechsel verknüpft. Unsere Daten eröffnen neue Sichtweisen auf die Energiegewinnung der Schliesszellen und weisen den Weg für zukünftige Forschung.
TABLE OF CONTENT

GENERAL INTRODUCTION .................................................................................................................
Stomatal guard cells – master regulators of plant water use efficiency ......................................... 1
The mechanisms of stomatal movements – a historical perspective ............................................ 3
Guard cell starch metabolism – unique features ........................................................................... 5
Stomatal opening – a matter of light quality ................................................................................. 8
Closing the gap – mechanisms of stomatal closure ..................................................................... 13
References ...................................................................................................................................... 19

AIMS OF THIS THESIS ......................................................................................................................... 31

CHAPTER I - Guard cell starch degradation yields glucose for rapid
stomatal opening in Arabidopsis ........................................................................................................
Abstract ........................................................................................................................................... 34
Introduction ....................................................................................................................................... 35
Results .............................................................................................................................................. 38
Discussion ....................................................................................................................................... 53
Methods ........................................................................................................................................... 59
References ....................................................................................................................................... 67
Supplemental Data .......................................................................................................................... 73

CHAPTER II - Glucose uptake to guard cells via STP transporters
provides carbon sources for stomatal opening and plant growth .............................................
Abstract ........................................................................................................................................... 86
Introduction ....................................................................................................................................... 87
Results .............................................................................................................................................. 89
Discussion ....................................................................................................................................... 99
Material and Methods .................................................................................................................. 103
References ..................................................................................................................................... 111
Expanded view Figures .................................................................................................................. 115

CHAPTER III - Mesophyll-derived sugars are positive regulators of light-
driven stomatal opening ....................................................................................................................
Summary ........................................................................................................................................ 129
Introduction ..................................................................................................................................... 130
Origin and fate of malate during blue light-induced stomatal opening ....................................... 131
Glucose is the main starch-derived metabolite under blue light ............................................... 132
Mesophyll-derived glucose and sucrose have distinct functions during stomatal
opening at dawn .............................................................................................................................. 134
Conclusion and Outlook ............................................................................................................... 137
References ..................................................................................................................................... 140
This is a cumulative dissertation consisting of seven original and independent research manuscripts and one method paper (Appendix). Paper I, II and VII were published in peer-reviewed journals. Paper III is an invited review and is currently under revision. Papers IV, V and VI are in preparation to be submitted. A general introduction highlights their commonalities and provides a broad background for the presented manuscripts. The manuscripts are followed by a general discussion and outlook highlighting the findings and open questions of my PhD work. The style of writing and formatting might vary among the manuscripts as they were or are to be published in different journals.
**General Introduction**

Stomatal guard cells – master regulators of plant water use efficiency

In the mind of most plant biologists, the word stomata is intimately linked to the exchange of gases between the interior of a plant and the surrounding atmosphere. However, these small pores are assumed to have first evolved to promote the desiccation of reproductive tissues on sporophytes of mosses and hornworts over 400 million years ago, which have likely diverged before the emergence of the vascular plants (Puttick *et al.*, 2018). In higher plants, stomata are found on most aerial parts and have a dual role in facilitating the uptake of CO$_2$ for photosynthesis and restricting water loss through transpiration. By integrating information derived from endogenous signals with cues from the environment, the guard cells delimiting the stomatal pore, control its aperture and allow a rapid adaptation to the surrounding environmental conditions (Hetherington & Woodward, 2003). Although only 0.3 - 5% of the total leaf surface is occupied by guard cells, stomata exert profound effects at a global level (Willmer & Fricker, 1996). $32 \times 10^{18}$ g of water vapour and $440 \times 10^{15}$ g CO$_2$ are estimated to traverse stomata every year (Hetherington & Woodward, 2003). This means that about 60% of all the precipitation that falls on terrestrial ground is taken up by plants and transpired through stomatal pores (Morison, 2003; Hetherington & Woodward, 2003). Ever since stomata emerged, they have changed markedly in size and morphology. Two broad stomatal morphologies are distinguished – the dumb-bell-shaped stomata of the grasses and the kidney-shaped stomata found in other species (Fig. 1; Willmer & Fricker, 1996).

![Figure 1. Different stomatal morphologies](image)

*Figure 1. Different stomatal morphologies.* Representative confocal micrographs of propidium iodide-stained guard cells are shown. Pictures were taken randomly during the day. Upper panel depicts examples of dicot species (*Arabidopsis thaliana, Kalanchoe fedtschenkoi* and *Mesembryanthemum crystallinum*) with kidney-shaped stomata, whereas the lower panel shows dumb-bell-shaped stomata of grass species (*Hordeum vulgare, Oryza sativa* and *Zea mays*). Pictures are courtesy of Eduard Bruderer and Sabrina Flütsch.
The dumb-bell-shaped stomata are surrounded by either graminaceous or tetracytic subsidiary cells (Lawson & Matthews, 2020), which provide them with a mechanical benefit resulting in faster stomatal opening (Franks & Farquhar, 2007). Species with stomata that adjust their aperture more rapidly have an obvious advantage in balancing CO₂ uptake with water vapour loss especially under water-limiting conditions (Lawson & Vialet-Chabrand, 2019). This directly translates into grass species having increased water use efficiencies (WUE) and maximized photosynthesis compared to other species with kidney-shaped stomata and the concurrent lack of subsidiary cells.

McAusland et al., (2016) quantified the direct impact of slow stomatal movements through a combination of gas exchange measurements and quantitative modelling in a range of species including several grass species. They found an average 10% limitation of photosynthesis by slow stomatal opening, potentially negatively impacting yield. In contrast slow stomatal closure caused considerable decreases in intrinsic WUE. Therefore, a close coupling of stomatal movements and photosynthesis is essential in enhancing carbon gain and WUE. Moreover, the authors could attribute the rapidity of stomatal responses in grass species to their guard cell morphology, whilst in kidney-shaped stomata, other factors than morphology determine stomatal kinetics (McAusland et al., 2016). Such other factors are intrinsic guard cell characteristics important for guard cell turgidity, including size and density of guard cells, capacity of solute transport and metabolic rearrangements (Lawson et al., 2014; Horrer et al., 2016; Lawson & Vialet-Chabrand, 2019; Lawson & Matthews, 2020). Size and density of stomata, which are themselves influenced by environmental factors determine the maximum theoretical conductance (Dow et al., 2014). However, manipulation of stomatal size and density to modulate stomatal responses and photosynthesis have often met with limited success imposed by the complex relationship underlying the two traits. The manipulation of physical attributes can be counterbalanced by modification in functions as demonstrated by several studies (Büssis et al., 2006; Lawson & Blatt, 2014; Tanaka et al., 2013). Other targets of manipulation are the transporters at the plasma membrane. The density and activity of transport proteins at the guard cell plasma membrane determine the total transport capacity of solutes required for turgor regulation and therefore affect stomatal movement speed (Faralli et al., 2019). Indeed, optogenetic manipulation of stomatal kinetics by the expression of a synthetic, light-gated K⁺ channel BLINK1 accelerated stomatal opening thereby driving 2.2-fold increases in biomass without water loss by the plants (Papanatsiou et al., 2019). Hence, these authors managed successfully to overcome the stomatal aperture/density trade-off and have focused on the
manipulation of other characteristics directly affecting the speed of stomatal movements. Similar results were obtained from genetic manipulation of genes affecting the sensitivity to the plant hormone abscisic acid (ABA) resulting in reduced transpiration in response to ABA providing the transgenic canola plants an advantage under drought conditions (Wang et al., 2005). Therefore, breeding for or engineering species with more speedy and efficient stomata represents a concrete possibility to genetically modulate WUE and generate more resilient plants (Nunes et al., 2020).

The mechanisms of stomatal movements – a historical perspective

Species differ considerably in the way they regulate stomatal movements depending on the environmental conditions in which they have evolved. Whereas most C3 and C4 species open their stomata in response to light and close them in darkness, crassulacean acid metabolism (CAM) species open their stomata at night to assimilate CO₂ into malic acid (Kollist et al., 2014). However, the basic principle of stomatal movements is common to all of them and lies in the rapid accumulation (to open) and release (to close) of osmotically active solutes, which affects the turgidity of guard cells by promoting inflow/efflux of water (Willmer & Fricker, 1996). Guard cells have evolved cell walls of unique physical strength and considerable elasticity to allow the reversible changes in size and shape to occur during stomatal movements (Jones et al., 2003; Rui et al., 2018). The volume of guard cells changes equally to their surface area, which needs the expansion of the plasma membrane surface by about 40% during stomatal opening (Diekmann et al., 1993). This is achieved through membrane internalization (Shope et al., 2003; Shope & Mott, 2006). Moreover, guard cell vacuoles undergo dynamic organisational changes during the adjustment of the stomatal pore (Gao et al., 2005, 2009; Bak et al., 2013; Andrés et al., 2014).

Hugo von Mohl (1856) was the first to appreciate that stomatal movements were due to turgor changes in guard cells which were osmotically regulated. The photosynthetic’ hypothesis formulated by von Mohl suggested that guard cell photosynthesis was responsible for the provision of osmotic species required for the observed adjustments in guard cell turgor (von Mohl, 1856). At the beginning of the 20th century, Lloyd further elaborated this hypothesis and proposed the starch-sugar theory. Lloyd noticed that guard cell starch granules were present in the dark but not in the light, and that their amount was inversely correlated with the aperture of the stomatal pore. Thus, he proposed that starch within guard cells is mobilized to yield sugars at dawn to increase their osmotic potential, while sugars are condensed back to starch at dusk (Lloyd, 1908). This starch-sugar hypothesis was then slowly replaced
INTRODUCTION

by the ion theory when several reports provided convincing evidence for substantial potassium (K\(^+\)) ion accumulation during stomatal opening linked to the observed changes in osmolarity (Imamura, 1943; Fischer, 1968; Fischer & Hsiao, 1968; Humble & Raschke, 1971). Soon after the K\(^+\) paradigm emerged, research into a counter-ion for K\(^+\) revealed chloride (Cl\(^-\)) ions as the main compensators for the intake of the positively charged K\(^+\) (Raschke & Fellows, 1971; Raschke & Schnabl, 1978). Yet, based on the massive changes in osmotic pressure, K\(^+\) accumulation and anion content of guard cells, the need for additional counter-ions to Cl\(^-\) was proposed. Malate\(^2-\) was put forward as an organic anion which in addition to Cl\(^-\) would balance K\(^+\) uptake (Allaway, 1973; Pearson, 1973; Raschke & Humble, 1973; Outlaw & Lowry, 1977; Raschke & Schnabl, 1978). The research into K\(^+\) and its counter-ions as guard cell osmotic solutes led to the formulation of the K\(^+\)-malate\(^2-\) hypothesis, which temporarily replaced the starch-sugar theory. In the subsequent years, researchers hypothesised that guard cell starch is converted into malate\(^2-\) during stomatal opening to promote guard cell turgidity (Raschke & Schnabl, 1978; Outlaw & Manchester, 1979; Schnabl, 1980; Vavasseur & Raghavendra, 2005). At the same time, new evidence brought the starch-sugar theory back to life (Outlaw & Kennedy, 1978; MacRobbie & Lettau, 1980; Tallman & Zeiger, 1988; Talbott & Zeiger, 1993). MacRobbie and Lettau (1980) observed that the changes in contents of K\(^+\) and its counter-ions are much too small to account for the osmotic changes needed to drive stomatal opening and suggested that organic solutes, perhaps sugars, are responsible for the observed rearrangements (MacRobbie & Lettau, 1980). Poffenroth et al., (1992) went even one step further and proposed a model for the accumulation of sugars depending on the applied light stimulus: a starch degradation pathway delivering sugars induced by blue light and a photosynthetic sugar production pathway mediated by red light (Poffenroth et al., 1992). This hypothesis got soon supported by findings from Talbott and Zeiger (1993). Since the red light response depends on photosynthesis (Sharkey & Raschke, 1981), researchers began to examine the contribution of mesophyll cells to this type of stomatal opening. Both K\(^+\) and sugar accumulation have been observed in response to red light (Tallman & Zeiger, 1988; Talbott & Zeiger, 1996; Olsen et al., 2002). Three paths for the origin of sugars were suggested, namely that sugars are provided through guard cell starch degradation (Outlaw & Manchester, 1979), imported from the apoplast (Hite et al., 1993; Lu et al., 1995) or generated from autonomous CO\(_2\) fixation (Gotow et al., 1988). Although research into guard cell carbon metabolism has spanned several decades, the origin of sugars in guard cells remains still elusive. In general, contradicting results led to yet
INTRODUCTION

no consensus about the importance of guard cell carbon metabolism for stomatal movements.

**Guard cell starch metabolism – unique features**

Starch is present in chloroplasts of guard cells and its degradation has been linked to stomatal movements for over a century (Lloyd, 1908). Mainly due to the lack of suitable experimental techniques, the exact role of guard cell starch mobilization, its regulation and the enzymes involved remained elusive for another century. Changes in stomatal starch concentrations were observed in some species, such as *Ocimum basilicum*, *Commelina communis* and *Arabidopsis thaliana* (Arabidopsis), based on iodide-stained guard cell chloroplasts (Tallman & Zeiger, 1988; Vavasseur & Raghavendra, 2005; Kang *et al.*, 2007; Valerio *et al.*, 2011). However, this method only provides information about presence/absence of starch, but not about the temporal dynamics of guard cell starch metabolism. It was only recently that the temporal pattern of starch turnover was revealed in guard cells of Arabidopsis (Horrer *et al.*, 2016). The combination of the staining of guard cell starch granules with the fluorophore propidium iodide and high-resolution imaging on a cell-by-cell basis by confocal microscopy enabled the quantitative analysis of guard cell starch contents (Horrer *et al.*, 2016).

![Figure 2. Diurnal starch turnover in mesophyll and guard cells.](image) Figure is taken from the review Santelia & Lunn, (2017).
The timing of starch deposition and mobilization in guard cells differs in many ways from the rest of the leaf (Fig. 2; Horrer et al., 2016; Santelia & Lunn, 2017). Whereas mesophyll cells accumulate starch gradually during the light period and degrade it in a near-linear manner at night (Stitt & Zeeman, 2012), guard cells contain substantial amounts of starch during the dark phase. In fact, starch contents increase for several hours after dusk before a slow decrease of starch amounts is observed towards dawn. However, considerable amounts of starch remain in the guard cell chloroplasts at the end of night, which get rapidly mobilized upon illumination. After the almost complete hydrolysis of starch, the contents rise continuously into the early hours of the night (Fig. 2; Horrer et al., 2016; Santelia & Lunn, 2017). Interestingly, the degradation of stomatal starch directly correlates with an increase in stomatal aperture (Fig. 3a; Horrer et al., 2016; Santelia & Lawson, 2016).

Figure 3. Guard cell starch breakdown and synthesis. (A) Guard cell starch contents throughout the 12 h light phase. Data shown are means ± SEM; n = 110. EoN = End of Night; EoD = End of Day. Data are redrawn from Horrer et al., (2016). (B) Models of Guard cell starch degradation induced by blue light and starch synthesis upon red light. For details see text. Whole figure is taken from Santelia & Lawson, (2016).
Guard cell starch is mobilized by a highly specific set of enzymes, with β-amylase 1 (BAM1) and α-amylase 3 (AMY3) being the main players (Fig. 3b left guard cell; Horrer et al., 2016; Santelia & Lawson, 2016). These two starch degrading enzymes are usually not required for night-time starch metabolism in the leaves, suggesting that they have acquired specialized functions compared to the other isoforms of the gene families they belong to (Seung et al., 2013; Monroe et al., 2014). BAM3, the main β-amylase isoform involved in night-time starch degradation in the leaves seems to be regulated differently compared to BAM1, which might explain their sub-functionalization. BAM1 and BAM3 are adapted for distinct pH ranges. BAM1 is more active than BAM3 at high pH reflecting their roles for day-time starch degradation in the guard cells and night-time starch breakdown in leaves as the pH of the guard cell stroma should be elevated compared to the night (Monroe et al., 2014). In addition, whereas BAM1 is redox regulated, BAM3 is not, the required cysteines for the formation of a disulfide bridge are not conserved in BAM3 (Sparla et al., 2006). More so, also AMY3 was shown to be redox regulated (Seung et al., 2013), indicating that BAM1 and AMY3 are coregulated to fulfil their function in guard cells. To fully hydrolyze starch, BAM1 and AMY3 work together with other starch degrading enzymes, such as the debranching enzymes Limit Dextrinase (LDA) and Isoamylase 3 (ISA3; Horrer et al., 2016). Whereas LDA has no clear function in mesophyll starch degradation, ISA3 is the major debranching enzyme in night-time leaf starch degradation (Delatte et al., 2006).

Guard cell starch degradation occurs upon blue light illumination and is mediated by the guard cell blue light signaling cascade that gets initiated through the perception of blue light by the plasma membrane-associated photoreceptors Phototropins 1 and 2 (PHOTs; Kinoshita et al., 2001). The signal is transduced via kinase signaling to the plasma membrane-localized H+-ATPase upstream of the starch degrading enzymes, resulting in its activation (Kinoshita & Shimazaki, 1999; Horrer et al., 2016). The signal linking the activity of the proton pump at the plasma membrane and the starch hydrolyzing enzymes in the chloroplast is still elusive. Prior to the work done in this thesis, the end product of starch degradation was unknown. For long, it was suggested that carbon skeletons resulting from the mobilization of starch might be converted into malate$^2$ in the cytosol through glycolysis to act as counter-ions for the intake of apoplastic K$^+$ ions or to promote guard cell turgor. Alternatively, sugars could be produced from starch, which might be used as an energetic source or as osmotic species during stomatal opening (Fig. 3b left guard cell; Horrer et al., 2016; Santelia & Lawson, 2016).
While we begin to understand the molecular mechanisms underlying stomatal starch degradation, starch synthesis remains under investigated. The specific pattern of starch formation observed in guard cells suggests that its regulation is independent from the transition between light and dark and other factors might control this process. Red light is one such factor efficiently inducing the accumulation of starch (Horrer et al., 2016; Santelia & Lawson, 2016). Prior to the work done in this thesis, it was unclear whether the carbon skeletons for starch synthesis derive from autonomous guard cell photosynthesis or from import of mesophyll-derived substrates or a combination of both processes. Recently, starch was put forward to act as a sink for osmotically active solutes required to be retrieved from the guard cells during high CO$_2$-induced stomatal closure (Azoulay-Shemer et al., 2016).

**Stomatal opening – a matter of light quality**

Stomatal opening is triggered by increasing light or temperature (up to an optimum of about 40°; Kostaki et al., 2020), high humidity and low CO$_2$ concentrations. Plants experience a range of light intensities and qualities throughout the diel period, with peaks of blue light at the beginning and end of the day, and high intensities of red light in between (Matthews et al., 2020). Stomatal responses to light are controlled by two distinct pathways; the photosynthesis-dependent red light response and the photosynthesis-independent blue light response (Shimazaki & Zeiger, 1987; Assmann & Shimazaki, 1999; Roelfsema & Hedrich, 2005; Hiyama et al., 2017; Inoue & Kinoshita, 2017; Matthews et al., 2020).

Blue light is among the most effective triggers of stomatal opening, especially when superimposed on a strong background of red light (Shimazaki et al., 2007). Blue light at low fluence rates (5 – 10 µmol m$^{-2}$ s$^{-1}$) acts as a signal evoking stomatal opening through the initiation of a specific guard cell blue light signaling cascade (Shimazaki et al., 2007; Inoue et al., 2010; Kollist et al., 2014). The blue light response of guard cells is of special importance during stomatal opening at dawn, facilitating photosynthesis when the light spectrum is enriched in blue wavelengths (Zeiger, 1984). Blue light is perceived by the two blue light photoreceptor protein kinases Phototropins 1 and 2 (PHOTs), initiating their activation through autophosphorylation (Fig. 6; Kinoshita et al., 2001). The phosphorylation of two Ser resides within the kinase domain of the PHOT proteins is required for downstream signaling, likely through substrate recognition (Inoue et al., 2008, 2010). The protein kinase Blue Light Signaling 1 (BLUS1; Takemiya et al., 2013a) is directly phosphorylated by the two PHOTs (Fig. 6) and from there the signal is transmitted to a type 1 Protein...
Phosphatase (PP1) through a yet elusive way (Takemiya et al., 2006). The serin/threonine kinase PP1 is comprised of a catalytic subunit (PP1c) and a regulatory subunit PP1 regulatory subunit2-like protein 1 (PRSL1), which is required for the blue light response of guard cells (Fig. 6; Takemiya et al., 2013b). Whereas the PHOTs are involved in other blue light-initiated processes in the plant, such as phototropism (Christie & Briggs, 2001; Christie, 2007), photomorphogenesis (Briggs & Olney, 2001), flowering (Banerjee & Batschauer, 2005; Hart et al., 2019) and chloroplast movements (Banaś et al., 2012), BLUS1 is specifically expressed in guard cells and hence thought to confer signal specificity (Inoue & Kinoshita, 2017). Ultimately, a plasma membrane H⁺-ATPase gets activated through phosphorylation of a penultimate Thr residue in the C-terminus and subsequent binding of a 14-3-3 protein downstream of PP1 (Fig. 6; Kinoshita & Shimazaki, 1999, 2002).

![Image](image.png)

Figure 6. Stomatal opening in response to blue and red light. Figure is taken from Matthews et al., (2020). For details see text.
INTRODUCTION

Work from this earlier this year revealed that high temperature signaling in guard cells requires several components of the blue light-signaling cascade, suggesting cross talk among temperature and light signaling. In particular, the authors proposed a mechanism, which involves phototropin-mediated activation of the H⁺-ATPase both in a BLUS1-dependent and -independent manner (Kostaki et al., 2020). Similarly, the pathways of blue light and low CO₂-induced stomatal opening have been reported to converge. Hiyama et al., (2017) demonstrated that the two kinases Convergence of Blue Light and CO₂ 1 and 2 (CBCs) interact with both PHOTs and that PHOT1 phosphorylates CBC1 in vitro suggesting that they are part of the blue light signal cascade in guard cells (Fig. 6). More so, both CBCs interact with and get phosphorylated by the kinase High Temperature 1 (HT1; Hashimoto et al., 2006), which is required to respond to low CO₂ concentrations (Fig. 6). In both signaling pathways the phosphorylation of CBCs ultimately leads to the inhibition of S-type anion channels required for stomatal closure (Fig. 6; Hiyama et al., 2017), thereby promoting the opening of the stomatal pore.

The Arabidopsis genome encodes 11 genes for the H⁺-ATPase (AHAs; Falhof et al., 2016). All of them are expressed in guard cells with high expression levels of AHA1, AHA2 and AHA5 (Ueno et al., 2005). However, AHA1 was shown to be the main isoform involved in stomatal movement regulation while AHA2 and AHA5 do not play essential roles in this process (Yamauchi et al., 2016). Recent work further revealed a new component of the blue light signaling pathway, a Raf-like protein kinase Blue Light-Dependent H⁺-ATPase Phosphorylation (BHP; Fig. 6). BHP directly binds to BLUS1 to form a complex, which is required for the activation of the proton pump, however BHP likely does not directly phosphorylate the H⁺-ATPase (Hayashi et al., 2017). The activation of the proton pump results in the ATP-consuming translocation of large quantities of H⁺ across the plasma membrane leading to the hyperpolarization of the membrane (Fig. 6; Roelfsema et al., 2001). This membrane hyperpolarization provides the driving force for the uptake of osmotically active solutes, such as K⁺, Cl⁻, NO₃⁻, malate²⁻ and sugars (Fig. 7).
Guard cells express at least five genes coding for K\textsuperscript{+} in channels, including the Shaker-type major guard cell K\textsuperscript{+} channel KAT1 (Fig. 7; Schachtman et al., 1992; Nakamura et al., 1995) and its close homolog KAT2 (Pilot et al., 2001; Lebaudy et al., 2008), the high-affinity HAK-type K\textsuperscript{+}-H\textsuperscript{+} symporters (Fig. 7; Rodriguez-Navarro et al., 1986; Blatt & Slayman, 1987) and finally the two homologs AKT1 and AKT2 (Lebaudy et al., 2008; Chen & Blatt, 2010). K\textsuperscript{+} in channels are regulated by membrane voltage, pH, concentrations of K\textsuperscript{+}, H\textsuperscript{+} and Ca\textsuperscript{2+}, GTP-binding proteins and phosphorylation (Thiel & Wolf, 1997). Indeed, a recent study suggests that the blue light activation of K\textsuperscript{+} in channels directly depends on their phosphorylation by a Calcineurin B-Like Interacting Protein Kinase 23 (CIPK23; Inoue et al., 2020). The increased uptake of K\textsuperscript{+} feeds directly into their transport across the tonoplast via mass action as K\textsuperscript{+} the concentration in the cytosol increases (Jezek & Blatt, 2017). K\textsuperscript{+} uptake into the vacuole occurs against both a concentration gradient and the electrical potential; therefore, the transport of K\textsuperscript{+} happens in antiport with H\textsuperscript{+} through the K\textsuperscript{+}, Na\textsuperscript{+}/ H\textsuperscript{+} exchangers NHX1 and NHX2 (Barragán et al., 2012; Andrés et al., 2014).

The uptake of anions into guard cells also occurs against the concentration gradient and the membrane potential and therefore is likely to happen in symport with H\textsuperscript{+}. To date, the Arabidopsis Nitrate Transporter 1.1 (NRT1.1) has been identified that promotes NO\textsubscript{3}\textsuperscript{-} uptake to guard cells (Guo et al., 2003), but no such transporter is known for Cl\textsuperscript{-} (Kollist & Nuhkat, 2014). The ATP-Binding Cassette (ABC) Transporter B14 mediates the intake of apoplastic malate\textsuperscript{2-} in symport with H\textsuperscript{+} and at the expense of ATP (Lee et al., 2008). The uptake of Cl\textsuperscript{-} into the vacuole is mediated by both the malate-activated ALMT9 (De Angeli et al., 2013) and the Chloride Channel c (CLCc; Jossier et al., 2010). Transporters for malate\textsuperscript{2-} at the vacuolar membrane remain
INTRODUCTION

unknown. Although sugar accumulation during stomatal opening has been observed (Tallman & Zeiger, 1988; Talbott & Zeiger, 1996; Olsen et al., 2002), the existence and identity of guard cell plasma membrane and vacuolar sugar transporters were entirely unclear prior to work done in this thesis. The vacuolar uptake of ions and other osmotically active solutes decreases the water potential of guard cells causing the inflow of water leading to stomatal opening (Eisenach & De Angeli, 2017; Jezek & Blatt, 2017; Inoue & Kinoshita, 2017; Matthews et al., 2020).

In parallel to the activation of membrane ion transport through the H⁺-ATPase, guard cell starch is broken down to provide carbon skeletons for energetic purposes and/or the synthesis of malate\(^2\)− and sugars (Fig. 6). This process is an integral part of the blue light signaling cascade, as the activity of the H⁺-ATPase is required for the stimulation of the starch degrading enzymes (Horrier et al., 2016). Moreover, a study from this year demonstrated that stomatal starch degradation is also induced by the phytohormone brassinosteroid (BR) through hydrogen peroxide (H\(_2\)O\(_2\)) signaling promoting stomatal opening (Li et al., 2020). This study corroborates the earlier report about BR signaling inducing stomatal opening, where BR synthesis mutants were found to exhibit normal H⁺-ATPase activities, but suffered from reduced K⁺ uptake (Inoue et al., 2017).

Another source of energy during stomatal opening is the blue light-mediated β-oxidation of lipids. Triacylglycerols stored in lipid droplets are degraded to provide ATP (McLachlan et al., 2016). The low number of chloroplasts found in guard cells and the elevated contents of mitochondria, along with the high respiratory rates, led to the hypothesis that guard cell mitochondria provide the main bulk of energy for stomatal opening (Fig. 6; Shimazaki et al., 1983, 2007; Vani & Raghavendra, 1994; Willmer & Fricker, 1996). More so, if mitochondrial respiration is repressed through oligomycin, KCN or low O\(_2\) concentrations, guard cell ATP levels drop and subsequently blue light-mediated proton pumping, and stomatal opening is reduced (Shimazaki et al., 1983; Zeiger, 1984; Gautier et al., 1991; Mawson, 1993). A bulk of recent studies further demonstrated that both malate\(^2\)− and sucrose are degraded within guard cells to fuel the fluxes through the mitochondrial Tricarboxylic Acid (TCA) cycle, yielding ATP and NADH (Daloso et al., 2016; Antunes et al., 2017; Robaina-Estévez et al., 2017; Medeiros et al., 2018). In addition, linear electron transport within guard cell chloroplasts has been reported to take place (Hipkins et al., 1983; Shimazaki & Zeiger, 1985; Lawson et al., 2002, 2003) and was suggested to provide ATP and NADPH for blue light-induced stomatal opening (Tominaga et al., 2001; Suetsumg & et al., 2014; Azoulay-Shemer et al., 2015). However, the capacity of guard cells for electron
transport and photosynthetic carbon fixation is still debated due to contradictory literature and remains one of the big open questions concerning stomatal movements.

In contrast to the blue light response, the receptor the guard cell red light response is still elusive (Fig. 6). Chlorophyll was put forward as an acceptor, given that red light-induced stomatal opening can be abolished by the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), suggesting that it depends on photosynthesis (Fig. 6; Sharkey & Raschke, 1981; Assmann & Shimazaki, 1999; Tominaga et al., 2001; Messinger et al., 2006). Moreover, there is no consensus about the location of the red light response – guard cells (Olsen et al., 2002) or the mesophyll cells (Lawson et al., 2014). Red light is assumed to link mesophyll photosynthesis to stomatal regulation via CO$_2$ signaling (Fig. 6; Mott, 1988; Roelfsema et al., 2002). This idea is further supported by a study demonstrating that stomatal opening to red light partly depends on components of the CO$_2$ signaling pathway in guard cells (Fig. 6; for details see section about stomatal closure; Matrosova et al., 2015). However, in parallel, evidence accumulated against CO$_2$ being the main signal of the red light response (Raschke, 1975; Sharkey & Raschke, 1981; Baroli et al., 2008; Lawson et al., 2008). Research over the past 30 years yielded a considerable amount of putative signals coordinating photosynthesis and stomatal behavior under red light, including Ribulose Bisphosphate (RuBP), ATP, NADPH, malate, and sugars (Lee & Bowling, 1992; Hedrich & Marten, 1993; Hedrich et al., 1994; Lee Joon-Sang & Bowling, 1995; Lu et al., 1997; Tominaga et al., 2001; Outlaw, Jr., 2003; Kang et al., 2007). Equally to blue light-induced stomatal opening, the red light-mediated changes in aperture require the uptake and release of osmolytes. Early studies revealed that K$^+$ accumulates as a result of H$^+$-ATPase activity under red light (Fig. 6; Serrano et al., 1988; Olsen et al., 2002). Subsequent patch clamp experiments could not replicate the red light-driven activation of the H$^+$-ATPase (Taylor & Assmann, 2001). However, the use of a newly developed immunohistochemical technique to detect phosphorylation levels of the H$^+$-ATPase revealed the red light-dependent phosphorylation of the H$^+$-ATPase in guard cells (Fig. 6; Ando & Kinoshita, 2018, 2019). Besides K$^+$, sugars were reported to accumulate upon red light exposure (Talbott & Zeiger, 1993), which could be either provided through starch degradation (Fig. 6; Outlaw & Manchester, 1979), imported from the mesophyll cells (Fig. 6; Lu et al., 1995) or through guard cell photosynthesis (Fig. 6; Lawson, 2009). Likely starch is not degraded to provide sugars due to red light illumination, as in a recent report Horrer et al. (2016) showed that red light rather promotes the accumulation of starch in guard cells (Horrer et al., 2016). The contribution of the other two proposed pathways remains unknown. Prior to the work done in this thesis, no guard cell sugar found to play a role in red light response. However, in parallel, evidence accumulated against CO$_2$ being the main signal of the red light response (Raschke, 1975; Sharkey & Raschke, 1981; Baroli et al., 2008; Lawson et al., 2008). Research over the past 30 years yielded a considerable amount of putative signals coordinating photosynthesis and stomatal behavior under red light, including Ribulose Bisphosphate (RuBP), ATP, NADPH, malate, and sugars (Lee & Bowling, 1992; Hedrich & Marten, 1993; Hedrich et al., 1994; Lee Joon-Sang & Bowling, 1995; Lu et al., 1997; Tominaga et al., 2001; Outlaw, Jr., 2003; Kang et al., 2007). Equally to blue light-induced stomatal opening, the red light-mediated changes in aperture require the uptake and release of osmolytes. Early studies revealed that K$^+$ accumulates as a result of H$^+$-ATPase activity under red light (Fig. 6; Serrano et al., 1988; Olsen et al., 2002). Subsequent patch clamp experiments could not replicate the red light-driven activation of the H$^+$-ATPase (Taylor & Assmann, 2001). However, the use of a newly developed immunohistochemical technique to detect phosphorylation levels of the H$^+$-ATPase revealed the red light-dependent phosphorylation of the H$^+$-ATPase in guard cells (Fig. 6; Ando & Kinoshita, 2018, 2019). Besides K$^+$, sugars were reported to accumulate upon red light exposure (Talbott & Zeiger, 1993), which could be either provided through starch degradation (Fig. 6; Outlaw & Manchester, 1979), imported from the mesophyll cells (Fig. 6; Lu et al., 1995) or through guard cell photosynthesis (Fig. 6; Lawson, 2009). Likely starch is not degraded to provide sugars due to red light illumination, as in a recent report Horrer et al. (2016) showed that red light rather promotes the accumulation of starch in guard cells (Horrer et al., 2016). The contribution of the other two proposed pathways remains unknown. Prior to the work done in this thesis, no guard cell sugar
transporters were characterized for the uptake of mesophyll-derived sugars in Arabidopsis. Similarly, the function and contribution of the guard cell chloroplasts to both carbon and energy metabolism is still unclear.

**Closing the gap – mechanisms of stomatal closure**

Stomatal closure is evoked by darkness, low humidity, high CO$_2$ concentrations, phytohormones and plant pathogens (Kollist *et al*., 2014; Jezek & Blatt, 2017). Among these factors, the plant hormone abscisic acid (ABA) is one of the most efficient in triggering the closure of stomata. ABA is known as a central player in the plant’s defense against drought, salinity and cold stress (Zhu, 2016). In the 1960s, ABA was first recognized as a factor mediating stomatal closure (Mittelheuser & van Steveninck, 1969; Wright & Hiron, 1969). At that time, the general opinion was that stomatal opening is an active process requiring coordination and energy for transport, whereas stomatal closure works passively (Jezek & Blatt, 2017). Flux studies with $^{86}$Rb$^+$ (MacRobbie, 1981) along with the observation of K$^+$ efflux from guard cells during stomatal closure (Schroeder *et al*., 1987) turned this opinion on its head.

Even though the synthesis of ABA mainly occurs in vascular tissues of roots and leaves, ABA is known to move inside the plant to its sites of action, including the guard cells (Boursiac *et al*., 2013). Interestingly, guard cells seem also capable of autonomous ABA synthesis, as demonstrated by the fact that the entire set of genes related to ABA de novo biosynthesis is expressed in these cells. More so, the wilting phenotype of the ABA synthesis mutant aba3-1 could be complemented by guard cell-specific expression of ABA3, demonstrating that guard cell autonomous ABA synthesis occurs (Bauer *et al*., 2013). The movement of ABA throughout the plant requires its translocation across membranes. Several ABA transporter proteins are encoded by plants, including the ATP-binding Cassette (ABC) Transporters G25 and G40 (Kang *et al*., 2010; Kuromori *et al*., 2010) and the NPF4.6, a member of the Nitrate Transporter 1/Peptide Transporter (NTR1/PTR) Family (NPF; Kanno *et al*., 2012).
ABA is perceived by cytosolic proteins acting as ABA receptors (Fig. 4), the Pyrabactin Resistance 1/PYR1-like/Regulatory Components of the ABA Receptors (PYR/PYL/RCAR; Ma et al., 2009; Park et al., 2009). Upon binding of ABA, PYR/PYL/RCAR mediates the inhibition of A type 2C Protein Phosphatases (PP2C; Melcher et al., 2009; Nishimura et al., 2009). Increasing levels of ABA therefore abolish the PP2C-mediated repression of Sucrose Non-Fermenting 1 (SNF1)-Related protein Kinase 2 (SnRK2; Soon et al., 2012), which gets activated upon phosphorylation (Fig. 4). Open Stomata 1 (OST1/SnRK2.6) is the main SnRK2 kinase family member expressed in guard cells (Mustilli et al., 2002; Yoshida et al., 2002). OST1 is known to phosphorylate several downstream target proteins, including ion channels and transcription factors, thereby controlling nuclear responses (Fig. 4; Sirichandra et al., 2010). Other PP2C-interacting proteins were found in guard cells, such as the Calcium-Dependent Protein Kinases (CDPKs) and Calcineurin B-like Interacting Protein Kinases (CIPKs; Fig. 4; Cotelle & Leonhardt, 2019). Upon ABA perception, OST1 activates the R-type and S-type anion channels that cause membrane depolarization and subsequent export of K⁺ through K⁺out channels including GORK (Fig. 4; Hosy et al., 2003). Moreover, OST1 inhibits the activity of the K⁺in channels KAT1 and the two K⁺ Uptake transporters 6 and 8 (KUPs; Osakabe et al., 2013). The S-type channel Slow activating Anion Channel 1 (SLAC1) and its close homolog SLAC1 Homolog 3 (SLAH3) mediate the export of Cl⁻ and nitrate (NO₃⁻; Negi et al., 2008; Geiger et al., 2009, 2011; Brandt et al., 2012). Moreover, recent work...
demonstrated that the anion channels SLAC1 and SLAH3 prevent stomatal opening through protein-protein interactions with KAT1, inhibiting the $K^{+}$ channel in a yet unknown manner. This way ABA-induced stomatal closure is directly linked to the inhibition of stomatal opening, providing a fast and energy-efficient mechanism of closure (Fig. 4; Zhang et al., 2016). The guard cell R-type anion channel is encoded by a member of the Aluminum-activated Malate Transporter (ALMT) family, ALMT12, also referred to as Quick activating Anion Channel (QUAC) and is associated to malate efflux (Sasaki et al., 2010; Meyer et al., 2010).

Rises in ABA levels also result in the accumulation of cytosolic Ca$^{2+}$ and Reactive Oxygen Species (ROS). OST1 phosphorylates the plasma membrane-localized NADPH oxidases Respiratory Burst Oxidase Homolog D and F (RBOHs) evoking ROS bursts (Sirichandra et al., 2009; Acharya et al., 2013), which in turn activate Ca$^{2+}$ channels (Schroeder & Hagiwara, 1990; Murata et al., 2001; Mori & Schroeder, 2004). The raise in cytosolic Ca$^{2+}$ further promotes the activity of SLAC1 through CDPKs (or CPKs; Geiger et al., 2010; Brandt et al., 2012; Scherzer et al., 2012) and inhibits the activity of the $H^{+}$-ATPase (Kinoshita et al., 1995) and $K^{+}$ channels (Fig. 4; Grabov & Blatt, 1999). Interestingly, the activation of anion channels also occurs without elevated cytosolic Ca$^{2+}$ concentrations (Marten et al., 2007), but Ca$^{2+}$ enhances their efficiency (Huang et al., 2019). Therefore, ABA signaling occurs via a Ca$^{2+}$-independent pathway involving OST1, which is modulated by a Ca$^{2+}$-dependent mechanisms (Cotelle & Leonhardt, 2019; Huang et al., 2019). The apoplastic ROS generated by RBOHs are transported passively to the cytosol by the membrane water channels aquaporin PIP2;1 (Rodrigues et al., 2017). Moreover, a leucine-rich repeat receptor-like kinase (LRR-RLK) Guard cell Hydrogen peroxide-Resistant 1 (GHR1) was identified as a key component in sensing apoplastic ROS (Fig. 4; Hua et al., 2012).

Early studies demonstrated that ABA can enhance CO$_2$-evoked stomatal closing and vice versa (Raschke, 1975; Raschke et al., 1976). Elevated CO$_2$ concentrations lead to the activation of anion channels causing stomata to close (Brearley et al., 1997; Raschke et al., 2003). Work with Arabidopsis mutants affected in components of the ABA signal transduction pathway including $slac1$ and $ost1$ reaveled that on top of their insensitivity to ABA they were also impaired in responding to CO$_2$, indicating that some components are shared among the signaling pathways (Leymarie et al., 1998; Israelsson et al., 2006; Kim et al., 2010). Recently, a pathway of guard cell CO$_2$ signaling was revealed, suggesting that ABA and CO$_2$-signals converge at the activation of OST1 (Tian et al., 2015). Although the CO$_2$ sensor in
guard cells remains elusive, recent work has demonstrated that the aquaporin PIP2;1 facilitates the uptake of CO$_2$ into guard cells (Fig. 5; Wang et al., 2016).

CO$_2$ and water are then converted into bicarbonate (HCO$_3^-$) by β-carboxy anhydrases 1 and 4 (βCAs; Fig. 5; Hu et al., 2010; Xue et al., 2011). βCA4 was also shown to directly interact with PIP2;1 thereby promoting the uptake of CO$_2$ (Wang et al., 2016). Thereafter, high levels of HCO$_3^-$ are sensed by a Multidrug And Toxic compound Extrusion (MATE)-like protein Resistant to High Carbon dioxide 1 (RHC1; Tian et al., 2015) inhibiting the downstream protein kinase High Temperature 1 (HT1; Fig. 5). HT1 itself negatively regulates OST1. Hence, RHC1-mediated inhibition of HT1 allows the activation of S-type anion channels such as SLAC1 (Fig. 5; Hashimoto et al., 2006). Alternatively, downstream of CO$_2$, the Mitogen Activated Protein Kinases 4 and 12 (MPKs) form an essential node for CO$_2$-induced stomatal closure (Fig. 5). Their activity was found to be highly specific to the response evoked by CO$_2$, whereas they are not required for stomatal closure induced by ABA, low humidity and ozone. MPKs further inhibit the activity of HT1 resulting in anion channel activation (Marten et al., 2008;
Jacóbson et al., 2016; Hórak et al., 2016; Töldsepp et al., 2018). Recently, two more kinases were found to directly interact with HT1, the activity of the Convergence of Blue light and CO₂ 1 and 2 (CBCs) gets modulated upon phosphorylation by HT1 (Fig. 5; Hiyama et al., 2017) resulting in the repression of SLAC1 in an unknown manner (Fig. 5). Another new player in guard cell CO₂ signaling was identified with the BIG protein, which distinguishes the processes of CO₂-induced stomatal closure and inhibition of stomatal opening by CO₂. BIG mediates S-type anion channel activation by bicarbonate (He et al., 2018).

Many critical components of CO₂-induced stomatal closure have been elucidated over the last years, however, the exact mechanisms and sequence of events remain unknown. Most importantly, recent work indicates that ABA and CO₂ signaling converge downstream of OST1. Hsu et al., (2018) showed that high CO₂-induced stomatal closure is not mediated by a rapid increase in ABA concentrations and OST1 kinase activity is not induced upon exposure to high CO₂ concentrations (Hsu et al., 2018). More so, a report published earlier this year further corroborates that OST1 does only get activated by ABA and not by high CO₂ concentrations. Using a FRET kinase sensor, SNACS, the authors highlighted that basal SnRK2 activity functioning in parallel to the CO₂ signal transduction pathway is needed for and/or enhances stomatal CO₂ signaling (Zhang et al., 2020). Therefore, future research will be required to disentangle if and where the ABA and CO₂ responses converge.

Stomatal closure also requires the efflux of ions from the guard cell vacuoles. A channel mediating K⁺ efflux from the vacuole is a member of the Two-Pore K⁺ (TPK) family (Gobert et al., 2007; Latz et al., 2007). TPK1 is regulated by Ca²⁺, 14-3-3 proteins and gets phosphorylated by the protein kinase KIN7 that mediates the activation of K⁺ channels during stomatal closure (Gobert et al., 2007; Latz et al., 2007; Isner et al., 2018). ALMT4, an anion channel required for malate export from the vacuole, was recently identified to function during ABA-induced stomatal closure, but not during darkness (Eisenach et al., 2017). Other processes occurring at the guard cell tonoplast during stomatal closure are currently uninvestigated, such as the release of other anions (e.g. Cl⁻ and NO₃⁻) or sugars. Moreover, the fate of the released solutes from the guard cell vacuoles – export from guard cells or metabolism within guard cells - is not fully resolved.
INTRODUCTION

REFERENCES


INTRODUCTION


INTRODUCTION


INTRODUCTION


INTRODUCTION


INTRODUCTION


INTRODUCTION


INTRODUCTION


26
INTRODUCTION


INTRODUCTION


Plants experience changes in environmental factors including CO$_2$ concentrations, water availability as well as fluctuating light intensities and qualities, modulating their productivity. In order to respond to these factors, plants have acquired active control of gaseous exchange between the leaf interior and the surrounding atmosphere controlling photosynthesis and water loss. Stomata, delimited by a pair of guard cells, are the master regulators governing the exchange of gases with the atmosphere. However, stomatal responses to dynamic changes in the environment and the process of photosynthesis are often not synchronous resulting in reduced water use efficiency (WUE) and biomass production. This is especially concerning species with C3 photosynthesis and kidney-shaped stomata, to which some of the worldwide most cultivated crops belong. Oowed to the increasingly unpredictable patterns of rainfall, extended periods of drought and the need to increase crop yields, stomata represent major targets to efforts in improving photosynthesis and water use and the coordination of these two processes. Stomatal movements depend on a complex network of interactions among membrane ion transport, metabolism and solute partitioning. However, our knowledge of the exact mechanisms underlying this network and the metabolic rearrangements required for stomatal movements are still fragmentary.

Recent work revealed that guard cell starch is degraded upon blue light illumination to promote stomatal opening at dawn. This process is induced upon the activation of an upstream plasma membrane H$^+$-ATPase and therefore intimately integrates with membrane ion transport. Nevertheless, the exact mechanisms underlying this interplay of metabolism and ion translocation are unknown.

Guard cells possess many characteristics of heterotrophic tissues and therefore it has long been hypothesized that their metabolism largely relies on substrates derived from mesophyll photosynthesis. Early biochemical studies have shown the uptake of sugars into guard cells, however the nature of the involved transporters remained elusive. More so, guard cell starch synthesis is poorly understood and whether this process requires the import of mesophyll-derived carbon substrates is unclear. Finally, recent research suggests that guard cell starch synthesis plays a major role during stomatal closure acting as a sink for osmolytes, which have been sequestered in the guard cell vacuoles earlier during the day to
promote the opening of stomata. Yet, additional experimental support for these findings is lacking and the molecular course of events is uninvestigated.

During my PhD work seven main question arose which are the basis of the seven chapters presented in this thesis. The main aims were to address these questions to provide a better understanding of the physiological role of both guard cell starch degradation and synthesis and to identify possible target genes for a future manipulation of the trade-off regarding stomatal movements and photosynthesis.

**Regarding the role of guard cell starch degradation:**

i) How does guard cell starch degradation impact on stomatal opening? And how does it integrate with membrane ion transport?

**Regarding mesophyll-derived sugars:**

ii) Are mesophyll sugars required for stomatal functioning? And does guard cell starch synthesis depend on the uptake of such sugars?

iii) What is the fate of sugars within guard cells?

**Regarding starch synthesis:**

iv) Which additional carbon sources are used for starch synthesis? And are vacuolar carbons converted into starch during stomatal closure?

v) Which enzymes are involved in the biosynthetic pathway of starch in guard cells?

**Regarding guard cell energy production:**

vi) How do guard cell chloroplasts obtain energy?

**Regarding stomatal movements in other species:**

vii) How are stomatal movements achieved in a CAM species? Therefore, *Kalanchoe fedtschenkoi* was used as a model system.
CHAPTER I

HOW DOES GUARD CELL STARCH DEGRADATION IMPACT ON STOMATAL OPENING?

Guard Cell Starch Degradation Yields Glucose for Rapid Stomatal Opening in Arabidopsis

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ABSTRACT

Starch in Arabidopsis (Arabidopsis thaliana) guard cells is rapidly degraded at the start of the day by the glucan hydrolases α-AMYLASE3 (AMY3) and β-AMYLASE1 (BAM1) to promote stomatal opening. This process is activated via phototropin-mediated blue light signaling downstream of the plasma membrane H⁺-ATPase. It remains unknown how guard cell starch degradation integrates with light-regulated membrane transport processes in the fine control of stomatal opening kinetics. We report that H⁺, K⁺, and Cl⁻ transport across the guard cell plasma membrane is unaltered in the amy3 bam1 mutant, suggesting that starch degradation products do not directly affect the capacity to transport ions. Enzymatic quantification revealed that after 30 min of blue light illumination, amy3 bam1 guard cells had similar malate levels as the wild type, but had dramatically altered sugar homeostasis, with almost undetectable amounts of Glc. Thus, Glc, not malate, is the major starch-derived metabolite in Arabidopsis guard cells. We further show that impaired starch degradation in the amy3 bam1 mutant resulted in an increase in the time constant for opening of 40 min. We conclude that rapid starch degradation at dawn is required to maintain the cytoplasmic sugar pool, clearly needed for fast stomatal opening. The conversion and exchange of metabolites between subcellular compartments therefore coordinates the energetic and metabolic status of the cell with membrane ion transport.
CHAPTER I

INTRODUCTION

Stomata are microscopic pores in the plant epidermis bounded by a pair of guard cells. The appearance of stomata was a major evolutionary innovation for the transition of plants to life on land (Hetherington and Woodward, 2003; Berry et al., 2010); they interrupt the impermeable waxy cuticle and open to facilitate CO\textsubscript{2} diffusion into the leaves for photosynthesis (CO\textsubscript{2} assimilation, $A$). This process, however, also allows water to diffuse out of the leaf through the evapotranspiration stream, risking desiccation. The capacity of stomata to enable CO\textsubscript{2} uptake or water loss is known as stomatal conductance ($g_s$) and measured as a mole flux per unit area (mol m\textsuperscript{-2} s\textsuperscript{-1}). To optimize daytime water use efficiency (WUE; amount of carbon fixed per unit water loss, $A$/evapotranspiration) and survive the harsh terrestrial environment, plants have evolved the capacity to actively control the stomatal pore aperture and change $g_s$ in response to fluctuating environmental conditions (Haworth et al., 2011). Plants generally open their stomata (increase in $g_s$) in response to light and low CO\textsubscript{2} concentrations, while they close them (decrease in $g_s$) in darkness, in response to high CO\textsubscript{2} concentrations, and under adverse environmental conditions (Murata et al., 2015).

In the steady state, changes in $A$ are often strongly associated with $g_s$ dynamics, leading to a near-optimal balance of carbon gain and water loss (Wong et al., 1979). In fluctuating environments, however, stomatal responses to changing conditions, especially light and temperature, are generally slower than photosynthetic responses (Lawson and Blatt, 2014; Lawson and Vialt-Chabrand, 2019). For example, upon changes in photosynthetic photon flux density during sun/shade flecks caused by passing clouds or overlapping leaves in a canopy, $A$ adapts quickly by reaching a new steady state within several tens of seconds to minutes, whereas changes in $g_s$ can take minutes to hours (Barradas and Jones, 1996; Ooba and Takahashi, 2003; Vico et al., 2011; McAusland et al., 2016; Vialt-Chabrand et al., 2016; Lawson and Vialt-Chabrand, 2019). Despite considerable variation in the magnitude and time scales of opening and closing responses across species and environmental conditions (Barradas and Jones, 1996; Vico et al., 2011; McAusland et al., 2016; Qu et al., 2016), stomatal delays to light fluctuations have a well-documented impact on the economics of leaf gas exchange, with important implications in terms of $A$ and transpiration and, hence, leaf WUE (Naumburg et al., 2001; Lawson and Blatt, 2014; Vialt-Chabrand et al., 2017b; Lawson and Vialt-Chabrand, 2019).

Many studies have explored stomatal anatomy, size, and density as strategies for increasing or decreasing $g_s$, based on the assumption that high densities of small
stomata can alter aperture faster than fewer, larger stomata (Hetherington and Woodward, 2003; Franks and Beerling, 2009; Drake et al., 2013; Raven, 2014). These approaches have often met with limited success. Modifications of stomatal density that result in gain in A through increases in gs can occur at the expense of WUE (Tanaka et al., 2013). Furthermore, manipulation of physical attributes may be counterbalanced by unpredicted modifications in function (Büssis et al., 2006). This holds true especially for species with elliptical (or kidney-shaped) guard cells, such as Arabidopsis spp. and many crop plants, in which differences in gs responses cannot simply be explained by the size of stomata (Elliott-Kingston et al., 2016; McAusland et al., 2016).

A less obvious and a relatively unexplored approach exploits stomatal movement kinetics to facilitate coordinated gs responses with mesophyll demands for CO2 (Viallet-Chabrand et al., 2017a). Modeled synchronous behavior in gs and A in Phaseolus vulgaris subjected to dynamic light has been shown to theoretically increase WUE by 20% (Lawson and Blatt, 2014; Lawson and Viallet-Chabrand, 2019). Furthermore, optogenetic manipulation of stomatal kinetics by expression of the synthetic, light-gated potassium (K+) channel BLINK1 in Arabidopsis guard cells demonstrated a 2-fold enhancement of WUE (Papanatsiou et al., 2019). These gains, in turn, would substantially boost plant growth and yield. A current limitation of this strategy is that a full mechanistic understanding of the molecular components determining gs kinetics is still lacking.

Stomatal opening is powered by the blue light (BL)-activated H+-ATPase (AHA1) at the guard cell plasma membrane (PM), which hyperpolarizes the membrane potential by pumping H+ out of the guard cells (Assmann et al., 1985; Shimazaki et al., 1986). The hyperpolarization drives K+ uptake through the inward-rectifying K+ channels, with accumulation of malate2− (Mal), chloride (Cl−), and nitrate, driving changes in cell turgor and stomatal aperture (Inoue and Kinoshita, 2017; Jezek and Blatt, 2017). BL simultaneously inhibits the S-type anion channel activities via CONVERGENCE OF BL AND CO2 protein kinases to support stomatal opening (Marten et al., 2007; Hiyama et al., 2017).

In parallel to the activation of membrane ion transport, starch in guard cell chloroplasts is degraded within the first hour of light, contributing to a rapid increase in stomatal aperture (Horrer et al., 2016). In the amy3 bam1 double mutant, which lacks the glucan hydrolases β-amylase1 (BAM1) and α-amylase3 (AMY3) needed to break down this starch, stomata open more slowly and to a lesser extent (Horrer et al., 2016). Early studies hypothesized that carbon skeletons derived from starch degradation are used to synthesize Mal. This hypothesis is based on experiments
linking changes in guard cell protoplast (GCP) volume to changes in Mal content (Schnabl, 1980a; Schnabl et al., 1982) as well as loss of starch in guard cells in the light (Lloyd, 1908; Outlaw and Manchester, 1979).

Interestingly, starch degradation is triggered by low levels of BL through the phototropin-mediated signaling cascade (Tallman and Zeiger, 1988; Horrer et al., 2016). Arabidopsis mutants lacking AHA1 also show defective guard cell starch degradation, indicating a coordinate requirement for the PM H⁺-ATPase (Horrer et al., 2016). This previously unexpected connection between light-regulated membrane ion transport and guard cell starch metabolism prompted us to investigate how these processes integrate in the fine control of stomatal opening kinetics. We found that H⁺, K⁺, and Cl⁻ transport across the guard cell PM is unaltered in the Arabidopsis stomatal starch-degrading mutant amy3 bam1, suggesting that starch degradation products do not directly affect the capacity to transport ions. Despite the long-held view that Mal derives from starch degradation, we observed that Mal levels in guard cells of amy3 bam1 were similar to those of the wild type, whereas Glc levels were greatly reduced. Rapid starch degradation at dawn is therefore very likely required to maintain sugar homeostasis during stomatal opening. By comparing gs kinetics with guard cell starch dynamics in plants subjected to pulses of light and darkness under common light growth conditions (150 µmol m⁻² s⁻¹), we further show that the amounts of starch and the ability to promptly break it down upon transition to light are associated with fast stomatal opening kinetics. Impaired guard cell starch degradation in the amy3 bam1 or aha1 mutant caused up to 40 min delay to reach 63% of the maximal gs amplitude compared with the wild type, which was not linked with stomatal size and density. In response to pulses of higher light intensity (400 µmol m⁻² s⁻¹) or in response to red light (RL; 300 µmol m⁻² s⁻¹), under which the plant is photosynthesis-saturated, gs kinetics did not depend on starch degradation. Finally, we provide evidence that fast gs kinetics under RL are primarily driven by photosynthetic production of Suc in the mesophyll and the import to guard cells, as energized by the PM H⁺-ATPase AHA1. Our findings provide insights into the molecular mechanisms determining fast stomatal opening kinetics to light in Arabidopsis, showing that they depend on a tight coordination between membrane ion transport and metabolic rearrangements.
RESULTS

BL-Induced Proton Pumping, K\(^+\) and Cl\(^-\) Channel Activities Are Unaltered in *amy3 bam1* Mutants

The major starch-degrading enzyme in Arabidopsis guard cells is BAM1, an exoamylase that attacks the nonreducing end of the glucan chains to release maltose. Upon illumination, BAM1 rapidly mobilizes starch in conjunction with the chloroplastic AMY3, an endoamylase that hydrolyzes \(\alpha\)-1,4 bonds within glucan chains. Simultaneous loss of BAM1 and AMY3 in the *amy3 bam1* double mutant leads to elevated guard cell starch levels throughout the diurnal cycle, severely affecting stomatal opening (Horrer *et al*., 2016). Guard cell starch is thought to be converted to Mal (Raschke and Schnabl, 1978; Schnabl, 1980a). Therefore, we hypothesized that reduced Mal synthesis and H\(^+\) accumulation associated with the inhibition of starch degradation in *amy3 bam1* would suppress H\(^+\)-ATPase activity and reduce membrane voltage, and consequently limit the driving force for ion uptake, potentially explaining the defective stomatal opening response of *amy3 bam1* (Blatt, 2016).

To test this hypothesis, we first examined the activity of the H\(^+\)-ATPase in GCPs in response to BL illumination (10 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) BL superimposed to 50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) RL). Recordings of H\(^+\) pumping in wild-type GCPs yielded an average H\(^+\) flux of 0.72 nmol h\(^{-1}\) \(\mu\)g protein\(^{-1}\) (Figures 1A and 1B), which is in line with H\(^+\) fluxes reported in other studies (Ueno *et al*., 2005; Hiyama *et al*., 2017). The H\(^+\) extrusion measured here is also consistent with the H\(^+\)-ATPase activity needed to drive solute uptake for stomatal opening for intact guard cells (Wang *et al*., 2012, 2017), as supported by the estimation of the H\(^+\) extrusion rate presented in Supplemental File 1. To our surprise, GCPs from *amy3 bam1* plants showed similar rates of BL-induced H\(^+\) pumping to those of the wild type (0.68 nmol h\(^{-1}\) \(\mu\)g protein\(^{-1}\); Figures 1A and 1B), as well as similar levels of phosphorylation of the H\(^+\)-ATPase (Figure 1C; Supplemental Figure 1A). While we did not detect differences in H\(^+\) pumping, *amy3 bam1* plants showed slow \(g_s\) kinetics and reduced amplitudes when exposed to the same light conditions (Supplemental Figure 1B). These results indicate a fully functional proton pump, even in the absence of starch degradation.
CHAPTER I

Figure 1. Membrane Ion Transport in Wild-Type and amy3 bam1 Guard Cells. (A) BL-dependent H⁺ pumping in GCPs. GCPs were exposed to RL (50 µmol m⁻² s⁻¹) for 2 h, after which BL (10 µmol m⁻² s⁻¹) was applied for 30 min. One representative experiment out of five experiments is shown. WT, wild type. (B) BL-dependent H⁺ pumping quantification. Values represent means ± SE (n = 5). (C) Immunoblots of BL-dependent H⁺-ATPase phosphorylation in GCPs. The upper blot displays the detection of the phosphorylation level of the H⁺-ATPase by immunoblot using the anti-phospho-Thr947 antibody (p-Thr). The lower blot shows detection of the H⁺-ATPase using a specific antibody against the C terminus of the H⁺-ATPase. Each lane contained 1.5 to 3.5 mg of guard cell proteins. (D) Steady-state currents recorded under voltage clamp for I_K,in and I_K,out in isolated guard cells. Solid curves are fittings of the wild type (n = 8) and amy3 bam1 (n = 8) to a Boltzmann function. Data are mean ± SE. The insets show measurements that were typically obtained by clamping in cycles with a holding voltage of -100 mV and 6-s steps either to voltages from -120 to -240 mV for I_K,in or voltages from -80 to +40 mV for I_K,out. (E) Instantaneous current voltage curves for I_Cl recorded in the wild type (n = 8) and amy3 bam1 (n = 11). Data are means ± SE. Solid curve shows an empirical fitting to the second-order polynomial function and is included for clarity. The insets show representative I_Cl traces during 7-s clamp steps to voltages from +30 mV to -160 mV after a 10-s clamp step at +30 mV.

Next, we recorded K⁺ and Cl⁻ channel currents under voltage clamp from intact guard cells. These methods bring membrane voltage under direct experimental control, thereby separating channel activity from complications of changes in membrane energization. Our voltage-clamp recordings detected no differences in K⁺ and Cl⁻ currents, their activation kinetics, or their conductances between the wild type and amy3 bam1, indicating that the mutation did not alter the capacity for K⁺ or Cl⁻ uptake (Figures 1D and 1E).

BL-Induced Guard Cell Starch Degradation Yields Glc

In our current model, Mal in guard cells is postulated to derive mainly from BL-induced starch degradation. This model is based on early publications (Raschke and Schnabl, 1978; Schnabl, 1980a), mostly correlative in nature, and lacks biochemical validation. To assess whether starch is indeed converted to Mal, we quantified Mal by enzymatic...
methods in wild-type and *amy3 bam1* guard-cell-enriched epidermal peels exposed to BL (75 µmol m⁻² s⁻¹) for 30 min. Mal levels in peels harvested at the end of the night (EoN) were similar in both genotypes (Figure 2A; Supplemental Table 1). Mal then decreased to a similar extent when isolated peels floating in opening buffer were illuminated with BL (Figure 2A; Supplemental Table 1), presumably as it was further metabolized to energize stomatal opening. However, Mal levels remained unchanged if the peels were kept in the dark for 30 min (Supplemental Figure 2A), indicating that the decrease in Mal was specifically induced by BL illumination. As a control, we measured Mal content in the leaves at the EoN and found no differences between wild-type and *amy3 bam1* plants (Supplemental Figure 2B). Altogether, these data suggest that Mal is metabolized in guard cells in response to BL, most likely for energy production. Furthermore, starch degradation in Arabidopsis guard cells does not directly result in Mal production, likely explaining why membrane ion transport is unaltered in *amy3 bam1* (Figure 1).

Given that there were no differences in Mal content, we reasoned that degradation of starch might directly influence soluble sugar homeostasis. We therefore quantified Glc, Fru, and Suc in guard-cell-enriched epidermal peels exposed to BL as detailed above. The wild type had substantial amounts of Glc at the EoN and lower quantities of Fru and Suc (Figure 2B; Supplemental Figure 2C; Supplemental Table 2). Fru and Suc then decreased when isolated peels floating in opening buffer were exposed to BL, while Glc levels did not significantly change (Figure 2B; Supplemental Table 2). Notably, *amy3 bam1* guard cells had only half as much Glc as those of the wild type at the EoN (Figure 2B; Supplemental Figure 2C), and almost undetectable amounts after the light treatment (Figure 2B). Fru levels were similar to the wild type, but, surprisingly, *amy3 bam1* guard cells had 3- to 4-fold more Suc than the wild type at the EoN (Figure 2B; Supplemental Figure 2C; Supplemental Table 2). Interestingly, *amy3 bam1* plants contained elevated amounts of Suc at the EoN in the leaves as well, possibly explaining the high Suc content in guard cells, whereas leaf levels of Glc and Fru were similar to the wild type (Supplemental Figure 2D). Suc in guard cells was then depleted in both the wild type and *amy3 bam1* after 30 min of BL illumination (Figure 2B), consistent with the idea that Suc is a substrate for light-induced stomatal opening (Lima *et al*., 2018; Medeiros *et al*., 2018). If isolated wild-type guard cells were kept in the dark for 30 min, Glc levels decreased to less than half (Supplemental Figure 2C), suggesting that the sustained levels of Glc in wild-type guard cells under BL result from BL-induced starch degradation. Glc was also partially metabolized in *amy3 bam1* guard cells during dark incubation (Supplemental Figure 2C). Fru levels decreased in wild-type guard cells but not in that of *amy3 bam1* (Supplemental Figure 2C), while
Suc remained unchanged during the 30 min of dark incubation (Supplemental Figure 2C). This result further supports the role of Suc as an energy supplier during light-induced stomatal opening. Overall, our findings suggest that under BL, isolated Arabidopsis guard cells release Glc from starch degradation. Thus, the slow and reduced stomatal opening of \textit{amy3 bam1} at the start of the day (Horrer \textit{et al.}, 2016) is a consequence of altered guard cell sugar homeostasis.

Fast Stomatal Opening Kinetics Are Associated with the Rate of Guard Cell Starch Degradation in Arabidopsis Plants Subjected to Alternating Pulses of Light and Darkness

Our electrophysiological and metabolite measurements ruled out a forward impact of starch degradation on membrane ion transport and suggest that the rapid conversion of starch to Glc might directly influence \(g_s\) kinetics. To test this hypothesis, we examined kinetics of \(g_s\) and \(A\) in relation to guard cell starch dynamics in wild type, \textit{amy3 bam1}, and \textit{aha1} plants. Given that loss of AHA1 H\(^+\)-ATPase in Arabidopsis impairs both membrane transport activities and starch metabolism (Horrer \textit{et al.}, 2016; Yamauchi \textit{et al.}, 2016), investigating the responses of \textit{aha1} should help in understanding the interaction between metabolism and ion transport in the control of \(g_s\) kinetics. Plants were subjected to a two-pulsed–light treatment, during which plants were given pulses of light and darkness of 2 h each. This began at the EoN, after 30 min of dark adaptation under 150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) white light illumination, which is common for Arabidopsis (George \textit{et al.}, 2018). Given the purpose of our gas exchange
measurements to compare stomatal opening kinetics between genotypes, $g_s$ and $A$ were normalized to the values at the EoN to facilitate the comparison of the velocity in the increase of the two parameters. Raw data for each experiment are provided in the Supplemental Figures.

Wild-type plants opened and closed their stomata in response to the alternating pulses of light and darkness (Figure 3A; Supplemental Figures 3A and 3B). However, stomatal opening during the second light pulse (4.5 h after dawn) was reduced and much slower compared with the first pulse (Figure 3B; Supplemental Figures 3A and 3B). Modeling the temporal responses of $g_s$ to light consistently revealed a two-fold increase in the time constant, $\tau$, for $g_s$ response between the second and the first light pulse, corresponding to an increase in the half-time for opening of 19 min (Figure 3C); whereas the maximum slope ($S_{\text{max}}$), a parameter that combines rapidity and amplitude of the $g_s$ response (Vialet-Chabrand et al., 2013), decreased by half (Figure 3D). The $amy3$ $bam1$ and $aha1$ mutants also responded to the fluctuations of light and darkness by opening and closing their stomata (Figure 3A; Supplemental Figures 3A and 3B), but their $g_s$ kinetics were slow during both light pulses, particularly in the case of $amy3$ $bam1$ (Figure 3A; Supplemental Figures 3C to 3H). Compared with the wild type, the $amy3$ $bam1$ and $aha1$ mutants showed significantly higher $\tau$ values for the first light pulse ($\tau_{amy3\;bam1} = 57 \pm 7$ min; $\tau_{aha1} = 25 \pm 2$ min; versus $\tau_{WT} = 17 \pm 1$ min), with a concomitant reduction in $S_{\text{max}}$, corresponding to 10 min to 40 min slower opening kinetics (Figures 3E and 3F; Supplemental Table 3). Note that the $g_s$ responses to the second light pulse were similar between all genotypes, with calculated $\tau$ for opening of 36 ± 2 min for the wild type, 48 ± 4 min for $amy3$ $bam1$, and 56 ± 7 min for $aha1$ (Figures 3A and 3C; Supplemental Figures 3E and 3F; Supplemental Table 3).

The differences in stomatal opening kinetics impacted on photosynthetic rates. $A$ in the wild-type plants increased rapidly during the first light pulse and reached a final steady state after ~ 20 min of light, while $A$ reached steady state only after ~ 50 min of light in response to the second light pulse (Figure 3G; Supplemental Figure 3I). Compared with the wild type, $amy3$ $bam1$ mutants had lower $A$ rates during the first light pulse (Figure 3G; Supplemental Figure 3I), in line with previous reports (Horrer et al., 2016). Estimations of $C_i/C_a$, describing the changes in the ratio of intercellular to ambient CO$_2$ concentrations throughout the treatment, assuming the resistance for CO$_2$ uptake being the same as for water efflux, confirmed that differences in photosynthetic rate in response to dark-to-light transition were driven largely by stomatal behavior. In wild-type plants, $C_i/C_a$ values during the first pulse initially decreased when light was turned on due to photosynthetic consumption of CO$_2$,
followed by an increase in $C_i/C_a$ due to stomatal opening (Supplemental Figure 3J). In response to the second light pulse, $C_i/C_a$ values after the initial drop increased more slowly due to the slower $g_s$ kinetics (Supplemental Figure 3J). It is well established that light-induced activation of ribulose-1,5-bis-phosphate carboxylase/oxygenase influences the kinetics of $A$ (Woodrow and Mott, 1989, 1992), particularly during the first 10 min, which is illustrated by the initial decrease in $C_i/C_a$. However, the absence of a difference in the $C_i/C_a$ response during the first minutes of light between the two pulses and that the subsequent increase in $C_i/C_a$ associated with the increase in $g_s$ suggests stomatal limitation of $A$ in our experimental conditions. The $C_i/C_a$ dynamics in $amy3$ bam1 and aha1 plants followed a similar trend to that of the wild type, but $amy3$ bam1 showed lower $C_i/C_a$ values during the first light pulse due to the diffusive stomatal limitations imposed by the slow $g_s$ responses (Supplemental Figure 3J).

As anticipated, rapid starch degradation occurred in wild-type guard cells during the first light pulse (Figure 3H). The second stomatal opening was, surprisingly, associated with a net increase in starch content up until the middle of the light pulse, followed by starch mobilization (Figure 3H). In the case of the $amy3$ bam1 mutant, starch content remained high for the entire duration of the experiment, with little or no turnover (Figure 3H). The aha1 mutant showed an intermediate phenotype, with slight starch degradation occurring during the second half of both light pulses (Figure 3H). A possible explanation for this observation is that other $H^+\text{-ATPase}$ isoforms may partially subsume the role of AHA1 in its absence. Consistent with this interpretation, we found that AHA5 and to some extent AHA2, which are preferentially expressed in guard cells (Yamauchi et al., 2016), were upregulated in guard-cell-enriched epidermal peels of the aha1 mutant when compared with the wild type at the EoN (Supplemental Figure 4). Altogether, these results show that the differences in stomatal opening kinetics between the first and the second light pulse in the wild type, and between the wild type and the mutants, which affected $A$ rates, were consistent with the underlying differences in guard cell starch metabolism. As a control, we measured starch content in the leaves, and found no differences between the wild type and the mutants (Supplemental Figure 5). In all cases, starch accumulation occurred in the light, while only a modest degradation of starch was observed in the wild type in response to the second dark period 6.5 h after dawn (Supplemental Figure 5). Thus, the delayed starch degradation in the wild-type guard cells during the second light pulse (Figure 3H) can explain the slow opening response.
Figure 3. Stomatal Opening Kinetics and Guard Cell Starch Dynamics in Plants Subjected to a Two-Pulsed–Light Treatment. (A) Normalized whole-plant recordings of \( \Delta g_s \sim g_{\text{initial}} \) values in dark-adapted (30 min) plants in response to 2-h L, 2-h D, 2-h L, and 2-h D (L, light; D, darkness). Plants were illuminated with 150 mmol m\(^{-2}\) s\(^{-1}\) white light. The \( g_s \) values were normalized to values at the EoN. Number of measured plants per genotype \( n \geq 3 \); values presented are means. WT, wild type. (B) Changes in \( g_s \sim g_{\text{initial}} \) values in response to a shift from dark to light in wild-type plants. Data are taken from (A). (C) to (F) Rapidity of the stomatal response estimated using a time constant \((t) [\text{C}]\) and the maximum slope of \( g_s \) increase \((S_{\text{max}}) [\text{D}]\) during the two consecutive pulses of light in the wild type \([\text{C}]\) and \([\text{D}]\) and during the first pulse in all three genotypes \([\text{E}]\) and \([\text{F}]\). Unpaired student's t test determined statistical significance between the indicated comparisons \((^*P < 0.05; \text{n.s., not significant}). (G) Normalized whole-plant recordings of \( A \sim A_{\text{initial}} \) values from plants under the same light regime as given in (A). Values for \( A \) were normalized to values at the EoN. Number of measured plants per genotype \( n \geq 3 \); values presented are means. (H) Guard cell starch dynamics of plants under the same light regime as given in (A). Each value represents mean ± SE of three biological replicates of >110 individual guard cells obtained from three independent experiments. Different letters indicate statistically significant differences among time points for the given genotype for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey's test.

To test the connection between guard cell starch degradation and \( g_s \) kinetics further, we extended the length of the first light pulse from 2 to 3 h (Figure 4). We reasoned that guard cell starch content might recover sufficiently to reach a threshold level that would allow immediate starch breakdown at the onset of the second light pulse and again promote fast stomatal opening. Indeed, in response to this modified two-pulsed–light treatment, wild-type guard cells degraded starch at the beginning of the second pulse (occurring this time 5.5 h after dawn; Figure 4A), and stomata opened more rapidly (Figures 4B and 4C; Supplemental Figures 6A and 6B). The changes in \( n \) and \( S_{\text{max}} \) between the second and the first light pulse this time corresponded to a reduction in the half-time for opening of 13 min (Figures 4D and 4E; Supplemental Table 3), showing that by extending the first light pulse, opening during the second was accelerated by almost 7 min when compared with the original two-pulsed–light treatment (Figures 4C and 4D; Supplemental Table 3). In line with
the $g_s$ kinetics, we observed no differences in $A$ and $C_i/C_a$ dynamics between the second and the first pulse (Figure 4F; Supplemental Figures 6I and 6J).

By contrast, the pattern of starch accumulation in the $amy3$ $bam1$ and $aha1$ mutants remained unchanged, resembling that of the original two-pulsed–light treatment (Figure 4A versus Figure 3H) and again resulted in reduced stomatal opening with slow $g_s$ kinetics compared with the wild type (Figure 4B; Supplemental Figures 6A to 6G). The $aha1$ mutant also showed a decrease in $g_s$ amplitude roughly 2.5 h after dawn, suggesting that this mutant does not maintain the stomata open under prolonged illumination (Figure 4B). As a result, $A$ rates in $aha1$ were reduced, particularly during the second light pulse (Figure 4F). The $C_i/C_a$ dynamics followed a similar trend to that of wild-type plants, but this time both $aha1$ and $amy3$ $bam1$ mutants showed reduced $C_i/C_a$ values compared to the wild type after the initial drop (Supplemental Figure 6J), matching the extremely slow $g_s$ kinetics and reduced amplitude (Figure 4B; Supplemental Figures 6A to 6H). This further highlights how changes in $C_i/C_a$ dynamics are linked to $g_s$ kinetics.

Taken together, our two-pulsed–light experiments and guard cell metabolite measurements suggest that the acceleration of stomatal opening above a baseline rate is associated with the amount of starch that is degraded to Glc, presumably needed to maintain proper guard cell sugar homeostasis.

Guard Cell Starch Dynamics in Response to Changes in Light Regime Do Not Depend on the Time of Day

To examine whether the changes in starch dynamics in response to the extension of the first light pulse from 2 to 3 h might simply reflect a time-of-day-dependent effect on guard cell starch metabolism, we subjected wild-type plants to a second regime of modified two-pulsed-light treatment. The first light pulse (2 h) was followed by 3 h of darkness, such that the beginning of the second light pulse still occurred 5.5 h after dawn (Figure 5). Under these conditions, stomatal opening during the second light pulse was accompanied by substantial guard cell starch accumulation, resulting in slow $g_s$ kinetics (Figures 5A to 5C; Supplemental Figure 7A). The time constant of the
Figure 4. Stomatal Opening Kinetics and Guard Cell Starch Dynamics in Plants Subjected to a Modified Two-Pulsed-Light Treatment. (A) Guard cell starch dynamics in dark-adapted (30 min) plants in response to 3-h L, 2-h D, 2-h L, and 2-h D (L, light; D, darkness). Plants were illuminated with 150 µmol m⁻² s⁻¹ white light. Each value represents mean ± SE of three biological replicates of >110 individual guard cells obtained from three independent experiments. Different letters indicate statistically significant differences among time points for the given genotype for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. WT, wild type. (B) Normalized whole-plant recordings of gs values in plants exposed to the same light regime as given in (A). The gs values were normalized to values at the EoN. Number of measured plants per genotype n ≥ 3; values presented are means. (C) Changes in A-A_initial values from plants under the same light regime as given in (B). Values for A were normalized to values at the EoN. Number of measured plants per genotype n ≥ 3; values presented are means.

The second pulse increased 4-fold, while the maximum slope decreased by 2.6-fold, corresponding to an increase in the half-time for stomatal opening of ~ 1 h (Figures 5D and 5E). A was also affected by the change in the light treatment, showing slower rates compared with the first light pulse (Figure 5F; Supplemental Figure 7B). Thus, the rearrangements of guard cell starch metabolism observed in our experiments were directly linked to the applied light regime, excluding the possibility that the decline in starch content in response to the second light pulse in the modified two-pulsed-light treatment (Figure 4A) was affected by the time of day.
Stomatal Anatomical Features of *amy3 bam1* and *aha1* Mutants Do Not Explain Their Slow $g_s$ Kinetics

We also examined foliar stomatal anatomy to assess its contribution to the altered $g_s$ response to light of *amy3 bam1* and *aha1* mutants. For this purpose, we calculated the maximum theoretical conductance ($g_{\text{max}}$). Anatomical $g_{\text{max}}$ defines the potential maximum rate of $g_s$ to water vapor as determined by the size and density of stomata in a diffusion-based equation (Dow et al., 2014). Compared with the wild type, both *amy3 bam1* and *aha1* mutant plants had more stomata per unit leaf area (143 ± 3 mm$^{-2}$ and 127 ± 8 mm$^{-2}$, respectively, versus 98 ± 6 mm$^{-2}$; Figure 6A). They also showed a smaller pore area ($a_{\text{max}}$; 152 ± 4 mm$^2$ for *amy3 bam1* and 134 ± 3 mm$^2$ for *aha1* compared with 161 ± 5 mm$^2$ for the wild type; Figure 6B), primarily because of a smaller aperture rather than reductions in pore length or depth (Figures 6C to 6E),
indicating that the guard cell membrane surface in *amy3 bam1* and *aha1* was likely similar to that of the wild type. The physical attributes of *amy3 bam1* and *aha1* with highly dense stomata and smaller pore area should theoretically allow increased stomatal conductance values compared with the wild type (Drake et al., 2013). Indeed, our calculations yielded significantly higher $g_{\text{smax}}$ values for both mutants (Figure 6F). The higher $g_{\text{smax}}$ of the *amy3 bam1* and *aha1* mutants, however, did not match the observed $g_s$ responses. These results suggest that the effect of starch and proton pumping on $g_s$ kinetics of Arabidopsis stomata is independent of anatomical features such as size and density. We interpret the elevated density of stomata in *amy3 bam1* and *aha1* mutants as an adaptive developmental response to the limited capacity to open the pore.

![Figure 6. Stomatal Anatomical Features.](image)

**Figure 6.** Stomatal Anatomical Features. Stomatal physical attributes of wild-type, *amy3 bam1*, and *aha1* plants were determined using micrographs of epidermal peels from the abaxial side of leaf number 6, harvested 2 h into the light phase. (A) Stomatal density. (B) Stomatal pore size ($a_{\text{max}}$). (C) Stomatal aperture. (D) Pore length. (E) Pore depth. (F) Anatomical maximum $g_{\text{smax}}$ as determined by stomatal size and density. Data are means ± SE of $n = 365$ stomata for the wild type, $n = 377$ stomata for *aha1*, and $n = 481$ stomata for *amy3 bam1* from three independent experiments. Different letters indicate statistically significant differences among genotypes for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.

**Fast $g_s$ Kinetics under Saturating Photosynthetic Active Radiation Are Independent of Guard Cell Starch Degradation, But Require the PM H^+-ATPase**

It is well established that guard cell osmoregulation is driven by different processes depending on the light quality and intensity (Talbott and Zeiger, 1996). The early morning BL response, which is non-photosynthetic, is associated with H^+-ATPase-dependent uptake of K^+ and Cl^−, synthesis/uptake of Mal, and degradation of starch...
The photosynthetic or RL response, which is induced by light intensities that saturate photosynthesis, is supposed to be accompanied by an increased sugar concentration, mainly Suc (Talbott and Zeiger, 1993), and to be independent of starch degradation (Poffenroth et al., 1992). Furthermore, \( g_s \) is determined by the capacity of the mesophyll tissue to fix carbon (Wong et al., 1979).

To test further the connection between guard cell starch degradation and stomatal opening kinetics under light intensities that saturate photosynthesis (400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for Arabidopsis; George et al., 2018), we subjected wild-type, \textit{amy3 bam1}, and \textit{aha1 pla} plants to a typical two-pulsed-light treatment with alternating pulses of white light and darkness of 2 h each with a light intensity of 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

**Figure 7.** Effect of Saturating Photosynthetic Active Radiation on Guard Cell Starch Metabolism and Stomatal Kinetics. (A) Normalized whole-plant recordings of \( g_s - g_{\text{max}} \) in dark-adapted (30 min) plants in response to 2-h L, 2-h D, 2-h L, and 2-h D (L, light; D, darkness). Plants were illuminated with 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) white light. The \( g_s \) values were normalized to values at the EoN. Number of measured plants per genotype \( n \geq 3 \); values presented are means. WT, wild type. (B) Normalized whole-plant recordings of \( A - A_{\text{max}} \) values from plants under the same light regime as given in (A). Values for \( A \) were normalized to values at the EoN. Number of measured plants per genotype \( n \geq 3 \); values presented are means. (C) Guard cell starch dynamics of plants under the same light regime as given in (A). Each value represents mean \( \pm \) SE of three biological replicates of >110 individual guard cells obtained from three independent experiments. Different letters indicate statistically significant differences among time points for the given genotype for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test.

Under these conditions, all genotypes achieved higher steady-state \( g_s \) compared with plants illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Figure 7A; Figures 3A and 4B for comparison), and a greater \( A \) (Figure 7B; Figures 3G and 4F for comparison). Wild-type stomata opened rapidly in response to both light pulses, with the speed of \( g_s \) responses resembling those of the first pulse at a fluence rate of 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Figure 7A; Supplemental Figures 8A to 8C). However, in this case, stomatal opening in the wild type was accompanied by starch accumulation (Figure 7C), suggesting that when plants are carbon-saturated, fast stomatal opening kinetics are independent of guard cell starch degradation and its derived metabolites.
This observation was supported by data from the *amy3 bam1* mutant. Guard cell starch dynamics in this mutant were unaffected by the changes in light intensity, and starch content remained high throughout the experiment (Figure 7C). Nonetheless, the *amy3 bam1* mutant showed rapid increases in *g*ₙ as well as wild-type-like *A* rates and *Cᵢ/Cᵣ* values (Figures 7A and 7B; Supplemental Figures 8A to 8D). Surprisingly, under these saturating light intensities, the *g*ₙ response of the *aha1* mutant differed from that of the *amy3 bam1* mutant. The *aha1* mutant displayed slower stomatal opening kinetics and reduced steady-state *g*ₙ during both light periods, which resulted in lower *A* rates (Figures 7A and 7B; Supplemental Figure 8E and 8F). The *aha1* mutant also showed reduced *Cᵢ/Cᵣ* values compared with the wild type and the *amy3 bam1* mutant (Supplemental Figure 8G).

The fact that *amy3 bam1*, but not *aha1*, behaved similar to the wild type suggests that: (1) carbon sources for stomatal opening under photosynthesis-saturating light conditions do not derive from starch degradation, and (2) photosynthesis-driven opening response depends on the activity of the PM H⁺-ATPase.

**PM H⁺-ATPase Activity Is Required for Fast Stomatal Opening Kinetics and Guard Cell Starch Accumulation under RL**

To uncover the reasons for the stomatal phenotype of *aha1* under saturating white light irradiation, we examined stomatal opening kinetics under RL, which avoids the non-photosynthetic BL responses (Shimazaki *et al*., 2007). The RL response is abolished by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (an inhibitor of PSII; Olsen *et al*., 2002; Messinger *et al*., 2006), and, by contrast to BL, is associated with net guard cell starch accumulation (Tallman and Zeiger, 1988; Horrer *et al*., 2016). As anticipated, illumination of wild-type plants with 300 µmol m⁻² s⁻¹ of RL resulted in rapid stomatal opening with elevated steady-state *g*ₙ, and was accompanied by efficient guard cell starch accumulation, which was sustained for the entire duration of the treatment (Figures 8A and 8B; Supplemental Figures 9A to 9C). Compared to the wild type, both *amy3 bam1* and *aha1* mutants showed reduced *g*ₙ amplitude, with *aha1* having exceptionally slow *g*ₙ kinetics (Figure 8A; Supplemental Figures 9A to 9C). The *aha1* mutant showed no changes in guard cell starch content, while the *amy3 bam1* mutant showed slight, but significant net increase in starch (Figure 8B). These differences, however, did not affect *A* rates, which were similar in all three genotypes (Supplemental Figures 9D and 9E).
Figure 8. Effect of RL on Guard Cell Carbohydrate Metabolism and Stomatal Kinetics. (A) Normalized whole-plant recordings of $g_{s\text{-inn}}$ in dark-adapted (30 min) plants in response to 6-h illumination with 300 µmol m$^{-2}$ s$^{-1}$ of RL. The $g_s$ values were normalized to values at the EoN, n = 3; values presented are means. WT, wild type. (B) Starch accumulation in guard cells. (C) Starch content in wild-type guard cells of intact leaves. (D) Starch content in wild-type isolated guard cells. (E) Stomatal aperture in wild-type guard cells of intact leaves. (F) Stomatal aperture in wild-type isolated guard cells. (G) Starch content in wild-type isolated guard cells with or without treatment (10 mM of Fc applied after 3 h of light exposure). (H) Stomatal aperture in wild-type isolated guard cells with or without treatment (10 mM of Fc; applied after 3 h of light exposure). (I) Soluble sugar and (J) total sugar contents of wild-type and aha1 guard-cell-enriched epidermal peels at the EoN and after 2-h illumination with 300 µmol m$^{-2}$ s$^{-1}$ of RL. Data for two independent experiments are shown (means ± SE; n ≥ 11). FW, fresh weight. (B) to (D) and (G) Same light conditions as given in (A). Each value represents mean ± SE of three biological replicates of >100 individual guard cells obtained from three independent experiments. Different letters indicate statistically significant differences among time points for the given genotype. Asterisk (*) indicates statistically significant difference between genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (E) to (H) Same light conditions as given in (A). Each value represents mean ± SE of four biological replicates of >200 individual stomata obtained from two independent experiments. Different letters indicate statistically significant differences among time points for the given genotype. Asterisk (*) indicates statistically significant difference between genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test.
Based on these observations and earlier research suggesting that stomatal opening under saturating light likely depends on the import of mesophyll-derived sugars (Poffenroth et al., 1992), we reasoned that the differences in starch accumulation under RL between the wild type and aha1 may reflect the capacity of their guard cells to import apoplastic sugars. Firstly, we compared the ability to accumulate starch in response to RL of wild-type guard cells of intact leaves and wild-type guard cells in isolated epidermal peels in which there is no connection with the mesophyll. We observed starch accumulation in both cases; however, guard cells of intact leaves, despite a short delay in the onset of synthesis, accumulated substantially more starch than isolated guard cells, showing a 4-fold increase in starch content by the end of the treatment (Figures 8C and 8D). We also observed that stomata of intact leaves efficiently opened in response to RL, whereas isolated stomata remained closed (Figures 8E and 8F). To verify that the isolated guard cells were still responsive to external stimuli after floating for several hours in the buffer, we performed a control experiment in which we treated isolated peels with fusicoccin (Fc). Fc is a chemical activator of the PM H⁺-ATPase and, in turn, of guard cell starch degradation (Horrer et al., 2016). After 3 h of RL illumination, exogenous application of Fc resulted in efficient starch degradation and induction of stomatal opening, as determined after 1 h and 3 h of treatment (Figures 8G and 8H). Altogether, these findings show that starch in guard cells under RL is made primarily from imported sugars; and they further support the idea that a mesophyll-derived signal (presumably sugars) is required for RL-induced stomatal opening (Lee and Bowling, 1992; Mott et al., 2008).

Having established the importance of mesophyll-derived sugars for RL-mediated responses, we next determined soluble sugar content in wild-type and aha1 guard cells of intact leaves at the EoN and after 2 h of RL illumination (300 µmol m⁻² s⁻¹). Glc and Fru levels were similar in the two genotypes and remained constant under RL (Figure 8I; Supplemental Table 4). Notably, Suc levels at the EoN were 2-fold higher in the wild type compared to aha1 (Figure 8I; Supplemental Table 4) and Suc doubled in wild-type guard cells under RL, whereas it remained low in aha1 (Figure 8I; Supplemental Table 4). Quantification of total amount of soluble sugars further showed that in wild-type guard cells, sugar content rose under RL by ~ 30%, but only ~ 12% in aha1 (Figure 8J).

Taken together, the severely impaired gs kinetics of aha1 under RL, along with impaired guard cell Suc accumulation and lack of starch synthesis, strongly suggest that the RL stomatal response depends on Suc supply from the mesophyll and that the uptake of mesophyll-derived Suc is mediated by the PM H⁺-ATPase, presumably via energization of Suc transporters.
DISCUSSION

Integration of Guard Cell Starch Metabolism with Membrane Ion Transport during BL-Induced Stomatal Opening

Loss of AHA1 H⁺-ATPase in Arabidopsis directly translates into a reduction of proton extrusion by the guard cells, impairing both membrane transport activities and starch metabolism, and causing reduced stomatal opening (Horrer et al., 2016; Yamauchi et al., 2016). Despite this seemingly tight connection between guard cell starch metabolism and ion transport, here we showed that starch degradation does not directly affect H⁺ flux or the capacity for K⁺ and Cl⁻ transport (Figure 1; Supplemental Figure 1). These findings have important implications. Firstly, besides the energy stored in starch, other metabolic processes, such as the electron transport chain in the chloroplast (Suetsugu et al., 2014), oxidative phosphorylation in mitochondria (Daloso et al., 2015), or BL-dependent b-oxidation of lipids (McLachlan et al., 2016), can contribute to the energy requirements of stomatal opening. Secondly, the presence of a functional H⁺-ATPase and of unaffected K⁺ and Cl⁻ channel currents in amy3 bam1 show that their transport activities alone do not limit light-induced stomatal opening. We found the amy3 bam1 mutant capable of driving H⁺ flux like the wild type under BL illumination and sufficient to energize the ion uptake needed for the increase in inorganic solute content during stomatal opening (Wang et al., 2012, 2017; Jezek and Blatt, 2017), yet gs increased only slowly in response to BL (10 µmol m⁻² s⁻¹) superimposed on RL (50 µmol m⁻² s⁻¹; Supplemental Figure 1B). We conclude that starch degradation in guard cells is not primarily required for energy production to drive stomatal opening, and does not directly affect the ability of membrane ion transport.

Glc Is the Major Starch-Derived Metabolite during BL-Induced Stomatal Opening

In the early 20th century, starch-to-sugar conversion was the most widely accepted theory explaining the osmotic changes leading to alterations in guard cell turgor (Lloyd, 1908; Scarth, 1927). However, soon after the importance of K⁺ in stomatal movement was revealed (Fischer, 1968; Fischer and Hsiao, 1968), the starch–sugar theory was put aside. Since then, K⁺ has been recognized as the major osmoticum in guard cells, with Mal and/or Cl⁻ and nitrate acting as the counterions (Humble and Raschke, 1971; Allaway, 1973; Outlaw and Lowry, 1977; Travis and Mansfield, 1977; Schnabl and Raschke, 1980). According to this model, Mal is synthesized within the guard cells using starch as a source of carbon skeletons. Experimental support for this model...
comes from studies in *Vicia faba* linking changes in GCP volume to changes in Mal and starch contents. Mal was determined enzymatically in GCPs incubated under white light and CO$_2$-free air (Schnabl, 1980a, 1980b; Schnabl et al., 1982). Further studies have reported an increase in Mal content in guard cells due to white light illumination (Allaway, 1973; Travis and Mansfield, 1977). These reports are based on measurements from *Vicia faba* or *Commelina communis* guard cells of intact leaves (Allaway, 1973) or epidermal fragments (Travis and Mansfield, 1977), not excluding the possibility of Mal import from the mesophyll.

Here, we quantified enzymatically Mal and sugars in wild-type and *amy3 bam1* isolated guard cells before and after floating them in opening buffer under BL for 30 min. We showed that defective starch degradation in *amy3 bam1* had no impact on Mal accumulation in guard cells at the EoN and after the BL treatment when compared to the wild type (Figure 2A; Supplemental Table 1). In response to BL illumination, Mal decreased in both genotypes (Figure 2A; Supplemental Figure 2A), indicating that Mal is a substrate for BL-induced stomatal opening in Arabidopsis. However, we cannot exclude that a transient peak in Mal accumulation was missed due to our experimental setup. Simultaneous synthesis and use of Mal in guard cells makes it difficult to detect fine changes in the amount of this metabolite. The fact that there were no differences in Mal content between the wild type and *amy3 bam1* leads us to conclude that Mal is not the major starch-derived metabolite in Arabidopsis guard cells during BL-induced stomatal opening.

Sugar homeostasis, on the other hand, was dramatically altered in *amy3 bam1* guard cells. Already at the EoN, *amy3 bam1* had half as much Glc as the wild type, but accumulated 4-fold more Suc (Figure 2B). After the BL treatment, Glc (7-fold less than the wild type) was almost undetectable, while wild-type guard cells still contained high amounts of Glc (Figure 2B; Supplemental Table 2). This was not the case if isolated guard cells were dark-incubated for 30 min (Supplemental Figure 2C). Glc levels in both genotypes decreased during darkness, demonstrating that Glc derives from guard cell starch degradation specifically under BL.

These unexpected findings suggest that Glc is the major-starch derived metabolite in Arabidopsis guard cells. We conclude that it is unlikely that Mal is synthesized from carbon skeletons derived from starch degradation, and propose that Mal is more likely produced from anaplerotic CO$_2$ fixation within the guard cells (Asai *et al.*, 2000; Robaina-Estévez *et al.*, 2017) or directly imported from the apoplast via the ABC transporter ABCB14 (Lee *et al.*, 2008) to fulfill its function as an allosteric activator, counterion, and osmotically active solute.
Even though the starch-sugar hypothesis got short shrift, recent evidence has again pointed to the significance of carbohydrates, in addition to K⁺ and anions, during the build-up of the guard cell turgor (reviewed by Daloso et al., 2016, 2017; Santelia and Lawson, 2016; Santelia and Lunn, 2017; Lima et al., 2018; Granot and Kelly, 2019). Our data further support this view. A first consideration is that ion transport across the PM requires a significant amount of energy in the form of ATP. One of the roles of guard cell carbohydrate metabolism is to meet this energetic demand. Suc was long thought to act as an osmolyte (Poffenroth et al., 1992; Talbott and Zeiger, 1993; Amodeo et al., 1996), but more recent reports suggest that Suc is broken down to fuel the tricarboxylic acid cycle and provide energy for stomatal opening (Daloso et al., 2015, 2016; Medeiros et al., 2018). In line with this hypothesis, we observed, in both wild-type and amy3 bam1 guard cells, Suc depletion under light (Figure 2B) but not in darkness (Supplemental Figure 2C). A second consideration is that sequestration of K⁺ in the vacuole (in the form of K₂Mal or KCl) requires cytosolic volume to be maintained. This can be achieved through import or synthesis of sugars. Thus, the cytoplasmic sugar pool must be replenished during stomatal opening to maintain cellular homeostasis and provide carbon skeletons for energy production. The reduced levels of Glc along with the slow and reduced stomatal opening in amy3 bam1 suggests that fast starch degradation at dawn is required for sufficient and continuous provision of sugars. Reduced levels of Glc in amy3 bam1 invokes compensatory Suc uptake and may explain why, at the EoN, amy3 bam1 accumulated 4-fold more Suc than the wild type along with the increased amount of available Suc from amy3 bam1 mesophyll cells.

The metabolic pathways within the mitochondria, chloroplasts, and cytosol are in a delicate balance. The rapid conversion and exchange of metabolites between these subcellular compartments is a cardinal event in guard cells, which ultimately coordinate the energetic and metabolic status of the cell with membrane ion transport activity.

**BL-Induced Guard Cell Starch Degradation Promotes Fast Stomatal Opening Kinetics under Common Lighting Conditions**

In response to fluctuations in environmental parameters, plants try to coordinate stomatal opening with the mesophyll demand for CO₂ and stomatal closure with the need to minimize water loss through transpiration. An important limitation in this process is the rate at which stomata open and close, which is usually more than an order-of-magnitude slower compared with photosynthetic responses (Lawson and
The intercellular CO$_2$ concentration ($C_i$) was long considered to be the factor mediating the coordination between $A$ and $g_s$ (reviewed in Lawson et al., 2014). However, recent research reporting increases in $g_s$ with light despite high $C_i$ or after reaching steady-state $A$ (Lawson et al., 2008; Matrosova et al., 2015) raises questions about the role of $C_i$ as primary driver of $A$-$g_s$ coordination. Furthermore, there is increasing evidence that for species with kidney-shaped stomata, such as Arabidopsis, anatomical features, including size and density, are not directly correlated with the speed nor the amplitude of stomatal responses (Franks and Farquhar, 2007; Elliott-Kingston et al., 2016; McAusland et al., 2016). Therefore, it has been hypothesized that characteristics other than stomatal anatomy may influence the $g_s$ kinetics in this type of stomata (McAusland et al., 2016).

In this study, we provide evidence that identifies guard cell starch metabolism as a key determinant of fast stomatal opening kinetics under common light conditions. We show that guard starch degradation in Arabidopsis helps to accelerate stomatal opening above a baseline rate. The temporal responses of $g_s$ to light showed that inhibiting guard cell starch degradation in $amy3$ $bam1$ or $aha1$ mutants resulted in slow stomatal opening kinetics compared to the wild type, with a calculated increase in the time constant for opening of up to 40 min (Figures 3E and 3F). The fact that the $amy3$ $bam1$ and $aha1$ mutants have a higher potential $g_{s_{max}}$, despite the observed reduced $g_s$ amplitude (Figure 6F), further demonstrates that the effect of starch on $g_s$ of Arabidopsis stomata was independent of size and density. Similar alterations in anatomical features that cannot explain the different temporal responses of $g_s$ were found in other mutants, such as the outward rectifying K$^+$ channel mutant $gork1$-1 (Vialet-Chabrand et al., 2017a). Thus, metabolism - and its coordination with membrane ion transport - overrides anatomy in the control of stomatal opening kinetics in kidney-shaped stomata. We propose that the manipulation of $g_s$ kinetics by controlling guard cell starch dynamics could be a potential tool to improve the coordination of stomatal opening with mesophyll demand for CO$_2$ that may be exploited to enhance plant WUE.

**H$^+$-ATPase Energizes Sugar Uptake for Fast $g_s$ Kinetics during Photosynthesis-Mediated Stomatal Responses**

We showed that when plants are photosynthetic-rate-saturated (i.e., at 400 µmol m$^{-2}$ s$^{-1}$ of light), fast stomatal opening kinetics are independent of guard cell starch degradation. Consistent with this idea, the $amy3$ $bam1$ mutant showed $g_s$ responses similar to the wild type when illuminated with 400 µmol m$^{-2}$ s$^{-1}$ of light (Figure 7A; Supplemental Figure 8). It is plausible that the osmolytes normally deriving from starch
degradation were replaced by the high photosynthetic sugar production in the mesophyll, which, in concomitance with K\(^+\) uptake and inhibition of anion channels (Marten *et al*., 2008), was sufficient to promote rapid guard cell turgor and stomatal opening. The fact that the *aha1* mutant had reduced and slow \(g_s\) responses under these saturating light intensities (Figures 7A and 7C) led us to conclude that the activity of the PM H\(^+\)-ATPase is necessary to promote the uptake of K\(^+\) and/or mesophyll-derived sugars for stomatal opening. Our discovery of the slow \(g_s\) kinetics and reduced amplitude of *aha1* stomata under RL illumination (Figure 8A; Supplemental Figure 9), which eliminates the BL-dependent response, further defines an essential role for PM H\(^+\)-ATPase in photosynthesis-mediated stomatal responses. This finding is in line with recent studies reporting that RL induces photosynthesis-dependent phosphorylation of PM H\(^+\)-ATPase in guard cells to promote stomatal opening in whole leaves (Ando and Kinoshita, 2018).

The RL response is partially driven by the accumulation of photosynthetically derived sugars, synthesized by the guard cell itself or imported from the mesophyll (Poffenroth *et al*., 1992; Talbott and Zeiger, 1993; Lu *et al*., 1995; Olsen *et al*., 2002), and is associated with net guard cell starch accumulation (Tallman and Zeiger, 1988; Horrer *et al*., 2016). It was indeed demonstrated that RL can stimulate stomatal opening via K\(^+\) accumulation and starch breakdown (i.e., the classic BL-dependent response) only under low CO\(_2\) conditions, when photosynthetic rates are low (Olsen *et al*., 2002). Because guard cell photosynthesis can provide only limited amounts of carbon (Tarczynski *et al*., 1989; Reckmann *et al*., 1990), mesophyll-derived sugars have long been considered as the most important source of osmotica for RL-mediated stomatal opening (reviewed in Lawson *et al*., 2014). In this study, we provide evidence supporting the role of mesophyll sugars in the RL response. When illuminated with 300 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) of RL, guard cells in isolated epidermal peels accumulated only \(\sim 25\%\) of starch amounts compared with guard cells of intact leaves (Figures 8C and 8D). Furthermore, stomata of intact leaves efficiently opened in response to RL, whereas isolated stomata remained closed (Figures 8E and 8F).

Early biochemical studies suggested that Suc produced by mesophyll photosynthesis is transported to the guard cells via the apoplast and is taken up into the guard cells, apparently in symport with protons (Dittrich and Raschke, 1977; Lu *et al*., 1997; Ritte *et al*., 1999). In agreement with this hypothesis, we showed that guard cells of wild-type and *aha1* plants contained different amounts of soluble sugars at the EoN, with wild-type plants showing elevated contents for all three sugars (Figures 8I and 8J; Supplemental Table 4). After the plants have been exposed to RL (300 \(\mu\)mol
m² s⁻¹) for 2 h, this difference became more pronounced, especially for Suc. Wild-type guard cells doubled their Suc content, whereas aha1 guard cells failed to increase Suc levels (Figure 8I; Supplemental Table 4). This, together with the fact that aha1 mutant guard cells did not accumulate starch in response to RL irradiation (Figure 8B), lead us to conclude that the activity of H⁺-ATPase under RL or saturating photosynthetic active radiation is essential to energize Suc uptake for guard cell turgor generation and starch biosynthesis. Our results are in line with research demonstrating a role for the PM H⁺-ATPase (PHA1; AHA1 from potato [Solanum tuberosum]) in Suc-starch metabolism in stolons of potato (Stritzler et al., 2017). It is conceivable that RL-induced CO₂ fixation provides the precursors needed for starch synthesis, but whether or not this accumulation of starch is required for RL stomatal responses remains unclear.
METHODS

Plant Material and Growth Conditions
All experiments were performed with four-week-old, non-flowering Arabidopsis (Arabidopsis thaliana) plants in the Columbia (Col-0) background. The Arabidopsis mutants used in this study, aha1-8 (Salk_118350C) and amy3 bam1, were described by Horrer et al. (2016). Plants were grown in soil in controlled-climate chambers (either KKD Hiross from CLITEC, or a Fitoclima 1200 or Fitoclima 2500 from Aralab) under a 12-h light/12-h dark photoperiod at a constant temperature of 21°C/19°C (day/night) and a relative humidity of 45/55% (day/night). Plants were illuminated with a total photon flux density of 150 µmol m⁻² s⁻¹ with a combination of white (Biolux; Osram) and purple (Fluora; Osram) halogen lamps. Alternatively, plants were illuminated with a Fitoclima 2500 light-emitting diode (LED) panel (Aralab) or Fitoclima 2500 LED tubes (Aralab). Guard cell starch quantification and gas exchange measurements were performed at the indicated time points on plants subjected to a two-pulsed-light treatment. In a typical experimental setup, plants were given pulses of light and darkness of 2 h each. Alternatively, plants were subjected to modified versions of the two-pulsed-light treatment, in which the first light pulse or the first dark pulse were extended to 3 h. In all cases, the experiment started at the EoN when plants were directly transferred from the climate chamber to the whole-plant Arabidopsis chamber, 6400-17 (LI-COR Biosciences), and given 30 min of dark adaptation before the beginning of the first light pulse. Plants were illuminated with standard growth light conditions (150 µmol m⁻² s⁻¹) or saturating photosynthetic active radiation (400 µmol m⁻² s⁻¹). For the RL experiments, plants were transferred at the EoN from the climate chamber to a reach-in climate chamber equipped with LED light sources (Fytoscope FS130; Photon Systems Instruments) and illuminated with 300 µmol m⁻² s⁻¹ RL.

Guard Cell Starch Quantification
Guard cell starch content was quantified as described by Flütsch et al. (2018). In brief, at the indicated time points, epidermal peels obtained from leaf number 5 or 6 were fixed immediately in fixative solution (50% [v/v] methanol and 10% [v/v] acetic acid). Alternatively, leaves number 5 and 6 from eight individual plants were blended using a kitchen blender (ProBlend Avance, Philips). Isolated guard cells were collected using a 200-mm nylon mesh (Sefar) and incubated in 1 mL of basic opening buffer (5 mM of MES- bistrispropane at pH 6.5, 50 mM of KCl, and 0.1 mM of CaCl₂). Isolated guard cells were dark-incubated for 1 h in a reach-in climate chamber (Fytoscope FS130; Photon Systems Instruments). The isolated guard cells were subsequently exposed to 300 µmol m⁻² s⁻¹ RL for 6 h. In a modified version of this experiment, isolated guard
cells were treated with 10 µM of Fc (Sigma-Aldrich) after 3 h of RL illumination. After
the incubation and at the indicated time points, isolated guard cells were fixed in 50%
(v/v) methanol and 10% (v/v) acetic acid. After fixation, starch granules were stained
using the modified pseudo-Schiff propidium iodide staining (Truernit et al., 2008;
Flütsch et al., 2018). To oxidize the hydroxyl groups of the Glc entities, samples were
incubated in 1% (w/v) periodic acid solution. The epidermal peels were stained with
propidium iodide (1 mg mL⁻¹ [w/v]) and Schiff reagent (100 mM of sodium
metabisulphite and 5 N of HCl). After destaining in distilled water, the samples were
covered with chloral hydrate solution on a microscopy slide. Finally, epidermal peels
were fixed with Hoyer’s solution after an overnight dark incubation. Guard cell starch
contents were visualized using a TCS SP5 confocal laser-scanning microscope (Leica
Microsystems). Starch granule area was determined using the software ImageJ v1.48
(NIH; http://rsbweb.nih.gov/ij/).

Stomatal Aperture and Trait Analysis
Stomatal morphological parameters (aperture, guard cell length, guard cell width, and
stomatal density) were measured from the 5. or the 6. leaf after 2 h of light, when
stomata are fully open, as previously described by Horrer et al. (2016). For the time
course of stomatal aperture, images were taken at the indicated time points. Briefly,
leaf number 5 or 6 was fixed on an adhesive tape with the abaxial epidermis facing
the tape. The mesophyll cell layer and the adaxial epidermis were removed using a
razor blade. The abaxial epidermal cell layer remaining on the tape was washed with
a 10-mM MES-KOH, pH 6.15 solution and subsequently fixed on a microscopy slide.
Stomata were immediately imaged using an inverted microscope (Eclipse TS100;
Nikon) at 40x magnification. Alternatively, isolated guard cells from leaves number 5
and 6 obtained as described in “Guard Cell Starch Quantification” were transferred to
microscopy slides at the indicated time points with or without treatment with 10 µM of
Fc (Sigma-Aldrich), and immediately imaged. Stomatal anatomical traits were
determined using the software ImageJ v1.48 (NIH).

Anatomical g_{smax} Calculation
The anatomical g_{smax} to water vapor (mol m⁻² s⁻¹) was determined according to the
double end-corrected version of the equation by Franks and Farquhar (2001):

\[ g_{smax} = \frac{d_w S_d a_{max}}{v(l + \frac{\pi}{2} \sqrt{\frac{a_{max}}{\pi}})} \]
where $d_w$ is the diffusivity of water vapor in air (m$^2$ s$^{-1}$) at 22°C, $v$ is the molar volume of air (m$^3$ mol$^{-1}$) at 1 atm and 22°C, $S_o$ is the stomatal density (m$^{-2}$), and $l$ represents the guard cell pore depth (m).

Maximum stomatal pore area ($a_{max}$) was calculated as $\pi \left( \frac{L}{2} \right)^2$. The maximum stomatal pore area was an ellipse with the main axis equal to pore length $L$ (m) and the minor axis equal to $L/2$.

**GCP Isolation and H$^+$ Pumping**

GCPs were enzymatically prepared from Arabidopsis wild-type or amy3 bam1 mutant leaves as described previously by Yamauchi and Shimazaki (2017). Isolated, overnight dark-adapted GCPs were illuminated with 50 µmol m$^{-2}$ s$^{-1}$ of RL for 2 h, after which BL (10 µmol m$^{-2}$ s$^{-1}$) was applied for 30 min. BL-dependent H$^+$-extrusion was determined using a glass pH-electrode as described by Yamauchi and Shimazaki (2017). The reaction mixture (0.8 mL) was composed of 0.125 mM of MES-KOH at pH 6.0, 10 mM of KCl, 0.4 M of mannitol, 1 mM of CaCl$_2$, and Arabidopsis GCPs (50 to 80 µg of protein).

**Measurements of K$^+$ Currents**

Currents were recorded using double-barreled microelectrodes as described by Chen et al. (2012) using Henry’s EP software (http://www.psrg.org.uk). To record inwardly rectifying K$^+$ currents ($I_{K,in}$) and outwardly rectifying K$^+$ currents ($I_{K,out}$), electrodes were filled with 200 mM of K-acetate at pH 7.5 to avoid anion leakage from the microelectrode (Blatt and Slayman, 1983; Chen et al., 2012). Microelectrodes were constructed to give tip resistances >500 MΩ for Arabidopsis guard cell impalements. Guard cells from epidermal peels were treated with depolarizing buffer and light of 150 µmol m$^{-2}$ s$^{-1}$ before recording K$^+$ currents in standard bathing solution of 5 mM of Ca$^{2+}$-MES at pH 6.1 containing 10 mM of KCl. Voltage and currents were recorded using a µP electrometer amplifier (WyeScience) with an input impedance of >2 x10$^{11}$ Ω (Blatt, 1987). Surface area and volume of impaled guard cells were estimated from the cell length and diameter, assuming a spheroid geometry.

**Measurements of Cl$^-$ Currents**

Voltage-clamp recordings were performed from Arabidopsis intact guard cells in epidermal peels using Henry’s EP Software Suite (http://www.psrg.org.uk). Double-barreled microelectrodes were filled with 200 mM of CsCl at pH 7.5, and the tissue was superfused with 5 mM of MES-Ca$^{2+}$ at pH 6.1, containing 15 mM of CsCl and 15 mM of tetraethylammonium chloride. Surface areas of impaled guard cells were
calculated assuming a spheroid geometry (Blatt and Slayman, 1983) and voltages were analyzed using Henry’s EP Software Suite. For clarity, the data of instantaneous current were fitted with a second-order polynomial function: \( I = y_0 + ax + bx^2 \).

### Gas Exchange Measurements

Whole-plant gas exchange measurements were performed using a 6400 XT Infrared Gas Analyzer equipped with a 6400-18 light source and the whole-plant Arabidopsis 6400-17 chamber (all from LI-COR Biosciences). To prevent any CO\(_2\) diffusion and water vapor from the soil, the pots were sealed with a clear film. All measurements were performed at 22°C, 50% relative humidity, and 400 µg mL\(^{-1}\) CO\(_2\). Before measurements, plants were equilibrated in darkness for 30 min. Measurements of net \( A \) and \( g_s \) values were performed on at least three different plants per genotype and light treatment, starting always at the same time of the diurnal cycle (EoN). Whole rosette area was determined using the software ImageJ v1.48 (NIH). The \( g_s \) and \( A \) values were normalized by subtracting the conductance values EoN (set as 0 = initial values for \( g_s \) or \( A \)) as described by Baroli et al. (2008). In all experiments, normalized \( g_s \) values during the dark pulses were lower compared with the \( g_s \) values at time 0 due to stomatal preopening during the last hours of the night period (Lebaudy et al., 2008). Calculation of gas-exchange parameters were made, according to von Caemmerer and Farquhar (1981) with \( C_i \) corrected for water vapor efflux from the leaf.

### Temporal Response of \( g_s \)

The temporal response of \( g_s \) to light has been described by a time constant (\( \tau \)) estimated using an exponential equation:

\[
g_s = g_{\text{max}} + (g_{\text{min}} - g_{\text{max}}) e^{-t/\tau}
\]

with \( g_{\text{min}} \) and \( g_{\text{max}} \) being the minimum and maximum \( g_s \). The time constant represents the time to reach 63% of the total \( g_s \) variation, and was used to estimate the maximum slope (\( S_{\text{max}} \)) using the maximum derivative:

\[
S_{\text{max}} = \frac{g_{\text{max}} - g_{\text{min}}}{\tau}
\]

Equation 1 was fitted on the observed data of each genotype collected in the different experiments using a non-linear mixed effect model. Fixed effects were set for \( g_{\text{min}} \), \( g_{\text{max}} \), and \( \tau \), and random effect were set for \( g_{\text{min}} \) and \( g_{\text{max}} \). The average and confidence interval estimated with this model was calculated for each genotype and experimental conditions. The analysis was performed using the R (v3.4.1) package nlme (v3.1) and
the nlme function. Initial parameter values were approximated using the initial and final $g_s$ observed, and the time to reach 63% of the observed variation for $\tau$.

**RNA Isolation and Quantitative PCR Analysis**

For RNA extraction from leaf material, three entire rosettes per genotype and time point (three biological replicates) were harvested and frozen in liquid nitrogen. For RNA extraction from epidermal peels, the middle veins of 12 rosettes per genotype and time point (one biological replicate) were excised and the remaining leaf material was blended in 100 mL of ice-cold water using a blender (ProBlend Avance, Philips). A total of three biological replicates per genotype and time point were used for one experiment. The blended material was passed through a 200-µm nylon mesh (Sefar) and the remaining epidermal peels were dried, collected, and immediately frozen in liquid nitrogen. Subsequently, the epidermal peels were ground using a tissue grinder (Mix Mill MM-301; Retsch). Total RNA was extracted from 30 mg of ground tissue using the SV Total RNA Isolation Kit (Promega) following the manufacturer’s instructions. RNA quality and quantity were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A total of 1 mg of RNA was used for cDNA first-strand synthesis using the M-MLV Reverse Transcriptase RNase H Minus Point Mutant and oligo(dT)15 primer (Promega). Transcript levels were examined by RT-qPCR using the SYBR Green Master Mix (Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems). RT-qPCR was performed in triplicates. Transcript levels were calculated according to the comparative CT method (Livak and Schmittgen, 2001) and were normalized against the expression of the Actin2 gene (ACT2; At3g18780). Error calculation was done according to Applied Biosystems guidelines (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf). Primers and PCR efficiencies for RT-qPCR are listed in Supplemental Table 5.

**Mesophyll Starch Extraction and Quantification**

Mesophyll starch contents were determined enzymatically according to Hostettler et al. (2011). In brief, entire Arabidopsis rosettes were harvested at the indicated time points and immediately frozen in liquid nitrogen. Rosettes were homogenized using a tissue grinder (Mix Mill MM-301; Retsch) and resuspended in 0.7M perchloric acid. Insoluble material was washed three times with 70% (v/v) ethanol and subsequently resuspended in water. Starch was solubilized by heating (95°C) and thereafter digested to Glc via enzymatic reactions (a-amylase and amyloglucosidase, both from Roche) at 37°C. The amount of Glc equivalents was determined using the enzymes hexokinase (Roche) and Glc-6-phosphate dehydrogenase (Roche), which convert
NAD to NADH in an equimolar ratio. The increase in NADH was determined spectrophotometrically (Synergy H1; BioTek) by monitoring the absorption spectrum at 340 nm.

**Guard Cell Soluble Sugar Quantification**

To extract soluble sugars from guard-cell-enriched epidermal peels, six rosettes per genotype, corresponding to one biological replicate, were collected at the EoN or after the plants were exposed to 300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of RL for 2 h, and the petiole was removed using scissors. The remaining leaf material was blended in 100 mL of ice-cold water using a kitchen blender (Avance Collection, Philips). The blended material was filtered through a 200-\( \mu \)m nylon mesh and the remaining epidermal peels were dried, collected in a tube, and immediately frozen in liquid nitrogen. Alternatively, epidermal peels from EoN samples were collected from the nylon mesh and incubated in basic opening buffer (5 mM of MES-bistrispropane at pH 6.5, 50 mM of KCl, and 0.1 mM of CaCl\(_2\)) for 30 min under 75 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of BL or darkness. To remove residual sugars from the guard cell apoplast, the samples were washed with 2 L of Milli-Q water (Merck Millipore) according to Daloso et al. (2015) and refrozen in liquid nitrogen. Subsequently, guard-cell-enriched epidermal peel materials were ground into a fine powder with a ball mill (Mix Mill MM-301; Retsch). Up to six biological replicates per genotype and time point were harvested for one experiment. Two independent experiments were performed. Soluble sugars were extracted as described by Thalmann et al. (2016). After the extraction, the samples were lyophilized in a freeze-dryer (Lyovac GT1; Lybold) and resuspended in 60 mL of Milli-Q water (Merck Millipore). Guard cell soluble sugars were quantified based on the protocol for quantification of root soluble sugars described by Thalmann et al. (2016) using 50 \( \mu \)L of neutralized soluble fraction obtained from the lyophilized and resuspended initial perchloric acid extraction as starting material.

**Leaf Soluble Sugar Quantification**

Leaf soluble sugars were determined enzymatically according to Thalmann et al. (2016). In brief, entire Arabidopsis rosettes were harvested at the EoN and immediately frozen in liquid nitrogen. Rosettes were homogenized using a tissue grinder (Mix Mill MM-301; Retsch) and resuspended in 0.7 M perchloric acid. After pelleting the insoluble material, 600 \( \mu \)L of clear supernatant was transferred to a fresh 1.5-mL Eppendorf tube. The soluble fraction was neutralized with neutralization buffer (400 mM of MES and 2 M of KOH), and 600 \( \mu \)L of clear supernatant was kept for analysis. Eight rosettes per genotype were harvested for one experiment. Soluble
sugars were quantified using 20 µL of neutralized soluble fraction obtained from the initial perchloric acid extraction.

**Guard Cell Malate Quantification**

To quantify the amount of malate from guard-cell-enriched epidermal peels, six rosettes per genotype, corresponding to one biological replicate, were collected at the EoN and the leaf material was blended in 100 mL of ice-cold water using a kitchen blender (Avance Collection, Philips). The blended material was filtered through a 200-µm nylon mesh and either dried, collected, and immediately frozen in liquid nitrogen, or incubated in basic opening buffer (5 mM of MES-bistrispropane at pH 6.5, 50 mM of KCl, and 0.1 mM of CaCl₂) for 30 min under 75 µmol m⁻² s⁻¹ of BL or darkness. The samples were washed extensively with 2 L of Milli-Q water to remove residual organic acids from the guard cell apoplast according to Daloso et al. (2015). Afterwards, guard-cell-enriched epidermal peel materials were ground into a fine powder with a ball mill (Mix Mill MM-301; Retsch). Up to six biological replicates per genotype and time point were harvested for one experiment. Two independent experiments were performed. To extract organic acids, 1 mL of Milli-Q water was added to the ground tissues and the samples were incubated at 95°C for 15 min, followed by 10 min of centrifugation at 16,000 g to collect the supernatant. After the extraction, samples were lyophilized in a Lyovac GT1 freeze-dryer (Lybold) and resuspended in 60 µL of Milli-Q water. L-malate content was determined using the K-LMAL-116A kit (Megazyme) following the manufacturer’s protocol using 50 µL of the lyophilized and resuspended organic acid extract.

**Malate Quantification in Leaves**

Malate content of leaves was determined using the K-LMAL-116A kit (Megazyme) following the manufacturer’s protocol. Entire Arabidopsis rosettes were harvested at the EoN and immediately frozen in liquid nitrogen. Malate was extracted as described above in “Guard Cell Malate Quantification.” Eight rosettes per genotype were harvested for one experiment. L-malate content was determined using 10 µL of the initial organic acid extract.

**Statistical Analysis**

Statistical differences between genotypes and time points were determined by ANOVA with post hoc Tukey’s Honest Significant Difference test (P-value < 0.05) or by the unpaired Student’s t test. Statistical significance was marked as follows: *P < 0.05; **P
< 0.01; ***P < 0.001. All data are indicated as means ± SE. Details are given in Supplemental File 2.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At3g23920 (*BAM1*), At1g69830 (*AMY3*), and At2g18960 (*AHA1*).

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**AUTHOR CONTRIBUTIONS**

D.S., M.R.B., and S.F. designed the research; S.F., Y.W., A.T., S.R.M.V.-C., M.K., and A.N. performed the research; A.H. added software utilities for data analysis; S.F., T.L., M.R.B., and D.S. analyzed the data; D.S., S.F., T.L., and M.R.B. wrote the article with approval from all authors.
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SUPPLEMENTAL DATA

Supplemental Figure 1. Quantification of H⁺-ATPase phosphorylation levels and \( g_s \) kinetics under blue light illumination superimposed on red light. (Supports Figure 1) (A) Quantification of phosphorylation levels of H⁺-ATPase in WT and \( amy3 \) \( bam1 \) isolated guard cell protoplasts illuminated with blue light (10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) blue light superimposed on 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) red light). H⁺-ATPase and the phosphorylated form of H⁺-ATPase were detected by immunoblotting using an antibody against H⁺-ATPase (anti-pThr) and the phospho-specific antibodies against the penultimate Thr, respectively. Densitometry analysis (ImageJ software) of three replicate blots was used to quantify the band intensities. Values are expressed relative to the mean band intensity of H⁺-ATPase. Each value is the mean ± SEM of 4 biological samples. Each sample was blotted in 3 technical replicates. Unpaired student’s \( t \) test determined statistical significance between the indicated comparisons (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); n.s., not significant).

(B) Whole-plant recordings of stomatal conductance (\( g_s \) - \( g_{\text{initial}} \)) in dark-adapted plants in response to 2 h red light illumination (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) followed by 30 min blue light (10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) superimposed on red light (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) illumination. \( g_s \) values were normalized to values after 110 min of red light illumination, number of measured plants per genotype \( n = 3 \); values presented are means ± SEM. \( g_{\text{initial}} \) ranged between 81 and 197 mmol m\(^{-2}\) s\(^{-1}\).
Supplemental Figure 2. Metabolite quantification in guard cells and leaves under darkness. (Supports Figure 2)

(A) Malate and (C) soluble sugar contents of WT and amy3 bam1 guard cell-enriched epidermal peels at the end of the night (EoN) and after 30 min of incubation in stomatal opening buffer in the dark (D = darkness). FW = fresh weight. Number of measured samples per genotype n ≥ 3; means ± SEM are shown. Different letters indicate significant statistical differences amongst genotypes for the given time point. Asterisk (*) indicates significant statistical difference between time points for the given genotype for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.

(B) Malate and (D) soluble sugar contents of WT and amy3 bam1 whole rosettes at EoN. FW = fresh weight. Number of measured samples per genotype n ≥ 4; means ± SEM are shown. Different letters indicate significant statistical differences amongst genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.
Supplemental Figure 3. Stomatal opening responses of WT, amy3 bam1 and aha1 plants subjected to a “2-pulse-light” treatment. (Supports Figure 3) (A) Whole-plant recordings of stomatal conductance ($g_s$) in dark-adapted (30 min) plants in response to 2 h L – 2 h D – 2 h L – 2 h D (L = light; D = darkness). Plants were illuminated with 150 µmol m$^{-2}$ s$^{-1}$ white light. Number of measured plants per genotype $n \geq 3$; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(B) Stomatal aperture in dark-adapted (30 min) plants in response to the same light treatment as in (A). Each value represents mean ± SEM of 4 biological replicates of more than 200 individual stomata obtained from 2 independent experiments. Unpaired student’s $t$ test determined statistical significance between the indicated comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

(C-D) Changes in stomatal conductance ($g_s - g_{initial}$) in response to a shift from dark to light in amy3 bam1 (C) and aha1 (D). Data are taken from Fig. 3A.

(E-F) Speed of increase in stomatal conductance estimated using a time constant ($\tau_i$) and (G-H) the maximum slope of $g_s$ increase ($S_{max}$) during the two consecutive pulses of light in amy3 bam1 (E and G) and aha1 (F and H).

(I) Whole-plant recordings of CO$_2$ assimilation ($A$) from plants under the same light regime as in (A). Number of measured plants per genotype $n \geq 3$; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(J) Whole-plant estimations of $C_i/C_a$ dynamics from plants under the same light regime as in (A). Number of measured plants per genotype $n \geq 3$; values presented are means.
**Supplemental Figure 4. Gene expression of AHA2 and AHA5 in guard cells of aha1 mutant.** (Supports Figure 3)

**(A)** Expression levels of β-amylase 3 (BAM3), MYB60 and KAT1 genes in guard cell-enriched epidermal peels compared to leaves from WT plants. MYB60 and KAT1 were used as guard cell-specific markers, while BAM3 was used as a leaf-specific marker. **(B)** Guard cell-specific expression of AHA2 and AHA5 in WT-guard cell-enriched epidermal peels compared to peels from aha1 plants harvested at the end of the night. Actin (ACT2) was used as housekeeping gene for normalization. Values are means of five independent experiments ± fold change range. For details about error calculation see Materials & Methods. Primer sequences and efficiencies are given in Table S5.

**Supplemental Figure 5. Leaf starch content of plants subjected to a “2-pulse-light” treatment.** (Supports Figure 3)
Mesophyll starch content of four-week-old WT, aha1 and amy3 bam1 plants after exposure to the same light treatment as in (Fig. 3). Number of measured plants per genotype n ≥ 6; values presented are means ± SEM. FW = fresh weight. Unpaired student’s t test determined statistical significance between the indicated comparisons (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant).
Supplemental Figure 6. Stomatal opening responses of WT, amy3 bam1 and aha1 plants subjected to a modified “2-pulse-light” treatment. (Supports Figure 4)

(A) Whole-plant recordings of stomatal conductance \( g_s \) in dark-adapted (30 min) plants in response to 3 h L – 2 h D – 2 h L – 2 h D (L = light; D = darkness). Plants were illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) white light. Number of measured plants per genotype \( n \geq 3 \); values presented are means \( \pm \) SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(B) Stomatal aperture in dark-adapted (30 min) plants in response to the same light treatment as in (A). Each value represents mean \( \pm \) SEM of 4 biological replicates of more than 200 individual stomata obtained from 2 independent experiments. Unpaired student’s \( t \) test determined statistical significance between the indicated comparisons (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); n.s., not significant).

(C-D) Changes in stomatal conductance \( g_s - g_{initial} \) in response to a shift from dark to light in amy3 bam1 (C) and aha1 (D). Data are taken from Fig. 4B.

(E-F) Speed of increase in stomatal conductance estimated using a time constant \( \tau \) and the maximum slope of \( g_s \) increase \( S_{\max} \) during the two consecutive pulses of light in amy3 bam1 (E and G) and aha1 (F and H).

(I) Whole-plant recordings of CO₂ assimilation \( A \) from plants under the same light regime as in (A). Number of measured plants per genotype \( n \geq 3 \); values presented are means \( \pm \) SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(J) Whole-plant estimations of C/\( \text{C}_a \) dynamics from plants under the same light regime as in (A). Number of measured plants per genotype \( n \geq 3 \); values presented are means.

77
Supplemental Figure 7. Effect of time-of-day on WT stomatal kinetics and photosynthesis. (Supports Figure 5)

(A) Whole-plant recordings of stomatal conductance ($g_s$) in dark-adapted (30 min) plants in response to 2 h L – 3 h D – 2 h L – 2 h D (L = light; D = darkness). Plants were illuminated with 150 $\mu$mol m$^{-2}$ s$^{-1}$ white light. Number of measured plants per genotype n = 3; values presented are means ± SEM.

(B) Whole-plant recordings of CO$_2$ assimilation (A) from plants under the same light regime as in (A). Number of measured plants per genotype n = 3; values presented are means ± SEM.
Supplemental Figure 8. Stomatal opening responses of WT, amy3 bam1 and aha1 plants subjected to a “2-pulse-light” treatment under saturating photosynthetic active radiation. (Supports Figure 7)

(A) Whole-plant recordings of stomatal conductance ($g_s$) in dark-adapted (30 min) plants in response to 2 h L – 2 h D – 2 h L – 2 h D (L = light; D = darkness). Plants were illuminated with 400 µmol m$^{-2}$ s$^{-1}$ white light. Number of measured plants per genotype n = 3; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(B) Stomatal aperture in dark-adapted (30 min) plants in response to the same light treatment as in (A). Each value represents mean ± SEM of 4 biological replicates of more than 200 individual stomata obtained from 2 independent experiments. Unpaired student’s t test determined statistical significance between the indicated comparisons (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant).

(C–E) Changes in stomatal conductance ($g_s - g_{initial}$) in response to a shift from dark to light in WT (C), amy3bam1 (D) and aha1 (E). Plants were illuminated with 400 µmol m$^{-2}$ s$^{-1}$ white light. Data are taken from Fig. 7A.

(F) Whole-plant recordings of CO$_2$ assimilation ($A$) from plants under the same light regime as in (A). Number of measured plants per genotype n = 3; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(G) Whole-plant estimations of $C_i/C_a$ dynamics from plants under the same light regime as in (A). Number of measured plants per genotype n = 3; values presented are means.
Supplemental Figure 9. Stomatal opening responses of WT, amy3 bam1 and aha1 plants in response to red light.

(A) Whole-plant recordings of stomatal conductance ($g_s$) in dark-adapted (30 min) plants in response to 6h illumination with 300 µmol m$^{-2}$ s$^{-1}$ of red light. Number of measured plants per genotype $n = 3$; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(B) Stomatal aperture in dark-adapted (30 min) plants in response to the same light regime as in (A). Each value represents mean ± SEM of 4 biological replicates of more than 200 individual stomata obtained from 2 independent experiments. Unpaired student’s $t$ test determined statistical significance between the indicated comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

(C) Relative increase in stomatal aperture during 6 h of illumination with red light given in percentage (value at end of the night (EoN), was set as 100%).

(D) Whole-plant recordings of CO$_2$ assimilation ($A$) from plants under the same light regime as in (A). Number of measured plants per genotype $n = 3$; values presented are means ± SEM. No significant statistical differences were detected amongst genotypes at selected time points by one-way ANOVA.

(E) Normalized whole-plant recordings of CO$_2$ assimilation ($A$-$A_{initial}$) from plants under the same light regime as in (A). $g_s$ values were normalized to values at the end of the night (EoN). Number of measured plants per genotype $n = 3$; values presented are means.
### Supplemental Table 1. (Supports Figure 2)
Malate content of guard-cell-enriched epidermal peels of WT and *amy3 bam1* treated with blue light (BL). Values represent means ± SEM.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time point</th>
<th>Malate content (mg g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>End of night</td>
<td>0.049 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>30 min of BL</td>
<td>0.024 ± 0.01</td>
</tr>
<tr>
<td><em>amy3bam1</em></td>
<td>End of night</td>
<td>0.047 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>30 min of BL</td>
<td>0.021 ± 0.009</td>
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### Supplemental Table 2. (Supports Figure 2)
Soluble sugar content of guard-cell-enriched epidermal peels of WT and *amy3 bam1* treated with blue light (BL). Values represent means ± SEM.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time point</th>
<th>Glucose content (mg g⁻¹ FW)</th>
<th>Fructose content (mg g⁻¹ FW)</th>
<th>Sucrose content (mg g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>End of night</td>
<td>0.074 ± 0.013</td>
<td>0.01 ± 0.003</td>
<td>0.005 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>30 min of BL</td>
<td>0.059 ± 0.012</td>
<td>0.002 ± 0.001</td>
<td>0.001 ± 0.0007</td>
</tr>
<tr>
<td><em>amy3bam1</em></td>
<td>End of night</td>
<td>0.034 ± 0.015</td>
<td>0.009 ± 0.004</td>
<td>0.02 ± 0.006</td>
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<tr>
<td></td>
<td>30 min of BL</td>
<td>0.008 ± 0.003</td>
<td>0.001 ± 0.0008</td>
<td>0</td>
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</tbody>
</table>

### Supplemental Table 3. (Supports Figures 3, 4 and 5)
Temporal responses of stomatal conductance in WT, *amy3 bam1* and *aha1* subject to different “2-pulse-light” treatments.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Experiment</th>
<th>Pulse</th>
<th>Tᵢ (min)</th>
<th>Slmax (mmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2-pulse</td>
<td>1ˢᵗ</td>
<td>17.05 ± 0.94</td>
<td>0.094 ± 0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2ⁿᵈ</td>
<td>36.2 ± 2.32</td>
<td>0.044 ± 0.005</td>
</tr>
<tr>
<td><em>aha1</em></td>
<td>2-pulse</td>
<td>1ˢᵗ</td>
<td>24.93 ± 1.76</td>
<td>0.054 ± 0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2ⁿᵈ</td>
<td>55.66 ± 6.87</td>
<td>0.032 ± 0.022</td>
</tr>
<tr>
<td><em>amy3bam1</em></td>
<td>2-pulse</td>
<td>1ˢᵗ</td>
<td>57.22 ± 6.87</td>
<td>0.025 ± 0.012</td>
</tr>
<tr>
<td></td>
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<td>2ⁿᵈ</td>
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<td>0.028 ± 0.008</td>
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<td>WT</td>
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<td>0.087 ± 0.025</td>
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<tr>
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<tr>
<td><em>aha1</em></td>
<td>2-pulse modified</td>
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<td>82.34 ± 9.02</td>
<td>0.036 ± 0.011</td>
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CHAPTER I

<table>
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<th>Genotype</th>
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<th>Glucose content (mg g⁻¹ FW)</th>
<th>Fructose content (mg g⁻¹ FW)</th>
<th>Sucrose content (mg g⁻¹ FW)</th>
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Supplemental Table 4. (Supports Figure 8)
Soluble sugar content of guard-cell-enriched epidermal peels of WT and aha1 treated with red light (RL). Values represent means ± SEM.

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<tr>
<th>Gene</th>
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<td>At3g18780</td>
<td>CGTACAACCGGTATTTGCT</td>
<td>GTAATCAGTAAAGTCACGTC</td>
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<td>AGAGCGATGGTCGAGTGCAG</td>
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<td>Yamauchi et al., 2016</td>
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Supplemental Table 5. (Supports Supplemental Figure 4)
Sequences of primers used for qRT-PCR (Cominelli et al., 2011; Horrer et al., 2016; Yamauchi et al., 2016; Jalakas et al., 2017).

Supplemental References


H⁺ extrusion rate reported in Figure 1B of this study: 0.72 nmol H⁺ h⁻¹ µg⁻¹ protein

Relation of protein content and guard cell number: 4.3 x 10⁷ guard cells contain 1 mg of protein

Protein content of a guard cell estimation: 2.3 x 10⁻⁵ µg protein

H⁺ extrusion rate per guard cell estimation: 2.3 x 10⁻⁵ µg protein x 0.72 nmol H⁺ h⁻¹ µg⁻¹ protein = 0.017 pmol H⁺ h⁻¹

Guard cell length (µm): 15-20

Guard cell diameter (µm): 4-6

Guard cell volume estimation Arabidopsis (pl): Volume for a spheroid = \( \frac{4}{3} \pi bc^2 \), where 

b is the radius of the major (rotational) axis and where c is the radius of the minor axis.

If we use b = 10 µm (maximal radius based on maximal guard cell length) and c = 3 µm (maximal radius based on maximal guard cell diameter) we obtain the following guard cell volume:

\[ \frac{4}{3} \pi \cdot 10 \cdot 3^2 = 377 \mu m^3 = 0.377 \text{ pl} \]

No data are available on H⁺ contents in guard cells during stomatal opening. For this reason, K⁺ content data from Vicia faba were used for the following calculations, assuming a 1:1 exchange of H⁺ and K⁺.

Guard cell volume Vicia faba: 5 pl

Change in K⁺ content during stomatal opening in Vicia faba (no Arabidopsis data): 0.36 pmol K⁺ µm⁻¹

Change in K⁺ content = H⁺ content during stomatal opening in Arabidopsis: 0.36 pmol H⁺ µm⁻¹ / 5 pl x 0.377 pl = 0.027 pmol H⁺ µm⁻¹

The 0.027 pmol H⁺ µm⁻¹ estimated H⁺ extrusion rate based on parameters from Vicia faba and maximal size parameters for Arabidopsis guard cells is well in the range of the proton extrusion rate of 0.017 pmol H⁺ h⁻¹ (based on estimations of guard cell protein content) reported in this study. Even though, the 0.027 pmol H⁺ µm⁻¹ H⁺ extrusion is most likely overestimated due to Vicia faba values.
References for Calculations


CHAPTER II

ARE MESOPHYLL SUGARS REQUIRED FOR STOMATAL FUNCTIONING?

Glucose uptake to guard cells via STP transporters provides carbon sources for stomatal opening and plant growth

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ABSTRACT

Guard cells on the leaf epidermis regulate stomatal opening for gas exchange between plants and the atmosphere, allowing a balance between photosynthesis and transpiration. Given that guard cells possess several characteristics of sink tissues, their metabolic activities should largely depend on mesophyll-derived sugars. Early biochemical studies revealed sugar uptake into guard cells. However, the transporters that are involved and their relative contribution to guard cell function are not yet known. Here, we identified the monosaccharide/proton symporters Sugar Transport Protein 1 and 4 (STP1 and STP4) as the major plasma membrane hexose sugar transporters in the guard cells of Arabidopsis thaliana. We show that their combined action is required for glucose import to guard cells, providing carbon sources for starch accumulation and light-induced stomatal opening that are essential for plant growth. These findings highlight mesophyll-derived glucose as an important metabolite connecting stomatal movements with photosynthesis.

Keywords glucose; guard cells; plant growth; stomatal opening; sugar transport protein
CHAPTER II

INTRODUCTION

Stomata are microscopic pores on the plant leaf epidermis surrounded by a pair of guard cells. These vital cells adjust pore aperture in response to numerous endogenous and exogenous factors, allowing uptake of carbon dioxide (CO$_2$) for photosynthesis ($A$), while preventing excessive water loss through transpiration ($E$). By controlling the trade-off between photosynthesis and transpiration, stomata play a critical role in determining water-use efficiency (WUE = amount of carbon fixed per unit water loss, $A/E$) and, hence, plant growth and productivity (Lawson & Vialet-Chabrand, 2019).

Stomatal opening and closure results from reversible changes in guard cell volume and shape. At the molecular level, this is driven primarily by the activity of the plasma membrane H$^+$-ATPase which stimulates the movement of large quantities of ions (mainly potassium, chloride, malate$^{2-}$, and nitrate) into and out of the guard cells and consequent osmotic water flow for their swelling or shrinking (Inoue & Kinoshita, 2017; Jezek & Blatt, 2017).

For more than a century, it has been known that guard cells possess modified carbohydrate metabolic pathways compared to the rest of the leaf, but their significance for stomatal function has long remained obscure. Only recently it became clear that guard cell starch metabolism integrates with signaling and membrane ion transport to regulate stomatal movements (Daloso et al., 2017; Santelia & Lawson, 2016). At the start of the day, the rapid breakdown of guard cell starch is activated by phototropin-mediated signaling downstream of the plasma membrane H$^+$-ATPase to promote efficient stomatal opening (Horrer et al., 2016). The major guard cell starch-derived metabolite is glucose (Glc), which is needed to maintain the cytoplasmic sugar pool contributing to fast stomatal opening (Flütsch et al., 2020). Starch formation induced during high CO$_2$-mediated stomatal closure has been proposed to facilitate the dissipation of the accumulated organic solutes leading to changes in guard cell osmotic potential for water efflux (Penfield et al., 2012; Azoulay-Shemer et al., 2016). Soluble sugars also regulate stomatal movements, but the way they do so remains still rather controversial (Daloso et al., 2016a). Initial studies suggested that sucrose (Suc) and its derived sugars (Glc; fructose, Fru) induce stomatal opening as direct osmotic (Outlaw & Manchester, 1979; Poffenroth et al., 1992; Talbott & Zeiger, 1993; Amodeo et al., 1996). More recently, it was revealed that Suc promotes stomatal opening by serving as a substrate for glycolysis and mitochondrial respiration (Daloso et al., 2015, 2016b; Medeiros et al., 2018). Sugars can also induce stomatal closure either as osmolytes in the guard cell apoplast (Lu et al., 1997; Outlaw & De Vlieghere-
He, 2001; Kang et al, 2007) or as signaling molecules through phosphorylation by hexokinase within the guard cells (Kelly et al, 2013; Lugassi et al, 2015).

Despite these studies shed light on the importance of carbohydrate metabolism for stomatal movements, experiments have not yet answered basic questions about the source of sugars in guard cells. Given that CO$_2$ fixation within guard cells is limited (Outlaw et al, 1979; Outlaw, 1989; Reckmann et al, 1990) and that photosynthesis in the mesophyll cells is the main source of sugars at the whole-plant level, it is likely that symplastically isolated guard cells rely mostly on mesophyll-derived Suc to fulfill their metabolic needs. Suc can be taken up directly via Suc transporters or in the form of hexoses via monosaccharide transporters following Suc hydrolysis by a cell wall invertase. Transcriptomics studies suggest that several sugar transporters are highly expressed in guard cells (Leonhardt et al, 2004; Wang et al, 2011; Bates et al, 2012; Bauer et al, 2013), but the relative contribution of this transport system to guard cell function is not yet known.

Here, we identified the monosaccharide/proton symporters Sugar Transport Protein 1 and 4 (STP1 and STP4) as the major plasma membrane hexose sugar transporters in the guard cells of Arabidopsis thaliana. We show that their combined action is required for Glc import to guard cells, providing carbon sources for starch accumulation and light-induced stomatal opening that are essential for plant growth. These findings highlight that a tight coordination between mesophyll and guard cell carbohydrate metabolism promotes optimal plant growth through regulation of stomatal opening.
CHAPTER II

RESULTS

Sugar Transport Protein 1, 4, and 13 are highly expressed in guard cells

Higher plants possess three types of plasma membrane carriers for the intercellular transport of sugars: MSTs (monosaccharide transporters), SUCs or SUTs (Suc transporters) and SWEETs (hexose and Suc transporters). Angiosperm genomes usually contain several paralogs of each class of transporters, most of which serve distinct physiological roles (Chen et al., 2015). Through literature and database searches, we identified 40 plasma membrane sugar transporters in the Arabidopsis genome, covering all three types of carriers (Appendix Table S1). To select potential candidates for our study, we performed in silico analysis of gene expression levels in Arabidopsis guard cells using publicly available expression data (Fig EV1A). As expected, several transporters were highly expressed in guard cells, for instance, sucrose transporters 1, 2, and 3 (SUC1, SUC2, SUC3); Sugars will eventually be exported transporters 1, 5, 11, and 12 (SWEET1, SWEET5, SWEET11, SWEET12); sugar transport proteins 1, 4, 5, and 13 (STP1, STP4, STP5, STP13); and polyol/monosaccharide transporters 4, 5 and 6 (PMT4, PMT5, PMT6) (Fig EV1A). We focused on STP1, 4, and 13, as their gene expression in guard cells was on average 15 to 40 times higher compared to other sugar transporters (Fig EV1A). We confirmed their high and preferential expression in guard cell-enriched epidermal peels by qPCR (Fig EV1B and Appendix Table S2). In a previous study, STP1 was shown by in situ hybridization and immunohistochemistry to localize to guard cells (Stadler et al., 2003), further supporting our results.

STPs are high-affinity monosaccharide/proton symporters responsible for the transport of Glc, Fru, galactose, mannose, arabinose, and xylose from the apoplastic space into the cytosol (Büttner & Sauer, 2000; Büttner, 2010; Poschet et al., 2010; Rottmann et al., 2016, 2018b). These transporters are mostly found in sink tissues or symplastically isolated cells, such as pollen tubes, developing embryo, or guard cells (Stadler et al., 2003; Büttner, 2010; Rottmann et al., 2018a). It has been shown that STPs fulfill three main functions in plants: uptake of monosaccharides for the nutrition of sink cells (Sherson et al., 2000); re-absorption of monosaccharides from damaged roots under abiotic stress (Yamada et al., 2011); and antibacterial defense by competing with pathogens for extracellular sugars (Yamada et al., 2016).
Light-induced stomatal opening is impaired in Arabidopsis plants lacking both STP1 and STP4 transporters

To assess the contribution of the selected STPs to stomatal function, we obtained homozygous Arabidopsis T-DNA insertion lines at the STP1 (stp1-1; SALK_048848 and stp1-2; SALK_139194), STP4 (stp4-1; SALK_049432 and stp4-2; SALK_091229) and STP13 (stp13, SALK_0455494) loci. qPCR analyses revealed disruption of the STP1 gene expression in the stp1-1 mutant line (Fig EV1C), and a reduction of STP1 transcripts of 60% in the stp1-2 mutant (Fig EV1D). Furthermore, STP4 and STP13 transcript levels were reduced by approximately 40 and 80% in their respective mutant backgrounds compared to wild type (WT; Fig EV1C and D). To uncover putative functional relationship between the different STP isoforms, we generated the double mutant combinations stp1stp4 (from stp1-1 and stp4-1), stp1stp13 (from stp1-1), stp4stp13 (from stp4-1).

To describe morpho-physiological performance of the mutant lines in vivo, we used the automated phenotyping platform Plant-Screen™ Compact System (PSI, Czech Republic). We established a robust phenotyping protocol to quantify daily, over a period of 8 days, plant morphological, physiological, and biochemical traits. Infrared thermography revealed that stp1stp4 plants had statistically significant higher leaf surface temperature compared to WT and all tested mutant combinations, even though the overall differences in surface temperatures were small (Fig 1A and B; Appendix Table S3). Given that leaf temperature is an indicator of stomatal aperture (Merlot et al, 2002), we hypothesized that stp1stp4 mutant plants may have closed stomata. Indeed, infrared gas analysis of stomatal conductance (gₛ) responses showed that light-induced stomatal opening was severely impaired in stp1stp4 plants (Fig 1C). Stomatal closure in response to darkness was also affected in this mutant (Fig 1C). The stp1-1 single mutant had a reduced steady-state gₛ. However, stp1-1 plants reached a similar overall gₛ amplitude as WT, but stomatal opening kinetics were slow (Fig 1C), well visible if gₛ values were normalized to values at the end of the night (EoN; Fig EV2A). The slow opening phenotype of stp1-1 single mutants was further confirmed in a second mutant allele stp1-2 (Fig EV2C and D). The mild stomatal opening phenotype of stp1 mutants can be explained by a strong upregulation of STP13 in the guard cells of mutant plants (Appendix Fig S1). STP13 might partially compensate for the loss of STP1 in the stp1 mutant.
Figure 1. Stomatal function is impaired in stp1stp4 plants.

A  Representative false color images of leaf surface temperature captured by a thermal camera from WT, stp1-1, stp4-1, stp1stp4, stp13, stp1stp13, and stp4stp13 plants. B  Normalized leaf surface temperature over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point. C  Whole-plant recordings of changes in stomatal conductance (gs) from WT, stp1-1, stp4-1, and stp1stp4 plants. Data shown are means ± SEM; n ≥ 3 per genotype. D  Whole-plant recordings of changes in stomatal conductance (gs) from self-grafted donor lines (WT/WT, stp1stp4/stp1stp4) and reciprocal grafting of shoot/root (WT/stp1stp4, stp1stp4/WT) plants. Data shown are means ± SEM; n = 3 per genotype.

Data information: (C, D) Plants have been illuminated with 150 μmol/m²/s white light after the end of the night (EoN) under ambient-air CO₂ concentrations. (B) Asterisk (*) indicates significant statistical difference between WT and stp plants for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (C, D) Different letters indicate significant statistical differences among genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test.

Interestingly, stp4-1 single mutants also had a reduced steady-state gs, but reached a greater overall gs amplitude compared to WT plants and showed similar stomatal opening kinetics (Figs 1C and EV2A). In addition, stp4-2 showed a similar elevated gs amplitude as the stp4-1 (Fig EV2C and D), indicating that mutation in the stp4 locus is responsible for the observed phenotype. Altogether, the phenotype of the single stp1-1 and stp4-1 mutants and their respective additional mutant alleles (stp1-2 and stp4-2), with gs amplitudes and stomatal opening kinetics similar to WT, suggests that STP1 and STP4 are both required to promote stomatal opening at the start of the day (Figs 1A–C and EV2A, C and D; Appendix Table S3). Despite the high expression of STP13 in guard cells (Fig EV1), the lack of functional STP13 in the stp13 single mutant did not cause a reduced gs amplitude nor slow opening kinetics. stp13 mutants behaved similar to the stp4 mutant alleles (Figs 1A and B, and EV2E and F; Appendix Table S3). To investigate possible reasons behind the lack of phenotypes in stp4, stp13, stp1stp13, and stp4stp13, we performed STP gene expression analyses on guard cell-
enriched epidermal peels of WT, *stp4-1*, and *stp13* plants. Intriguingly, we found that *STP1* was upregulated in guard cells of *stp4-1* and *stp13* plants (Appendix Fig S1), suggesting that *STP1* might partially take over the role of *STP4* and *STP13* in their absence. In addition, *STP13* is upregulated in response to pathogen infections or treatments with bacterial elicitors (Büttner, 2010), and it is the only *STP* gene inducible by osmotic stress, high salinity and abscisic acid (Yamada et al, 2011). *STP13* function in guard cells may therefore become critical under stress conditions.

Previous studies reported the expression of *STP1* and *STP4* in Arabidopsis roots and their involvement in the uptake of monosaccharides from the rhizosphere (Truernit et al, 1996; Sherson et al, 2000; Yamada et al, 2011). To rule out the possibility that simultaneous knock-out of *STP1* and *STP4* in the roots contributed to the severe impairment of stomatal opening in *stp1stp4*, we measured *gₛ* in WT/ *stp1stp4* and *stp1stp4*/WT grafted plants. Plants with WT shoots and *stp1stp4* roots showed stomatal conductance comparable to WT, whereas plants bearing *stp1stp4* shoots and WT roots displayed a *stp1stp4*-like phenotype (Figs 1D and EV2B). The genetic identity of the roots from the reciprocal grafted plants was confirmed by molecular genotyping (Fig EV2G). Our grafting experiments indicate that the stomatal phenotype of *stp1stp4* is independent from the function of these transporters in the roots and further support their essential role in guard cells.

*stp1stp4* guard cells have reduced levels of glucose

We reasoned that inhibition of light-induced stomatal opening in *stp1stp4* mutants might be a direct consequence of impaired Glc and/or Fru import to guard cells. To test this hypothesis, we measured soluble sugar content in guard cells of intact leaves of WT and *stp1stp4* mutant plants at the EoN and after 40 min of light (Fig 2A). In WT guard cells, the levels of Glc and Suc were unaltered in response to the light treatment, while Fru levels decreased to half due to illumination (Fig 2A). The sustained levels of Suc are likely due to continuous Suc import from the mesophyll, which is consistent with the high expression of *SUC1* and *SUC3* transporters in guard cells at the EoN (Fig EV3A). Notably, *stp1stp4* guard cells had significantly lower amounts of Glc and Fru at the EoN compared to WT, and Glc levels were almost undetectable after 40 min of light (Fig 2A).
Suc surprisingly accumulated to higher levels (Fig 2A), perhaps as a result of reduced consumption, sequestration in the vacuole or SUC transporters upregulation to compensate for the loss of STPs. In contrast to stp1stp4 double mutants, guard cells
of single \textit{stp1-1} and \textit{stp4-1} mutants contained similar amounts of sugars as that of WT guard cells (Fig EV3B). However, there was a trend in \textit{stp1-1} guard cells towards reduced Glc levels compared to WT (Fig EV3B), which is in line with the mild impairment in stomatal opening in these plants.

These data demonstrate \textit{in vivo} the function of STP1 and STP4 in the coordinate transport of Glc and to a lesser extent Fru at the guard cell plasma membrane during light-induced stomatal opening and likely explain the inability of \textit{stp1stp4} to open stomata in the light. Based on the phenotype of the \textit{stp1stp4} mutant, which cannot open stomata despite the high levels of Suc, we suggest that imported Glc provides a major source of carbon for light-induced stomatal opening at the start of the day.

\textbf{\textit{stp1stp4} guard cells are devoid of starch}

Given that guard cells possess several characteristics of sink tissues with fewer and smaller chloroplasts and low photosynthetic rates, we next investigated whether the import of apoplastic Glc by STPs influenced guard cell starch metabolism. As we reported previously (Horrer \textit{et al}, 2016), starch was degraded in WT guard cells when the plants were illuminated, coinciding with the opening of the stomata (Fig 2B and C); after falling to near zero in the first hour after dawn, starch levels then began to rise again (Fig 2B and C). Notably, at the EoN, \textit{stp1stp4} guard cells were essentially devoid of starch, and no starch synthesis occurred during the first 3 h of light (Fig 2B and C). The single \textit{stp} mutants displayed a milder phenotype. Although they degraded the starch in guard cells upon transition to light similar to WT, they failed to resynthesize it (Figs 2B and C, and EV3C and D).

The fact that \textit{stp1stp4} guard cells were unable to make starch even in the presence of high amounts of Suc led us to hypothesize that at the start of the day mesophyll-derived Glc imported by STPs is the precursor for guard cell starch biosynthesis. If this hypothesis is correct, Arabidopsis plants lacking SUC transporters should be able to make starch under the investigated conditions. Indeed, as we show in Fig 2B and C, guard cells of \textit{suc1} and \textit{suc3} mutants had essentially normal starch turnover during the first 3 h of light (Fig 2B and C).
**stp1stp4 stomatal phenotype impacts on plant photosynthesis and growth**

Nearly all CO$_2$ needed for mesophyll photosynthesis enters the plant waxy leaf epidermis through the stomatal pores. Consequently, we predicted that reduced stomatal aperture in *stp1stp4* should have a major impact on intercellular CO$_2$ concentrations ($C_i$, μmol CO$_2$ mol/air) and photosynthetic performance. As anticipated, estimations of $C_i$ of illuminated leaves were lower in *stp1stp4* compared to WT and the single *stp* mutants (Fig EV4A). Kinetic chlorophyll fluorescence imaging further revealed that photosystem II (PSII) operating efficiency ($\Phi_{PSII}$) (Figs 3A and B, and EV4B and C), maximum quantum yield of PSII photochemistry in the dark-adapted ($F_v/F_m$) and the light adapted ($F_v'/F_m'$) states, and photochemical quenching (qP) (Appendix Table S4) were reduced in *stp1stp4* compared to WT and all tested mutant combinations. Independent measurements of CO$_2$ assimilation rates ($A$) confirmed these observations, both for mutant and grafted plants (Figs 3C and D, and EV4D–I).

Consistent with the reduced rate of photosynthesis, we found that Glc, Fru, and Suc levels in *stp1stp4* leaves at the end of the day (EoD) were, respectively, 40, 60, and 65% lower than that of WT (Fig 3E–G). EoD leaf starch content was also reduced by 60%, indicating that *stp1stp4* failed to accumulate starch efficiently in mesophyll cell chloroplasts (Fig 3H). In contrast to *stp1stp4*, the single *stp* mutants and the other *stp* double mutant combinations had sugar and starch levels similar to that of WT (Figs 3E–G and EV4J–M).

Using top-view RGB imaging, we determined growth performance of the plants. The *stp1stp4* mutants had 60% reduction in growth and a lower growth rate compared to WT (0.175 versus 0.211 per day; Appendix Table S5), while *stp1-1*, *stp4-1*, and *stp13* showed an intermediate phenotype, with only 20% reduction in growth (Figs 3I and J, and EV4N and O; Appendix Table S5). The *stp1stp13* and *stp4stp13* double mutants were indistinguishable from WT (Fig EV4N and O; Appendix Table S5); this again could be explained by a compensating mechanism between different STP isoforms when one is missing (Appendix Fig S1). No morphological differences among WT and *stp* mutants were observed, with the exception of reduced rosette perimeter and leaf slenderness in the case of *stp1stp4* (Appendix Table S5).
Figure 3. *stp1* *stp4* plants have reduced photosynthetic growth.

A Representative false color images of photosystem II (PSII) operating efficiency ($\Phi_{PSII}$) captured by chlorophyll fluorescence imaging from WT, *stp1*-*1*, *stp4*-*1* and *stp1* *stp4* plants. $\Phi_{PSII}$ was measured at a photosynthetically active radiation (PAR) of 440 µmol/m²/s. B $\Phi_{PSII}$ quantified over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point. C Whole-plant recordings of changes in CO$_2$ assimilation (A) from WT, *stp1*-*1*, *stp4*-*1*, and *stp1* *stp4* plants. Data shown are means ± SEM; n ≥ 3 per genotype. D Whole-plant recordings of changes in CO$_2$ assimilation (A) from self-grafted donor lines (WT/WT, *stp1* *stp4*/ *stp1* *stp4*) and reciprocal grafting of shoot/root (WT/*stp1* *stp4*/WT, *stp1* *stp4*/WT) plants. Data shown are means ± SEM; n = 3 per genotype. E Quantification of leaf glucose (Glc). Data shown are means ± SEM; n = 8 per genotype and time point. F Quantification of leaf fructose (Fru). Data shown are means ± SEM; n = 8 per genotype and time point. G Quantification of leaf sucrose (Suc). Data shown are means ± SEM; n = 8 per genotype and time point. H Quantification of leaf starch. Data shown are means ± SEM; n = 8 per genotype and time point. I Representative Red Green Blue (RGB) images of 3-(day 0) and 4-week-old (day 7) WT, *stp1*-*1*, *stp4*-*1*, and *stp1* *stp4* plants. Scale bar, 10 mm. J Projected rosette area over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point.

Data information: (B and J) Asterisk (*) indicates significant statistical difference between WT and *stp* plants for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (C and D) Plants have been illuminated with 150 µmol/m²/s white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. Different letters indicate significant statistical differences among genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test.
Elevated ambient-air CO\(_2\) restores \textit{stp1stp4} leaf carbohydrate metabolism and photosynthetic growth

Diffusional CO\(_2\) limitation on \(A\) imposed by stomatal conductance can be balanced off by subjecting plants to elevated ambient-air CO\(_2\). With this in mind, we reasoned that exposing \textit{stp1stp4} to high external CO\(_2\) concentrations would improve its photosynthetic capacity. Notably, when ambient-air CO\(_2\) was increased from 400 to 600–1,000 ppm, \textit{stp1stp4} steady-state \(A\) values fully recovered and were even slightly elevated compared to that of WT (Fig 4A). Furthermore, \textit{stp1stp4} plants grown in controlled environment under 600 ppm CO\(_2\) accumulated WT levels of leaf soluble sugars and starch (Fig 4B–E) and displayed a growth rate similar to that of WT (0.17 versus 0.18 per day; Fig 4F and G). The high CO\(_2\) treatment also increased starch accumulation in guard cells of WT at the EoN, but had no effect on \textit{stp1stp4} guard cells, which were still devoid of starch (Fig 4H and I). The lack of starch in \textit{stp1stp4} guard cells under elevated CO\(_2\) suggests that this phenotype is a direct consequence of the genetic defect in this mutant and further supports our conclusion that starch in guard cells is primarily made using mesophyll-derived Glc imported via STPs.

Overall, these results indicate that elevated CO\(_2\) concentrations could restore \textit{stp1stp4} leaf carbohydrate metabolism and photosynthetic growth to WT levels, implying the presence of a fully functional photosynthetic apparatus in \textit{stp1stp4}. Indeed, measurements by visible-near-infrared (VNIR) hyperspectral imaging of Normalized Difference Vegetation Index (NDVI), a commonly used estimator of chlorophyll content (Rouse \textit{et al}, 1974), indicated that NDVI of \textit{stp1stp4} plants was comparable to that of WT and the other \textit{stp} mutants (Fig EV5A and B; Appendix Table S3). Moreover, estimation of the variation in rosette green colors using the greenness hue abundance automatically computed from color-segmented RGB images showed no significant differences in paler hues of green (3, 4, and 5) for \textit{stp} plants and between \textit{stp1stp4} and WT plants (Fig EV5C–I; Appendix Table S5). Chlorophyll content was also unchanged (Fig EV5J). Based on these observations, we conclude that simultaneous mutation of \textit{STP1} and \textit{STP4} genes in Arabidopsis results in diffusive stomatal limitation that constrains CO\(_2\) availability for mesophyll photosynthesis, explaining the low photosynthetic performance, the alterations in mesophyll carbohydrate metabolism, and the resulting defective growth phenotype of \textit{stp1stp4} mutant.
Figure 4. Elevated levels of CO$_2$ rescue the stp1stp4 photosynthetic growth phenotype.

A Photosynthetic CO$_2$ assimilation (A) in dark-adapted WT and stp1stp4 plants in response to a step increase in CO$_2$ concentrations from 0 to 1,000 ppm. Data shown are means ± SEM; n = 3 per genotype. B Quantification of leaf glucose (Glc). Data for two independent experiments are shown; means ± SEM; n ≥ 17 per genotype and time point. C Quantification of leaf fructose (Fru). Data for two independent experiments are shown; means ± SEM; n ≥ 17 per genotype and time point. D Quantification of leaf sucrose (Suc). Data for two independent experiments are shown; means ± SEM; n ≥ 17 per genotype and time point. E Quantification of leaf starch. Data for two independent experiments are shown; means ± SEM; n ≥ 17 per genotype and time point. F Representative Red Green Blue (RGB) images of 3-(day 0) and 4-week-old (day 6) WT and stp1stp4 plants grown under 600 ppm CO$_2$. Scale bar, 10 mm. G Projected rosette area over the phenotyping period. Data shown are from two independent experiments; means ± SEM; n ≥ 24 per genotype and time point. H Representative confocal laser microscopy images of propidium iodide-stained guard cell starch granules of intact leaves of WT and stp1stp4 plants grown under 600 ppm CO$_2$. Scale bar, 10 µm. I Starch dynamics in guard cells of intact leaves of WT and stp1stp4 plants grown under 600 ppm CO$_2$ at the end of the night (EoN) and after 1 and 3 h of illumination with 150 µmol/m$^2$/s of white light. Data for two independent experiments are shown; means ± SEM; n = 80 individual guard cells per genotype and time point.

Data information: (B–E) Metabolites are from entire rosettes of WT and stp1stp4 plants at the end of the night (EoN) and end of the day (EoD) in a 12-h light/12-h dark cycle. Plants were grown under 600 ppm CO$_2$. FW, fresh weight. Asterisk (*) indicates significant statistical difference between time points for the given genotype. Different letters indicate significant statistical differences among genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (G) Asterisk (*) indicates significant statistical difference between genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (I) Different letters indicate significant statistical differences among time points for the given genotype for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test.
CHAPTER II

DISCUSSION

Due to limited autonomous CO$_2$ fixation capacity (Outlaw, 1989; Reckmann et al, 1990) and the lack of functional plasmodesmata (Wille & Lucas, 1984), it was long hypothesized that guard cells import mesophyll-derived sugars as a source of nutrition and osmotica. A few $^{14}$C-pulse-labeling studies have documented sugar uptake into guard cells, providing some evidence that it occurs in symport with H$^+$ in a process that is energy dependent, enhanced in the presence of fusicoccin (an activator of the plasma membrane H$^+$-ATPase), and inhibited by uncouplers (Dittrich & Raschke, 1977; Reddy & Das, 1986; Lu et al, 1995, 1997; Ritte et al, 1999). In these early experiments, the identity of the involved sugar transporters had not been determined, and our current knowledge is still mostly based on transcriptomic studies. The "omics" approach has provided a long list of sucrose and hexose transporter genes that are highly expressed in guard cells compared to mesophyll cells (Leonhardt et al, 2004; Wang et al, 2011; Bates et al, 2012; Bauer et al, 2013), but experimental evidence to support their function in guard cell regulation is essentially lacking.

In the present study, we identified the high-affinity monosaccharide/H$^+$ symporters STP1 and STP4 as major guard cell plasma membrane hexose transporters in Arabidopsis. We provide genetic, physiological and biochemical evidence that STP1 and STP4 cooperate in the import of mesophyll-derived Glc at dawn (Fig 2A), delivering to guard cells the carbon sources needed to promote light-induced stomatal opening (Fig 1), guard cell starch accumulation (Fig 2B and C), and plant growth (Fig 3).

STP1 was the first higher plant hexose transporter to be characterized (Sauer et al, 1990; Boorers et al, 1994). STP1 was initially found in germinating seeds and seedling roots, where it was shown to mediate the uptake of extracellular hexoses (Sherson et al, 2000). Stadler et al (2003) reported high expression of STP1 also in guard cells, but could not detect phenotypic changes in stp1 mutants, perhaps due to the low resolution of their gas exchange measurements. Heterologous expression studies in yeast cells demonstrated that STP4 is also an energy-dependent monosaccharide-H$^+$ symporter (Truernit et al, 1996). STP4 was reported to be expressed in root tips and pollen grains (Truernit et al, 1996), and was recently implicated in the uptake of Glc into pollen tube, where Glc serves as a signaling molecule for pollen tube growth (Rottmann et al, 2018a). Our work significantly advances knowledge on plant hexose transporters by revealing a novel function for STP1 and STP4 in Glc uptake to guard cells. Reciprocal WT/stp1stp4 grafting experiments (Figs 1D and 3D) ruled out that the stp1stp4 stomatal phenotype was
linked to the function of these transporters in the roots, further supporting a novel role for STPs in guard cells. Reduced Glc import at dawn impaired stomatal opening (Fig 1) and caused a reduction in intracellular CO$_2$ concentrations (Fig EV4A), which in turn impacted on photosynthetic capacity and plant growth (Fig 3). However, these CO$_2$ diffusional limitations could be compensated by growing the plants in elevated ambient-air CO$_2$ concentrations (Fig 4).

Single stp1 and stp4 mutants displayed mild phenotypes. In particular, stp1 mutants showed slightly slower stomatal opening kinetics compared to WT, whereas stp4 had greater overall $g_s$ amplitude (Figs 1C and EV2A, C and D). Given the opposite phenotypes of the single stp1 and stp4 mutants, we suggest that STP1 and STP4 are not redundant in guard cells, but rather have an intricate functional relationship. Like the distantly related Glc transporters from human (Hamill et al, 1999) or the SUT transporters in plants (Reinders et al, 2002), STP1 and STP4 may form a complex at the guard cell plasma membrane. Hetero-oligomerization of these transporters would be a fast mean to regulate sugar transport properties to guard cells and adjust the loading capacity according to supply and demand. Further studies are required to assess the potential of STP transporters to form hetero-oligomers and whether/how hetero-oligomerization affects STP transport properties.

Our discovery of a novel function for STP1 and STP4 in the regulation of stomatal opening has notable implications. Firstly, we shed new light on the unresolved issue concerning the potential sources of sugar in guard cells. In our current model, mesophyll-derived Suc is postulated to be the most important carbon source, which is broken down during stomatal opening to fuel the tricarboxylic acid (TCA) cycle (Daloso et al, 2015, 2016b; Medeiros et al, 2018). Our finding of reduced levels of Glc in stp1stp4 guard cells at the beginning of the day (Fig 2A) provides compelling evidence that imported Glc significantly contributes to the pool of sugars during dark-to-light transitions. This observation is in line with an early study which identified a monosaccharide-H$^+$ symporter activity in pea (Pisum sativum) guard cell protoplasts in addition to Suc uptake (Ritte et al, 1999). Interestingly, in the same study, it was reported that Suc uptake was much slower than that of Glc, suggesting that guard cells import carbohydrates mainly in the form of hexoses. However, during time of high CO$_2$ assimilation and transpiration (e.g. around noon), Suc uptake rates increased significantly (Lu et al, 1997; Ritte et al, 1999). These differences imply that there is a considerable flexibility in the extent and the means by which guard cells metabolize carbohydrates. It seems likely that in the early morning guard cells predominantly import Glc, while Suc is expected to become prominent among the sugars taken up when it is released in high amounts by the mesophyll, swept to the
epidermis with the transpiration stream, and accumulates in high concentrations around the guard cells.

Secondly, our work provides new exciting evidence on the role of soluble sugars in guard cell function. The defective stomatal opening of stp1stp4, despite the presence of high levels of Suc (Fig 2A), suggests that Glc import via STPs at dawn significantly contributes to the carbon sources fueling guard cell metabolism for stomatal opening. Apart from playing an osmotic role, Glc can be condensed in the cytosol to make hexose phosphates. These can then enter glycolysis and produce pyruvate to feed the TCA cycle; the reduced levels of Glc and Fru found in guard cells of stp1stp4 plants might therefore cause a decreased energy status (i.e. low ATP/ADP ratio) contributing to the impaired stomatal opening in this mutant. Hexose phosphates can also be used for the synthesis of uridine diphosphate glucose (UDP-Glc), which is a precursor of cellulose and most of the other cell wall polysaccharides, or they can be translocated to the chloroplast to make starch. The lack of starch in stp1stp4 guard cells (as we show in Fig 2B and C), and the fact that cellulose microfibrils in the cell wall are reorganized during guard cell movement in a xyloglucan and cellulose synthesis-dependent manner (Rui & Anderson, 2016), further supports the essential role of Glc as carbon source for guard cell metabolism regulating stomatal movements. The importance of Glc presented here is in line with latest research demonstrating that Glc is the major guard cell starch-derived metabolite which helps to accelerate stomatal opening above a baseline rate (Flütsch et al, 2020).

The increased quantities of Suc in stp1stp4 guard cells could be explained by upregulation of SUC transporters in the absence of functional STP1/STP4 or by reduction in Suc consumption due to feedback inhibition. Either way, Suc might enter a futile cycle of metabolic reactions, in which Suc is re-synthesized from UDP-Glc by the activity of the sucrose synthase Susy (Robaina-Estévez et al, 2017). Despite synthesis/degradation/import of Suc in guard cells is a dynamic process that has been shown to control stomatal movement, the existence of such Suc futile cycle might be interpreted as the closest steady-state solution to an underlying dynamic process, in which synthesis/degradation alternate according to the need of the cell (Robaina-Estévez et al, 2017). Furthermore, the presence of high Suc but very low starch in stp1stp4 mutants suggests that the pathway from Suc towards starch is nearly blocked in guard cells. The reason for such blocking is unclear. It can either result from guard cell-specific properties of the corresponding enzymes or it can be due to sequestration of Suc in the vacuole, presumably via the action of the vacuolar TST-type carriers (e.g. TMT1 and TMT2) that have been demonstrated to be capable of efficient proton-coupled Suc transport (Schulz et al, 2011; Jung et al, 2015).
In conclusion, our study demonstrates that guard cells have insufficient carbon reduction capacity and their function strongly depends on carbon supply from the mesophyll mediated by STP proteins. The tight correlation between mesophyll and guard cell carbohydrate metabolism as we now show puts Glc forward as an important metabolite connecting $g_s$ and $A$, and explains how small symplastically isolated guard cells could have critical roles in regulating $CO_2$ uptake for fine-tuned photosynthetic growth.
MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana* accession Columbia (Col-0) was used as wild type (WT) in this study. The transfer DNA (T-DNA) insertion lines SALK_048848 (*stp1-1*) (Yamada *et al.*, 2016), SALK_139194 (*stp1-2*), SALK_049432 (*stp4-1*), SALK_091229 (*stp4-2*) (Truernit *et al.*, 1996), SALK_045494 (*stp13*) (Norholm *et al.*, 2006; Schofield *et al.*, 2009; Yamada *et al.*, 2016), SALK_41553 (*suc1*), and SALK_037223 (*suc3*) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Alonso *et al.*, 2003), and the homozygous lines were isolated by molecular genotyping (for primer sequences see (Appendix Table S2). *stp1*-*stp13*, and *stp4*-*stp13* double mutant lines were generated by crossing through standard techniques and isolated by molecular genotyping (for primer sequences see Appendix Table S2). Seeds were sown on soil (Profisubstrat, Einheitserde, Classic), stratified at 4°C for 3 days in the dark, and transferred to the growth chamber for 7 days. Seedlings of similar size were then transplanted into single pots and cultivated in climate-controlled chambers (KKD Hiross, CLITEC Boulaguim, Root, Switzerland; Fitoclima 1200 or Fitoclima 2500, Aralab, Rio de Mouro, Portugal) under 12 h/12 h light/dark photoperiod, with a temperature of 21°C/19°C, a relative humidity of 45%/55%, and an irradiance of 150 µmol/m²/s using a combination of white (Osram Biolux) and purple (Osram Fluora) halogen lamps (in KKD Hiross) or LED tubes (in Fitoclima 1200) or panels (in Fitoclima 2500). In the case of the high CO₂ experiments, plants were cultivated in the Fitoclima 2500 climate chamber equipped with an external CO₂ gas tank. Unless otherwise stated, experiments were performed with 4-week-old non-flowering plants.

High-throughput phenotypic characterization

The high-throughput phenotypic characterization of WT and *stp* plants was carried out at Photon Systems Instruments (PSI) Research Center (Drásov, Czech Republic).

Plant growth conditions

Plants were cultivated as described in (Awlia *et al.*, 2016) with the following modifications. Seeds were sown on soil (Substrate 2, Klasmann-Deilmann GmbH, Germany), stratified at 4°C for 3 days in the dark, and transferred to a climate-controlled chamber (FytoScope FS WI, PSI, Drásov, Czech Republic) under 12 h/12 h, 22°C/20°C light/dark photoperiod with a relative humidity of 60% and an irradiance of 150 µmol/m²/s (cool-white LED and far-red LED). Seven days after stratification (DAS), seedlings of similar size were transplanted into single pots prepared the day
before with 60 g of sieved soil and watered up to the maximum soil water holding capacity. Plants were cultivated in the climate-controlled chamber as described above. 18 and 20 DAS plants were watered up to 60% soil water content using the automated weighing and watering unit of PlantScreen™ Compact System (PSI, Drásov, Czech Republic).

High-throughput automated non-invasive phenotyping platform in controlled environment

Plant phenotypic measurements were performed using Plant-Screen™ Compact System installed in controlled environment (FytoScope FS_WI, PSI, Drásov, Czech Republic). The platform is equipped with four robotic-assisted imaging units, an acclimation tunnel, and a weighing and watering unit. Plants set in trays are transported to the individual units by conveyor belts. The system is located in a climate-controlled chamber with a temperature of 23°C ± 1°C and a relative humidity of 55% ± 5%.

Phenotyping protocol and imaging sensors

A total of 10 plants per genotype were randomly distributed in specific trays of 20 pots each. Plant imaging started 21 DAS (day 0, 3-week-old plants) and continued until 28 DAS (day 7, 4-week-old plants). Plants were imaged daily using the following protocol. Briefly, plants were manually transferred from the climate-controlled growth chamber to the conveyor belt of the acclimation tunnel of PlantScreen™ Compact System and adapted for 30 min to the controlled environment under an irradiance of 150 µmol/m²/s (cool-white LED and far-red LED). Subsequently, plants were automatically phenotyped for around 2 h using thermal imaging, Red Green Blue (RGB) imaging, kinetic chlorophyll fluorescence imaging, and hyperspectral imaging in the listed order. At the end of the phenotyping protocol, plants were manually moved back to the climate-controlled growth chamber until the subsequent day. Using the automatic timing function of PlantScreen™ Scheduler (PSI Drásov, Czech Republic), the phenotyping protocol was programmed to start always at the same time of the diurnal cycle (after 2 h of illumination in the climate-controlled growth chamber). The acquired images were automatically processed using Plant Data Analyzer (PSI, Drásov, Czech Republic), and the raw data exported into CSV files were provided as input for analysis.

RGB imaging and processing were carried out as described in (Awlia et al, 2016) with the following modifications. Plant growth rate was calculated by fitting an exponential function to the interval of the projected leaf area increase over time (day
For leaf greenness evaluation, 9 hues of green were automatically generated using as input RGB images captured during the phenotyping period (day 0 – day 7). After a preliminary leaf greenness analysis, the 5 most representative hues were selected and used to estimate the variations in rosette colors.

Kinetic chlorophyll fluorescence imaging described in (Awlia et al, 2016) was optimized using a single photon irradiance level of 440 µmol/m²/s with a duration of 240 sec in the light curve protocol to quantify the rate of photosynthesis.

A thermal infrared camera (FLIR A615, FLIR Systems Inc.) with a resolution of 640 x 480 pixels mounted on a robotic arm was employed to automatically acquire top-view infrared images. The trays were automatically transported from the acclimation tunnel to the thermal imaging cabinet. Single snapshot image of one tray was acquired, and leaf surface temperature of each plant was automatically extracted with Plant Data Analyzer by pixel-by-pixel integration of values across the entire rosette. In order to subtract background from plant tissue, binary masks resulting from RGB image analysis were used. To minimize the influence of the environmental variability and the difference in the image acquisition timing among individual trays, the raw temperature of each plant (°C) was normalized by the average temperature (°C) of all plants present in the corresponding tray. The values in Fig 1B are shown as normalized leaf surface temperature.

Visible-near-infrared (VNIR) hyperspectral camera HC-900 Series (PSI, Drásov, Czech Republic) was used to acquire the spectral reflectance profiles of each plant. HC-900 camera operates in line scan mode in a wavelength range of 350–900 nm with a spectral resolution of 0.8 nm FWHM. The camera is mounted on robotic arm with implemented halogen tube light source (600 W) for homogenous and spectrally appropriate sample illumination during image acquisition. Prior to each measurement, two calibration measurements were performed automatically: dark current and radiometric using reflectance standard. Acquired hyperspectral data were processed using pixel-by-pixel analysis implemented in Hyperspectral Analyzer (PSI, Drásov, Czech Republic), featuring radiometric and dark noise calibration, background subtraction, and automated vegetation indices computation.

Leaf and guard-cell-enriched epidermal peel RNA isolation and qPCR
To extract RNA from leaf material, one full rosette per genotype, corresponding to one biological replicate, was harvested at the end of the night and immediately frozen in liquid nitrogen. To extract RNA from guard-cell-enriched epidermal peels, the middle veins of fully developed leaves from 12 rosettes per genotype, corresponding to one biological replicate, were excised at the end of the night at 4°C in the dark and the
remaining leaf material was blended in 100 ml ice-cold water using a kitchen blender (Philips, Avance Collection). The blended material was filtered through a 200 µm nylon mesh, and the remaining epidermal peels were dried, collected in a tube, and immediately frozen in liquid nitrogen. Subsequently, leaf and guard-cell-enriched epidermal peel materials were ground into a fine powder with a ball mill (Mix Mill MM-301, Retsch). Two or three biological replicates per genotype were harvested for one experiment. Two independent experiments were performed for each extraction (leaves and guard cell-epidermal peels). Total RNA was isolated from 30 mg of grinded tissue using the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions. RNA quantity and quality were determined using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). 1 µg of RNA was used for first-strand synthesis of cDNA using the M-MLV Reverse Transcriptase RNase H Minus Point Mutant (Promega) and oligo(dT)15 primer (Promega). Transcript levels were determined by qPCR using SYBR Green PCR Master Mix (Applied Biosystems) with the 7500 Fast Real-Time PCR System (Applied Biosystems). qPCR reactions were performed in triplicates. ACT2 was used as housekeeping gene for normalization. Transcript levels were calculated using the comparative CT (DDCT) method (Livak & Schmittgen, 2001). Raw data (CT values) processing and error calculation were performed according to Applied Biosystems guidelines (http://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042380.pdf). Primer sequences and PCR amplification efficiencies are listed in Appendix Table S2.

Leaf chlorophyll quantification
To quantify chlorophyll from leaf material, one full rosette per genotype, corresponding to one biological replicate, was harvested at the end of the night and immediately frozen in liquid nitrogen. Entire rosettes were ground in liquid nitrogen using mortar and pestle. Chlorophyll from approximately 100 mg of ground plant material was extracted at -20°C for 2 h in pre-cooled 90% acetone/10% 0.2 M Tris–HCl pH 8. The supernatant was diluted 1:20 in chlorophyll extraction buffer. Samples were transferred to a glass cuvette, and the OD at 649 and 665 nm was measured spectrophotometrically. Chlorophyll (Chl) a, b and a+b concentrations (µg/ml) were calculated according to the following formulas:

\[
\text{Chl a} = 11.63 \times \text{OD665} - 2.39 \times \text{OD649} \\
\text{Chl b} = 20.11 \times \text{OD649} - 5.18 \times \text{OD665} \\
\text{Chl a + b} = 6.45 \times \text{OD665} + 17.72 \times \text{OD649}
\]
Three biological replicates per genotype were harvested for one experiment. Three independent experiments were performed.

**Gas exchange measurements**

For gas exchange measurements, plants were grown in a Fitoclima 2500 under 8 h/16 h light/dark photoperiod, with a temperature of 21°C/19°C, a relative humidity of 45%/55%, and an irradiance of 150 µmol/m²/s. Whole-plant gas exchange measurements were carried out using the LI-6400XT System (LI-COR Biosciences) equipped with the 6400-17 whole-plant Arabidopsis chamber and the integrated 6400-18A RGB light source. To prevent any CO₂ diffusion and water vapor from the soil, pots containing Arabidopsis were sealed with clear film. Stomatal conductance ($g_s$) and net CO₂ assimilation ($A$) were measured at 22°C ± 2°C, with a relative humidity of 50% and a CO₂ concentration of 400 ppm. The plant was equilibrated in darkness within the chamber until all the parameters had stabilized (30 min). After the reading was constant for 10 min, an irradiance of 150 µmol/m²/s was applied to the rosette for 2 h, followed by 30 min of darkness. The parameters were recorded every min. $g_s$ and $A$ were normalized by subtracting the values at the end of the night (set as $0 = g_{initial}$ or $A_{initial}$). At least three independent plants per genotype were measured on different days starting at the same time of the diurnal cycle (end of the night). The time point after 2 h of illumination corresponds to the same time of the diurnal cycle at which thermal images were taken. Rosette area was determined using ImageJ version 1.48 (NIH USA, http://rsbweb.nih.gov/ij/). To evaluate the effect of increasing concentrations of ambient-air CO₂ on $A$, photosynthetic CO₂ assimilation under irradiance of 150 µmol/m²/s was measured by applying a step increase in CO₂ concentrations (from 0 to 1,000 ppm) after plants reached steady-state conditions for $A$.

**Guard cell starch quantification**

Epidermal peels were manually prepared at the end of the night and after 1 and 3 h of illumination. Guard cell starch granules were stained and fixed as described in (Flütsch et al, 2018). Guard cell starch granules were visualized using confocal laser-scanning microscope Leica TCS SP5 (Leica Microsystems), and their area was quantified using ImageJ version 1.48 (NIH USA, http://rsbweb.nih.gov/ij/). Four biological replicates per genotype were measured for each time point for one experiment. Three independent experiments were performed.
Leaf starch and soluble sugar quantification
Starch and soluble sugars (glucose, fructose, and sucrose) were extracted as described in (Thalmann et al., 2016) from entire rosettes harvested at the end of the night or at the end of the day. Leaf starch content was quantified as described in (Thalmann et al., 2016). Leaf soluble sugars were quantified based on the protocol for quantification of root soluble sugars described in (Thalmann et al., 2016) using as starting material 15 µl of neutralized soluble fraction obtained from the initial perchloric acid extraction. At least eight biological replicates per genotype were measured for each time point.

Guard cell soluble sugar quantification
To extract soluble sugars from guard-cell-enriched epidermal peels, six rosettes per genotype, corresponding to one biological replicate, were collected at the end of the night or after the plants were exposed to white light of 150 µmol/m²/s for 40 min and the petiole was removed using scissors. The remaining leaf material was blended in 100 ml ice-cold water using a kitchen blender (Philips, Avance Collection). The blended material was filtered through a 200 µm nylon mesh, and the remaining epidermal peels were dried, collected in a tube, and immediately frozen in liquid nitrogen. To remove residual sugars from the guard cell apoplast, the samples were washed with 2L of MilliQ water (Daloso et al., 2015) and refrozen in liquid nitrogen. Afterward, guard-cell-enriched epidermal peel materials were ground into a fine powder with a ball mill (Mix Mill MM-301, Retsch). Six biological replicates per genotype and time point were harvested for one experiment. Two independent experiments were performed.

Soluble sugars were extracted as described in (Thalmann et al., 2016). After the extraction, the samples were lyophilized in a freezedryer (Lyovac GT1, Lybold) and resuspended in 60 µl of MilliQ water. Leaf soluble sugars were quantified based on the protocol for quantification of root soluble sugars described in (Thalmann et al., 2016) using 50 µl of neutralized soluble fraction obtained from the initial perchloric acid extraction as starting material.

Arabidopsis grafts
Sterilized seeds were germinated on half-strength MS vertical plates, containing 2% (w/v) phytoagar and sealed with 3M™ Micropore™ Tape (3M Company). After 2 days of stratification at 4°C in the dark, plates were transferred to a climate-controlled chamber (Konstantraum 5, University of Zurich, Switzerland) under 16 h/8 h, light/dark photoperiod with a constant temperature of 21°C and an irradiance of 70 µmol/m²/s
(Osram Fluora L58W/77 and Osram Biolux L58W/965 in a 1:1 ratio). Grafts of WT and stp1stp4 plants were generated by micrografting technique (Turnbull et al., 2002) under a stereomicroscope (Nikon, SMZ1500) using 3/4-day-old seedlings. Micro-scissors with angled tip and a cutting edge of 2.5 mm (Fine Science Tools) were used to perform a neat horizontal cut in the upper region of the hypocotyl of the donor lines WT and stp1stp4 plants. A graft union between shoot and root of the donor lines was performed to generate the self-grafted donor lines WT/WT and stp1stp4/stp1stp4, and the reciprocal grafted lines WT/stp1stp4 and stp1stp4/WT. Grafted plants were grown horizontally for 3–4 days in a climate-controlled chamber (Fytoscope FS130, Photon Systems Instruments, PSI, Drásov, Czech Republic) located next to the stereomicroscope to minimize movements and promote the rapid formation of the graft union. Grafted plants were grown for additional 3 days vertically under the same conditions as described above. Grafts were monitored daily. 6–7 days after grafting, successful grafts (robust graft connection and no adventitious roots) were transplanted in soil and grown in a Fitoclima 2500 or KKD Hiross under 8 h/16 h, 21°C/19°C, light/dark photoperiod with a relative humidity of 45%/55% and an irradiance of 150 μmol/m²/s. Approximately 5–6 weeks after grafting, grafted plants were used to perform gas exchange measurements as described above. Subsequently, roots of grafted plants were excised and collected for molecular genotyping in order to validate the success of the grafting procedure.

Data and statistical analysis

Data were processed using specifically developed R scripts (R Core Team, 2015). Statistical differences between genotypes and time points were determined by one-way analysis of variance (ANOVA) with post hoc Tukey’s Honest Significant Difference (HSD) test (P < 0.05) performed using appropriate R scripts (R Core Team, 2015). Data are displayed as means ± SEM.

Data availability

Material and data set produced and analyzed for this study are available upon request to the corresponding author. Sequence data from this study can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACT2, AT3G18780; BAM3, AT4G17090; KAT1, AT5G46240; MYB60, AT1G08810; STP1, AT1G11260; STP4, AT3G19930; STP13, AT5G26340; SUC1, AT1G71880 and SUC3, AT2G02860.
ACKNOWLEDGEMENTS

We thank Diana Pazmino for technical support, Patricie Pakostová and Jaromír Pytela for plant material preparation, Zbyněk Pospíchal and Plant-Screen™ systems development team for technical support and optimization of phenotyping platform, Petr Polach for re-processing raw images, Magdalena M. Julkowska for providing R scripts, Tracy Lawson and Enrico Martinoia for helpful discussion. We further thank Michele Moles and Luca Distefano for help with preparation of guard cell-enriched material, Cyril Zipfel and Stefan Hörtensteiner from University of Zürich for providing us with growth chambers and laboratory equipment during our transition to ETH Zürich. Data produced in this paper were partially generated in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich. This work was supported by European Union’s Seventh Framework Program for research, technological development and demonstration under grant agreement no PITN-GA-2013-608422—IDP BRIDGES (to D.S.), the Swiss National Science Foundation SNSF-Grant 31003A_166539 and SNSF-Grant 310030_185241 (to D.S.), European Union’s Horizon 2020 research and innovation program under grant agreement no 722338—PlantHUB (to D.S.), the ETH Zürich and the University of Zürich.

AUTHOR CONTRIBUTIONS

DS conceptualized research; DS, KP, SF, and AN designed experiments; SF, AN, FC and MTh performed research; SF, AN, JF, MTr and KP analyzed the data; DS, SF and AN wrote the paper.
CHAPTER II

REFERENCES


111


CHAPTER II


R Core Team (2015) *R: a language and environment for statistical computing*. Vienna, Austria: R Core Team


Figure EV1. **STP1, STP4 and STP13 are highly and preferentially expressed in guard cells.**

**A** *In silico* analysis of plasma membrane sugar transporter gene expression levels in Arabidopsis guard cells. Arabidopsis eFP browser (http://bar.utoronto.ca/). Arabidopsis guard cell protoplasts (Yang et al, 2008). **B** STP1, STP4 and STP13 gene transcript levels in WT guard cell-enriched epidermal peels compared to WT rosette leaves at the end of the night. *KAT1* and *MYB60* were used as guard cell-specific markers, whereas *BAM3* was used as leaf-specific marker. Data for two independent experiments are shown; means ± fold change range n ≥ 6. **C** STP1, STP4 and STP13 gene transcript levels in WT rosette leaves compared to *stp1-1*, *stp4-1* and *stp13* rosette leaves at the end of the night. Data for two independent experiments are shown; means ± fold change range n ≥ 6. **D** STP1, STP4 and STP13 gene transcript levels in WT rosette leaves compared to *stp1-2* and *stp4-2* rosette leaves at the end of the night. Data for two independent experiments are shown; means ± fold change range n ≥ 5.

Data information: (B, C and D) *ACT2* was used as a housekeeping gene for normalization. For details about fold change and error calculations see Materials and Methods section. Primer sequences and efficiencies are given in Appendix Table S2.
Figure EV2. Stomatal function in stp13 plants and genetic identity of WT-stp grafted plants.

A Normalized whole-plant recordings of changes in stomatal conductance ($g_s$-$g_{\text{initial}}$) from WT, stp1, stp4 and stp1stp4 plants. Data shown are means ± SEM; n ≥ 3 per genotype. B Normalized whole-plant recordings of changes in stomatal conductance ($g_s$-$g_{\text{initial}}$) from self-grafted donor lines (WT/WT, stp1stp4/stp1stp4) and reciprocal grafting of shoot/root (WT/stp1stp4, stp1stp4/WT) plants. Data shown are means ± SEM; n ≥ 3 per genotype. C Whole-plant recordings of changes in stomatal conductance ($g_s$) from WT, stp1-2 and stp4-2 plants. Data shown are means ± SEM; n = 4 per genotype. D Normalized whole-plant recordings of changes in stomatal conductance ($g_s$) from WT, stp1-2 and stp4-2 plants. Data shown are means ± SEM; n = 4 per genotype. E Whole-plant recordings of changes in stomatal conductance ($g_s$) from WT and stp13 plants. Data shown are means ± SEM; n = 3 per genotype. F Normalized whole-plant recordings of changes in stomatal conductance ($g_s$) from WT and stp13 plants. Data shown are means ± SEM; n = 3 per genotype. G Representative molecular genotyping of roots from reciprocal grafted WT/stp1stp4 and stp1stp4/WT (shoot/root) plants. Roots from WT/WT and stp1stp4/stp1stp4 were used as a control. Genomic DNA extracted from roots was amplified using the genotyping primers listed in Table S2. For each grafted plant, PCR products were loaded according to the following order: STP1 gene-specific band, STP1 T-DNA-specific band, STP4 gene-specific band, STP4 T-DNA-specific band.

Data information: (A - F) Plants have been illuminated with 150 μmol m$^{-2}$ s$^{-1}$ white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. (A, B, D and F) $g_s$ values were normalized to values at the end of the night (EoN; $0 = g_{\text{initial}}$). (C and E) Different letters indicate significant statistical differences amongst genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey's test. (E and F) WT recordings are taken from Fig. 1C.
Figure EV3. Guard cell metabolites and gene expression in stp mutants.

A SUC1 and SUC3 gene transcript levels in WT guard cell-enriched epidermal peels compared to WT rosette leaves at the end of the night. KAT1 and MYB60 were used as guard cell-specific markers, whereas BAM3 was used as leaf-specific marker. Data for two independent experiments are shown; means ± fold change range n ≥ 5. B Content of soluble sugars in guard cell-enriched epidermal peels of WT, stp1-1 and stp4-1 plants at the end of the night (EoN) and after 40 min of illumination with white light at 150 µmol m⁻² s⁻¹ following the EoN. Data shown are means ± SEM; n = 3 for WT and n ≥ 5 for the mutants per time point. C Representative confocal laser microscopy images of propidium iodide-stained guard cell starch granules of intact leaves of WT and stp13 plants. Scale bar, 10 µm. D Starch dynamics in guard cells of intact leaves of WT and stp13 plants at the end of the night (EoN) and after one and three hours of illumination with 150 µmol m⁻² s⁻¹ of white light. Data for three independent experiments are shown; means ± SEM; n = 120 individual guard cells per genotype and time point.

Data information: (A) ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations see Materials and Methods section. Primer sequences and efficiencies are given in Appendix Table S2. (B, D) Different letters indicate significant statistical differences amongst genotypes for the given time point. Asterisk (*) indicates significant statistical differences amongst time points for the given genotype for P < 0.05 determined by one-way ANOVA with post hoc Tukey's test. (C, D) WT data are taken from Fig. 2B and C.
Figure EV4. Carbohydrate metabolism in the leaves of WT and stp mutant plants.

A. Whole-plant estimations of the internal CO₂ concentration (Cᵢ) from WT, *stp1*, *stp4* and *stp1stp4* plants. Data shown are means ± SEM; n ≥ 3 per genotype. B. Representative false colour images of photosystem II (PSII) operating efficiency (ΦPSII) captured by chlorophyll fluorescence imaging from WT, *stp13*, *stp1stp13* and *stp4stp13* plants. ΦPSII was measured at a photosynthetically active radiation (PAR) of 440 µmol m⁻² s⁻¹. C. ΦPSII quantified over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point. D. Normalized whole-plant recordings of changes in CO₂ assimilation (A) from WT, *stp1*, *stp4* and *stp1stp4* plants. Data shown are means ± SEM; n ≥ 3 per genotype. E. Normalized whole-plant recordings of changes in CO₂ assimilation (A) from self-grafted donor lines (WT/WT, *stp1stp4*/*stp1stp4* and reciprocal grafting of shoot/root (WT/*stp1stp4*, *stp1stp4*/WT) plants. Data shown are means ± SEM; n = 3 per genotype. F. Whole-plant recordings of changes in CO₂ assimilation (A) from WT, *stp1-2* and *stp4-2* plants. Data shown are means ± SEM; n = 4 per genotype. G. Normalized whole-plant recordings of changes in CO₂ assimilation (A) from WT, *stp1-2* and *stp4-2* plants. Data shown are means ± SEM; n = 4 per genotype. H. Whole-plant recordings of changes in CO₂ assimilation (A) from WT and *stp13* plants. Data shown are means ± SEM; n = 3 per genotype. I. Normalized whole-plant recordings of changes in CO₂ assimilation (A) from WT and *stp13* plants. Data shown are means ± SEM; n = 3 per genotype. J. Quantification of leaf starch. Data shown are means ± SEM;
n = 8 per genotype and time point. **K** Quantification of leaf glucose (Glc). Data shown are means ± SEM; n = 8 per genotype and time point. **L** Quantification of leaf fructose (Fru). Data shown are means ± SEM; n = 8 per genotype and time point. **M** Quantification of leaf sucrose (Suc). Data shown are means ± SEM; n = 8 per genotype and time point. **N** Representative Red Green Blue (RGB) images of 3-(day 0) and 4-week-old (day 7) WT, stp13, stp1stp13 and stp4stp13 plants. **O** Projected rosette area over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point.

Data information: (A and D – I) Plants have been illuminated with 150 μmol m$^{-2}$ s$^{-1}$ white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. (D, E, G and I) A values were normalized to values at the end of the night (EoN; 0 = $A_{\text{initial}}$). (F and H) Different letters indicate significant statistical differences amongst genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test. (H and I) WT recordings are taken from Fig. 3C and 3D. (J – M) Metabolites are from entire rosettes of WT, stp13, stp1stp13 and stp4stp13 plants at the end of the night (EoN) and end of the day (EoD) in a 12-h-light/12-h-dark cycle. FW, fresh weight. Asterisk (*) indicates significant statistical difference between time points for the given genotype. Different letters indicate significant statistical differences amongst genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.
Figure EV5. Variation in rosette colours and chlorophyll quantification in WT and stp plants.

A Representative false colour images of normalized difference vegetation index (NDVI) captured by a visible-near-infrared (VNIR) hyperspectral camera. B NDVI measured over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point. C-I Dynamic relative changes in greenness hue abundance over the phenotyping period in (C) WT; (D) stp1; (E) stp4; (F) stp13; (G) stp1stp4; (H) stp1stp13; (I) stp4stp13. The 5 most representative hues of green are shown in false colour scale as percentage of the rosette area (pixel counts); n = 10. J Chlorophyll a, b and a+b content in rosette leaves of WT and stp plants. Data for three independent experiments are shown; mean ± SEM; n ≥ 8.

Data information: (B) Asterisk (*) indicates significant statistical difference between WT and stp plants for P < 0.05 determined by one-way analysis of variance (ANOVA) with post hoc Tukey’s test. (J) FW, fresh weight.
APPENDIX

Table of Contents:

Appendix Table S1: List of putative plasma membrane sugar transporter genes. Page 2
Appendix Table S2: Oligonucleotides used in this study. Page 3
Appendix Table S3: Leaf surface temperature, normalized leaf surface temperature and normalized differences in vegetation index NDVI. Page 4
Appendix Table S4: Chlorophyll fluorescence parameters. Page 5
Appendix Table S5: RGB parameters. Page 6
Appendix Figure S1: Gene Expression of STPs in stp mutant backgrounds. Page 7
Appendix References List Page 8
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Appendix Table S1. List of (putative) plasma membrane sugar transporter genes in *Arabidopsis*.
Gene | AGI code | Forward primer | Reverse primer | PCR efficiency | Source  
--- | --- | --- | --- | --- | ---  
ACT2 | AT3G18780 | CCGTACCAACCGGTATTTGCTG | GATAATCAGTAAAGGTGTTCA | 2.18 | This study  
BAM3 | AT4G17090 | TTTAGCTTCTGCCTGCTCCT | GAATTCCGAGATAATCTCAGT | 2.07 | Ref 1  
KAT1 | AT5G46240 | AGATCAGTGAGTGGAGAATG | AGAGGACTCGTTCGGAATTCG | 1.88 | Ref 1  
MYB60 | AT1G08810 | CATGAGATTTGCTAGTCG | TTCCATTGAGCCGCCCTAG | 1.98 | Ref 1  
STP1 | AT1G11260 | GCTAATCGAGCGCTGCACT | CAACCGCTCTTAGATCTGCG | 2.09 | This study  
STP4 | AT3G19930 | TGCTTTGCTCTCAGTCGCTA | GCCACGATTAGTTGCCCCAC | 2.01 | This study  
STP13 | AT5G26340 | TCAAGCCGTCTCGGCTTCT | CTTTAACTCGGCTGCTCCA | 1.96 | This study  
SUC1 | AT1G71880 | CAGCATTCTTCACAGTCAAC | ACCAGATTGCTTTGAGAT | 2.10 | Ref 1  
SUC3 | AT2G02860 | CAAAGACCGAGCGCTTAATC | CTTGACGCGACCCGAAAT | 1.85 | Ref 1  

**qPCR PRIMERS** for amplification of gene-specific band  
STP1-1 | AT1G11260 | ATTTTGCAGTTCGAGAG | TCAATGCTCAATTTCTTGAG | SIGnAL  
STP4-1 | AT3G19930 | ATTTTGCAGTTCGAGAG | AAGACCGAGAGCAAATGTC | SIGnAL  
STP1-2 | AT1G11260 | TCGATGAGACACAGGCTC | CCAGTTGTAATGAGCTTG | SIGnAL  
STP4-2 | AT3G19930 | ATGTAATGAAAAGCTGAC | AAGACCGAGAGCAAATGTC | SIGnAL  
STP13 | AT5G26340 | ATTTTGCAGTTCGAGAG | AAGACCGAGGCAATGAGC | SIGnAL  

**GENOTYPING PRIMERS** for amplification of T-DNA specific band  
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STP4-1 | AT3G19930 | ATTTTGCAGTTCGAGAG | AAGACCGAGAAACATGTC | SIGnAL  
STP1-2 | AT1G11260 | TCGATGAGACACAGGCTC | CCAGTTGTAATGAGCTTG | SIGnAL  
STP4-2 | AT3G19930 | ATGTAATGAAAAGCTGAC | AAGACCGAGAAACATGTC | SIGnAL  
STP13 | AT5G26340 | ATTTTGCAGTTCGAGAG | AAGACCGAGAAACATGTC | SIGnAL  

### Appendix Table S2. Oligonucleotides used in this study.  

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### Appendix Table S3. Leaf surface temperature (Temp), normalized leaf surface temperature (Temp norm) and normalized difference vegetation index (NDVI).  
Parameters were calculated at the beginning (day 0, 3-week-old plants) and at the end (day 7, 4-week-old plants) of the phenotyping period in WT, stp1-1, stp4-1, stp13, stp1stp4, stp1stp13 and stp4stp13 plants. Data shown are means ± SEM; n = 10. Units are indicated next to each parameter. If absent, dimensionless. Within the same row and for the specified day, asterisk (*) indicates significant statistical difference between WT and stp plants for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. If absent, no statistical differences between the genotypes.
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Appendix Table S4. Chlorophyll fluorescence (ChlF) parameters.

Parameters were measured at the beginning (day 0, 3-week-old plants) and at the end (day 7, 4-week-old plants) of the phenotyping period in WT, stp1-1, stp4-1, stp13, stp1stp4, stp1stp13 and stp4stp13 plants. Data shown are means ± SEM; n = 10. Apostrophe (') indicates that the parameter was recorded in the light-adapted state at a photosynthetically active radiation (PAR) of 440 µmol m⁻² s⁻¹. Within the same row and for the specified day, asterisk (*) indicates significant statistical difference between WT and stp plants for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. If absent, no statistical differences between the genotypes.
Appendix Table S5. RGB parameters.

Parameters were measured at the beginning (day 0, 3-week-old plants) and at the end (day 7) of the phenotyping period in WT, stp1-1, stp4-1, stp13, stp1stp4, stp1stp13 and stp4stp13 plants. Growth rates were determined using the exponential function. Data shown are means ± SEM; n = 10. Units are indicated next to each parameter. If absent, dimensionless. Within the same row and for the specified day, different letters indicate significant statistical differences for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test. If absent, no statistical differences amongst the genotypes.
Appendix Figure S1. Gene Expression of STPs in stp mutant backgrounds. STP1, STP4 and STP13 gene transcript levels in WT guard cell-enriched epidermal peels compared to stp1-1, stp4-1 and stp13 guard cell-enriched epidermal peels at the end of the night. ACT2 was used as a housekeeping gene for normalization. Data for two independent experiments are shown; means ± fold change range n ≥ 4. For details about fold change and error calculations see Material and Methods section. Primer sequences and efficiencies are given in Appendix Table S2.

Appendix References

Mesophyll-derived sugars are positive regulators of light-driven stomatal opening

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SUMMARY

Guard cell membrane ion transport and metabolism are deeply interconnected, and their coordinated regulation is integral to stomatal opening. Whereas ion transport is exceptionally well understood, how guard cell metabolism influences stomatal movements is still in its relative infancy. Organic metabolites, like malate and sugars, fulfill several functions in guard cells during stomatal opening as allosteric activators, counter-ions, energy source and osmolytes. However, their origin and exact fate remain debated. Recent work revealed that the guard cell carbon pool regulating stomatal function and plant growth is mostly of mesophyll origin, highlighting a tight correlation between mesophyll and guard cell carbohydrate metabolism. This review discusses latest research into guard cell carbon metabolism and its impact on stomatal function and whole plant physiology.

Key words: Starch, glucose, malate, sucrose, guard cells, blue light, stomatal opening, plant growth
INTRODUCTION

Stomata are tiny adjustable pores on the leaf epidermis. They have dual contrasting functions in maximizing CO$_2$ uptake for photosynthesis while restricting water loss via evapotranspiration. By balancing this inevitable trade-off, stomata impact on plant’s water status and productivity (Lawson & Matthews, 2020). Specialized guard cells surrounding the stomatal valve actively regulate pore aperture through reversible changes in their turgor pressure. A variety of osmotically active solutes accumulate during stomatal opening promoting water influx and guard cell swelling. Potassium (K$^+$), chloride (Cl$^-$) and nitrate (NO$_3^-$) are the main inorganic ions; malate$^2-$ (Mal) and sugars, mainly sucrose (Suc) and its derived monosaccharides glucose (Glc) and fructose (Fru), are the major organic solutes. While the inorganic ions are taken up from the apoplastic space, metabolites can derive from autonomous guard cell CO$_2$ fixation, guard cell starch degradation or from mesophyll photosynthesis and subsequent import to guard cells (Vavasseur & Raghavendra, 2005; Santelia & Lawson, 2016; Jezek & Blatt, 2017).

Stomatal opening is induced by light, including blue and red light, and distinct mechanisms underly stomatal responses to these different wavelengths (Assmann & Wang, 2001; Inoue & Kinoshita, 2017). The red light response requires elevated light intensities, prolonged illumination and is photosynthesis-dependent. Red light is considered to be the primary signal connecting stomatal dynamics with mesophyll photosynthesis. However, the exact sources of osmotica and the signaling metabolites involved in this process remain to be elucidated (Matthews et al., 2020). Blue light represents the most prominent signal inducing stomatal opening (Shimazaki et al., 2007). The blue light-specific response is inducible at low light intensities of short duration and is photosynthesis-independent (Ando & Kinoshita, 2018). Blue light in guard cells is perceived by the photoreceptors phototropin 1 and 2 (PHOT; Kinoshita et al., 2001), which initiate a signaling cascade that ultimately results in the activation of a plasma membrane H$^+$-ATPase (Fig. 1; Kinoshita & Shimazaki, 1999). Proton (H$^+$) extrusion by the H$^+$-ATPase and subsequent membrane hyperpolarization stimulate ion uptake from the apoplast (Inoue & Kinoshita, 2017). In parallel to the activation of membrane ion transport, blue light triggers starch degradation in guard cell chloroplasts through the same PHOT-mediated signaling cascade, establishing a previously unsuspected connection between starch metabolism and ion transport (Fig. 1; Horrer et al., 2016). Simultaneous mutation of the glucan hydrolases α-amylase 3 (AMY3) and β-amylase 1 (BAM1) in Arabidopsis thaliana (Arabidopsis) inhibits guard cell starch breakdown. As a consequence, stomata in the amy3bam1 mutant open
more slowly and to a lesser extent, indicating that light-induced mobilization of starch contributes to fast and efficient stomatal opening (Horrer et al., 2016).

In this review, we discuss recent unexpected discoveries in the metabolism of starch, sugars and organic acids in guard cells which identify mesophyll-derived sugars as key organic molecules connecting metabolism, signaling and ion transport in the regulation of stomatal opening in response to mesophyll demand for CO₂, ultimately influencing plant growth.

**Origin and fate of malate during blue light-induced stomatal opening**

Initial research into guard cell starch metabolism led to the hypothesis that blue light-induced starch degradation results in Mal accumulation, which contributes to stomatal opening by serving as a counterion to K⁺ and an osmolyte for turgor generation (Raschke & Schnabl, 1978). Experimental evidence from *Vicia faba* correlating changes in guard cell protoplast volume to alterations in Mal and starch contents further supported this view (Schnabl, 1980). However, biochemical validation of this interconversion has long been hindered by the lack of suitable analytical methods and genetic material. Flütsch et al. (2020a) has combined the use of the *amy3bam1* double mutant, which is defective in guard cell starch degradation, with physiological and biochemical assays to challenge these earlier assumptions. Enzymatic quantification of metabolites in isolated guard cell-enriched epidermal peels unexpectedly revealed that *amy3bam1* guard cells have Mal levels equivalent to those of wild type, even after exposure to blue light at dawn. Thus, guard cell starch degradation does not directly result in Mal production (Flütsch et al., 2020a).

This finding raises questions about the origin of Mal in guard cells. Recent elegant ¹³C-feeding experiments in isolated guard cell-enriched epidermal fragments demonstrated that both anaplerotic carbon fixation by phosphoenolpyruvate carboxylase (PEPc) and reflux of carbon from glycolysis via the activity of the tricarboxylic acid (TCA) cycle are important contributors to Mal synthesis in guard cells (Daloso et al., 2015; Robaina-Estévez et al., 2017). Direct import of apoplastic Mal mediated by the ATP-binding cassette transporter ABCB14 may represent an additional source of Mal (Fig. 1; Lee et al., 2008). The relative contribution of each of the above-mentioned pathways in shaping the pool of Mal in guard cells is still not fully clear and deserves further investigations.
Isotope labeling has been a valuable approach also in determining the fate of carbon derived from Mal. It was reported that $^{13}$C-enrichment into Mal decreased between 30 and 60 min of illumination (Robaina-Êstévez et al., 2017). Although the possibility of Mal efflux from guard cells cannot be excluded, the concomitant increase in the $^{13}$C label in some intermediates from the TCA cycle has led to the conclusion that most of the accumulated Mal is used as a respiratory energy source during light-induced stomatal opening (Daloso et al., 2015; Robaina-Êstévez et al., 2017). The idea of Mal metabolization under light was very recently corroborated by independent enzymatic measurements showing that Mal decreased in response to blue light in both amy3bam1 and wild-type isolated guard cells (Flütsch et al., 2020a).

These recent studies shed new light on the origin and fate of Mal in guard cells during stomatal opening. Future experiments aiming at detecting fine changes in dark-to-light guard cell Mal concentrations (e.g. with higher spatio-temporal resolution) are a priority if we are to fully understand the biological function of this fundamental metabolite.

**Glucose is the main guard cell starch-derived metabolite under blue light**

Besides Mal, sugars represent another end product of guard cell starch mobilization and have been linked to stomatal dynamics ever since the starch-sugar theory emerged. In the early 20th century, reports investigating guard cell osmoregulation during stomatal opening proposed Suc resulting from the breakdown of starch as the main osmolyte (Lloyd, 1908; Scarth, 1927). The original starch-sugar hypothesis was then revisited after the importance of $K^+$ was revealed (Fischer & Hsiao, 1968), shifting the attention to $K^+$-Mal. It was suggested that $K^+$ and its counterions Mal (mainly derived from starch degradation), $Cl^-$ and $NO_3^-$ promote stomatal opening early in the day, while Suc accumulates later in the diel period to maintain stomatal aperture (Talbott & Zeiger, 1996). Interestingly, latest research provided new evidence pointing again to the significance of starch-derived carbohydrates for guard cell osmoregulation at dawn.

A comparative study measuring soluble sugar contents in isolated guard cells of wild type and the starch-degrading mutant amy3bam1 exposed to blue light showed that Glc, not Suc, is the major starch-derived metabolite at dawn (Flütsch et al., 2020a). Already in darkness, amy3bam1 guard cells had half as much Glc as the wild type. After exposure to low levels of blue light, Glc was almost undetectable in amy3bam1 guard cells. Support for this new surprising finding comes from feeding experiments analyzing the fate of $^{13}$C-sucrose in isolated guard cells under light. After
60 min of illumination, high incorporation of $^{13}$C was found in Fru (fully labelled) whereas Glc labelling reached only 80% of the total labelling possible (Medeiros et al., 2018). The authors speculated that the reduced $^{13}$C enrichment in Glc is the result of unlabeled Glc residues deriving from starch breakdown, which are, by contrast, not incorporated in Fru.

Although starch-derived Glc appears to be essential, its fate within guard cells remains unclear. More than 90% of the cytosolic osmolytes taken up or produced are generally sequestered into the vacuole (Martinoia, 2018). It is therefore reasonable to hypothesize that at least a fraction of the starch-derived Glc accumulates in this organelle. Transport experiments on isolated vacuoles demonstrated uptake of sugars (Schulz et al., 2011), but direct evidence that this process occurs in guard cells is lacking. Potential vacuolar Glc transporter candidates are members of the tonoplast monosaccharide transporter family (TMTs; Wormit et al., 2006); the glucose transporter 1 (VGT1; Aluri & Büttner, 2007); and the hexose transporters Sugar Will Be Eventually Exported 16 and 17 (SWEET16 and SWEET17), which are located at the tonoplast, with SWEET16 acting predominantly as a glucose facilitator (Chardon et al., 2013; Guo et al., 2014). Future work investigating the impact of guard cell-specific lack of these Glc vacuolar transporters will reveal their potential contribution to stomatal function.

Alternatively, starch-derived Glc might be key in maintaining the cytoplasmic carbohydrate pool needed for fast stomatal opening at times when photosynthesis-derived sugars are not yet available. Whether this starch-derived carbon is then used as a source of energy or to preserve cytosolic volume when ions are sequestered in the vacuole is not fully understood. Available experimental evidence indicates that starch degradation products do not directly affect the capacity to transport H$^+$, K$^+$ and Cl$^-$ across the guard cell plasma membrane (Flütsch et al., 2020a), suggesting that they are not primarily required for energy production. Thus, the phenotype of amy3bam1, showing slow and reduced stomatal opening in the presence of fully functional ion transport, leads to the conclusion that ion transport activities alone do not limit light-induced stomatal opening (Box 1). This is a highly regulated process, where conversion and exchange of metabolites between subcellular compartment coordinate the energetic and metabolic status of the cell with membrane ion transport.
Upon exposure to blue light, guard cell membrane ion transport is stimulated via the PHOT-mediated activation of the H^+-ATPase (AHA1), resulting in $\text{K}^+$, $\text{Cl}^-$, $\text{NO}_3^-$ and $\text{Mal}^2$ uptake from the apoplastic space. In parallel, guard cell starch is broken down by the synergistic activity of BAM1 and AMY3 in response to the activation of the proton pump. Starch degradation yields Glc required to maintain guard cell sugar homeostasis during stomatal opening. Photosynthetically active radiation promotes Suc synthesis in mesophyll cells, part of which is transported to the guard cell apoplast. Apoplastic Suc will enter the guard cells partially via SUCs or SWEETs, but the main bulk of Suc will undergo hydrolysis by cwINV to be taken up in the form of hexoses via the coordinate action of STP1 and STP4. The large Glc pool in guard cells resulting from STP-dependent import and guard cell starch degradation can support stomatal opening in a direct or indirect manner. Glc can accumulate in the vacuole to help generating the turgor pressure necessary for guard cell swelling and stomatal opening. Glc can also act as a respiratory and glycolytic substrate for energy generation in the mitochondrial metabolism or serve as a precursor for guard cell starch biosynthesis. Red light induces the uptake of mesophyll-derived sugars, mainly Suc, in an AHA1-dependent fashion to promote stomatal opening. INV, cytosolic invertase; cwINV, cell wall invertase; SUS, sucrose synthase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; NAD-MDH, NAD-dependent malate dehydrogenase; FUM, fumarase; Fum, fumarate; 2-OG, 2-oxoglutarate; Gln, glutamine; Glu, glutamate; TCA, tricarboxylic acid cycle; CBC, Calvin Benson Cycle.

Mesophyll-derived glucose and sucrose have distinct functions during stomatal opening at dawn

Sugars have long been implicated in stomatal opening, but their exact function has been a matter of debate. Initial research suggested that sugars induce stomatal opening as direct osmotica (Poffenroth et al., 1992; Talbott & Zeiger, 1993). Other studies found a weak correlation between the increase in sugar content and stomatal...
aperture (Pearson, 1973), thereby shedding considerable doubts on the presumed role of sugars as the main osmolytes that increase the osmotic pressure of guard cells at dawn (Granot & Kelly, 2019). If anything, evidence accumulated towards a role for sugars, primarily sucrose, to promote stomatal closure. Lu et al. proposed a mechanism for stomatal closure that is driven by the accumulation of mesophyll-derived sucrose at the guard cell apoplast, removing water from the guard cells, subsequently reducing stomatal aperture (Lu et al., 1997). In support of this hypothesis, an unrelated study described hexokinases (HXK) within guard cells as sensors of sugars, initiating a signaling cascade for stomatal closure (Kelly et al., 2013). By now, an increasing number of reports documented that the overexpression of guard cell HXKs improves water use efficiency under diverse conditions independent of the species (Granot & Kelly, 2019; Kelly et al., 2019; Lugassi et al., 2019).

Moreover, recent work using modern molecular technology, such as cell-specific promoters and isotope labeling, provided new evidence that Suc does not act as an osmolyte during light-induced stomatal opening, but rather is degraded within guard cells to provide energy through glycolysis and mitochondrial respiration (Daloso et al., 2016a; Lima et al., 2018). A first evidence came from manipulation of the activity of Suc synthase (SUS), which catalyzes the conversion of Suc to Fru and UPD-Glc (Fig. 1). Potato (Solanum tuberosum) plants with reduced SUS3 expression had altered sucrolytic activity and impaired stomatal opening (Antunes et al., 2012). Guard cell-specific overexpression of SUS3 in tobacco (Nicotiana tabacum) resulted in elevated steady-state stomatal conductance and lower guard cell Suc contents (Daloso et al., 2016b). Kinetic isotope labeling experiments in tobacco and Arabidopsis further revealed that the carbon released from Suc breakdown within guard cells during dark-to-light transition was incorporated in Glc, Fru, Mal and intermediates of the TCA cycle, ultimately promoting glutamine (Gln) biosynthesis (Daloso et al., 2015, 2016b; Medeiros et al., 2018). Given that Gln is a positive effector of fumarase (FUM; Zubimendi et al., 2018), the authors speculated that Gln may act as a signal to activate FUM, leading to fumarate (Fum) accumulation (Fig 1; Medeiros et al., 2018; Lima et al., 2018). In line with these studies, Flütsch et al. (2020a) recently observed Suc depletion in isolated guard cells at dawn illuminated with blue light (Flütsch et al., 2020a). Interestingly, Suc seems to retain a prominent role as an osmolyte for stomatal opening specifically under red light (Box 2).

An additional long-standing controversy concerns the sources of sugars for light-induced stomatal opening. We have already discussed previously that a fraction of the cytosolic Glc derives from guard cell starch degradation. It is doubtful that a significant
amount of the remainder sugars is produced by autonomous photosynthesis, as guard
cell capacity to fix CO$_2$ via the Calvin cycle is notoriously limited compared to
mesophyll cells (Lawson, 2009). This observation, along with the fact that guard cells
lack functional plasmodesmata (Wille & Lucas, 1984), led to the hypothesis that
mesophyll-imported sugars represent the major source of carbon for guard cells. Early
$^{14}$C pulse-labeling studies provided some evidence of sugar uptake to guard cells,
showing that it occurs in symport with H$^+$ in a process which is energy dependent (Lu
et al., 1997; Ritte et al., 1999). However, it was only very recently that the identity and
the function of some of the involved guard cell plasma membrane sugar transporters
was revealed.

Antunes et al. (2017) demonstrated that tobacco plants with reduced Sucrose
Transporter 1 (SUT1) expression in guard cells contained less Suc, which was
associated with decreased steady-state stomatal conductance compared to wild type
(Antunes et al., 2017). Apoplastic Suc can also be taken up in the form of hexoses
following Suc hydrolysis by a cell wall invertase (cwINV; Fig. 1). This alternative
transport route has long been overlooked. Flütsch et al. (2020b) used a reverse
genetic approach in Arabidopsis and demonstrated that Glc import to guard cells at
dawn via the coordinated action of the monosaccharide-H$^+$ symporters Sugar
Transport Protein 1 and 4 (STP1 and STP4) is essential for light-induced stomatal
opening and plant growth (Flütsch et al., 2020b). Guard cells of stp1stp4 double
mutants had almost undetectable amounts of Glc under light, were nearly devoid of
starch, but surprisingly accumulated Suc to higher levels compared to wild type
(Flütsch et al., 2020b). Imported Glc via STPs likely joins the cytosolic pool of guard
cell starch-derived Glc and significantly contributes to maintaining homeostatic
carbohydrate levels needed for stomatal opening. This mesophyll-derived Glc also is
the main precursor for guard cell starch biosynthesis at the start of the day. The excess
of Suc in stp1stp4 guard cells, while clearly not used for making starch, may result
from reduced consumption due to feedback inhibition.

Imported Glc was also shown to be crucial for stomatal opening in grasses,
which have morphologically distinct stomata with guard cells flanked by a pair of
subsidiary cells. A member of the Sugars Will Eventually be Exported Transporters
(SWEET) gene family, Closed Stomata 1 (CST1), was identified in a forward genetic
screen in maize (Zea mays) as a subsidiary cell membrane-localized glucose
transporter which positively regulates stomatal opening and photosynthesis (Wang et
al., 2019).
While these studies have significantly progressed our knowledge on the sources and function of sugars in guard cells, they only represent the tip of the iceberg. Available transcriptomic data show that several other sugar transporters are highly expressed in guard cells (Leonhardt et al., 2004; Wang et al., 2011; Bates et al., 2012; Bauer et al., 2013), suggesting a potential intricate network of transport routes at the guard cell plasma membrane.

Conclusions and Outlook

This review described recent exciting discoveries which have challenged long-standing theories in guard cell carbon metabolism and led to the redrawing of our current model concerning the influence of starch and sugars during light-induced stomatal opening. Most of the sugars for guard cell function come from the mesophyll and they have multiple roles, which may vary depending on the type of sugar and time of day. At dawn, rapid degradation of starch releases Glc promoting efficient and fast stomatal opening for CO\(_2\) uptake when photosynthesis-derived sugars are not yet available. Subsequent import of apoplastic Suc and Glc fuels guard cell metabolic rearrangements needed to produce ATP and carbon intermediates to sustain membrane ion transport and guard cell turgor generation. This tight correlation between mesophyll and guard cell carbohydrate metabolism highlights sugars as important metabolites regulating CO\(_2\) uptake for fine-tuned photosynthetic growth.

Future research should investigate how rearrangements in carbon fluxes within guard cells induce downstream responses required for fast stomatal opening. This knowledge will be important to design sophisticated strategies to integrate guard cell signaling, ion transport and metabolism for optimal stomatal function.

Box 1: The speed of stomatal opening depends on the tight coordination between guard cell metabolism and membrane ion transport

Stomatal responses to environmental changes, including light, are generally slower than photosynthetic responses, resulting in CO\(_2\) diffusional limitations and unnecessary water loss (McAusland et al., 2016). Manipulation of stomatal movement kinetics has been suggested as a novel approach to improve the coordination between stomatal conductance and photosynthesis, enhancing plant water use efficiency (WUE) and productivity (Lawson & Vialet-Chabrand, 2019).

The extent and velocity of stomatal responses are intrinsically linked to guard cell characteristics, including the capacity for membrane solute transport and osmolyte...
availability. How these processes relate to turgor generation determining fast stomatal opening kinetics remains largely unknown. The Blatt and Christie labs have recently demonstrated the potential to manipulating the capacity of ion transport to increase stomatal movement kinetics. The overexpression of the synthetic blue light-gated K\(^+\) channel BLINK1 in guard cells of Arabidopsis enhanced K\(^+\) conductance in the plasma membrane and accelerated stomatal responses, leading to increase in biomass as a direct consequence of improved WUE (Papanatsiou et al., 2019).

Membrane ion transport is tightly coordinated with - and dependent on - guard cell starch metabolism (Horrer et al., 2016; Flütsch et al., 2020a). The release of Glc from starch in guard cells is therefore crucial for rapid stomatal opening. This conclusion is further supported by the results of a series of comparative analyses of stomatal opening kinetics and guard cell starch dynamics, demonstrating that the speed of stomatal opening is strictly correlated with the ability of the guard cells to rapidly degrade starch in response to light (Flütsch et al., 2020a). These studies highlight that a coordinated manipulation of guard cell starch degradation and BLINK1-mediated K\(^+\) conductance has the potential to enhance the speed of stomatal responses even further, thereby synchronizing gas exchange with photosynthesis for improved plant WUE.

**Box 2: Role of mesophyll-derived Suc during red light-induced stomatal opening**

It is well established that the red light response of stomata is photosynthesis dependent. Several studies suggest that it occurs in the mesophyll chloroplast, but the signal that is transferred from the mesophyll to the guard cells has not been fully elucidated (Matthews et al., 2020). The decrease in the intercellular CO\(_2\) concentration (C\(_i\)) as a result of mesophyll CO\(_2\) consumption was long considered to be the driver of stomatal opening under red light (Roelfsema et al., 2002). However, the observation that stomatal conductance increases with light despite high C\(_i\) or after reaching steady-state photosynthesis (Messinger et al., 2006; Lawson et al., 2008; Matrosova et al., 2015) prompted to consider other signals. Both an aqueous metabolic signal, with potential candidates such as ribulose bisphosphate (RuBP), ATP, NADPH, malate and sugar, and a vapor phase ion have been suggested (Matthews et al., 2020).

Interestingly, recent research points towards a prominent role of mesophyll-derived Suc as a signal and osmolyte for stomatal opening under red light. It was reported that Arabidopsis guard cells of intact leaves illuminated with red light for 2h doubled their Suc content in an H\(^+\)-ATPase-dependent manner (Flütsch et al., 2020a).
Ando & Kinoshita further demonstrated that red light induces phosphorylation of the guard cell plasma membrane H\(^+\)-ATPase, promoting stomatal opening in whole leaves (Ando & Kinoshita, 2018) in a fluence-dependent manner (Ando & Kinoshita, 2019). These studies also reported that stomata of intact leaves, which are connected with the mesophyll, opened in response to red light, whereas isolated stomata remained mostly closed (Ando & Kinoshita, 2018; Flütsch et al., 2020a). Despite a direct guard cell response to red light has been established (Zhu et al., 2020), it seems likely that the uptake of mesophyll-derived Suc, as energized by the H\(^+\)-ATPase, provides the main osmotica for red light-induced stomatal opening. Notably, a fraction of this imported Suc is further metabolized within the guard cells to produce ATP for ion transport and provide carbon skeletons for starch biosynthesis (Daloso et al., 2015; Medeiros et al., 2018; Flütsch et al., 2020a).
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Guard cell starch synthesis involves import of hexose sugars via STP and SWEET proteins and the gluconeogenic conversion of vacuolar solutes

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SUMMARY

Stomata have twofold and contrasting functions, maximizing the intake of CO$_2$ for leaf photosynthesis while restricting water efflux via transpiration. Guard cells enclosing the stomatal valve actively regulate stomatal movements through transport of ions and metabolic rearrangements affecting guard cell turgidity. The synthesis of starch in guard cells affects the plant's ability to close their stomata. However, our knowledge on the function of starch synthesis and the carbon sources for this process in guard cells is still fragmented. Besides the already described Sugar Transport Proteins 1 and 4 (STPs), here, we demonstrate that several plasma membrane members of the Sugars Will Eventually be Exported Transporter (SWEET) family provide mesophyll-derived sugars to guard cells, which serve as carbon sources for starch accumulation. We further show that the release of solutes from the guard cell vacuole via Aluminum activated Malate Transporter 4 (ALMT4), Early Response to Dehydration Like 6 (ERDL6) and sucrose-proton symporter 4 (SUC4) and their subsequent gluconeogenic conversion into starch are integral to the total stomatal starch pool. Finally, we demonstrate that guard cell starch acts as a sink for osmotically active solutes, promoting efficient stomatal closure. Our work reveals new essential players driving stomatal responses to environmental cues.

Key words: starch, glucose, guard cells, vacuole, sugar transport, malate, stomatal movements
INTRODUCTION

Starch is an insoluble, osmotically inert storage carbohydrate composed of \(\alpha\)-glucose polymers. In plants, starch plays a dual role in carbon allocation. It acts both, as a source, releasing energy for growth and development, and as a sink, contributing to carbohydrate reserves and sink strength (Pfister & Zeeman, 2016). Starch pools are present in a variety of photosynthetic and heterotrophic plant tissues and thus have a profound impact on whole plant physiology. Starch synthesis and degradation controls the availability of free sugars glucose (Glc), fructose (Fru) and sucrose (Suc), which in turn regulate various physiological processes (Sulpice et al., 2009; MacNeill et al., 2017). In photosynthetic tissues, starch gradually accumulates during the day and is mobilized at night to sustain metabolism in the absence of photosynthesis (Stitt & Zeeman, 2012). Such transitory starch is also found within the leaf epidermis in stomatal guard cells. The diurnal pattern of starch deposition and mobilization, however, differs markedly from that of the mesophyll cells. Guard cell starch is abundant during the night and is rapidly degraded within 1 h after dawn. Starch breakdown in guard cells is triggered by blue light during stomatal opening (Horrier et al., 2016), involving the action of phototropin-signaling (Kinoshita et al., 2001), ultimately leading to the activation of a plasma membrane H\(^+\)-ATPase (Kinoshita & Shimazaki, 1999). Only recently it was discovered that guard cell starch degradation yields Glc for fast stomatal opening at dawn and therefore plays a crucial role in controlling the trade-off between photosynthesis and transpiration, ultimately impacting on plant growth and productivity (Flütsch et al., 2020a).

In mesophyll cells, starch synthesis follows a distinct pathway converting the Calvin cycle intermediate fructose-6-phosphate (F6P) into the activated glucosyl donor ADP-glucose via three enzymatic steps (Stitt & Zeeman, 2012). In contrast, the synthesis of this vital carbohydrate in guard cells is poorly understood. The accumulation of starch is observed after its near complete hydrolysis and gradually continues for the rest of the day and the first hours of the night (Horrier et al., 2016). CO\(_2\) fixation is limited within guard cells (Outlaw, 1989; Lawson, 2009) and therefore it was long hypothesized that guard cells rely upon supply of carbons from the neighboring mesophyll tissue as a source for starch biosynthesis (Raschke & Dittrich, 1977; Schnabl, 1980; Daloso et al., 2016; Santelia & Lawson, 2016). Due to a lack of suitable experimental techniques, evidence for such processes, however, was scarce and incomplete. Only earlier this year, we identified the high-affinity proton-coupled monosaccharide transporters Sugar Transport Proteins 1 and 4 (STPs) as the major plasma membrane hexose importers in guard cells of Arabidopsis thaliana. STP1 and
CHAPTER IV

STP4 cooperate in the delivery of Glc to guard cells, providing carbon sources for starch biosynthesis and light-induced stomatal opening (Flütsch et al., 2020b).

Arabidopsis mutants simultaneously lacking STP1 and STP4 also show delayed stomatal closure (Flütsch et al., 2020a). Various environmental and physiological cues such as darkness, low photosynthetic rates, the plant hormone abscisic acid (ABA) and high CO₂ concentrations evoke stomatal closure (Roelfsema & Hedrich, 2005). The release of large quantities of anions via R-type and S-type anion channels (Schroeder & Keller, 1992) and the activation of outward-rectifying potassium channels (Blatt, 1993) is integral to stomatal closure. Interestingly, guard cell starch synthesis has been proposed to facilitate the retrieval of organic osmolytes during stomatal closure to help induce the required changes in guard cell turgor for water efflux (Raschke & Dittrich, 1977; Penfield et al., 2012; Azoulay-Shemer et al., 2016). However, the relative contribution to stomatal function and the molecular mechanisms of this process are yet unknown.

Here, we thoroughly analyzed the contribution of sugar transport to stomatal starch accumulation and function. We provide evidence that i) in addition to STP1 and STP4, other plasma membrane-localized hexose transporters from the SWEET family supply the guard cells with mesophyll-derived sugars, which are partly used as carbon sources for starch synthesis; ii) guard cell starch acts as a sink for vacuolar-derived osmotically active solutes during stomatal closure and iii) vacuolar solutes are an important source of carbons for the built-up of the total guard cell starch pool during the second half of the day. Our work provides new insights into the role of guard cell starch metabolism for stomatal movements and whole plant physiology. Highlighting the dual role of starch in guard cells acting as a source and sink for carbons utilized during stomatal opening and closure, therefore contributing to a better understanding of these vital processes.
RESULTS

Guard cells synthesize starch using different carbon sources depending on the time of the day

We previously reported that early morning Glc uptake into guard cells via STP1 and STP4 is essential for guard cell starch synthesis, as stp1stp4 double mutants had no starch at the end of the night (EoN) and failed to synthesize it during the first 3 h of light (Flütsch et al., 2020b).

To investigate whether apoplastic hexose import via STPs is required for guard cell starch biosynthesis throughout the 24 h of the day/night cycle, we quantified guard cell starch contents in the stp1/stp4 single and double mutants (Fig. 1a and Fig. S1a). Consistent with previous observations, starch was rapidly mobilized at dawn in wild-type guard cells and re-synthesized after it was almost completely degraded 1 h after the EoN (Fig. 1a and Fig. S1a; Horrer et al., 2016). stp1stp4 guard cells were devoid of starch up to 6 h of light (Fig. 1a), while a progressive increase in starch contents was observed in the stp single mutants after 3 h into the day (Fig. S1a). Notably, stp1, stp4 single and the stp1stp4 double mutants synthesized a substantial amount of starch after 6h of light (Fig. 1a and Fig. S1a). Starch synthesis rate in stp1stp4 guard cells was considerably higher between 6 and 9 h of light compared to wild-type guard cells (stp1stp4, 5.96; wild type, 0.51; Table S2), whereas the starch synthesis rates were near zero in the mutant until this time point (Table S2). Nocturnal starch dynamics were similar for wild type and stp1stp4 guard cells (Fig. 1a and Table S2). Single stp1 and stp4 mutants behaved similarly to stp1stp4 mutants, although stp4 guard cells showed an elevated starch synthesis rate between 3 and 6 h of illumination rather than between 6 and 9h (Table S2).

The fact that stp1stp4 guard cells accumulated starch after 6h of light (Fig. 1a) suggests that guard cells make starch in the afternoon using carbon sources other than apoplastic Glc.

Several SWEET and SUC sugar transporters are preferentially expressed in guard cells

Given the diurnal pattern of starch accumulation of stp1stp4 guard cells (Fig. 1a), we hypothesized that other plasma membrane sugar carriers may be involved in supplying the guard cells with carbon sources for starch synthesis.
Arabidopsis plants possess three kinds of sugar transporters: Monosaccharide transporters (MSTs), which comprises the STPs; the H\(^+\)-coupled Suc symporters (SUCs); and the Suc and hexose facilitators Sugars Will Eventually be Exported Transporters (SWEETs). Through *in silico* expression profiling using publicly available data, we previously identified several transporters that are preferentially expressed in guard cells such as *SUC1*, *SUC2*, *SUC3* as well as *SWEET1*, *SWEET4*, *SWEET5*, *SWEET11* and *SWEET12* (Flütsch *et al.*, 2020b). Since SWEET11 and 12 have been reported to function redundantly in Suc phloem loading and are the predominant
SWEET transporters in leaves (Chen et al., 2012), we excluded them from further analyses. Similarly, SUC2 is known to be the major Suc transporter that loads the phloem in Arabidopsis (Gottwald et al., 2000) and was therefore not further considered.

Members of the recently described SWEET super family play crucial roles in various developmental processes, such as pollen nutrition (Sun et al., 2013), phloem loading (Chen et al., 2012) and seed filling (Chen et al., 2015). SWEETs are uniporters, facilitating diffusion of sugars across cell membranes. Therefore, their main function is to translocate carbohydrates to different tissues. The Arabidopsis SWEET1 protein was the first member described and identified as a low-affinity Glc uniporter, which is highly expressed in flowers (Chen et al., 2010). SWEET4 was reported as another plasma membrane-localized Glc uniporter, expressed in the stele of roots, the veins of leaves and flowers (Liu et al., 2016). In contrast, substrates and transport characteristics as well as expression of SWEET5 are largely unknown (Liu et al., 2016).

Using RT-qPCR on guard cell-enriched epidermal peels, we confirmed the predicted preferential guard cell expression of SWEET1, SWEET4 and SWEET5. Their transcript levels were similar to the guard cell-specific marker genes K+ channel in Arabidopsis (KAT1) and Myb transcription factor 60 (MYB60; Fig. 1b and Table S1). SWEET2 was reported to be a tonoplast-localized sugar transporter highly expressed in roots and leaves (Chen et al., 2015) and was included in our experiments as a negative control. As expected, SWEET2 showed pronounced preferential expression in leaves, similarly to β-amylase 3 (BAM3; Fig. 1b and Table S1). Preferential guard cell gene expression for both SUC1 and SUC3 was already reported by (Flütsch et al., 2020b).

Single sweet and suc mutants turnover guard cell starch like the wild type

To assess whether the selected SWEETs play a role in guard cell starch accumulation, presumably after 6 h into the light, we examined starch contents in guard cells of homozygous transfer-DNA (T-DNA) lines of SWEET1 (sweet1; SALK_029479), SWEET2 (sweet2; SALK_034060), SWEET4 (sweet4; SALK_200835) and SWEET5 (sweet5; SALK_04204) during the diurnal 12 h light period (Fig. 1c and Fig. S1b). To increase the resolution of the guard cell starch measurements, we added an additional harvest time point at 2 h into the day to our experimental set-up.
Despite the high guard cell expression of SWEET1, 4 and 5, the lack of either of these SWEET proteins did not considerably alter guard cell starch accumulation compared to wild type (Fig. 1c). Starch degradation occurred in all four genotypes to a similar level between 1 and 2 h of illumination (Fig. 1c and Table S3). After this time point, starch synthesis was observed in wild-type and sweet1 guard cells, while starch contents stayed the same in sweet4 and sweet5 over the subsequent hour (Fig. 1c and Table S3). After 3 h of light, all four genotypes continuously synthesized starch, such that the mutants and wild-type guard cells contained similar amounts of starch at the end of the day (EoD; Fig. 1c and Table S3). As anticipated, guard cell starch amounts were comparable in wild-type and sweet2 plants throughout the 12 h light phase (Fig. S1b and Table S3).

We previously reported that suc1 and suc3 single mutants had normal starch turnover during the first 3 h of light (Flütsch et al., 2020b). Here, we further observed that after 3 h into the day, guard cell starch dynamics were essentially the same in suc single mutants and wild-type plants, suggesting that apoplastic Suc is not a carbon source for guard cell starch synthesis (Fig. 1d and Table S3).

Coordinate action of SWEET1 and SWEET5 is essential for early morning guard cell starch synthesis

Given the lack of a guard cell starch phenotype in sweet and suc single mutants, we generated the double mutant combinations sweet1sweet5, sweet1sweet4, sweet4sweet5 and suc1suc3 to investigate a potential functional redundancy between the different isoforms. The generated double mutants were examined for their guard cell starch amounts during the 12 h light period (Fig. 2a, b, d and Fig. S2a).

To our surprise, starch was almost undetectable in guard cells of sweet1sweet5 plants at the EoN compared to wild type (Fig. 2a), indicating that SWEET1 and SWEET5 are both required for guard cell starch synthesis during the early morning. Furthermore, starch synthesis was only observed after 6 h of illumination in this double mutant (Fig. 2a). Interestingly, the guard cell starch phenotype of sweet1sweet5 plants nearly mimicked that of stp1stp4 mutants, although with slightly elevated starch contents throughout the day compared to stp1stp4 guard cells. In both double mutants, starch levels rose substantially between 6 and 9 h of light as supported by the starch synthesis rates (wild type, 0.3; sweet1sweet5, 2.25; Table S4 and wild type, 0.51; stp1stp4, 5.96; Table S2).
These data demonstrate that coordinately SWEET1 and SWEET5 along with STP1 and STP4 supply guard cells with the hexose sugars needed to synthesize starch during the early morning.

Fig. 2 Stomatal phenotype of sweet double mutants. Starch dynamics in guard cells of intact leaves of wild-type and (a) sweet1sweet5, (b) sweet1sweet4, (d) sweet4sweet5 plants during the light period in 12 h/12 h day/night light regime. Plants were illuminated with 150 μmol m⁻² s⁻¹ of white light. Data from two independent experiments are shown; means ± SEM; n = 80 individual guard cells per genotype and time point. EoN = end of night. Same wild-type data are plotted in a, b and d. (c) SWEET1, SWEET2, SWEET4 and SWEET5 gene expression in wild-type guard cell-enriched epidermal peels harvested at 6 h into the day relative to wild-type guard cell-enriched epidermal peels harvested at 3 h into the day. Data from two independent experiments are shown; means ± fold change range; n = 6. ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. (e) Whole-plant recordings of changes in stomatal conductance (gs) from wild-type, sweet1sweet4, sweet1sweet5 and sweet4sweet5 plants in response to a shift from dark to light and from ambient CO₂ concentration to 1000 μl L⁻¹ CO₂ after 2h of illumination. Data shown are means ± SEM; n = 3 per genotype. EoN = end of night. (f) Whole-plant recordings of changes in CO₂ assimilation (A) from wild-type, sweet1sweet4, sweet1sweet5 and sweet4sweet5 plants in response to a shift from dark to light and from ambient CO₂ concentration to 1000 μl L⁻¹ CO₂ after 2h of illumination. Data shown are means ± SEM; n = 3 per genotype. EoN = end of night. (g) Photosynthetic CO₂ assimilation (A) in dark-adapted wild-type, sweet1sweet4, sweet1sweet5 and sweet4sweet5 plants in response to a step increase in CO₂ concentrations from 0 to 1,000 μl L⁻¹ CO₂. Data shown are means ± SEM; n = 3 per genotype. No statistical differences were detected among genotypes for the given time points. (a-f) Letters (*) indicate significant statistical difference between genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (a-g) WT = wild type.
SWEET1 and SWEET4 provide apoplastic sugars for guard cell starch synthesis after 3 h into the day

Given the substantial increases in starch contents in the guard cells of stp1stp4 and sweet1sweet5 mutants after 6 h into the day, we wondered whether other SWEET transporters may mediate the import of carbon sources for starch synthesis later into the day. Loss of SWEET1 and SWEET4 caused a particular pattern of guard cell starch accumulation during the 12 h day (Fig. 2b), with essentially normal turnover between the EoN and 3 h of light (Fig. 2b and Table S4). However, beyond this time of the day, sweet1sweet4 guard cells showed a net decline in starch amounts between 3 and 6 h of light and low starch synthesis between 6 and 9 h of the day in comparison to wild-type guard cells (Fig. 2b and Table S4). Interestingly, SWEET1 and SWEET4 gene expression was strongly induced at 6 compared to 3 h into the day in wild-type guard cell-enriched epidermal peels (Fig. 2c and Table S1), coinciding with the reduced starch biosynthetic activity in sweet1sweet4 guard cells during midday. Starch levels at the EoD were only mildly reduced in sweet1sweet4 double mutants compared to wild type (Fig. 2b), as the double mutant accumulated significantly more starch between 9 and 12 h of illumination (wild type; 0.66 and sweet1sweet4; 2.71, Table S4), suggesting that at this time of the day, guard cells use alternative carbon sources for starch synthesis than those provided by SWEET1 and SWEET4. Hence, our data suggest that SWEET1 and SWEET4 are required for the supply of hexoses for guard cell starch synthesis specifically around midday.

Further specific alterations in starch accumulation were observed for sweet4sweet5 double mutant guard cells (Fig. 2d). Despite wild type-like starch contents at the EoN, starch amounts were particularly low between 2 h and 6 h, as well as between 9 h and 12 h into the day (Fig. 2d and Table S4). Notably, SWEET5 was not transcriptionally upregulated at 6 h into the day (Fig. 2c and Table S1). Loss of both, SUC1 and SUC3 did not significantly alter starch dynamics in comparison to wild type (Fig. S2a), suggesting that Suc imported via SUCs is not a substrate for starch synthesis during daytime.

Collectively, our guard cell starch quantifications in sweet and suc double mutants demonstrate that SWEET1, SWEET4 and SWEET5 together deliver mesophyll-derived sugars for guard cell starch synthesis. Their activity seems to be highly regulated as indicated by our gene expression data and they seem to be active during specific periods of the day.
Stomatal movement kinetics are altered in sweet double mutants

Guard cell starch degradation integrates with light-regulated membrane ion transport processes yielding Glc to maintain sugar homeostasis during stomatal opening, thereby fine-tuning stomatal opening kinetics (Flütsch et al., 2020a). Starch synthesis has been also implicated in stomatal movement regulation. Stomatal closure in response to an increase in CO₂ concentrations was impaired in Arabidopsis mutants lacking starch in guard cells (Azoulay-Shemer et al., 2016). Therefore, transitory starch in guard cells plays a key role in determining the magnitude and velocity of stomatal movements.

To investigate the impact on stomatal function of impaired guard cell starch accumulation in sweet mutants, we recorded stomatal conductances (gs) and photosynthetic assimilation rates (A) in response to a shift from dark to light (150 µmol m⁻² s⁻¹), followed by a shift from ambient CO₂ concentrations (400 µL L⁻¹) to 1000 µL L⁻¹ CO₂ (Fig. 2e - g and Fig. S2b-f). Given that the purpose of our infrared gas analysis was to compare stomatal opening and closure kinetics amongst genotypes, gs and A were normalized to the values at the EoN (for the opening) and the last 10 min of the light-response under ambient CO₂ (for the closure). Normalized data are presented in the Supplemental Information.

As anticipated, light-induced stomatal opening was severely affected in sweet1sweet5 plants, with slower gs kinetics and reduced steady-state values (Fig. 2e). The time to reach half of the maximum gs response (half time of opening) was increased by 7 min in the double mutant compared to WT (Table S5 and Fig. S2b). Furthermore, the amplitude of the total gs response to light was only ~ 65% of that of WT (Table S5). Similarly, stomatal closure was affected by increasing CO₂ concentrations (Fig. 2e), with slower stomatal closure kinetics and reduced amplitudes (Fig. S2c and Table S6). Sweet1sweet4 double mutants also had impaired stomatal movements, showing a reduced steady-state gs with initially comparable gs kinetics to wild type, albeit reduced amplitudes (Fig. 2e and Table S5). We observed that an early closure response was initiated in sweet1sweet4 double mutant plants (Fig. 2e). Furthermore, this mutant had particularly slow stomatal closure kinetics and was impaired in maintaining the stomata closed (Fig. S2c and Table S6). Interestingly, sweet4sweet5 mutants had wild type-like steady-state gs and opening amplitudes, but slower opening kinetics (20 min versus 17 min; Fig. 2e and Table S5). Stomatal closure was unaffected in this mutant (Fig. S2c and Table S6).
Despite high guard cell transcript amounts of SUC1 and SUC3, the lack of both symporters did not cause a reduction in steady-state $g_s$ nor the $g_s$ amplitude (Fig. S2d and Tables S5 and S6). Suc1suc3 mutants had essentially identical $g_s$ responses to wild type (Fig. S2e and S2f), suggesting that they are not involved in regulation of stomatal movements.

Altogether, the slower stomatal movement kinetics and reduced amplitudes of the stomatal responses in the sweet double mutants, suggest that SWEET1, SWEET4 and SWEET5 are required to promote both light-induced stomatal opening and CO$_2$-induced stomatal closure.

Based on the slower stomatal movement kinetics and reduced $g_s$ amplitudes recorded for the sweet double mutants, we anticipated that CO$_2$ assimilation rates should be also affected. Indeed, $A$ was reduced in all three sweet double mutants compared to wild type (Fig. 2f and Fig. S3a). In contrast, $A$ was similar between wild type and suc1suc3 double mutant plants throughout the measurements (Fig. S3a and S3b). Surprisingly, sweet4sweet5 mutants assimilated more CO$_2$ relative to WT and the other two double mutants once the CO$_2$ concentration was raised to 1000 µl L$^{-1}$ (Fig. 2f and S3a).

Reduced stomatal conductance can limit CO$_2$ diffusion into the leaf, directly affecting photosynthetic capacity. To test whether the reduced $A$ observed in the sweet double mutants is linked to their altered stomatal movement kinetics and $g_s$ amplitudes, plants were exposed to increasing external CO$_2$ concentrations ranging from 0 to 1000 µl L$^{-1}$ CO$_2$. At 400 µl L$^{-1}$ CO$_2$ steady-state $A$ recordings were markedly reduced in mutants compared to wild type (Fig. 2g), while at CO$_2$ concentrations of 600 -1000 µl L$^{-1}$, $A$ was comparable among the genotypes (Fig. 2g). suc1suc3 double mutants had similar $A$ values already at 400 µl L$^{-1}$CO$_2$ as also observed in the beforementioned gas exchange recordings (Fig. S3c).

Taken together, our gas exchange measurements suggest that the photosynthetic apparatus is functional in the sweet double mutants and that the lower photosynthetic performance is caused by stomatal limitation of CO$_2$.

The vacuolar transporter genes ALMT4, ERDL6 and SUC4 are preferentially expressed in guard cells

Considering the substantial increases in starch contents in stp (Fig. 1a and Fig. S1a) and sweet double mutants (Fig. 1c, 1d and Fig. S1b) after approximately 6 to 9 h into the day, we hypothesized that carbon sources other than apoplastic sugars might be
used for starch synthesis during the second half of the day. Diurnal $g_s$ recordings from
Arabidopsis indicate that stomata tend to slowly close after 3 to 4 h of illumination
(Jakobson et al., 2016; Yaaran et al., 2019). Hence, osmotically active solutes need
to be removed from the guard cells to induce the required changes in turgor (Santelia
& Lunn, 2017). Highly specific efflux carriers for such metabolites have been identified
at both the guard cell vacuolar (Martinoia, 2018) and plasma membrane (Jezek & Blatt,
2017), which could mediate the required removal of osmolytes. Besides rapid efflux of
solutes, sugars and/or organic acids that accumulated in the vacuole earlier during the
day might be converted back into starch via gluconeogenesis. Thus, guard cell starch
may act as a sink for osmolytes and promote the removal of water necessary for
stomatal closure. To test this hypothesis, we selected potential candidate vacuolar
exporters based on literature search. The following transporters have been described
to localized to the tonoplast: the Aluminum activated Malate Transporter 4 (ALMT4;
Eisenach et al., 2017), a malate exporter; the Early Response do Dehydration-Like 6
(ERDL6; Poschet et al., 2011), a Glc exporter; and the Suc-H$^+$ symporter SUC4
(Endler et al., 2006; Schulz et al., 2011), a Suc exporter.

Firstly, we examined tissue-specific gene expression. ALMT4, ERDL6 and
SUC4 transcripts were highly abundant in guard cell-enriched epidermal peels relative
to intact leaves (Fig. 3a and Table S1). Of the three, ERDL6 was the most highly
expressed gene in guard cells, with values similar to that of the guard cell markers
KAT1 and MYB60 (Fig. 3a and Table S1). While ERD6 gene expression in guard cells
has not been investigated so far, our data for ALMT4 and SUC4 are in line with
previous reports (Eisenach et al., 2017b) (Zheng et al., 2019).

Secondly, we assessed gene expression in wild-type guard cell-enriched
epidermal peels at 6 compared to 3 h of light. Transcriptional upregulation was
observed for all three candidate genes after 6 h of light (Fig. 3b and Table S1), with
ALMT4 showing the strongest induction (Fig. 3b and Table S1).
Fig. 3 Guard cell gene expression of tonoplast transporter. (a) ALMT4, ERDL6 and SUC4 gene expression in WT guard cell-enriched epidermal peels relative to wild-type intact rosette leaves at the end of the night. KAT1 and MYB60 were used as markers for guard cell-specific expression, while BAM3 was used as a leaf-specific marker. Data for two independent experiments are shown; means ± fold change range; n = 6. Letters (“) indicate significant statistical difference between genes for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (b) ALMT4, ERDL6 and SUC4 gene expression in wild-type guard cell-enriched epidermal peels harvested 6 h into the day relative to WT guard cell-enriched epidermal peels harvested 3 h into the day. Data from two independent experiments are shown; means ± fold change range; n = 6. No statistical differences were detected among the genes. (a-b) ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1.

Export of sugar and organic acids from the guard cell vacuole provides carbon sources for starch synthesis during stomatal closure

Stomatal closure integrates with starch synthesis in guard cells (Azoulay-Shemer et al., 2016) and requires the release of osmotically active solutes from the guard cell vacuole (Eisenach & De Angeli, 2017). Consequently, we investigated guard cell starch accumulation during the 12 h light phase in T-DNA mutant lines for ALMT4 (almt4; SALK_086236), ERDL6 (erdl6; SALK_106049) and SUC4 (suc4; SALK_100140).

Guard cell starch mobilization and accumulation patterns were highly similar between the three mutants but distinct from the wild type (Fig. 4a). Starch degradation at dawn occurred at a reduced rate in mutant guard cells compared to wild type (Fig. 4a and Table S7). Furthermore, while wild-type guard cells synthesized substantial amounts of starch after the plants were illuminated for 3 h, starch contents in mutant guard cells remained low throughout the day (Fig. 4a and Table S7). almt4 guard cells displayed a net decrease of starch between 3 and 6 h of the day and a noticeable low starch synthesis activity between 9 and 12 h (Fig. 4a) as supported by the starch synthesis rates (wild type at 3-6 1.04, almt4 at 3-6 -0.31; wild type at 9-12 0.42, almt4 at 9-12 0.21; Table S7). Similar starch dynamics were observed for erdl6 guard cells with nearly zero starch synthesis between 3 and 6 h into the day and a significant loss of starch towards
the EoD (wild type \(3.6 \pm 1.04\), \(erdl6_{3.6} 0.04\); wild type \(9.12 \pm 0.42\), \(erdl6_{9.12} -0.27\); Table S7). Suc4 mutants displayed milder alterations in starch synthesis rates during 3 to 9 h compared to the other two mutants and wild type (WT\(3.6 \pm 1.04\), suc4\(3.6 \pm 0.04\); WT\(9.0 0.42\), suc4\(9.0 0.91\); Table S7). However, also in guard cells of suc4 mutants, starch amounts did not increase after 9 h (Fig. 4a and Table S7).

Collectively, these data reveal that sugars and organic acids released from the guard cell vacuole via ALMT4, ERDL6 and SUC4 are at least partially converted into starch, thereby contributing to the built-up of the total guard cell starch pool. Furthermore, the gluconeogenic conversion of vacuolar metabolites into starch seems to be of special importance towards the end of the day, presumably when the rate of stomatal closure increases, and the import of mesophyll-derived carbons becomes neglectable as plants are carbon saturated.

**Vacuolar exporter mutants have unaltered levels of guard cell metabolites**

Based on the pronounced guard cell starch phenotypes of the single vacuolar exporter mutants and their presumed role in solute release from the guard cell vacuoles, we investigated if the loss of either of the vacuolar transporters might cause changes in guard cell metabolite concentrations. Therefore, we measured guard cell soluble sugar contents in guard cell-enriched epidermal peels from wild type and single vacuolar exporter mutant plants that were illuminated for 6 and 9 h (Fig. 4b). The time points were chosen to specifically examine guard cell metabolite levels during stomatal closure and in coincidence with pronounced starch biosynthetic activity.

After 6 h of light, wild-type guard cells contained only half as much Glc (Fig. 4b) as at the EoN (EoN, 0.074 ± 0.013; 6 h, 0.029 ± 0.002; Flütsch et al., 2020a). However, counterintuitively, Glc levels did not decrease between 6 and 9 h of light (Fig. 4b). Interestingly, Fru contents were half of the levels at the EoN (EoN, 0.01 ± 0.003; 6 h, 0.005 ± 0.0006; Fig. 4b; Flütsch et al., 2020a) but raised substantially from 6 to 9 h of light (Fig. 4b). This suggests that at this time of the day, some starch synthesis from Suc occurs, metabolizing only Glc, while Fru is not used and accumulates. Suc amounts on the other hand were elevated compared to the early morning (EoN, 0.005 ± 0.002; 6 h, 0.009 ± 0.003; Fig. 4b; Flütsch et al., 2020a) but surprisingly were unaltered between 6 and 9 h (Fig. 4b). Notably, all three vacuolar exporter mutants had guard cell sugar levels similar to that of wild type at both time points (Fig. 4b); except for Fru, which only accumulated in wild-type guard cells and not in that of the mutants (Fig. 4b), coinciding with their reduced starch biosynthetic activity at this time of the day (Fig. 4a and Table S7). Since soluble sugar contents
were measured in guard cell enriched-epidermal peels and not guard cell vacuoles, we cannot exclude the possibility that differences in vacuolar soluble sugar amounts exist between the wild type and the three single mutants.

**Fig. 4** Guard cell metabolites in WT and vacuolar transporter mutants. (a) Starch dynamics in guard cells of intact leaves of wild-type, almt4, erdl6 and suc4 plants during the light period in 12 h/12 h day/night light regime. Plants were illuminated with 150 µmol m⁻² s⁻¹ of white light. Data from three independent experiments are shown; means ± SEM; n = 120 individual guard cells per genotype and time point. EoN = end of night. (b) Content of soluble sugars and (c) malate in guard cell-enriched epidermal peels of wild-type, almt4, erdl6 and suc4 plants after 6 h and 9 h of illumination with white light at 150 µmol m⁻² s⁻¹. Data from two independent experiments are shown; means ± SEM; n ≥ 6 per genotype and time point. (a-c) Letters (*) indicate significant statistical difference between genotypes for the given time point. (a-c) WT = wild type. (b-c) Asterisk (*) indicates significant statistical difference among time points for the given genotypes for P < 0.05 determined by one-way ANOVA with post hoc Tukey's test.

Since ALMT4 is a malate transporter, we further quantified malate contents in guard cell-enriched epidermal peels as detailed above. Wild-type guard cells contained 50 times less malate at 6 h into the day (Fig. 4c) compared to the EoN (EoN, 0.049 ± 0.004; 6 h, 0.008 ± 0.0002; Flütsch et al., 2020a). This observation is consistent with the idea that malate is required to be rapidly removed from guard cells during stomatal closure (Santelia & Lunn, 2017). Interestingly, malate levels rose between 6 and 9 h of light in wild-type guard cells (Fig. 4c). However, the malate levels were still more than 10 x lower compared to the early morning (Fig. 4c; Flütsch et al., 2020a). Malate contents at 6 h were significantly elevated in both erdl6 and suc4 mutants (Fig. 4c), while almt4 guard cells had wild type-like amounts (Fig. 4b). At the later time point, malate contents in the single mutants were significantly lower compared to wild type guard cells (Fig. 4c). Interestingly, we observed increases in malate contents in almt4 and erdl6, but not in suc4 guard cells during the late afternoon (Fig. 4c). Even though we detected differences in malate amounts in guard cells of wild type and the three mutants during the afternoon, the vacuolar levels of malate remain unknown. Therefore, further experimental support will be needed to elucidate the exact impact of the loss of these exporters on metabolite levels in different stomatal compartments.
Vacuolar exporter mutants are impaired in stomatal responses

To assess whether the altered starch dynamics observed in the single vacuolar exporter mutants affect their ability to open and close stomata in response to a shift from dark to light (150 µmol m\(^{-2}\) s\(^{-1}\)), followed by a shift from ambient CO\(_2\) concentrations (400 µl L\(^{-1}\)) to 1000 µl L\(^{-1}\) CO\(_2\), we recorded gas exchange parameters (Fig. 5).

Infrared gas analysis revealed reduced steady-state values of \(g_s\) for all three mutants, whereby the \(erdl6\) mutant was the most affected (Fig. 5a). Light-induced stomatal opening was severely impaired in \(almt4\) and \(erdl6\) mutants with slow \(g_s\) kinetics compared to wild type and \(suc4\) (Fig. 5a and Fig. S4a). The half times for opening were comparable among wild type (~ 15 min; Table S8), \(almt4\) (~ 15 min; Table S8) and \(erdl6\) (~ 14 min; Table S8), however, considering the reduced amplitudes of the opening responses in the mutants (wild type: 77 mmol m\(^{-2}\) s\(^{-1}\); \(almt4\): 52 mmol m\(^{-2}\) s\(^{-1}\) and \(erdl6\): 41.9 mmol m\(^{-2}\) s\(^{-1}\); Table S8), stomatal opening kinetics were clearly reduced in the two mutants. In contrast, half time of opening and amplitude were wild type-like in \(suc4\) mutants (Fig. S4a and Table S8).

Stomatal closure kinetics and amplitudes were also impaired in the mutants (Fig. 5a and Fig. S4b). Stomatal closure was delayed by ~ 3 min in both \(almt4\) and \(suc4\) mutants and even by ~ 5 min in \(erdl6\) mutants compared to wild type (Fig. S4b and Table S9). Interestingly, while \(almt4\) and \(erdl6\) plants displayed reduced ranges of the closure response (Table S9), the amplitude was increased for \(suc4\) and the mutant did not reach a steady-state \(g_s\) during 30 min of recording (Fig. 5a and Fig. S4b).

Next, we analyzed CO\(_2\) assimilation in response to the same light/CO\(_2\) treatment (Fig. 5b). The slow opening responses of \(almt4\) and \(erdl6\) impacted also on \(A\). In wild type, \(A\) increased rapidly during the first 20 to 25 min (Fig. 5b and Fig. S4c) while steady-state \(A\) values were only reached after ~ 50 min in the single mutants. Interestingly, also \(suc4\) plants had lower \(A\) compared to wild type, although their \(g_s\) response to light was similar to that of wild type (Fig. 5a and Fig. S4c). Upon the shift to an elevated CO\(_2\) concentration, the mutants assimilated almost equal amounts of CO\(_2\) as wild-type plants (Fig. 5a and Fig. S4c).

In conclusion, our gas exchange recordings demonstrate that the loss of either ALMT4, ERDL6 or SUC4 significantly impacts on stomatal function, delaying opening and closure responses, thereby affecting their ability to take up atmospheric CO\(_2\).
Leaf carbohydrate metabolism and growth are largely unaffected in tonoplast transporter mutants

A previous study reported the expression of ERDL6 in mesophyll tissues and a role in cellular sugar homeostasis (Poschet *et al.*, 2011). Furthermore, also for ALMT4 relatively high transcript abundance in rosettes was observed (Eisenach *et al.*, 2017b). Notably, SUC4 expression was found to be weak in the leaves (Schulz *et al.*, 2011) or absent (Schneider *et al.*, 2012).

To determine to which extent the selected vacuolar transporters are affected in leaf carbohydrate metabolism, we enzymatically quantified leaf soluble sugar, starch and malate contents at the EoN and EoD (Fig. 5c-g). Interestingly, we only detected differences in leaf soluble sugar contents among the genotypes for Glc (Fig. 5c-e). While we observed a non-significant trend in almt4 plants towards reduced Glc contents at EoN (Fig. 5c), erdl6 mutants contained almost double the amount of Glc compared to wild type at EoN (Fig. 5c). The latter finding agrees with the results presented in Poschet *et al.*, (2011), where erdl6 T-DNA lines were found to over-accumulate Glc in the leaves (Poschet *et al.*, 2011). Furthermore, Glc levels were elevated in all three mutants at EoD in comparison to wild type (Fig. 5c). This suggests that the loss of either of the vacuolar exporters results in altered leaf Glc metabolism during the day.

Interesting differences were also observed for leaf starch accumulation (Fig. 5f). Whereas erdl6 mutants had leaf starch contents similar to those of wild type at EoD, almt4 and suc4 mutants accumulated significantly more leaf starch during the day (Fig. 5f). In contrast, we did not detect significant differences in leaf malate contents among the genotypes at the EoN and EoD (Fig. 5g).

Using Red-Green-Blue (RGB) imaging, we examined growth over seven day, starting when plants were three-week-old (Fig. 5i-j). We observed that almt4 and erdl6 plants were obviously smaller at the beginning of the imaging period, whereas suc4 seemed to be slightly bigger (Fig. 5i). However, during the following 6 days, growth curves were similar for all four genotypes (Fig. 5j), suggesting that the loss of either of the vacuolar transporters did not affect growth rate. The different plant sizes recorded at day 0 of the experiment might therefore rather result from differential germination or seedling expansion among the genotypes. Finally, it should be noted that the imaged plants reached the stationary phase of plant growth at the beginning of our experiment and the growth curves are therefore relatively flat.
Fig. 5 Leaf metabolites in wild type and tonoplast exporter mutants. (a) Whole-plant recordings of changes in stomatal conductance ($g_s$) from wild-type, *almt4*, *erdl6* and *suc4* plants in response to a shift from dark to light and from ambient CO$_2$ concentration to 1000 µL L$^{-1}$ CO$_2$ after 2 h of illumination. Data shown are means ± SEM; n ≥ 3 per genotype. EoN = end of night. (b) Whole-plant recordings of changes in CO$_2$ assimilation (A) from wild-type, *almt4*, *erdl6* and *suc4* plants in response to a shift from dark to light and from ambient CO$_2$ concentration to 1000 µL L$^{-1}$ CO$_2$ after 2h of illumination. Data shown are means ± SEM; n ≥ 3 per genotype. EoN = end of night. (a-b) Plants were illuminated with 150 µmol m$^{-2}$ s$^{-1}$ white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. (c) Quantification of leaf glucose (Glc), (d) leaf fructose (Fru), (e) leaf sucrose (Suc), (f) leaf starch and (g) leaf malate in wild-type, *almt4*, *erdl6* and *suc4* plants. Data shown are means ± SEM; n ≥ 8 per genotype and time point. (a-g) Letters (*) indicate significant statistical difference between genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test. (i) Representative Red Green Blue (RGB) images of 3-(day 0) and 4-week-old (day 6) wild-type, *almt4*, *erdl6* and *suc4* plants. Scale bar, 10 mm. (j) Projected rosette area over the phenotyping period. Data shown are means ± SEM; n = 10 per genotype and time point. No statistically significant differences were detected among genotypes. (a-j) WT = wild type.
DISCUSSION

A battery of tightly regulated hexose sugar transporters is required for guard cell starch synthesis and stomatal movement regulation

Over the past few years, great progress has been made in elucidating the physiological roles of guard cell starch metabolism (Azoulay-Shemer et al., 2016; Horrer et al., 2016; Flütsch et al., 2020b; Li et al., 2020). However, how starch is made in guard cells is poorly understood. It has been a matter of debate whether guard cell photosynthetic CO$_2$ reduction and the resulting Calvin cycle products contribute to guard cell starch synthesis and stomatal regulation. Only recently we demonstrated that the accumulation of guard cell starch is largely independent from autonomous CO$_2$ fixation (Flütsch et al., 2020a), while it relies mostly upon the uptake of mesophyll-derived sugars (Flütsch et al., 2020b).

The high-affinity monosaccharide-H$^+$ symporters STP1 and STP4 are guard cell plasma membrane-localized and we previously showed that their cooperative action in the uptake of mesophyll-originating Glc is essential for guard cell starch synthesis. Guard cells of stp1stp4 plants are devoid of starch at EoN and fail to accumulate it until 3 h into the day (Flütsch et al., 2020b). Here, we report that guard cells of stp1stp4 surprisingly synthesize significant amounts of starch in their guard cells just after 6 h of light exposure (Fig. 1a). This finding has important implications. Firstly, there must be a fine regulation of STP1 and STP4 activity. Indeed, gene expression of STP1 and STP4 follows circadian oscillations, induced during the dark and progressively declining during light (Harmer et al., 2000; Stadler et al., 2003; Büttner, 2010). In particular, STP1 and STP4 transcripts are relatively abundant at dawn, while 4 h into the day a strong reduction of transcripts was observed; thereafter, transcript levels remained low for both genes until 8 h of light (Büttner, 2010). Interestingly, the pattern of transcriptional up- and downregulation of STP1 and STP4 matches not only with the temporal dynamics of guard cell starch contents in stp1stp4 mutants (Fig. 1a) but also with the reported repression of gene expression by Glc (Price et al., 2004; Cordoba et al., 2015; Rottmann et al., 2016). Assuming that Glc contents in the guard cell apoplast will rise during the morning as a consequence of mesophyll cell photosynthesis, STP1 and STP4 would get repressed during this accumulation. Besides transcriptional regulation also post-translational modifications could fine-tune the activities of STP1 and STP4, since a phosphopeptide was previously identified for STP1 (Nühse et al., 2004). In contrast, such evidence is lacking for STP4.
Secondly, carbon sources other than that of STP-imported hexoses are used for guard cell starch production the second half of the day. We found that other sugar carriers such as SWEET1, SWEET4 and SWEET 5 together with SUC1 and SUC3 are highly expressed in guard cells relative to leaves (Fig. 1b; Flütsch et al., 2020b). However, experimental evidence to support their function in guard cells was not investigated so far in Arabidopsis.

We show that diurnal guard cell starch contents in single sweet and suc mutants did not differ significantly from that of wild type (Fig. 1c and Fig. 1d). Hence, it is likely that SWEET and SUC isoforms act redundantly in guard cells. Indeed, sweet1sweet5 double mutants had guard cell starch amounts similar to those of stp1stp4 (Fig.1a and Fig. 2a), revealing that also SWEET1 and SWEET5 cooperatively contribute to starch biosynthesis in guard cells specifically at dawn. It is tempting to speculate that sweet1sweet5 mutant guard cells are also suffering from low Glc amounts at the EoN as previously demonstrated for the stp1stp4 mutants (Flütsch et al., 2020b). This point should be addressed in future research (see outlook at this end of this chapter).

Notably, sweet1sweet5 plants were also affected in stomatal opening (Fig. 2e and Fig. S2b), restricting the assimilation of CO₂ (Fig. 2f). The stomatal phenotypes of sweet1sweet5 and stp1stp4 might results from limited substrate availability caused by reduced CO₂ fixation imposed by the mutations. Indeed, stp1stp4 plants contained 40 to 65% less leaf soluble sugars and starch compared to wild type (Flütsch et al., 2020a), which in turn could constrain Suc fluxes to the guard cell apoplast and subsequent import into guard cells via SWEET1 and SWEET5. Therefore, future research should be guided towards the investigation of leaf carbohydrate metabolism in sweet1sweet5 plants, which may explain its phenotype despite functional STP proteins.

Besides the cooperative action of SWEET1 and SWEET5, both isoforms also act independently in combination with SWEET4. In Fig. 2, we show that both sweet1sweet4 and sweet4sweet5 double mutants were impaired in guard cell starch accumulation during the day, but, interestingly, with distinct temporal patterns. Loss of SWEET1 and SWEET4 resulted in particularly low starch amounts and starch biosynthetic activity around midday (Fig. 2b), while the loss of SWEET4 and SWEET5 affected starch contents towards the EoD (Fig. 2d). Altered guard cell starch dynamics and probably altered guard cell sugar homeostasis (to be tested) affected light-induced stomatal opening in the double mutants and impacted on A (Fig. 2e-f and Fig. S2b). These distinct stomatal phenotypes point again towards a tightly regulated activity of SWEET1, SWEET4 and SWEET5 in guard cells. We provide some evidence for
transcriptional regulation of SWEET genes, with specific induction of SWEET1 and 4 at 6 h relative to 3 h of light in wild-type guard cell-enriched epidermal peels (Fig. 2c). Other means of regulation could be hetero-oligomerization among the three different SWEET isoforms depending on post-translational modification and subsequent protein conformational as it was shown for other SWEETs (Xuan et al., 2013).

Our data also provide additional evidence that Suc imported via SUC1 and SUC3 is not a substrate for daytime guard cell starch synthesis (Fig. S2a). Indeed, recent work revealed a primarily energetic role for Suc in guard cells. Transgenic tobacco plants overexpressing the Suc degradation enzyme Sucrose Synthase 3 (SUS3) specifically in guard cells had elevated steady-state $g_s$ whereby Glc and Fru were not accumulated (Daloso et al., 2016). The opposite was observed for SUS3 antisense potato plants (Antunes et al., 2012). Moreover, kinetic isotope labeling experiments revealed fast and high $^{14}$C-enrichment in Glc and Fru, followed by malate and other tricarboxylic acid (TCA) intermediates, highlighting the role of Suc as a substrate for light-induced stomatal opening (Medeiros et al., 2018).

The lack of a guard cell starch and stomatal movement phenotype in suc1suc3 mutants, albeit the strong gene expression of SUC1 and SUC3 in guard cells (Meyer et al., 2004; Flütsch et al., 2020a), indicates that other SUC isoforms or Suc transporters are involved in the described mechanisms. SWEET11 and SWEET12, which have been shown to transport Suc (Chen et al., 2012) are highly expressed in guard cells (Flütsch et al., 2020b). Furthermore, transgenic plants expressing a guard cell-specific antisense construct against SUC2 had reduced Suc uptake to guard cells and altered stomatal movements (Antunes et al., 2017). Hence, these transporters represent suitable candidates for the further investigation of Suc metabolism in guard cells.

A recent study suggested a key role for guard cell starch synthesis in CO$_2$-induced stomatal closure, presumably as a sink for previously accumulated sugars and organic acids (Azoulay-Shemer et al., 2016). Since experimental support for this hypothesis is lacking, we examined stomatal responses in the starch synthesis-impaired sweet double mutants in response to a sudden exposure to elevated CO$_2$ concentrations (1000 µl L$^{-1}$; Fig. 2e and Fig. S2c). Stomatal closure was markedly slower in sweet1sweet5 and sweet1sweet4 mutants compared to wild type, whereas the simultaneous lack of SWEET4 and SWEET5 did not affect stomatal closure (Fig. S2c). The absence of a stomatal closing phenotype in sweet4sweet5 might be related to the experimental time frame; $g_s$ was recorded after the EoN, but our results suggest that the combined action of SWEET4 and SWEET5 is specifically required for stomatal starch accumulation during the second half of the day (Fig. 2d). Therefore, $g_s$ should
be monitored throughout the diurnal period to follow the natural pattern of stomatal closure in sweet4sweet5 and other starch-related mutants.

In summary, our genetic and physiological data on selected STP and SWEET sugar transporters demonstrate their requirement for guard cell starch accumulation and proper stomatal functioning. Hence, guard cells possess a highly specific set of plasma membrane sugar carriers mediating the uptake of Glc during daytime for guard cell starch accumulation and stomatal movement regulation. Moreover, the activity of such transporters is finely regulated to assure the continuous supply of mesophyll-derived Glc depending mesophyll photosynthetic activity and the subsequent production and partitioning of sugars to sink tissues. Our new evidence further emphasizes the idea of Glc as a master regulator of stomatal movements. Lastly, we provide support for a role of guard cell starch accumulation during CO₂-induced stomatal closure.

**ALMT4, ERDL6 and SUC4 are required for guard cell starch synthesis and stomatal closure**

The increased quantities of starch in guard cells of stp and sweet double mutants (Fig. 2a, 2b and 2d) towards the EoD, indicate that carbon sources other than mesophyll-derived sugars are used to make starch during the latest part of the day. Reportedly, gs and photosynthetic assimilation continuously decline from midday on until the EoD (Jakobson et al., 2016; Yaaran et al., 2019). This should affect apoplastic sugar levels towards the evening and could restrict the substrates for plasma membrane sugar transporters.

Stomatal closure requires the retrieval of solutes from the guard cell vacuole. Guard cell starch has been proposed to act as a sink for such solutes, which accumulated earlier during the day in the vacuole (Raschke & Dittrich, 1977; Azoulay-Shemer et al., 2016; Santelia & Lunn, 2017). In line with this hypothesis, we provide experimental evidence for the conversion of metabolites previously stored in the guard cell vacuole into starch. This process is mediated by the tonoplast-localized ALMT4, ERDL6 and SUC4 exporter proteins (Fig. 4a), which contribute to stomatal movement regulation (Fig. 5a-b).

ALMT4 was recently described as a vacuolar anion channel mediating malate efflux in Arabidopsis guard cells. ALMT4 activity is regulated by phosphorylation (Eisenach et al., 2017), which is required to mediate the large and fast changes in ion concentrations during stomatal closure (MacRobbie & Kurup, 2007). Eisenach et al. (2017) reported that ALMT4 is needed for ABA-induced stomatal closure, but not in
response to darkness. Latest research indicates that the expression of members of the ABA receptor family PYR/PYL/RCAR is stimulus-specific (Dittrich et al., 2019). Therefore, ALMT4 might be part of the ABA-induced pathway linked to the PYL2 receptor and depend on its activity. PYL2 is the receptor sufficient for ABA-induced stomatal closure, but not for closure in response to darkness (Dittrich et al., 2019).

Here, we further demonstrate that the ALMT4-mediated release of malate from the vacuole is also involved in high CO\textsubscript{2}-activated closure (Fig. 5a and Fig. S4b). Notably, it was reported that ALMT4 regulation is independent of the core ABA-kinase Open Stomata 1 (OST1/SnRK2.6), as channel currents recorded from tobacco plants expressing ALMT4-GFP fusion constructs were unchanged in response to treatment with recombinant OST1 (Eisenach et al., 2017). This finding agrees with the recent discovery that rapid CO\textsubscript{2} signal transduction leads to stomatal closure via an ABA-independent pathway downstream of OST1 (Hsu et al., 2018). Interestingly, steady-state \( g_s \) in response to light was also reduced in \textit{almt4} knockout mutants (Fig. 5a) and stomatal opening was delayed (Fig. S4a). In contrast, Eisenach et al. (2017) reported normal stomatal opening in \textit{almt4} mutants. However, their gas exchange recordings were performed under 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of white light, while we illuminated the plants with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). As we previously showed, stomatal movement regulation differs depending on light quality and quantity (Flütsch et al., 2020b). Moreover, the altered stomatal movements in \textit{almt4} mutants impacted on \( A \) (Fig. 5b and Fig. S4c) but had no effect on plant growth (Fig. 5i-j). Since ALMT4 is expressed throughout plant tissues, including leaves (Eisenach et al., 2017), it is not surprising that we observed alterations in leaf metabolites in \textit{almt4} mutants. Particularly, Glc, starch and malate accumulated to higher levels in the mutants compared to wild-type leaves at the EoD (Fig. 5c-g). This suggests an overall imbalance of the carbon metabolism in \textit{almt4} leaves caused by its reduced capability to store malate in vacuoles. It is likely that excess malate is partially converted into other carbon compounds, such as Glc and starch. Malate is not only an intermediate of the TCA cycle and an important regulator of pH (Fernie & Martinoia, 2009), but also a storage carbon molecule (Zell et al., 2010). In general, malate accumulates to higher levels in conditions of reduced starch accumulation (e.g. longer photoperiods) and \textit{vice versa} (Gibon et al., 2009). Interestingly, altered leaf carbohydrate metabolism had little or no effect on guard cell carbon metabolite levels in \textit{almt4} mutants (Fig. 4b-c).

Previous work revealed SUC4 expression in guard cells and linked SUC4 with mannitol-induced stomatal closure (Zheng et al., 2019). In line with these observations, we showed that SUC4 is also needed for CO\textsubscript{2}-induced stomatal closure as \textit{suc4} mutants failed to reach steady-state \( g_s \) during exposure to 1000 \( \mu \text{l mL}^{-1} \) of CO\textsubscript{2}.
for 30 min, whereas wild type did (Fig. 5a and Fig. S4b). Conversely, stomatal opening in response to light in *suc4* was similar to wild type in terms of speed (Fig. 5a) with increased *g*ₚ amplitudes (Fig. S4a). We observed no overaccumulation of Suc or other carbohydrates in leaves and guard cells (Fig. 4b-c and Fig. 5c-g) nor significant differences in plant growth (Fig. 5i-j). Thus, our whole plant physiological data further corroborate earlier observations of no phenotypic alterations among *suc4* and wild type. Schneider *et al.* (2012) analyzed carbohydrate contents in seedling of knockout mutants and did not detect differences from wild-type amounts. The authors explained the absence of a visible phenotype on the basis of a very low expression of SUC4 in leaves. The same study proposed that SUC4 is co-regulated with the Tonoplast Monosaccharide Transporters 1 and 2 (TMTs), which transport sugars in the opposite direction, to prevent a futile cycle of Suc (Schneider *et al*., 2012). Up to date, evidence for a role of the TMTs in guard cells is missing and should be investigated in the future. Moreover, Zheng *et al.* (2019) provided evidence for strict regulation of SUC4 transporter concentration through protein-sorting at the guard cell tonoplast.

Unlike ALMT4 and SUC4, ERDL6 was not previously linked to guard cells. ERDL6 is a vacuolar glucose exporter and its expression is specifically induced during darkness, heat stress or wounding and repressed during conditions that trigger Glc accumulation such as cold stress and supply of external sugars (Poschet *et al*., 2011). This would indicate that *ERDL6* gene expression is induced once apoplastic and subsequently cytosolic Glc concentrations decline and limit the uptake of Glc into the vacuole (e.g. during the early evening). Even though Poschet *et al.* (2011) demonstrated a wide-spread pattern of *ERDL6* expression in different plant tissues, here, we report that *ERDL6* transcripts are highly abundant in stomatal guard cells (Fig. 3a). We further provide evidence for ERDL6 involvement in stomatal movement regulation. We observed impaired responses for both light-induced stomatal opening (Fig. 5a and Fig. S4a) and CO₂-mediated stomatal closure in *erdl6* mutants (Fig. 5a and Fig. S4b). Interestingly, out of the three vacuolar exporter mutants studied in this report, *erdl6* mutants had the strongest phenotype under all investigated conditions (Fig. 3-5). Starch contents were significantly lower in *erdl6* compared to *almt4* and *suc4* guard cells at the EoN and EoD (Fig. 4a). Similarly, stomatal opening and closure were severely delayed and reduced, limiting *A* (Fig. 5b and Fig. S4c). Our discovery of this novel function of ERDL6 integrates with the recently proposed dominant role of Glc in guard cell sugar homeostasis and stomatal movement regulation (Flütsch *et al*., 2020b,a).

Lastly, it should be noted that it is likely that the vacuolar exporter mutants contain unusual amounts of metabolites in their guard cell vacuoles leading to the
altered stomatal starch and movement dynamics. However, due to experimental limitations we are currently unable to dissect metabolite concentrations in different guard cell compartments. Future research might employ non-invasive FRET-based (Förster resonance energy transfer) nanosensors for sugars to follow carbon fluxes into and out of guard cell vacuoles. Unfortunately, no such sensor exists for malate yet.

In summary, we show that the gluconeogenic conversion of carbons sequestered in the vacuole during stomatal opening significantly contributes to the total starch pool observed in guard cells. Our data provide evidence that guard cell starch acts as a sink for osmotically active solutes, gradually removing osmotic power during daytime to promote stomatal closure. Therefore, guard cell starch metabolism is not only essential for rapid stomatal opening, but likewise to promote and accelerate stomatal closure.
**MATERIALS AND METHODS**

**Plant material and growth conditions**

All experiments were performed with non-flowering, 4-week-old Arabidopsis (*Arabidopsis thaliana*) plants in the accession Columbia (Col-0 = WT) background. The transfer DNA (T-DNA) insertion lines SALK_048848 (*stp1-1*) (Yamada *et al.*, 2016), SALK_049432 (*stp4-1*) (Truernit *et al.*, 1996), SALK_41553 (*suc1*) (Flütsch *et al.*, 2020b), SALK_037223 (*suc3*) (Flütsch *et al.*, 2020b), SALK_029479 (*sweet1*), SALK_034060 (*sweet2*), SALK_200835 (*sweet4*) (Liu *et al.*, 2016), SALK_042040 (*sweet5*), SALK_086236 (*almt4*) (Eisenach *et al.*, 2017b), SALK_100140 (*suc4*) (Schulz *et al.*, 2011) and SALK_106049 (*erd16*) (Poschet *et al.*, 2011) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). *stp1*/*stp4* was previously described by Flütsch *et al.*, (2020b). *sweet1*/*sweet4*, *sweet1*/*sweet5*, *sweet4*/*sweet5* and *suc1*/*suc3* double mutant lines were generated through standard crossing techniques and isolated by molecular genotyping (for primer sequences see Table S1).

Plants were grown in soil in controlled-climate chambers (either Fitoclima 1200, Aralab; ClimeCab 1400, Kälte3000; Klimaschrank from Kälte3000) under a 12-h/12-h light/dark photoperiod, with a temperature of 21°C/19°C day/night, a relative humidity of 45%/55% day/night and an irradiance of 150 µmol m⁻² s⁻¹ using LED tubes (Fitoclima 1200), LED panels (ClimeCab 1400) and halogen lamps (Klimaschrank).

**Guard cell starch quantification**

Guard cell starch was quantified at the indicated time points. Epidermal peels were manually obtained from leaf number 5 or 6. Guard cell starch granules were fixed and stained as previously described (Flütsch *et al.*, 2018). Subsequently, guard cell starch granules were visualized and imaged using a confocal laser-scanning microscope Leica TCS SP5 (Leica Microsystems) or Zeiss LSM 780 (Zeiss) and their area was measured using ImageJ version 1.48 (NIH USA, http://rsbweb.nih.gov/ij/). For one experiment, four biological replicates were analyzed per genotype and time point.

**Leaf and guard cell RNA isolation and qPCR**

To extract leaf RNA, three entire rosettes per genotype and time point (three biological replicates) were harvested at the indicated time points and frozen in liquid nitrogen. To extract RNA from guard cell-enriched epidermal peels, the middle veins of 12 rosettes per genotype and time point (one biological replicate) were excised at the indicated time points and the remaining leaf material was blended in 100 ml ice-cold water using a kitchen blender (ProBlend Avance collection, Philips). The blended sample was filtered through a 200 µm nylon mesh (Sefar), and the remaining
epidermal peels were dried, collected in a tube, and immediately frozen in liquid nitrogen. Subsequently, the epidermal peels were ground to a fine powder using a tissue grinder (Mix Mill MM-301, Retsch). For one experiment two or three biological replicates per genotype and time point were harvested. Two independent experiments were performed for each extraction (leaves and guard cell-enriched epidermal peels). Total RNA was extracted from ≥ 30 mg of ground tissue using the SV Total RNA Isolation Kit (Promega) following the manufacturer’s instructions. RNA quality and quantity were analyzed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A total of 1 µg of RNA was used for cDNA first-strand synthesis using the M-MLV Reverse Transcriptase RNase H Minus Point Mutant and oligo(dT)15 primer (Promega). Transcript levels were examined by RT-qPCR using the SYBR Green Master Mix (Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems). RT-qPCR was performed in triplicates. Transcript levels were calculated according to the comparative CT method (Livak & Schmittgen, 2001) and were normalized against the expression of the Actin2 gene (ACT2; At3g18780). Error calculations were done according to Applied Biosystems guidelines (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/general_documents/cms_042380.pdf). Primers and PCR efficiencies for RT-qPCR are listed in Table S1.

**Mesophyll starch and soluble sugar extraction and quantification**

Mesophyll starch and soluble sugar contents were extracted as described in Thalmann et al., (2016) from entire rosettes harvested at the end of the night or at the end of the day. Leaf starch contents were determined enzymatically as described by Hostettler et al., (2013), leaf soluble sugar contents as reported by Thalmann et al., (2016) using 20 µl neutralized soluble fraction as starting material obtained from the initial perchloric acid extraction. At least 9 biological replicates per genotype and time point were analyzed.

**Guard cell soluble sugar quantification**

To extract soluble sugars from guard-cell-enriched epidermal peels, at least six rosettes per genotype, corresponding to one biological replicate, were collected after the plants were exposed to 150 µmol m⁻² s⁻¹ of white light for 6 h or 9 h, and the petiole was removed using scissors. Guard cell soluble sugars were extracted as described in Flütsch et al., (2020a). Up to six biological replicates per genotype and time point were harvested for one experiment. Two independent experiments were performed. Guard cell soluble sugars were quantified based on the protocol for quantification of
root soluble sugars described by Thalmann et al. (2016) using 50 µL of neutralized soluble fraction obtained from the lyophilized and resuspended initial perchloric acid extraction as starting material.

**Leaf malate quantification**

Malate content of leaves was determined using the K-LMAL-116A kit (Megazyme) following the manufacturer’s protocol. Entire Arabidopsis rosettes were harvested at the end of the night or end of the day and immediately frozen in liquid nitrogen. Malate was extracted as described in Flütsch et al., (2020a). Eight rosettes per genotype and time point were collected for one experiment. L-malate content was determined using 10 µL of the initial organic acid extract.

**Guard cell malate quantification**

To quantify the amount of malate from guard cell-enriched epidermal peels, at least six rosettes per genotype and time point, corresponding to one biological replicate, were collected after the plants were exposed to 150 µmol m⁻² s⁻¹ of white light for 6 h or 9 h, and the petiole was removed using scissors. Guard cell malate was extracted as described in Flütsch et al., (2020a). Up to six biological replicates per genotype and time point were harvested for one experiment. Two independent experiments were performed. L-malate content was determined using the K-LMAL-116A kit (Megazyme) following the manufacturer’s protocol using 50 µL of the lyophilized and resuspended organic acid extract.

**Gas exchange measurements**

For whole-plant gas exchange measurements, plants were grown in a Klimaschrank (Kälte3000) under 8 h/16 h light/dark photoperiod, with a temperature of 21°C/19°C, a relative humidity of 45%/55%, and an irradiance of 150 µmol m⁻² s⁻¹. Gas exchange measurements were carried out using a 6400 XT Infrared Gas Analyzer equipped with a 6400-18 light source and the whole-plant Arabidopsis 6400-17 chamber (LI-COR Biosciences). To prevent any CO₂ diffusion and water vapor from the soil, the pots were sealed with clear film. All measurements were performed at 22°C, 50% relative humidity, and 400 µg L⁻¹ CO₂. Before measurements, plants were equilibrated in darkness for 30 min until all parameters had stabilized. After the reading was constant for 10 min, an irradiance of 150 µmol m⁻² s⁻¹ was applied to the rosette for 2 h, followed by 1 h exposure to 1000 µg L⁻¹ CO₂. Measurements of net A and gₛ values were performed on at least three different plants per genotype, starting always at the same time of the diurnal cycle (end of night). Parameters were recorded every minute. Whole
rosette area was determined using the software ImageJ version 1.48 (NIH USA, http://rsbweb.nih.gov/ij/). The $g_s$ and $A$ values were normalized by subtracting the conductance values at the end of the night (set as $0 = \text{initial values for } g_s \text{ or } A$) as described by Baroli et al., (2008) or alternatively for stomatal closure by subtracting the conductance values at 10 minutes before increasing the CO$_2$ concentration (set as $0 = \text{initial values for } g_s \text{ or } A$). In order to evaluate the effects of dark/light transition and elevated CO$_2$ concentrations, the following parameters were calculated as described previously by Merilo et al., (2018): (i) opening/closure half times were obtained by scaling the whole 2 h or 1 h response respectively to a range from 0% to 100% and by calculating the time when 50% of the stomatal response was achieved. (ii) Amplitudes, calculated by the changes in the $g_s$ response as the differences between $g_{\text{max}} - g_{\text{min}}$, where $g_{\text{max}}$ is the maximum value of $g_s$ after transition from dark to light or the last $g_s$ value recorded before the shift to elevated CO$_2$ concentrations and $g_{\text{min}}$ is the last $g_s$ value before transition from dark to light or the last $g_s$ value after the plants were exposed to elevated CO$_2$ concentrations. To evaluate the effect of increasing concentrations of ambient-air CO$_2$ on $A$, photosynthetic CO$_2$ assimilation under irradiance of 150 µmol m$^{-2}$ s$^{-1}$ measured by applying a step increase in CO$_2$ concentrations (from 0 to 1,000 µg L$^{-1}$ CO$_2$) after plants reached steady-state conditions for $A$.

**Determination of growth**

For Red Green Blue (RGB; PSI) imaging of plants, one batch of seeds including wild type were stratified at 4°C for 2-3 day. Seven days after stratification, seedlings of similar size were transplanted into single pots containing 70 g of sieved soil. Plants were cultivated in ClimeCabs (Kälte3000) as described above. Plant imaging started when plants were 21 days old and lasted for 7 days. Plants were imaged daily at the same time and the acquired images were processed using Plant Data Analyzer (PSI). RGB imaging and processing were carried out as described in Flütsch et al., (2020b).

**Statistical Analysis**

Statistical differences between genotypes and time points were determined by ANOVA with post hoc Tukey’s Honest Significant Difference test ($P$-value < 0.05) All data are indicated as means ± SEM.
Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At3g23920 (BAM1), At3g18780 (ACT2), At5g46240 (KAT1), At1g08810 (MYB60), At1g11260 (STP1), At3g19930 (STP4), At1g21460 (SWEET1), At3g14770 (SWEET2), At3g28007 (SWEET4), At5g62850 (SWEET5), At1g71880 (SUC1), At2g02860 (SUC3), At1g25480 (ALMT4), At1g75220 (ERDL6), and At1g09960 (SUC4).

ACKNOWLEDGEMENTS

We thank Prof. Mark Stitt (MPI, Golm), Prof. Enrico Martinoia (UZH) and Prof. Alex Widmer (ETHZ) for helpful input on the interpretation of the presented data. Matthias Thalmann (JIC, Norwich), Michele Moles (ETHZ) and Luca Distefano (ETHZ) for helpful discussions. Data produced in this article were partially generated in collaboration with the Genetic Diversity Centre, ETHZ.
REFERENCES


Sun MX, Huang XY, Yang J, Guan YF, Yang ZN. 2013. Arabidopsis RPG1 is important for primexine deposition and functions redundantly with RPG2 for plant fertility at the late reproductive stage. Plant Reproduction 26: 83–91.


**SUPPORTING INFORMATION**

![Graphs](Image1.png)

**Fig. S1** Guard cell starch contents in *stp* and *sweet2* single mutants.

(a) Starch dynamics in guard cells of intact leaves of wild-type, *stp1-1* and *stp4-1* plants over the 24 h diel cycle (12h light/12h night). Wild-type data are taken from Fig. 1a. (b) Starch dynamics in guard cells of intact leaves of wild-type and *sweet2* plants over the 12 h light period. Wild-type data are taken from Fig. 1c and d. (a-b) Plants were illuminaed with 150 µmol m$^{-2}$ s$^{-1}$ of white light. Data for three independent experiments are shown; means ± SEM; n = 120 individual guard cells per genotype and time point. EoN = end of night. Letters (*) indicate significant statistical differences between genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test. WT = wild type.
Fig. S2 Stomatal phenotypes of sweet and suc double mutants.
(a) Starch dynamics in guard cells of intact leaves of wild-type and suc1suc3 plants plants over the 12 h light period. Plants were illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of white light. Wild-type data are taken from Fig. 2a, b and d. Data from two independent experiments are shown; means ± SEM; \( n = 80 \) individual guard cells per genotype and time point. EoN = end of night. Letters (*) indicate significant statistical difference between genotypes for the given time point for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test. (b-c and e-f) Normalized whole-plant recordings of changes in stomatal conductance \( (g_s) \) from (b-c) wild-type, sweet1sweet5, sweet1sweet4 and sweet4sweet5 plants and (e-f) wild-type and suc1suc3 plants. Data shown are means ± SEM; \( n = 3 \) per genotype. (b and e) Plants have been illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) white light after the end of the night (EoN) under ambient-air CO\(_2\) concentrations. \( g_s \) values were normalized to values at the end of the night (EoN; \( 0 = g_{\text{initial}} \)). (c and f) Plants have been illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) white light under ambient-air CO\(_2\) concentrations and a shift to 1000 \( \mu \text{L L}^{-1} \) CO\(_2\). \( g_s \) values were normalized to values 10 min before the shift in CO\(_2\) concentration (\( 0 = g_{\text{initial}} \)). (d) Whole-plant recordings of changes in stomatal conductance \( (g_s) \) from wild-type and suc1suc3 plants in response to a shift from dark to light and from ambient CO\(_2\) concentration to 1000 \( \mu \text{L L}^{-1} \) CO\(_2\). Wild-type data are taken from Fig. 2e. Data shown are means ± SEM; \( n = 3 \) per genotype. EoN = end of night. No statistically significant differences were detected between the genotypes. (a-f) WT = wild type.
Fig. S3 Photosynthetic assimilation in sweet and suc double mutants.

(a) Normalized whole-plant recordings of changes in CO₂ assimilation (A) from wild-type, sweet1sweet5, sweet1sweet4, sweet4sweet5 and suc1suc3 plants. Data shown are means ± SEM; n = 3 per genotype. Plants were illuminated with 150 μmol m⁻² s⁻¹ white light after the end of the night (EoN) under ambient-air CO₂ concentrations. After 2 h of illumination, CO₂ concentration was increased to 1000 µl L⁻¹. A values were normalized to values at the end of the night (EoN; 0 = A_initial). (b) Whole-plant recordings of changes in CO₂ assimilation (A) from wild-type and suc1suc3 plants in response to a shift from dark to light and from ambient CO₂ concentration to 1000 µl L⁻¹ CO₂. Wild-type data are taken from Fig. 2f. Data shown are means ± SEM; n = 3 per genotype. EoN = end of night. Plants have been illuminated with 150 μmol m⁻² s⁻¹ white light after the end of the night (EoN) under ambient-air CO₂ concentrations. (c) Photosynthetic CO₂ assimilation (A) in dark-adapted wild-type and suc1suc3 plants in response to a step increase in CO₂ concentrations from 0 to 1000 µl L⁻¹ CO₂. Wild-type data are taken from Fig. 2g. Data shown are means ± SEM; n = 3 per genotype. (b-c) No statistical differences were detected among genotypes for the given time points. (a-c) WT = wild type.
Fig. S4 Stomatal response in vacuolar transporter mutants.

(a-b) Normalized whole-plant recordings of changes in stomatal conductance ($g_s$) from wild-type, almt4, erdl6 and suc4 plants. Data shown are means ± SEM; n ≥ 3 per genotype. (a) Plants have been illuminated with 150 μmol m$^{-2}$ s$^{-1}$ white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. $g_s$ values were normalized to values at the end of the night (EoN; 0 = $g_{initial}$). (b) Plants have been illuminated with 150 μmol m$^{-2}$ s$^{-1}$ white light under ambient-air CO$_2$ concentrations and a shift to 1000 μl L$^{-1}$ CO$_2$. $g_s$ values were normalized to values 10 min before the shift in CO$_2$ concentration (0 = $g_{initial}$). (c) Normalized whole-plant recordings of changes in CO$_2$ assimilation ($A$) from wild-type, almt4, erdl6 and suc4 plants. Data shown are means ± SEM; n ≥ 3 per genotype. Plants have been illuminated with 150 μmol m$^{-2}$ s$^{-1}$ white light after the end of the night (EoN) under ambient-air CO$_2$ concentrations. After 2 h of illumination, CO$_2$ concentration was increased to 1000 μl L$^{-1}$. $A$ values were normalized to values at the end of the night (EoN; 0 = $A_{initial}$). (a-c) WT = wild type.
CHAPTER IV

Table S1. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Starch synthesis rates</th>
<th>Genotype</th>
<th>Time points</th>
</tr>
</thead>
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<td>1h light</td>
</tr>
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<td>WT</td>
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<tr>
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</tr>
<tr>
<td>stp4</td>
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Table S2. Starch synthesis rates of wild-type and stp mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.
**Table S3.** Starch synthesis rates of wild-type, *sweet* and *suc* single mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN-1h light</th>
<th>1h light</th>
<th>2h light</th>
<th>3h light</th>
<th>6h light</th>
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<tr>
<td>WT</td>
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<td>-0.55</td>
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<td>0.18</td>
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<tr>
<td>sweet2</td>
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<td>-0.02</td>
<td>0.54</td>
<td>0.34</td>
</tr>
<tr>
<td>sweet4</td>
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<td>-0.56</td>
<td>0.13</td>
<td>1.57</td>
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<td>0.16</td>
<td>0.57</td>
<td>0.36</td>
</tr>
<tr>
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<td>-0.03</td>
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<td>suc3</td>
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<td>-0.26</td>
<td>0.39</td>
<td>0.22</td>
<td>0.35</td>
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**Table S4.** Starch synthesis rates of wild-type, *sweet* and *suc* double mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN-1h light</th>
<th>1h light</th>
<th>2h light</th>
<th>3h light</th>
<th>6h light</th>
<th>9h light</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-0.57</td>
<td>-0.37</td>
<td>1.32</td>
<td>0.30</td>
<td>0.58</td>
<td>0.66</td>
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<tr>
<td>sweet1sweet5</td>
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<td>0.81</td>
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<tr>
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<tr>
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**Table S5.** Stomatal opening parameters of wild-type, *sweet* and *suc* double mutants. Opening half times were obtained by scaling the 2 h response to a range from 0 - 100% and by calculating the amount of time to reach 50% of the response. Amplitudes were estimated as the differences between $g_{\text{max}} - g_{\text{min}}$, where $g_{\text{max}}$ is the maximum value of $g_s$ after transition from dark to light. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Half times of opening (min)</th>
<th>$g_{\text{min}}$ (mmol m(^{-2}) s(^{-1}))</th>
<th>$g_{\text{max}}$ (mmol m(^{-2}) s(^{-1}))</th>
<th>$g_{50}$ (mmol m(^{-2}) s(^{-1}))</th>
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<tbody>
<tr>
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<td>89.2</td>
<td>71.2</td>
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<td>sweet1sweet4</td>
<td>~ 15</td>
<td>41.5</td>
<td>93.3</td>
<td>67.4</td>
<td>51.9</td>
</tr>
<tr>
<td>sweet1sweet5</td>
<td>~ 24</td>
<td>44.7</td>
<td>88.1</td>
<td>66.4</td>
<td>43.3</td>
</tr>
<tr>
<td>sweet4sweet5</td>
<td>~ 20</td>
<td>49.3</td>
<td>114.8</td>
<td>82.0</td>
<td>65.5</td>
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<td>125.9</td>
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<td>70.6</td>
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### Stomatal closure parameters

<table>
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<tr>
<th>Genotype</th>
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<th>g\textsubscript{max} (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
<th>g\textsubscript{50} (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
<th>Amplitudes (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
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<td>54.1</td>
<td>90.1</td>
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<tr>
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<td>105.9</td>
<td>80.9</td>
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**Table S6.** Stomatal closure parameters of wild-type, *sweet* and *suc* double mutants. Closure half times were obtained by scaling the 1 h response to a range from 0 - 100% and by calculating the amount of time to reach 50% of the response. Amplitudes were estimated as the differences between $g_{\text{max}} - g_{\text{min}}$, where $g_{\text{max}}$ is the maximum value of $g_s$ after transition ambient to high CO$_2$ concentrations. WT = wild type.

### Starch synthesis rates

<table>
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<th>Genotype</th>
<th>Time points</th>
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<td>0.42</td>
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</tr>
<tr>
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<td></td>
<td>-0.32</td>
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<td>0.87</td>
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<td>erdl6</td>
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</table>

**Table S7.** Starch synthesis rates of wild-type and vacuolar exporter mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

### Stomatal opening parameters

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Half times of opening (min)</th>
<th>g\textsubscript{min} (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
<th>g\textsubscript{max} (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
<th>g\textsubscript{50} (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
<th>Amplitudes (mmol m\textsuperscript{-2} s\textsuperscript{-1})</th>
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</thead>
<tbody>
<tr>
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<td>100.8</td>
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<td>74.9</td>
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<td>erdl6</td>
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<td>75.9</td>
<td>54.9</td>
<td>41.9</td>
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<td>39.6</td>
<td>122.1</td>
<td>79.4</td>
<td>82.5</td>
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</table>

**Table S8.** Stomatal opening parameters of wild-type and vacuolar exporter mutants. Opening half times were obtained by scaling the 2 h response to a range from 0 - 100% and by calculating the amount of time to reach 50% of the response. Amplitudes were estimated as the differences between $g_{\text{max}} - g_{\text{min}}$, where $g_{\text{max}}$ is the maximum value of $g_s$ after transition from dark to light. WT = wild type.
**Stomatal closure parameters**

<table>
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<th>$g_{\text{max}}$ (mmol m$^{-2}$ s$^{-1}$)</th>
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**Table S9.** Stomatal closure parameters of wild-type and vacuolar exporter mutants. Closure half times were obtained by scaling the 1 h response to a range from 0 - 100% and by calculating the amount of time to reach 50% of the response. Amplitudes were estimated as the differences between $g_{\text{max}} - g_{\text{min}}$, where $g_{\text{max}}$ is the maximum value of $g_{s}$ after transition ambient to high CO$_2$ concentrations. WT = wild type.

**Supplemental References**


OUTLOOK

The presented research is not yet finalized. Some datasets will profit from additional repetitions of experiments, e.g. gas exchange measurements of sweet double mutants (one more repetition is planned) and the leaf metabolite quantifications in vacuolar exporter mutants (one more repetition is planned).

As mentioned in the discussion, the quantification of guard cell and leaf metabolites in sweet double mutants is fundamental to draw conclusions about the i) the relationship of SWEET1, SWEET5 and STP1, STP4 transporters and ii) the role of SWEET proteins in stomatal metabolism and function. Furthermore, petiole-feeding bioassays (Lin et al., 2011) using different sugar sources (e.g. Glc and Suc) could indicate whether substrate limitation during the early morning, caused by reduced photosynthetic activity in \textit{stp1stp4} and \textit{sweet1sweet5} mutants, results in the low guard cell Glc levels and absence of starch despite the presence of functional STP (in \textit{sweet1sweet5}) and SWEET (in \textit{stp1stp4}) proteins. Finally, to complete the physiological characterization of the sweet double mutants, their growth will be assessed using RGB imaging as described in Material and Methods.

\textit{SWEET1} is ubiquitously expressed in Arabidopsis including the roots (Chen et al., 2010). Similarly, \textit{SWEET4} transcripts are found in diverse plant tissues (Liu et al., 2016). Furthermore, \textit{SWEET4} is strongly induced upon pathogen attack (Chen et al., 2010). Finally, \textit{SWEET5} (also known as \textit{VEX1}) gene expression is relatively uninvestigated. Engel \textit{et al.} (2005) reported its expression in the vegetative cell during pollen development. \textit{SWEET5} is also upregulated upon inoculation with \textit{Pseudomonas syringae} (Chen et al., 2010). Given their widespread gene expression, it is important to exclude pleotropic effects potentially resulting in the phenotypes of sweet double mutants described in this manuscript. Therefore, we plan to functionally complement the double mutants by expressing the respective full-length wild-type coding sequences (CDS) specifically in guard cells of the respective mutants using the guard cell-specific \textit{KST1} promoter (Kelly \textit{et al.}, 2013). Subsequently, transgenic plants will be examined for their guard cell starch contents over the 12 h light period. In line with these experiments, the cellular localization of SWEET5 has not yet been shown yet and we aim to confirm its predicted plasma membrane localization using transient expression assays in \textit{Nicotiana benthamiana}.

In Fig. 2g we demonstrated that photosynthetic CO$_2$ assimilation can be rescued by exposing the sweet double mutants to elevated CO$_2$ concentrations. Hence, to investigate whether the observed phenotypes are linked to impaired
stomatal function or other affected processes in the plants, we will conduct CO\textsubscript{2} complementation assays as previously reported in Flütsch et al., (2020b).

Similarly, to the SWEET proteins investigated in this study, the vacuolar exporter candidate genes, \textit{ALMT4}, \textit{ERDL6} and \textit{SUC4}, are expressed in all plant tissues including roots (Eisenach et al., 2017, Schneider et al., 2012, Poschet et al., 2011). Therefore, it is likewise important to exclude pleiotropic effects. \textit{almt4} mutants were previously complemented with the full genomic DNA sequence of \textit{ALMT4} (Eisenach et al., 2017). Thus, for the presented research, both, \textit{erdl6} and \textit{suc4}, will be functionally complemented using guard cell-specific expression of the respective wild-type CDS. The complementation lines will be further investigated for the role of \textit{ERDL6} and \textit{SUC4} in stomatal function.

\textbf{Outlook References}


CHAPTER V

WHICH ENZYMES ARE INVOLVED THE BIOSYNTHETIC PATHWAY OF STARCH IN GUARD CELLS?

Enzymatic pathway(s) of guard cell starch synthesis in Arabidopsis – traits of hetero- and autotrophism

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Author contributions: D.S. and S.F. conceptualized research. S.F. performed the majority of the experiments. D.H. did the crosses for the generation of the gpt1pgi and the apl3apl4 double mutants, and contributed to data in Fig. 1a, Fig. 2b, Figs. 3b-d, Fig. S1 and Fig. S3 A.N. isolated the homozygous gpt1 mutant line. S.F analyzed all the data. S.F. wrote the manuscript with support of D.S.
SUMMARY

Guard cells in the leaf epidermis of higher plants contain substantial amounts of starch throughout day and night. This starch plays a critical role for the osmotic adjustment of guard cell turgor for stomatal movements. However, our understanding how starch is synthesized in guard cells is nominal. Here, we thoroughly investigated the role of starch biosynthetic enzymes from the classical starch synthesis pathway found in leaf tissues as well as of alternative routes of starch formation using substrates derived from source tissues. We identify the Glucose-6-phosphate/Phosphate Translocator 1 (GPT1) responsible for the uptake of hexose-phosphates into chloroplasts as a main enzyme contributing to the accumulation of starch. More so, we demonstrate that guard cells show characteristics of heterotrophic and autotrophic tissues employing carbon sources derived from the guard cell Calvin cycle as well imported carbons of mesophyll origin for starch biosynthesis. Our work significantly enhances our knowledge about guard cell starch metabolism, which is a newly-emerged target in the manipulation of stomatal movement rapidity directly affecting Water Use Efficiency (WUE).

Key words: Guard cells, starch, hexoses, sugar transport, heterotrophism, chloroplast, Calvin cycle
**Introduction**

Starch is a semi-crystalline insoluble polymer consisting of α-1,4- and α-1,6-linked glucose (Glc) units. It is a commercially important carbohydrate and the major carbon reserve in plants. Starch is synthesized in both autotrophic and heterotrophic tissues. In the leaf chloroplasts, starch is gradually formed during the day using a fraction of the carbon fixed through photosynthesis; at night, starch is degraded to support non-photosynthetic leaf metabolism and the export of sucrose (Suc) (Martin & Smith, 1995). In heterotrophic tissues, Suc is imported from source tissues and used as a carbon source for energy production and starch biosynthesis (José Muñoz et al., 2006).

In the leaf mesophyll, starch is the end product of a biosynthetic pathway that exclusively takes place within the chloroplast and is linked to the Calvin cycle by means of the Phosphoglucose Isomerase (PGI) enzyme. PGI generates glucose-6-phosphate (G6P) from the primary photosynthetic product fructose-6-phosphate (F6P). Phosphoglucose Mutase (PGM) further converts G6P into glucose-1-phosphate (G1P), which is ultimately used for the ATP-consuming generation of the activated glucosyl donor ADP-glucose by the ADP-glucose Pyrophosphorylase (AGPase; Pfister & Zeeman, 2016). Each enzymatic step of this linear oligosaccharide synthesis is essential, as the loss of either PGI (Yu et al., 2000), PGM (Caspar et al., 1985) or the AGPase (Lin et al., 1988) results in almost undetectable or absent starch contents in leaf chloroplasts. The subsequent biosynthetic steps required for starch synthesis involve several Starch Synthase (SSs), Starch Branching (BEs) and Starch Debranching (DBEs) enzymes (Pfister & Zeeman, 2016).

The AGPase catalyzes a near rate-limiting step in the pathway of starch synthesis and therefore represents the main target of regulation (Neuhaus & Stitt, 1990). The heterotetrameric enzyme contains two large subunits (APLs) and two smaller subunits (APSs) (Lin et al., 1988; Crevillén et al., 2003, 2005). The activity of the AGPase is allosterically induced by 3-phosphoglyceric acid (3PGA) and inhibited by high cellular concentrations of Pi linking starch synthesis to the activity of the Calvin cycle (Crevillén et al., 2003, 2005). Moreover, the AGPase is subjected to posttranslational redox regulation, which involves the reversible formation of a disulfide bridge between two cysteines (Cys81) of the small subunits connecting its activity to the processes of the electron transport chain (Hendriks et al., 2003; Hädrich et al., 2012).
Although several biochemical steps of starch synthesis occurring in photosynthetic leaves are conserved in heterotrophic tissues, some are specific to sink organs. For instance, in amyloplasts of endosperms, starch is formed following the incorporation of Suc-derived sugar compounds entering the plastid via a G6P/Phosphate Translocator (GPT). This transmembrane protein was initially detected in the plastidial envelope membranes of maize endosperm (Kammerer et al., 1998). Since then, GPT cDNAs have been isolated from different plant species and in planta functional studies confirmed their function as G6P transporters, including Arabidopsis thaliana (Arabidopsis) (Niewiadomski et al., 2005) and Vitis vinifera (grapewine) (Noronha et al., 2015). Moreover, some plant species, import G1P into their plastids, as shown for potato tubers (Fettke et al., 2010). Alternatively, in heterotrophic tissues of rice, wheat and potato, G1P can be added directly to elongating glucan chains via the α-Glucan Phosphorylase (PHS1) (Satoh et al., 2008; Tickle et al., 2009; Fettke et al., 2010). Lastly, ADP-glucose is produced in the cytosol and subsequently imported into the chloroplast via Brittle1 (BT1) in cereal endosperm (Kirchberger et al., 2007).

Starch is also present in stomatal guard cells (Lloyd, 1908), which surround the stomatal pore on the leaf epidermis of higher plants. Guard cells control the exchange of CO₂ for photosynthesis and limit the loss of water through transpiration at the same time. These highly specialized cells possess several characteristics of heterotrophic tissues, such as reduced numbers of chloroplasts (Willmer & Fricker, 1996), low levels of Rubisco (Outlaw, 1989; Reckmann et al., 1990) and high respiratory rates (Willmer & Fricker, 1996). Guard cell starch shows a distinct temporal pattern of accumulation and degradation that differs in several aspects from that in mesophyll cells. Guard cell starch is abundant during the night, is rapidly mobilized within one hour of illumination and thereafter is accumulated continuously until the middle of the night (Horrer et al., 2016). Starch breakdown in guard cell coincides with stomatal opening (Horrer et al., 2016) and yields Glc to maintain sugar homeostasis during this process (Flütsch et al., 2020a). Interestingly, we previously showed that in isolated guard cells, where there is no connection with the beneath mesophyll, starch accumulation is limited compared to intact guard cells (Flütsch et al., 2020a). We also showed that guard cells rely on the uptake of mesophyll-derived Glc as a main carbon source for starch formation (Flütsch et al., 2020b). However, the relative contribution of either pathway and the enzymes involved in guard cell starch synthesis remain unknown.

In this study, we investigated the early steps of starch biosynthesis in Arabidopsis guard cells. We report the functional characterization of genes related to the classical pathway of starch biosynthesis, such as PGI, PGM and the AGPase, as
well as of genes linked to uptake of sugars to the chloroplast, including the \textit{GPTs}.
Although guard cells appear to be limited in photosynthetic activities, here we reveal that guard cell starch synthesis employs carbon substrates derived from both guard cell and mesophyll cell photosynthesis. Our data significantly advance the knowledge about starch synthesis in guard cells and therefore allow a better understanding of how guard cell starch metabolism integrates with stomatal functioning, thereby affecting whole plant productivity.
RESULTS

PGI is not strictly required for guard cell starch synthesis

The first committed step of leaf starch synthesis is the PGI-mediated conversion of the Calvin cycle intermediate F6P to G6P (Stitt & Zeeman, 2012). It has been previously reported that guard cells of pg1 mutants contain similar amounts of starch as wild-type ones. However, the authors visualized guard cell starch granules only at the end of the day (EoD) and therefore the role of PGI in guard cell starch synthesis remained uninvestigated (Azoulay-Shemer et al., 2016). To elucidate whether plastidial PGI is required for guard cell starch accumulation, we examined stomatal starch levels in ethyl methanesulfonate (EMS)-mutagenized pg1 plants (pg1-1; herein named pg1; Yu et al., 2000) throughout the 24 h day/night cycle (Fig. 1a). Since pg1 mutants are derived from an EMS mutagenesis, we confirmed the point mutation at the base 834 (C to T) by sequencing (Fig. S1).

As previously observed, starch was rapidly degraded in wild-type guard cells in response to light and after its almost complete hydrolyzation, resynthesis occurred from 2 h into the day onwards until the middle of the night (Fig. 1a; Horrer et al., 2016). Notably, pg1 guard cells contained elevated amounts of starch during the night, starting the day with significantly more starch relative to wild type (Fig. 1a). Upon light exposure, starch contents fell to a similar extent in pg1 and wild-type guard cells, consistent with the fact that the slope-derived starch synthesis rates were comparable between genotypes (WT0-1, -0.61; pg1-1, -0.56; Table S2). However, while wild-type guard cells substantially accumulated starch between 2 and 3 h of light (WT2-3, 1.27; Table S2), starch contents remained low in pg1 guard cells (Fig. 1a; pg12-3, 0.13; Table S2). Interestingly, starch synthesis rates and starch accumulation were significantly increased in pg1 mutant guard cells from 6 h onwards (Fig. 1a and Table S2), reaching markedly higher amounts of starch by the EoD compared to wild type (Fig. 1a).

A possible explanation for the mild reductions in guard cell starch contents in the pg1 mutant is the remaining PGI gene expression in the mutant background. We found ~25% residual expression in pg1 intact rosette leaves compared to that in wild type (Fig. 1b). This finding is consistent with the previously reported remaining enzyme activity of ~5% in the same mutant line (Yu et al., 2000) and that pg1 leaves still contain ~25% of wild-type starch contents (Niewiadomski et al., 2005). Another explanation for the mild alterations in guard cell starch contents is that G6P could be imported from the cytosol. This reaction is facilitated by GPT transporters at the inner chloroplast membrane. The Arabidopsis genome encodes two GPT genes for G6P uptake into the chloroplast, GPT1 and GPT2. Ectopic expression of the GPTs in pg1 leaves was
shown to rescue the starch deficiency phenotype of the \textit{pgi} mutant (Niewiadomski \textit{et al.}, 2005) and therefore, the reaction via PGI was circumvented.

![Figure 1](image_url)

**Fig. 1** Guard cell starch contents and \textit{GPT} gene expression in \textit{pgi} mutants.

(a) Starch dynamics in guard cells of intact leaves of wild-type and \textit{pgi} plants over the 24 h diel cycle. Plants were illuminated with 150 \(\mu\text{mol m}^{-2} \text{s}^{-1}\) of white light. Data from three independent experiments are shown; means ± SEM; \(n = 120\) individual guard cells per genotype and time point. EoN = end of night. Letters (*) indicate significant statistical difference between genotypes for the given time point for \(P < 0.05\) determined by one-way ANOVA with post hoc Tukey’s test. (b) \textit{PGI} gene expression in intact rosette leaves of wild-type and \textit{pgi} plants at the EoN. (c) \textit{GPT1} and \textit{GPT2} gene expression in \textit{pgi} guard cell-enriched epidermal peels relative to wild-type guard cell-enriched epidermal peels at the EoN. (b-c) Data from two independent experiments are shown; means ± fold change range; \(n = 6\). \textit{ACT2} was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. Letters (*) indicate significant statistical difference between genes for \(P < 0.05\) determined by one-way ANOVA with post hoc Tukey’s test. (a-c) \textit{WT} = wild type.

Consistent with this idea, we detected \(\sim 1.5\)-fold transcriptional upregulation of \textit{GPT1} in guard cell-enriched epidermal peels of \textit{pgi} mutants relative to wild type. In contrast, \textit{GPT2} was expressed to a similar level in wild-type and \textit{pgi} guard cells or even mildly downregulated (Fig. 1c and Table S1).

**\textit{GPT1} and \textit{GPT2} transcripts are highly abundant in guard cells**

The generation of G6P in the cytosol involves the cytosolic isoenzyme of PGI (cPGI), which is usually required for both the synthesis and breakdown of Suc and the interconversion of F6P into G6P (Kunz \textit{et al.}, 2014). Parts of this cytosolic G6P could be taken up by the functional G6P translocators GPTs in the plastidial envelope (Kammerer \textit{et al.}, 1998). Both genes are ubiquitously expressed at low levels across all plant tissues (Niewiadomski \textit{et al.}, 2005). While \textit{GPT1} transcripts are highly abundant in heterotrophic cell types and \textit{GPT1} is required for embryo sac development and pollen maturation, \textit{GPT2} expression is specifically induced in leaves during times of sugar accumulation (Lloyd & Zakhleniuk, 2004). \textit{GPT1} expression was
also observed in guard cells in transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the GPT1 promoter region (GPT1pro:GUS) (Niewiadomski et al., 2005). However, experimental support for a role of GPT1 and/or GPT2 in guard cells is still lacking.

We determined guard cell-specific gene expression of the GPTs. Transcript levels of GPT1 were ~4-fold higher in wild-type guard cell-enriched epidermal peels relative to leaves (Fig. 2a and Table S1). These findings are consistent with the earlier report of 10-fold upregulation of GPT1 in guard cell protoplasts compared to mesophyll...
cell protoplasts (Niewiadomski et al., 2005). Interestingly, GPT2 was expressed to similar levels as the guard cell marker genes $K^+$ channel in Arabidopsis (KAT1) and Myb transcription factor 60 (MYB60) and therefore displayed a more pronounced preferential guard cell expression than GPT1 (Fig. 2a and Table S1).

Loss of GPT1 function perturbs guard cell starch accumulation

To examine whether the GPTs are involved in G6P provision to the guard cell chloroplasts for starch accumulation, we quantified stomatal starch amounts in homozygous transfer-DNA (T-DNA) mutants of GPT1 (gpt1-3; herein named gpt1; SALK_021762) and GPT2 (gpt2; GABIKAT_454H06) during the 24 h light/dark cycle (Fig. 2b). Mutations affecting GPT1 were previously described to be embryo lethal, however, viable, homozygous T-DNA lines for the GPT1 locus are available (gpt1-3, gpt1-5 and gpt1-6). These lines were characterized to have unaltered GPT1 transcript amounts (Niewiadomski et al., 2005). In contrast, a more recent study used the gpt1-3 allele to investigate the function of GPT1 in starch turnover of Arabidopsis reproductive tissues and found highly significant reductions of GPT1 transcripts in different flower organs in the gpt1-3 mutants along with reduced starch contents (Hedhly et al., 2016). Hence, we decided to proceed with the gpt1-3 allele for our analyses.

We observed a similar overall pattern of guard cell starch synthesis and degradation in wild-type and gpt1 plants, but gpt1 guard cells contained significantly lower starch amounts throughout the day (Fig. 2b). In particular, starch breakdown during the first 1 h of light occurred to a similar extent in both genotypes (Fig. 2b; WT$_{0-1}$, -0.61 and gpt1$_{0-1}$, -0.82; Table S2), followed by comparable starch accumulation between 2 and 3 h of light (Fig. 2b; WT$_{2-3}$, 1.27; gpt1$_{2-3}$, 1.00; Table S2). After 3 h, starch levels were only slightly reduced in guard cells of gpt1, but then remained significantly lower until the EoD compared to wild type (Fig. 2b and Table S2). These data suggest that starch synthesis during this period of the day is promoted by import of cytosolic G6P via GPT1. This idea is further supported by the ~2-fold transcriptional upregulation of GPT1 at 6 h compared to 3 h in wild-type guard cell-enriched epidermal peels (Fig. 2c and Table S1). GPT1 high gene expression during the second half of the day matches with the observed starch dynamics in the gpt1 single mutants. Notably, during the first 3 h of darkness, gpt1 guard cells accumulated starch to wild-type levels, thereafter the amounts were similar for the remainder of the night (Fig. 2b and Table S2), implicating that sources other than G6P imported via GPT1 may be used for starch synthesis during nighttime.
Although GPT2 was highly expressed in guard cells relative to leaves (Fig. 2a), stomatal starch contents in gpt2 mutants were wild type-like throughout the majority of the 24 h light/dark cycle (Fig. 2b). gpt2 guard cells displayed a particular pattern of net increase and decrease of guard cell starch contents between 1 and 3 h of light (Fig. 2b and Table S2). However, after 6 h, gpt2 guard cell starch amounts were only mildly reduced compared to wild type and even rose to the same level after 9 h (Fig. 2b and Table S2). By the EoD and for the entire night, gpt2 guard cells had mildly elevated starch levels compared to wild type (Fig. 2b and Table S2). Surprisingly, we also observed induced GPT2 gene expression at 6 h compared to 3 h (Fig. 2c and Table S1), which had only a mild or no effect on starch contents in gpt2 plants. Given that GPT1 was also highly expressed at this time of the day, while PGI was not (Fig. 2b), GPT1 might be functionally compensating for the lack of GPT2.

In summary, our data on the single gpt mutants suggest that GPT1 is the dominant isoform in guard cells required to deliver cytosolic G6P to the chloroplast, which is used for guard cell starch accumulation. The observed formation of starch in gpt1 guard cells could be either due to guard cell photosynthetic activity and subsequent conversion of F6P into G6P via PGI or G6P import through the closely related GPT2 translocator.

gpt1gpt2 mutants phenocopy gpt1 single mutants

In order to assess whether GPT2 is responsible for the observed starch accumulation in gpt1 mutants, we generated the double mutant combination gpt1gpt2 and examined its guard cell starch amounts during the 12 h light phase (Fig. 2d).

To our surprise, the additional loss of GPT2 had essentially no effect on guard cell starch accumulation during the day compared to the gpt1 single mutant (Fig. 2d and for comparison Fig. 2b). In particular, starch contents at the end of the night (EoN) were comparable among both genotypes and starch degradation occurred at a similar rate until 2 h of light (Fig. 2d; WT0-1, -0.58; WT1-2, -0.24; gpt1gpt20-1, -0.64; gpt1gpt21-2, -0.05; Table S3). While wild-type guard cells continuously accumulated starch from 2 h onwards, we observed a lag phase of starch synthesis in the gpt1gpt2 double mutants between 2 and 3 h (Fig. 2d and Table S3). Thereafter, starch levels remained reduced in the double mutant in comparison to wild type (Fig. 2d and Table S3).

Given that the loss of GPT2 had no additive effect on the gpt1 guard cell starch phenotype, makes it unlikely that GPT1 and GPT2 work redundantly in G6P uptake to the guard cell chloroplasts, they might rather have distinct temporal patterns of activity.
gpt1pgi double mutants accumulate substantial amounts of guard cell starch

These similar patterns of starch formation and dissipation in gpt1 and gpt1gpt2 mutants (Fig. 2b and Fig. 2d), prompted us to test whether PGI activity may contribute to the observed remaining starch accumulation. We generated gpt1pgi double mutants by crossing homozygous gpt1 with pgi plants, followed by molecular genotyping (Fig. S1). After successful isolation of homozygous double mutant plants, their guard cell starch contents were examined during the diurnal 24 h light/dark cycle, as pgi single mutants had elevated amounts of starch during the entire night (Fig. 3a).

Simultaneous loss of GPT1 and PGI did not restrict starch accumulation to a greater extent than in gpt1 (Fig. 2b) or gpt1gpt2 (Fig. 2d) guard cells. Starch contents were essentially identical in wild type and gpt1pgi throughout the entire night (Fig. 3a and Table S2), This is surprising, as GPT1 and PGI should provide the majority of G6P needed for starch synthesis. Similarly to the pgi single mutants, starch synthesis in the double mutant was affected early in the day, with an even more pronounced lag phase (Fig. 3a and Fig. 1a for comparison). Hence, the starch synthesis rate was negative in the gpt1pgi double mutant between 2 and 3 h (gpt1pgi2-3, -0.11; Table S2), while the rate was slightly positive in pgi mutants (pgi2-3, 0.13; Table S2) and the highest throughout the 24 h in wild type (WT2-3, 1.27; Table S2). Interestingly, starch contents remained at reduced levels compared to wild type for the rest of the day (Fig. 3a), although stomatal starch increased markedly between 3 and 6 h in gpt1pgi and the starch synthesis rate was elevated compared to wild type and the single mutants (Fig. 3a; WT3-6, 0.65; pgi3-6, 0.46; gpt13-6, 0.44 gpt1pgi3-6, 2.00; Table S2). The lack of early starch synthesis (e.g. between 2 and 3 h) in combination with the reduced starch contents throughout the day indicate that both PGI and GPT1 are required for proper guard cell starch synthesis. An explanation for the remaining considerable amounts of starch in gpt1pgi mutants could be transcriptional upregulation of GPT2 in this genetic background. However, we did not observe elevated amounts of GPT2 transcripts in guard cell-enriched epidermal peels of gpt1pgi relative to wild-type at the EoN (Fig. S2 and Table S1).
Fig. 3 Guard cell starch contents in *gpt1pgi* double mutants and *PGI* silencing lines in *gpt1gpt2* backgrounds.

(a) Starch dynamics in guard cells of intact leaves of wild-type and *gpt1pgi* plants over the 24 h day cycle. Wild-type data are the same as in Fig. 1a. Data from three independent experiments are shown; means ± SEM; n = 120 individual guard cells per genotype and time point. (b) *PGI* gene expression in guard cell-enriched epidermal peels of artificial microRNA-induced silencing lines of *PGI* in the *gpt1gpt2* mutant background (amiRNA-*PGI*) relative to wild-type guard cell-enriched epidermal peels and in intact rosette leaves of amiRNA-*PGI* lines relative to wild-type intact rosette leaves at end of night (EoN). Data from one experiment are shown; means ± fold change range; n = 3. *ACT2* was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. No statistical test was used on this data set as only one experiment was performed. (c) Representative confocal images of propidium iodide-stained starch granules in guard cells of intact leaves of wild-type and amiRNA-*PGI* plants over the 12 h light period. Scale bar = 10 μm. (d) Starch dynamics in guard cells of intact leaves of wild-type and amiRNA-*PGI* plants over the 12 h light period. Data from one experiment are shown; means ± SEM; n = 40 individual guard cells per genotype and time point. (a and d) Plants were illuminated with 150 μmol m⁻² s⁻¹ of white light. EoN = end of night. Letters (*) indicate significant statistical difference between genotypes for the given time point for *P* < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (a-d) WT = wild type.

**Triple gpt1gpt2pgi mutants are nearly devoid of guard cell starch until 6 h into the day**

To explore if either GPT2 in *gpt1pgi* double mutants or PGI in *gpt1gpt2* double mutants partially complemented their guard cell starch phenotype, we generated *gpt1gpt2pgi* triple mutants. Due to the leaky nature of the *pgi* mutant described above, we employed artificial microRNA-based silencing (Schwab *et al.*, 2010) to transcriptionally downregulate *PGI* in the genetic background of *gpt1gpt2* mutants. PGI plays a vital role in mesophyll starch metabolism (Yu *et al.*, 2000) and its lack strongly affects plant growth (Fig. S3). Therefore, we restricted the expression of the silencing construct to...
guard cells by expressing the corresponding amiRNA-\( PGI \) construct under the control of the guard cell-specific promoter \( KST1 \) (Kelly et al., 2013).

qRT-PCR analyses on guard cell-enriched epidermal peels and intact rosette leaves confirmed the guard cell-specific downregulation of \( PGI \) in the \( gpt1gpt2 \) genetic background for two independent lines; amiR-\( PGI \) \#1 and \#2 (Fig. 3b and Table S1). Guard cell \( PGI \) transcripts were reduced by approximately 30 – 40% respectively, while \( PGI \) gene expression in the leaves was comparable to wild-type for both silencing lines (considering the fold change range; Fig. 3b and Table S1).

Next, we analyzed guard cell starch contents over the 12 h light period in plants carrying the \( PGI \) silencing constructs (Fig. 3c). In contrast to the single \( pg1, gpt1 \) and \( gpt2 \) mutants (Fig. 1a and Fig. 2b) and the two investigated double mutants \( gpt1gpt2 \) and \( gpt1pgi \) (Fig. 2d and Fig. 3a), guard cell starch contents were severely reduced at the EoN in the silencing lines (Fig. 3c and Fig. 3d). Moreover, guard cell starch was almost undetectable in both silencing lines between 1 and 6 h of the day, whereas wild-type guard cells had already replenished starch to half of the contents of the EoN (Fig. 3c and Fig. 3d). In between 6 and 9 h as well as 9 and 12 h both silencing lines accumulated starch up to contents that were ~ 8- and 4-fold higher, respectively, than those at EoN (Fig. 3c and Fig. 3d). These observations were further supported by the starch synthesis rates during the second half of the day (WT\(_{6-9}\); 0.59; WT\(_{9-12}\); 0.82; amiRNA-\( PGI \) \#1\(_{6-9}\); 8.28; amiRNA-\( PGI \) \#1\(_{9-12}\); 0.60; amiRNA-\( PGI \) \#2\(_{6-9}\); 1.62; amiRNA-\( PGI \) \#2\(_{9-12}\); 1.40; Table S4).

Collectively, these data reveal that guard cell starch synthesis up to 6 h into the day, uses G6P derived from \( PGI \)-mediated conversion of F6P as well as from G6P imported through GPT1 and GPT2, with a pronounced role for GPT1. The guard cell starch phenotypes of the investigated \( gpt1gpt2 \) and \( gpt1pgi \) double mutants likely resulted from partial functional complementation by the remaining GPT2 or \( PGI \) enzymes delivering G6P. Furthermore, the observed starch formation in guard cells of the \( PGI \) silencing lines specifically after 6 h of light suggests that i) other sources of G6P may exist in guard cells or that ii) uptake of G1P or ADPglucose into the guard cell chloroplasts may occur, thereby bypassing the chloroplastic conversion of G6P to G1P.

**PGM does not exclusively provide G1P in guard cells**

The PGM-catalyzed conversion of G6P to G1P is essential for starch biosynthesis in mesophyll cells. Arabidopsis plants lacking functional plastidial PGM, as the EMS mutant \( pgm1-1 \) (herein called \( pgm \)), are unable to synthesize starch in their leaf
chloroplasts (Caspar et al., 1985). These mutant plants also completely lack starch in guard cell chloroplasts (Lasceve et al., 1997; Horrer et al., 2016). Similarly to pgi mutants, plant health and growth are severely affected by the loss of PGM (Paparelli et al., 2013). To avoid pleiotropic effects on stomatal function, we silenced PGM specifically in guard cells of wild-type plants. We employed again artificial microRNA-based gene silencing (Schwabe et al., 2010) and the guard cell-specific KST1 promoter (Kelly et al., 2013), as we did for PGI.

In a first step, two independent PGM silencing lines, amiR-PGM #1 and #2, were isolated based on their guard cell-specific expression of PGM (Fig. 4a and Table S1). In both lines, PGM transcripts were reduced by ~60%, whereas their leaf PGM transcripts were comparable to wild-type and unaffected by the insertion of the silencing constructs (Fig. 4a and Table S1).

Fig. 4 Guard cell starch contents in PGM silencing lines in gpt1gpt2 backgrounds. (a) PGM gene expression in guard cell-enriched epidermal peels of artificial microRNA-induced silencing lines of PGM in wild-type genetic background (amiRNA-PGM) relative to wild-type guard cell-enriched epidermal peels and in intact rosette leaves of amiRNA-PGM lines relative to wild-type intact rosette leaves at EoN. Data from two experiments are shown; means ± fold change range; n = 6. Letters (*) indicate significant statistical difference between genes for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (b) Representative confocal images of propidium iodide-stained starch granules in guard cells of intact leaves of wild-type and amiRNA-PGM plants over the 12 h light period. Scale bar = 10 µm. (c) Starch dynamics in guard cells of intact leaves of wild-type and amiRNA-PGM plants over the 12 h light period. Data from two experiments are shown; means ± SEM; n = 80 individual guard cells per genotype and time point. Plants were illuminated with 150 µmol m⁻² s⁻¹ of white light. EoN = end of night. Letters (*) indicate significant statistical difference between genotypes for the given time point for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. (a-c) WT = wild type. (d) PGM gene expression in wild-type guard cell-enriched epidermal peels at 6 h relative to 3 h into the day. (e) G1PT1 and G1PT2 expression relative to PGM expression over the 12 h light period in wild-type guard cell-enriched epidermal peels. Data from one experiment are shown; means ± fold change range; n = 3. (a and d-e) ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1.
Having confirmed the guard-cell specific silencing of \textit{PGI}, we next examined guard cell starch contents during the day in the two silencing lines and in the wild type (Fig. 4b and Fig. 4c). Guard cell starch was present in both amiR-\textit{PGM} lines (Fig. 4b and Fig. 4c). Notably, starch levels were significantly elevated at the EoN in the amiR-\textit{PGM} guard cells compared to wild type (Fig. 4b and Fig. 4c), suggesting that \textit{PGM} is not primarily involved in nighttime starch synthesis in guard cells. Upon illumination, starch was degraded in all three genotypes, however, with a markedly lower pace in the silencing lines (WT\textsubscript{0.1}, -0.59; WT\textsubscript{1.2}, -0.53; amiR-\textit{PGM} \#1\textsubscript{0.1}, -0.57; amiR-\textit{PGM} \#1\textsubscript{1.2}, -0.15; amiR-\textit{PGM} \#2\textsubscript{0.1}, -0.44; amiR-\textit{PGM} \#2\textsubscript{1.2}, -0.30; Table S5) resulting in considerably higher amounts of starch after 2 h (Fig. 4b and Fig. 4c). Between 2 and 9 h, wild-type guard cells constantly accumulated starch, while starch contents did not increase in guard cells of the amiR-\textit{PGM} \#2 line and only mildly in the amiR-\textit{PGM} \#1 line (Fig. 4b, Fig. 4c and Table S5). Hence, guard cells seem to use \textit{PGM}-provided G1P for starch synthesis throughout the majority of the day. As demonstrated in Fig. 4a, the silencing of \textit{PGM} was not complete and therefore we cannot exclude that the remaining \textit{PGM} transcripts and the resulting proteins are responsible for the observed starch accumulation in the silencing lines. The particular patterns of guard cell starch loss and formation in the two transgenic lines, which differ from that of wild type (Fig. 4b and Fig. 4c), however, point towards distinct impairments in starch biosynthesis resulting from the silencing of \textit{PGM}. If the grade of downregulation would affect guard cell starch dynamics, we would expect to see a constitutive reduction of guard cell starch contents in these lines and not the observed specific lack of starch accumulation between 2 and 9 h.

Given that starch formation is impaired specifically around midday in the \textit{PGM} silencing lines, we also analyzed the expression of \textit{PGM} at this time of the day specifically in guard cells. We observed a \textasciitilde{} 3.5-fold upregulation of \textit{PGM} at 6 h compared to 3 h into the day (Fig. 4d and Table S1) further supporting the guard cell starch data. During this time, starch contents even declined in the case of the amiR-\textit{PGM} \#2 line (Fig. 4c and Table S5). Strikingly, after 9 h, starch synthesis rates were massively increased in both silencing lines (WT\textsubscript{9-12}, 0.27; amiR-\textit{PGM} \# 1\textsubscript{9-12}, 1.44; amiR-\textit{PGM} \# 2\textsubscript{9-12}, 1.68; Table S5) and at EoD, starch levels were essentially the same as in wild-type guard cells (Fig. 4b and Fig. 4c).

In summary, the stomatal starch measurements in the guard cell-specific silencing lines against \textit{PGM} suggests that, unlike mesophyll cells, guard cells may be able to utilize G1P derived both from the PGM-mediated conversion of G6P and imported from the cytosol. Guard cells may import G1P from the cytosol into the
chloroplasts, add G1P directly to growing starch granules via PHS1 or import ADP-glucose to circumvent the PGM-catalyzed reaction.

Other sources of G1P in guard cells

G1P transport was previously observed into Arabidopsis mesophyll cell protoplasts as well as isolated chloroplasts and G1P was rapidly incorporated into starch (Fettke et al., 2011). In a recent follow-up study, the same authors, identified two genes encoding UDP-rhamnose/UDP-galactose transporters, which are able to transport G1P; At1g34020 (herein called G1PT1) and At4g09810 (herein called G1PT2). Arabidopsis double mutant plants showed reduced transport of G1P along with mild alterations in leaf starch and sugar metabolism (Malinova et al., 2019).

The fact that starch granules appeared in the gpt1gpt2pgi triple mutants after 6 h of light exposure (Fig. 3c and Fig. 3d) and that amiR-PGM lines contained substantial amounts of starch both at the EoN and EoD as well as low levels throughout the entire day (Fig. 4b and Fig. 4c), suggests that G1P derives from several sources in guard cells and not exclusively from the PGM-catalyzed conversion of G6P within the chloroplast. G1P could be generated in the cytosol through the cytosolic isoforms of PGM (PGM2 and PGM3; Egli et al., 2010) and subsequently get translocated across the chloroplast membrane via the two putative G1P translocators (G1PTs).

To assess a potential activity of G1PTs in guard cells, we investigated their guard cell gene expression. As we were specifically interested in determining if these transporters play a complementary role to PGM, we compared their transcript levels to those of PGM in wild-type guard cell-enriched epidermal peels harvested throughout the 12 h light period (Fig. 4e and Table S1). Strikingly, we found both of the putative G1P transporters to be expressed at higher levels at all time points compared to PGM (Fig. 4e and Table S1). Interestingly, the expression of G1PT1 and G1PT2 was ~ 10-fold respectively ~ 8-fold higher compared to PGM at EoN, coinciding with the elevated amounts of guard cell starch in the PGM silencing lines (Fig. 4b and Fig. 4c). Notably, after 6h, G1PT1 gene expression was highly induced relative to PGM from ~ 16-fold at 6 h up to ~ 43-fold at the EoD (Fig. 4e and Table S1). This again matches with the striking formation of starch between 9 h and 12 h of light in the two transgenic lines (Fig. 4b and Fig. 4c). Compared to G1PT1, the expression of G1PT2 relative to PGM was not as pronounced but followed a similar pattern of upregulation and was ~ 14-fold induced at EoD (Fig. 4e and Table S1). Therefore, the two recently reported G1P transporters represent interesting candidates for future research on uptake of cytosolic G1P into guard cell chloroplasts for starch synthesis.
Besides the cytosolic formation of G1P and the succeeding uptake into the chloroplast, heterotrophic cells might form starch from the direct transfer of G1P onto growing starch granules with the help of PHS1. This pathway was shown to operate in heterotrophic storage tissues of potato, rice and wheat (Satoh et al., 2008; Tickle et al., 2009; Fettke et al., 2010). Usually PHS1 catalyzes the reversible phosphorolytic cleavage of $\alpha$-1,4-glycosidic bonds in leaf tissues (Zeeman et al., 2004). To assess whether PHS1 plays a role in guard cell starch biosynthesis, we firstly determined its gene expression in guard cell-enriched epidermal peels relative to intact rosette leaves of wild type (Fig. S4a and Table S1). We found that PHS1 transcripts were ~ 4-fold enriched in guard cells compared to leaves (Fig. S4a and Table S1). Interestingly, this had no profound effect on guard cell starch dynamics during the day in T-DNA phs1 single mutants (GABI_257A06; Fig. S4b and Table S6). Hence, we exclude that daytime guard cell starch synthesis involves the action of PHS1. Given its high guard cell gene expression it might fulfill other roles than the elongation of glucan chains.

Subunits of the AGPase in guard cells

The AGPase, responsible for the conversion of G1P into ADPglucose (Stitt & Zeeman, 2012), represents the main level of regulation of starch synthesis in mesophyll cells. The heterotetrameric enzyme is composed of two small subunits (APS1-2) and two large subunits (APL1-4). The leaf isoform is composed of two catalytic APS1 subunits. Furthermore, transcript and proteomic analyses revealed that the leaf AGPase consists of two regulatory APL1 proteins. In contrast, APL3 and APL4 are preferentially expressed in sink tissues and APL2 is considered to be generally expressed at neglectable levels (Crevillén et al., 2003, 2005). It is therefore likely that the catalytic activity of the AGPase depends on the composition of the regulatory large subunits and thus defines its role in source and sink tissues. The composition of the guard cell AGPase subunits is unknown.

In order to gain insights into the AGPase subunit organization in guard cells, we firstly determined the gene expression of the APL genes in wild-type guard cell-enriched epidermal peels relative to intact rosette leaves at EoN (Fig. 5a and Table S1). As previously reported, APL1 was preferentially expressed in leaf tissues similarly to the leaf marker gene $\beta$-amylase 3 (BAM3; Fig. 5a and Table S1; Crevillén et al., 2003). APL3 and APL4 on the other hand were both highly expressed in guard cells compared to leaves, with APL4 showing a more pronounced preferential guard cell expression compared to APL3 (Fig. 5a and Table S1).
Fig. 5 Guard cell gene expression of APLs and guard cell starch contents in apl mutants.

(a) APL1, APL3 and APL4 gene expression in wild-type guard cell-enriched epidermal peels relative to wild-type intact rosette leaves at EoN. KAT1 and MYB60 were used as markers for guard cell-specific expression, while BAM3 was used as a leaf-specific marker. Data from two experiments are shown; means ± fold change range; n = 6. ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. Letters (*) indicate significant statistical difference between genes for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test. Starch dynamics in guard cells of intact leaves of (b) wild type and apl1, apl4, (c) Was and apl3, (d) wild type and apl3apl4 plants. Data from four experiments are shown; means ± SEM; n = 160 individual guard cells per genotype and time point. Plants were illuminated with 150 µmol m\(^{-2}\) s\(^{-1}\) of white light. EoN = end of night. Wild-type data are the same as in Fig. 5b. Letters (*) indicate significant statistical difference between genotypes for the given time point for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test (b-d) WT = wild type.

Based on the promising qRT-PCR results, we proceeded to quantify stomatal starch contents in the EMS mutant apl1 (Lin et al., 1988) and the T-DNA insertion mutants apl3 (Wasiljevskaja = Was background; FLAG_458A07) and apl4 (SALK_108632) during the 12 h light period. Guard cell starch accumulation and degradation were highly similar between wild-type and the two apl1 and apl4 single mutants (Fig. 5b and Table S6). In contrast, starch contents were constitutively elevated in apl3 guard cells compared to its Was wild-type control (Fig. 5c), indicating deregulation of the AGPase enzyme in the absence of APL3. Furthermore, the wild-type and the apl3 mutant in the Was background contained elevated overall levels of
both guard cell starch (Fig. 5c) and leaf starch (Fig. S5) compared to wild-type of Columbia (Col-0) background. Starch contents in the leaves of apl3 single mutants were further elevated compared to Was control plants, supporting the idea of a deregulated enzyme (Fig. S5).

To exclude a functional overlap between the APL3 and APL4 subunits in guard cells, we generated the apl3apl4 double mutant through initial backcrossing of the apl3 mutation into Col-0 wild-type. The combined lack of APL3 and APL4 resulted in reduced overall guard cell starch amounts for the majority of the 12 h light period (Fig. 5d and Table S6). Wild-type and apl3apl4 guard cells contained comparable amounts of starch at EoN (Fig. 5d and Table S6), followed by identical loss of starch upon light exposure (Fig. 5d and Table S6). However, while wild-type guard cells gradually increased their starch contents from 1 h into the day on, the double mutant guard cells displayed a net decrease of starch in between 1 and 2 h (Fig. 5d; WT: 1-2, 0.30; apl3apl4: 1-2, -0.74; Table S7). Thereafter, starch contents remained at a lower level until the EoD in the mutant compared to wild-type guard cells (Fig. 5d and Table S6).

Thus, we conclude that the subunit composition of the AGPase is critical for guard cell starch synthesis. Furthermore, APL3 and APL4 seem to have partially redundant function in guard cell starch accumulation.

Brittle1 is not required for guard cell starch accumulation

Amyloplasts of maize endosperm were demonstrated to import extraplastidial synthesized ADPglucose (Endosperms et al., 1998), via the plastidic ADPglucose transporter BT1 (Kirchberger et al., 2007). Similar observations were made for rice endosperm (Li et al., 2017). The Arabidopsis genome encodes a BT1 gene (Brittle1), which is structurally similar to the one from maize. Brittle1 is localized to the plastidial membrane and was described to mediate AMP, ADP and ATP transport across the chloroplast membrane. In vitro experiments have further supported the idea that Brittle1 does not accept ADPglucose as a substrate (Kirchberger et al., 2008). However, we sought to investigate whether Brittle1 contributes to guard cell starch synthesis by translocating ADPglucose into the chloroplasts, potentially providing a precursor for starch synthesis at 6 to 9 h into the day.

We found Brittle1 gene expression to be almost 4-fold higher in wild-type guard cells relative to intact leaves (Fig. S6a and Table S1). However, when comparing guard cell starch contents of the T-DNA brittle1 single mutant (SALK_026943) with wild type, we did not detect differences throughout the 12 h light phase (Fig. S6b), except for the time point at 12 h, where the mutant guard cells contained significantly
less starch (Fig. S6b). Therefore, the starch dynamics in guard cells in brittle1 mutants led us to conclude that Brittle1 is not required for stomatal starch accumulation and might have a different function that importing ADPglucose. For example, it may work as a nucleotide phosphate transporter, as it was described for other plant tissues (Kirchberger et al., 2008).
DISCUSSION

Earlier this year, we reported that the accumulation of starch is limited in isolated Arabidopsis guard cells compared to guard cells of intact leaves. Starch contents increased by 51% in isolated guard cells during 6 h of red light illumination, whereas starch amounts in guard cells of intact leaves reached 206% of the initial value. We therefore concluded that autonomous CO$_2$ fixation in guard cells provides only a limited amount of carbon skeletons for starch biosynthesis (Flütsch et al., 2020a). Subsequently, we revealed that import of mesophyll-derived Glc via the monosaccharide-H$^+$ symporters Sugar Transport Proteins 1 and 4 (STPs) is essential for guard cell starch formation at the beginning of the day (Flütsch et al., 2020b). However, which enzymes are involved in guard cell starch biosynthesis and their relative impact on stomatal function remained uninvestigated.

The first step of starch synthesis – provision of G6P

*PGI is required for guard cell starch formation*

Previous reports suggested that the plastidial isoenzyme of PGI is essential for starch synthesis in photoautotrophic plant tissues, whereas it is not required in heterotrophic tissues (Yu et al., 2000; Kunz et al., 2010). However, here we demonstrate that guard cells of *pgi* single mutant plants contained starch, but in altered amounts compared to wild type, suggesting a deregulation of guard cell starch biosynthesis in the absence of PGI. Starch accumulation was specifically impaired during the early phase of guard cell starch synthesis (e.g. 2-3 h of light; Fig. 1a and Table S2). Hence, plastidial PGI is involved in guard cell starch accumulation and our data further support the idea that the guard cell Calvin cycle is functional and delivers at least some F6P for starch synthesis. Interestingly, we found that guard cell starch levels were significantly elevated at night, EoN and EoD in *pgi* mutants (Fig. 1a and Table S2), suggesting that guard cells use additional other carbon sources for starch biosynthesis, which are not derived from the PGI-catalyzed conversion of F6P. The guard cell starch phenotype of *pgi* coincides with the proposed dominant role of Glc import for guard cell starch accumulation (Flütsch et al., 2020b). In such a scenario, Glc imported from the apoplastic space would get phosphorylated through cytosolic hexokinases (HXKs) and would then get translocated across the plastidial envelope.
**GPT1 contributes significantly to guard cell starch accumulation**

Indeed, several authors have reported that the reaction via PGI can be bypassed through G6P import into the chloroplasts (Kammerer et al., 1998; Niewiadomski et al., 2005; Kunz et al., 2010). The Arabidopsis genome encodes six plastidial Phosphate Translocators (pPTs), of which two genes **GPT1** and **GPT2** are G6P/phosphate antiporters (Kammerer et al., 1998; Niewiadomski et al., 2005). The proposed role of the GPTs in heterotrophic tissues is the delivery of G6P to chloroplasts for incorporation into starch or to feed the oxidative pentose phosphate pathway (OPPP) (Niewiadomski et al., 2005; Kunz et al., 2010). Ectopic overexpression of either of the GPTs was reported to rescue the starch deficiency phenotype of **pgi** in mesophyll cells (Niewiadomski et al., 2005).

Here, we confirmed the previously observed high levels of **GPT1** guard cell transcripts (Fig. 2a; Niewiadomski et al., 2005). We show that the loss of **GPT1** severely impacted on daily guard cell starch accumulation, while nighttime starch levels were unaffected by this mutation (Fig. 2b and Table S2), suggesting that during the day guard cells take up G6P into chloroplasts and this import is mediated by **GPT1**. Strikingly, starch accumulation was strongly affected at different times of the day in **pgi** (e.g. 2 – 6h) and **gpt1** (e.g. 3 – 12 h) single mutants (Fig. 1a, Fig. 2b and Table S2). Moreover, the **gpt1pgi** double mutant guard cells displayed specifically reduced starch accumulation between 2 and 12 h (Fig. 3a and Table S2), suggesting that PGI and **GPT1** play complementary roles in guard cells. These findings imply that the two genes are regulated differently. We provide evidence for transcriptional regulation of **GPT1** and **PGI** with distinct temporal patterns (Fig. 2c and Table S1). Whereas **GPT1** transcripts were almost 2-fold enriched at 6 h compared to 3 h in wild-type guard cells, **PGI** transcript amounts did not increase towards 6 h (Fig. 2c and Table S1). Different factors might affect their gene expression, such as signals from light receptors, redox state of cellular compartments and the concentration of metabolites, predominantly sugars (Häusler et al., 2014). Finally, the elevated levels of starch observed during the entire night in **pgi** mutants (Fig. 1a and Table S2), were no longer detectable in **gpt1pgi** double mutants (Fig. 3a and Table S2). Hence, our data suggest that nighttime starch overaccumulation in **pgi** guard cells resulted from **GPT1** activity.

**GPT2 plays a minor role in guard cells**

The remaining high amounts of stomatal starch in **gpt1pgi** double mutants prompted us to investigate the function of **GPT2** in guard cells. Kunz et al. (2010) reported that **GPT2** was transcriptionally upregulated if starch metabolism was disrupted as in
mutants affected in PGI, PGM and the AGPase (Kunz et al., 2010). However, in the case of $gpt1pgi$ double mutant guard cells, we could not detect transcriptional induction of $GPT2$ (Fig. S2 and Table S1), which might have been related to the sampling time point at the EoN. $GPT2$ was described to be repressed in the dark (Kunz et al., 2010).

Whereas $GPT1$ gene expression was previously detected in stomatal guard cells (Niewiadomski et al., 2005), GPT2 was not yet investigated in this cell type. Here, we show that $GPT2$ was highly expressed in guard cells relative to leaves. Its preferential expression in guard cells was even more pronounced than the one of $GPT1$ (Fig. 2a and Table S1). Surprisingly, we did not observe large differences in starch dynamics among wild-type and the $gpt2$ single mutants throughout 24 h (Fig. 2b and Table S2). A possible explanation for the lack of a stomatal starch phenotype in $gpt2$ plants would be the functional compensation through either PGI or more likely the closely related $GPT1$. Indeed, leaf starch quantification in $gpt2pgi$ double mutants revealed that these plants were not devoid of starch but contained only slightly less compared to $pgi$ single mutants. Consequently, the authors concluded that $GPT1$ was responsible for the remaining starch formation (Kunz et al., 2010). Following this line of evidence, we examined starch amounts in guard cells of $gpt1gpt2$ double mutants during the light phase to test potential functional redundancy between $GPT1$ and $GPT2$ in guard cells (Fig. 2d and Table S3). The double mutant phenocopied $gpt1$ single mutants in terms of its guard cell starch levels and did not result in a more severe impairment of starch synthesis (Fig. 2d and Table S3), suggesting that $GPT1$ and $GPT2$ do not act redundantly in guard cells. Therefore, it seems likely that $GPT2$ gets active specifically upon the lack of either PGI or GPT1 to compensate for their loss, although our gene expression analyses do not support such hypothesis (Fig. 1c and Fig. S2). It is generally accepted that the response at the mRNA level does not necessarily reflect the response at the protein level or the level of enzyme activity. Furthermore, recent work revealed that the GPT isoforms strongly diverge at their N-termini, while their central protein regions responsible for substrate binding and specificity are highly conserved (Baune et al., 2020). Hence, posttranslational modifications likely play a role in specifying the activities of $GPT1$ and $GPT2$. Finally, the mild guard cell starch phenotype of $gpt1gpt2$ indicates that either PGI compensated for the loss of both GPTs in the double mutant or that guard cells used other carbon sources than G6P for starch accumulation.
Triple gpt1gpt2pgi mutants are not devoid of guard cell starch

In Fig. 3, we provide evidence for a PGI functional compensation in gpt1gpt2 mutants. The loss of GPT1 and GPT2 in combination with the guard cell-specific downregulation of PGI led to almost undetectable starch contents at EoN (Fig. 3c and Fig. 3d, Table S4). Guard cell starch was near zero in the two amiR-PI lines until 6 h into the light, demonstrating that during this period of the day, guard cell starch is made exclusively from G6P. G6P could derive from F6P interconversion through PGI or G6P uptake into the chloroplasts via GPT1 and GPT2.

Interestingly, the absence of guard cell starch at EoN in the triple mutants revealed that starch accumulation during the night depends on the action of PGI and the two GPTs. However, it is not trivial to imagine how PGI would be involved in nighttime starch formation, as F6P will not be delivered from light-dependent CO₂ assimilation. PGI, as well as GPT1, have both been implicated in the regeneration of G6P molecules in the OPPP in heterotrophic organs and non-illuminated tissues (Niewiadomski et al., 2005; Bahaji et al., 2015; Baune et al., 2020). The OPPP generates precursors for nucleic acids, fatty acids and phenolic compounds as well as reducing equivalents in the form of NADPH (Bahaji et al., 2015). The produced NADPH could be employed by the plastidial NADPH-dependent thioredoxin reductase C (NTRC), which was previously implicated in sugar metabolism in the dark, to reduce and activate the AGPase (Michalska et al., 2009; Lepistö et al., 2013; Daloso et al., 2017). In non-photosynthetic root tissues, loss of the plastidial NTRC resulted in decreased redox activation of the AGPase and starch synthesis in the dark after treatment with Suc (Michalska et al., 2009). Additionally, yeast two hybrid analysis revealed the direct interaction of the NTRC and AGPase enzymes (Lepistö et al., 2013). Hence, future research should address if this proposed route of nighttime starch synthesis involving PGI in a indirect way through its participation in the OPPP as well as the GPTs in supplying mesophyll-derived carbons to the guard cells as precursors for both starch synthesis and the OPPP contribute to nighttime starch accumulation in guard cells. Monitoring nightly guard cell starch amounts in ntrc knockouts should help to address this question. Furthermore, one should keep in mind that the complete lack of starch observed in the triple mutant during the first half of the day could also partially be a consequence of the involvement of PGI and the GPTs in the OPPP-mediated generation of reducing equivalents.

The accumulation of starch in both PGI silencing lines between 6 h and 12 h of light (Fig. 3c and Fig. 3d, Table S4), indicates that G6P can derive from yet another source in guard cells or the reactions involving G6P are circumvented. Another path of G6P production could be the incorporation of cytosolic Glc via the plastid-localized
glucose transporter (pGlcT) and subsequent phosphorylation by the plastidial HXK3 (Weber et al., 2000; Karve et al., 2008). However, pGlcT has been shown to rather function in the export of Glc at night than the uptake of cytosolic Glc (Weber et al., 2000; Cho et al., 2011) and pglct mutants accumulated wild type-like levels of starch in their leaves (Cho et al., 2011). Thus, it seems unlikely that guard cells make starch following the chloroplastic import of Glc, but to exclude this route of G6P provision, additional research would be needed.

The second step of starch synthesis – provision of G1P

There are at least two sources of G1P in guard cells

G1P can derive from the PGM-catalyzed conversion of G6P or it might be taken up into the chloroplasts to bypass the PGM reaction. In this report, we shed light on the role of PGM in guard cells. In contrast to previous studies, we used guard cell-specific silencing lines against PGM to investigate its function, whereas earlier experiments were done on the pgm null-mutants (Lasceve et al., 1997; Horrer et al., 2016). pgm mutants are compromised at the whole-plant level, suffering from severely reduced growth and disturbed carbohydrate metabolism (Caspar et al., 1985). It is therefore likely that the lack of starch and the resulting diurnal overaccumulation of sugars in the leaves of pgm directly affected stomatal function. By employing two independent silencing lines with 60% reductions of PGM guard cell transcripts, we demonstrate that PGM is not strictly essential for guard cell starch synthesis and that guard cells possess other sources of G1P (Fig. 3b and Fig. 3c, Table S5). Our data show that guard cells relied on PGM-derived G1P for starch formation during the majority of the day, however, at the EoN and EoD, PGM silencing lines contained higher or equal amounts of starch compared to wild-type guard cells (Fig. 3b and Fig. 3c, Table S5).

Almost ten years ago it was demonstrated that both mesophyll cell protoplasts and isolated chloroplast have the capacity to import G1P and metabolize it into starch (Fettke et al., 2011). Malinova et al. (2019) identified two UDP-rhamnose/UDP-galactose transporters, At1g34020 and At4g09810, which are able to transport G1P (Rautengarten et al., 2011; Malinova et al., 2019). Using transient expression in tobacco mesophyll protoplasts it was revealed that both transporters localize to the plasma membrane (Malinova et al., 2019). However, earlier reports along with the examination of N-terminal targeting peptides suggest that G1PT2 (At4g09810) is targeted to the plastid, whereas for G1PT1 (At1g34020) no such peptide was identified (Knappe et al., 2003; ChloroP 1.1: http://www.cbs.dtu.dk/services/ChloroP/). Interestingly, in Fig. S1C of Malinova et al., (2019), showing the subcellular localization
in transiently transformed protoplasts, a clear chloroplast signal is visible for G1PT1 (At1g34020). Therefore, the localization of these two transporters is not yet fully resolved and has to be addressed in future research.

Here, we show that both G1PTs were highly and at opposing times relative to PGM expressed in guard cells of wild-type (Fig. 4e). As their expression level was 43- (G1PT1) and 14-fold (G1PT2) elevated compared to that of PGM, we speculate that G1PTs may play a dominant role towards the EoD. Hence, examining guard cell starch contents in knockout mutants of G1PT1 (At1g34020) and G1PT2 (At4g09810) will certainly help to decipher their role in guard cell starch biosynthesis.

The third step of starch synthesis – provision of ADPglucose

AGPase subunit composition in guard cells differs from that of mesophyll cells

The AGPase catalyzes the third and limiting step of starch synthesis and is regulated allosterically by the levels of 3-PGA and inorganic phosphate in photosynthetic tissues, such as the spongy mesophyll (Preiss, 1988). The AGPase is also subject to redox regulation (Hendriks et al., 2003). Previous biochemical experiments have shown that the activity of the heterotetrametric enzyme depends on the combination of the small catalytic subunit APS1 with the large subunits APL1-APL4. Studies on the Arabidopsis leaf AGPase subunits, revealed that the APS1/APL1 heterotetramer shows the highest sensitivity towards the allosteric effectors, while heterotetramers containing APS1 in combination with APL2-APL4 only respond to dramatic changes in effector concentrations. The large subunits also influence the substrate affinity of the AGPase with APL1 showing the highest affinity towards ATP and G1P (Crevillén et al., 2003). In line with these findings, APL1 gene expression was shown to be the highest in leaves, whereas APL3 reaches its highest levels in sink organs such as inflorescences, fruits and roots (Crevillén et al., 2005). Interestingly, microarray analyses indicated that APL4 is the most abundant AGPase large subunit in guard cells of Arabidopsis (Leonhardt et al., 2004). Sugars can transcriptionally induces both APL3 and APL4, but not APL1 and APL2 and therefore the activity of the AGPase in sink tissues could be responding to sugar availability (Crevillén et al., 2005). Nevertheless, a functional characterization of the APL subunits in guard cells is lacking.

By investigating guard cell gene expression, we corroborate earlier results and show that both APL3 and, to a higher extent, APL4 were preferentially expressed in guard cells relative to leaves, whereas APL1 transcripts were highly abundant in leaf tissues (Fig. 5a). However, guard cell starch quantifications did not reveal differences
between wild type and the single mutant *apl4* (Fig. 5b and Table S6). As we show in Fig. 5c, the lack of APL3 interestingly caused a deregulation of starch synthesis, not only in guard cells (Fig. 5c and Table S6), but also in mesophyll cells (Fig. S4). The increased accumulation of starch in both cell types might have resulted from overexpression of either of the remaining APL subunits, leading to functional complementation. We therefore generated *apl3apl4* double mutants to investigate putative overlapping functions in guard cells. Stomatal starch levels were reduced in *apl3apl4* plants throughout the 12 h light phase, suggesting a role for these subunits in guard cells (Fig. 5d and Table S6). However, also in the case of the double mutant, APL1 or APL2 might partially complement the starch phenotype resulting in relatively high starch levels in *apl3apl4* guard cells. It would be worth to test the expression of APL1 and APL2 in *apl3apl4* guard cells in future experimental work. In addition, it would be interesting to gain insights into the sugar regulation of the guard cell AGPase, possibly by employing the *apl3apl4* double mutant.

*The lack of Brittle1 has no consequences for guard cell starch levels*

Unaffected levels of ADPglucose in mutants of PGM and AGPase prompted to rethink the classical model of starch biosynthesis (Muñoz et al., 2005). Several reports have suggested that ADPglucose is generated in the cytosol through the activity of Sucrose Synthases (SUS) and subsequently translocated across the chloroplast membrane (Baroja-Fernández et al., 2003; Muñoz et al., 2005; Muñoz et al., 2006). Interestingly, transcriptomic studies revealed high abundance of SUS3 in guard cells (Bates et al., 2012). Moreover, in maize endosperm, ADPglucose is imported into plastids via Brittle1 (Kirchberger et al., 2007) and Arabidopsis genome contains a highly conserved Brittle1 protein (Kirchberger et al., 2008). Nevertheless, the same authors could not detect ADPglucose transport activity for Brittle1, but they found that it can only exchange ATP, ADP and AMP (Kirchberger et al., 2008). Here, we provide additional support for the lack of ADPglucose transport activity of Brittle1, as we did not observe alterations in starch dynamics between *brittle1* and wild-type guard cells (Fig. S6a). Interestingly, the gene expression of Brittle1 was approximately 4-fold higher in guard cells relative to leaves (Fig. S6b and Table S1). We therefore suggest that Brittle1 might fulfill other functions in guard cells, such as mediate nucleotide transport. Finally, our data on the *brittle1* mutant cannot exclude ADPglucose transport across the guard cell plastid envelope by another transporter.

In conclusion, we have characterized the Arabidopsis enzymes annotated to the classical pathway of starch synthesis found in photosynthetic leaf tissues, as well
as enzymes (or Arabidopsis homologs), which are known to be important players in transitory starch biosynthesis in heterotrophic organs or in storage starch formation. We have found that both enzymes of the plastidic PGI-PGM-AGPase pathway and sugar transporters, especially GPT1, at the chloroplast membrane contribute to guard cell starch accumulation during day and night. We shed new light on the contribution of guard cell autonomous CO\textsubscript{2} fixation to starch accumulation in this cell type. However, our new evidence further strengthens the role of mesophyll-derived sugars for guard cell starch biosynthesis. Starch synthesis in guard cells and the involved enzymes appear to be a highly regulated and it seems impossible to generate plants lacking starch throughout the diurnal 24 h light/dark cycle. Hence, guard cell starch synthesis possibly employs substrates from many different origins. This is likely even more pronounced in backgrounds where a specific gene or several genes are missing. The mutation of one gene might cause cellular-wide effects and pave the way for the activation of other enzymes, which are usually not employed in a wild-type background, as it might be the case for GPT2.
Plant material and growth conditions
All experiments were performed with non-flowering, four-week-old Arabidopsis (Arabidopsis thaliana) plants in the accession Columbia (Col-0 = WT) background. The transfer DNA (T-DNA) insertion lines SALK_021762 (gpt1), SALK_026943 (brittle1), SALK_019405 (sus3), GABIKAT_454H06 (gpt2) and GABI_257A06 (phs1) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). SALK_108632 (apl4-3) line was provided by Samuel Zeeman (ETH Zürich, CH). Alison Smith (John Innes Centre, UK) provided the FLAG_458A07 (apl3-1) in the accession Was. The EMS mutants apl1 (adv2; Lin et al., 1988), pgm1-1 (Caspar et al., 1985) and pgi1-1 (Yu et al., 2000) were described previously.

The apl3-1apl4-3 double mutant was created firstly by backcrossing the apl3-1 mutant into the Col-0 WT in order to eliminate the Was background. Then, crosses using either the Col-0 WT or the backcrossed apl3-1 mutant as the pollen donor always yielded heterozygous plants in the expected ratio. In each generation heterozygous apl3-1 mutant plants were selected by genotyping using the primers listed in Table S1. Heterozygous apl3-1 mutants of the fourth generation were crossed with homozygous apl4-3 plants and double homozygous mutants selected by molecular genotyping using primers combination as listed in Table S1.

The gpt1pgi1-1 and gpt1gpt2 double mutant plants were generated through standard crossing techniques and isolated by molecular genotyping (for primer sequences see Table S1). Genotyping of the pgi1-1 point mutation was done by sequencing of the PCR product obtained with the primers listed in Table S1. 1 µl of purified genomic DNA was used in a PCR reaction, followed by column purification using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). 2 µl of the purified PCR reaction (around 100 ng) were used for sequencing. Sequencing chromatograms were analysed for the single C to T substitution at base 834 (Fig. S1; Yu et al., 2000).

Plants were cultivated in soil in controlled-climate chambers (either Fitoclima 1200, Aralab; ClimeCab 1400, Kälte3000; Klimaschrank from Kälte3000) under a 12-h/12-h light/dark photoperiod, with a temperature of 21°C/19°C day/night, a relative humidity of 45%/55% day/night and an irradiance of 150 µmol m⁻² s⁻¹ using LED tubes (Fitoclima 1200), LED panels (ClimeCab 1400) and halogen lamps (Klimaschrank).
Guard cell specific gene silencing

For the guard cell specific gene silencing of PGI and PGM, sequences of pre-miRNAs were designed using the Web MicroRNA Designer tool (WMD3; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The primer set listed in Table S1 was used to incorporate the 21-bp amiRNA sequence into the MIR319a vector (Schwab et al., 2010). Subsequently, the amiRNA construct was subcloned into BJ36 (Moore et al., 1998) containing the guard cell-specific promoter KST1 (Kelly et al., 2013). The resulting pSF10 (amiR-PGM) and pSF21 (amiR-PGI) constructs were transformed into Arabidopsis WT (pSF10) and gpt1gpt2 (pSF21) backgrounds followed by selection of stable, independent lines.

Guard cell starch quantification

Guard cell starch was quantified at the indicated time points. Epidermal peels were manually obtained from leaf number 5 or 6. Guard cell starch granules were fixed and stained as previously described in (Flütsch et al., 2018). Subsequently, guard cell starch granules were visualized and imaged using a confocal laser-scanning microscope Leica TCS SP5 (Leica Microsystems) or Zeiss LSM 780 (Zeiss) and their area was measured using ImageJ version 1.48 (NIH USA, http://rsbweb.nih.gov/ij/). Four biological replicates were analyzed per genotype and time point for one experiment.

Leaf and guard cell RNA isolation and qPCR

To extract leaf RNA, three entire rosettes per genotype and time point (three biological replicates) were harvested at the indicated time points and frozen in liquid nitrogen.

To extract RNA from guard cell-enriched epidermal peels, the middle veins of 12 rosettes per genotype and time point (one biological replicate) were excised at the indicated time points and the remaining leaf material was blended in 100 ml ice-cold water using a kitchen blender (ProBlend Avance collection, Philips). The blended sample was filtered through a 200 µm nylon mesh (Sefar), and the remaining epidermal peels were dried, collected in a tube, and immediately frozen in liquid nitrogen.

Subsequently, the epidermal peels were ground to a fine powder using a tissue grinder (Mix Mill MM-301, Retsch). For one experiment two or three biological replicates per genotype and time point were harvested. Two independent experiments were performed for each extraction (leaves and guard cell-enriched epidermal peels).

Total RNA was extracted from ≥ 30 mg of ground tissue using the SV Total RNA Isolation Kit (Promega) following the manufacturer’s instructions. RNA quality
and quantity were analyzed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A total of 1 µg of RNA was used for cDNA first-strand synthesis using the M-MLV Reverse Transcriptase RNase H Minus Point Mutant and oligo(dT)15 primer (Promega). Transcript levels were examined by RT-qPCR using the SYBR Green Master Mix (Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems). RT-qPCR was performed in triplicates. Transcript levels were calculated according to the comparative CT method (Livak & Schmittgen, 2001) and were normalized against the expression of the Actin2 gene (ACT2; At3g18780). Error calculations were done according to Applied Biosystems guidelines (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/general/documents/cms_042380.pdf). Primers and PCR efficiencies for RT-qPCR are listed in Table S1.

**Statistical Analysis**

Statistical differences between genotypes and time points were determined by ANOVA with post hoc Tukey’s Honest Significant Difference test ($P$-value < 0.05). All data are indicated as means ± SEM.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At3g23920 (BAM1), At3g18780 (ACT2), At5g46240 (KAT1), At1g08810 (MYB60), At5g54800 (GPT1), At1t61800 (GPT2), At4g24620 (PG1), At5t51820 (PGM), At3t29320 (PHS1), At4g39210 (APL1), At4g39210 (APL3), At2g21590 (APL4), At4g32400 (BRITTLE1), At1g34020 (G1PT1), At4g09810 (G1PT2).

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REFERENCES


**SUPPORTING INFORMATION**

**Fig. S1** Genotyping of *pgi* and *gpt1pgi* mutants.

Representative sequencing chromatograms of PCR amplicons around the base at position 834 (for primers see Table S1). *Pgi* mutants have a point mutation from C to T at position 834, which is labelled in red in the complementary strands visualized in the chromatograms. (a) wild type, (b) *pgi* and (c) *gpt1pgi*. 
Fig. S2 Gene expression of GPT2 in gpt1pgi mutants.

GPT2 gene expression in guard cell-enriched epidermal peels of wild-type and gpt1pgi plants at the end of night. Data from two independent experiments are shown; means ± fold change range; n = 6. ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. No statistical differences were detected among genotypes.

Fig. S3 Growth retardation of pgi mutants.

Images of 4-week-old wild-type and pgi plants grown under a 12 h light/12 h dark photoperiod.
Fig. S4 Guard cell gene expression of PHS1 and starch contents in phs1 mutants.

(a) PHS1 gene expression in wild-type guard cell-enriched epidermal peels relative to wild-type intact rosette leaves at EoN. KAT1 and MYB60 were used as markers for guard cell-specific expression, while BAM3 was used as a leaf-specific marker. Data for the marker genes are the same as in Fig. 2a. Data from two experiments are shown; means ± fold change range; n = 6. ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. Letters indicate significant statistical difference between genes for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test.

(b) Starch dynamics in guard cells of intact leaves of WT and phs1 plants over the 12 h light period. Plants were illuminated with 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of white light. Data from four independent experiments are shown; means ± SEM; n = 160 individual guard cells per genotype and time point. EoN = end of night. Wild type data are the same as in Fig. 5b and 5d. Letters indicate significant statistical differences between genotypes for the given time point for \( P < 0.05 \) determined by one-way ANOVA with post hoc Tukey’s test. WT = wild type.
Fig. S5 Leaf starch contents in apl mutants.

Leaf starch amounts in wild-type (Col-0), Was, apl1, apl3 and apl4 mutant plants at the end of the night (EoN) and end of the day (EoD). Data from one experiment are shown; means ± SEM; n = 8. Letters indicate significant statistical difference between genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.
Fig. S6 Guard cell gene expression of BRITTLE1 and starch contents in brittle1 mutants.

(a) BRITTLE1 gene expression in wild-type guard cell-enriched epidermal peels relative to wild-type intact rosette leaves at EoN. KAT1 and MYB60 were used as markers for guard cell-specific expression, while BAM3 was used as a leaf-specific marker. Data from two experiments are shown; means ± fold change range; n = 6. ACT2 was used as a housekeeping gene for normalization. For details about fold change and error calculations refer to Materials and Methods section. Primer sequences and efficiencies are given in Table S1. Letters indicate significant statistical difference between genes for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test.

(b) Starch dynamics in guard cells of intact leaves of WT and brittle1 plants over the 12 h light period. Plants were illuminated with 150 $\mu$mol m$^{-2}$ s$^{-1}$ of white light. Data from four independent experiments are shown; means ± SEM; n = 160 individual guard cells per genotype and time point. EoN = end of night. Wild type data are the same as in Fig. 5b and 5d. Letters indicate significant statistical differences between genotypes for the given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test. WT = wild type.
CHAPTER V

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Table S1. Oligonucleotides used in this study
**Table S2.** Starch synthesis rates of wild-type, *pgi* and *gpt* mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

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<th>12h light - 15h light</th>
<th>15h light - 18h light</th>
<th>18h light - 21h light</th>
<th>21h light - 24h light</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>-0.61</td>
<td>-0.21</td>
<td>1.27</td>
<td>0.65</td>
<td>0.20</td>
<td>0.002</td>
<td>0.09</td>
<td>0.003</td>
<td>0.03</td>
<td>-0.51</td>
</tr>
<tr>
<td><em>pgi</em></td>
<td>-0.56</td>
<td>-0.30</td>
<td>0.13</td>
<td>0.46</td>
<td>1.18</td>
<td>0.45</td>
<td>0.18</td>
<td>-0.08</td>
<td>-0.09</td>
<td>-0.56</td>
</tr>
<tr>
<td><em>gpt1</em></td>
<td>-0.62</td>
<td>0.04</td>
<td>1.00</td>
<td>0.44</td>
<td>0.59</td>
<td>0.08</td>
<td>0.34</td>
<td>0.01</td>
<td>-0.06</td>
<td>-0.62</td>
</tr>
<tr>
<td><em>gpt2</em></td>
<td>-0.62</td>
<td>0.63</td>
<td>-0.35</td>
<td>1.38</td>
<td>0.38</td>
<td>0.05</td>
<td>1.26</td>
<td>0.005</td>
<td>0.006</td>
<td>-0.58</td>
</tr>
<tr>
<td><em>gpt1</em>pgi</td>
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<td>-0.47</td>
<td>-0.11</td>
<td>2.00</td>
<td>0.79</td>
<td>-0.06</td>
<td>0.38</td>
<td>-0.04</td>
<td>0.02</td>
<td>-0.42</td>
</tr>
</tbody>
</table>

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**Table S3.** Starch synthesis rates of wild-type and *gpt1gpt2* guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN - 1h light</th>
<th>1h light - 2h light</th>
<th>2h light - 3h light</th>
<th>3h light - 6h light</th>
<th>6h light - 9h light</th>
<th>9h light - 12h light</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-0.58</td>
<td>-0.24</td>
<td>0.84</td>
<td>1.16</td>
<td>0.61</td>
<td>0.19</td>
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<tr>
<td><em>gpt1gpt2</em></td>
<td>-0.64</td>
<td>-0.05</td>
<td>-0.08</td>
<td>1.53</td>
<td>0.08</td>
<td>0.25</td>
</tr>
</tbody>
</table>

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**Table S4.** Starch synthesis rates of wild-type and *PGI* silencing lines guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN - 1h light</th>
<th>1h light - 2h light</th>
<th>2h light - 3h light</th>
<th>3h light - 6h light</th>
<th>6h light - 9h light</th>
<th>9h light - 12h light</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-0.79</td>
<td>-0.43</td>
<td>1.64</td>
<td>0.96</td>
<td>0.59</td>
<td>0.82</td>
</tr>
<tr>
<td>amiR-PGI/1</td>
<td>-0.81</td>
<td>6.15</td>
<td>-0.35</td>
<td>-0.42</td>
<td>8.28</td>
<td>0.60</td>
</tr>
<tr>
<td>amiR-PGI/2</td>
<td>-0.90</td>
<td>2.07</td>
<td>0.43</td>
<td>0.22</td>
<td>1.62</td>
<td>1.40</td>
</tr>
</tbody>
</table>
Table S5. Starch synthesis rates of wild-type and PGM silencing lines guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN-1h light</th>
<th>1h light-2h light</th>
<th>2h light-3h light</th>
<th>3h light-6h light</th>
<th>6h light-9h light</th>
<th>9h light-12h light</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
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<td>-0.53</td>
<td>2.69</td>
<td>0.68</td>
<td>0.14</td>
<td>0.27</td>
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<tr>
<td>amiR-PGM #1</td>
<td>-0.57</td>
<td>-0.15</td>
<td>0.21</td>
<td>0.13</td>
<td>0.32</td>
<td>1.44</td>
</tr>
<tr>
<td>amiR-PGM #2</td>
<td>-0.44</td>
<td>-0.30</td>
<td>0.18</td>
<td>-0.32</td>
<td>0.43</td>
<td>1.68</td>
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</tbody>
</table>

Table S6. Starch synthesis rates of wild-type, phs1 and apl mutant guard cells. Starch synthesis rates are derived from the slope in between time points. WT = wild type.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EoN-1h light</th>
<th>1h light-2h light</th>
<th>2h light-3h light</th>
<th>3h light-6h light</th>
<th>6h light-9h light</th>
<th>9h light-12h light</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>-0.64</td>
<td>0.30</td>
<td>-0.11</td>
<td>0.67</td>
<td>0.98</td>
<td>0.40</td>
</tr>
<tr>
<td>api1</td>
<td>-0.75</td>
<td>0.61</td>
<td>0.23</td>
<td>0.83</td>
<td>0.47</td>
<td>0.49</td>
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<tr>
<td>api4</td>
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<td>0.07</td>
<td>0.29</td>
<td>0.14</td>
<td>0.95</td>
<td>0.15</td>
</tr>
<tr>
<td>Was</td>
<td>-0.89</td>
<td>1.84</td>
<td>-0.11</td>
<td>1.71</td>
<td>0.15</td>
<td>0.98</td>
</tr>
<tr>
<td>api3</td>
<td>-0.44</td>
<td>0.29</td>
<td>0.02</td>
<td>0.08</td>
<td>0.81</td>
<td>0.21</td>
</tr>
<tr>
<td>api3ap4</td>
<td>-0.70</td>
<td>-0.74</td>
<td>2.96</td>
<td>0.79</td>
<td>1.08</td>
<td>0.37</td>
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<tr>
<td>phs1</td>
<td>-0.34</td>
<td>-0.59</td>
<td>1.37</td>
<td>0.05</td>
<td>0.67</td>
<td>0.66</td>
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</tbody>
</table>

Supplemental References


OUTLOOK

We have shown that guard cell starch synthesis requires a complex network of enzymes known from the classical path of mesophyll cell starch formation as well as several chloroplastic sugar transporters. However, additional experiments are required to strengthen the presented data as well as to elucidate the physiological role of the investigated enzymes.

The experiments presented in Fig. 3b and Fig. 3d (amiR-PGI lines) were only performed once and need further confirmation. Similarly, the starch quantification of the PGM silencing lines would profit from another experimental repetition (so far two experiments).

As we have learned from other chapters of this thesis, guard cell starch metabolism plays a fundamental role in the regulation of stomatal movements. Therefore, the most promising mutants – gpt1, amiR-PGI, amiR-PGM, apl3apl4 and putatively the g1pts – will be further examined for their physiological role using infrared gas analysis, RGB imaging, metabolite quantifications in both leaves and guard cells and finally if suitable, CO₂ complementation experiments as reported in Flütsch et al., (2020b).

Given that GPT1 is ubiquitously expressed in heterotrophic tissues and plays a critical role in embryo development and pollen nutrition (Niewiadomski et al., 2005; Andriotis et al., 2010), we plan to functionally complement GPT1 specifically in guard cells of gpt1 mutants using the KST1 promoter (Kelly et al., 2013). Consequently, the transgenic plants will be examined for their guard cell starch contents and possible other stomatal phenotypes.

In the discussion we mentioned that nighttime starch synthesis might be involving the plastidial NADPH-dependent thioredoxin reductase C (NTRC) and therefore investigating starch contents in ntrc mutants is fundamental to draw conclusions about i) starch synthesis at night in general and ii) to justify how PGI is involved in this process.

The putative G1P translocators G1PT1 (At1g34020) and G1PT2 (At4g09810) are promising candidates for G1P uptake into guard cell chloroplast as their expression profiles show (Fig. 4e). However, their localization is not fully clarified and needs additional experimental support. If their chloroplastic localization turns out to be true, mutants affected in the G1PTs should be investigated for their guard cell starch dynamics. Moreover, even if the localization of G1PT is restricted to the plasma membrane, it would be interesting to investigate stomatal phenotypes in a g1pt1 mutant as it appears to be very highly expressed in guard cells and guard cells might import phosphorylated hexoses for a range of metabolic process (Fig. 4e).
Finally, the investigation of ADPglucose sources should be expanded to SUS3. Even though, we did not observe changes in starch accumulation upon the lack of Brittle1, this does not necessarily exclude the possibility that guard cell chloroplast import ADPglucose provided through SUS3. Hence, sus3 mutants should be examined for their guard cell starch amounts.

Outlook References


Arabidopsis guard cell chloroplasts import cytosolic ATP via the NTT1 transporter

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Correspondence to: DS and BLL
*These authors contributed equally to this work.

This chapter is written for a submission to Nature or a similar internationally peer-reviewed journal.

Author contributions: BLL conceptualized research. BLL, SLL, SF, LD and DS designed the study. SF performed RNA extraction and quantitative RT-PCR as well as GC starch quantification under RL. JL carried out chloroplast isolation experiments. SL produced the transgenic iNAPs and SoNAR sensor lines and carried out all the other experiments. SLL and SF analyzed the data. BLL, SLL, wrote the manuscript draft. SF and DS are currently revising the manuscript.
ABSTRACT

Guard cells (GCs) control the exchange of carbon dioxide and water between the leaf and the atmosphere, thereby determining plant growth and productivity\(^1\). Unlike mesophyll cells, where starch accumulates in the light and is broken down in the dark\(^2\), starch in *Arabidopsis thaliana* GC chloroplasts (GCCs) is degraded at dawn to release glucose to fuel rapid stomatal opening\(^3\). Following its complete disappearance, starch levels in GCCs start to rise until reaching a peak few hours after dusk\(^2,4\). The carbon sources for guard cell starch synthesis are only partially known and it remains controversial whether GCCs carry out photosynthesis to fix CO\(_2\). By employing several fluorescence protein sensors, here we show that illumination stimulates detectable ATP and NADPH production in mesophyll chloroplasts but not in GCCs and therefore the capacity of GCs to photosynthetically fix carbons is limited. We further show that unlike mesophyll chloroplasts, which are impermeable to cytosolic ATP, GCCs import exogenous ATP through the NTT transporters. Our results demonstrate that the main role of GCCs is not photosynthesis, but to serve as a reservoir of starch, which allows the adjustment of sugar solute levels in the GCs during stomatal opening.
Stomatal opening allows the inflow of CO\textsubscript{2} and the diffusion of O\textsubscript{2} and water, which drives the transpiration stream from the roots\textsuperscript{5}. Stomata are closed when the pair of GCs is flaccid and they are open when the pair of GCs is turgid through influxes of potassium (K\textsuperscript{+}), chloride (Cl\textsuperscript{-}) and nitrate (NO\textsubscript{3}\textsuperscript{-}) ions, malate (Mal\textsuperscript{2-}) and sugars\textsuperscript{1,8}. Since GCs lack plasmodesmata, the fluxes of ions must be mediated by transporters or ion channels on the plasma membrane (PM)\textsuperscript{7}. After dawn, weak blue light (BL; e.g. 5 - 10 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}) induces the autophosphorylation of the PM-associated photoreceptor kinases phototropin 1 and 2 (PHOT 1 and 2)\textsuperscript{8,9}, which in turn phosphorylate the Ser/Thr protein kinase blue light signaling 1 (BLUS1)\textsuperscript{10,11}. An unidentified kinase downstream of BLUS1 phosphorylates the C-terminal penultimate Thr residue of the H\textsuperscript{+}-ATPase, which subsequently allows the binding of a 14-3-3 protein and the activation of the proton pump\textsuperscript{12}. The H\textsuperscript{+}-ATPase is deactivated by a class of protein phosphatases, which remove the phosphate group at the penultimate Thr residue\textsuperscript{13}. H\textsuperscript{+}-ATPase transports H\textsuperscript{+} from the cytosol to the apoplast, at the expense of hydrolyzing ATP, causing a hyperpolarization of the PM and activation of voltage-gated K\textsuperscript{+} channels\textsuperscript{1,14}. The influx of K\textsuperscript{+} ions accompanied by the influx of water increases the turgor pressure of the GCs and opens the stomata and Mal\textsuperscript{2-}, Cl\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} serve as counter-ions for K\textsuperscript{+}\textsuperscript{15,16}. Since PM-hyperpolarization largely relies on the activity of the H\textsuperscript{+}-ATPase, stomatal opening is an energy-expensive process that consumes significant amounts of cytosolic ATP\textsuperscript{4}.

Previous work demonstrated that in response to light, GCCs directly export ATP, or indirectly supply cytosolic ATP through exporting DHAP via the triose phosphate shuttle\textsuperscript{1,17}. More so, it is believed that GCC are able to generate ATP and NADPH through the chloroplastic electron transport chain\textsuperscript{1}. However, whether GCCs produce energy in the form of ATP/NADPH and directly fix CO\textsubscript{2} is a subject of contentious debate. Early studies implied that GCCs are unable to autonomously fix CO\textsubscript{2}\textsuperscript{18,19}, whereas some more recent studies argued otherwise\textsuperscript{20-22}. Further, it remains controversial whether GCCs and/or GC photosynthesis plays a direct role in stomatal movements\textsuperscript{1,23}. Previous studies have demonstrated that GCs in epidermal strips and protoplasts are less responsive to light than GCs of intact plants. This is likely due to the detachment from their natural cellular environment\textsuperscript{24}, which interrupts the connection to mesophyll cells\textsuperscript{25,26}. 
To determine to which extent GCCs produce energy in the form of ATP and NADPH, we examined dynamic changes of stromal ATP and NADPH levels in GCCs of Arabidopsis thaliana (Arabidopsis). Illumination of 3-week-old wild-type plants expressing a MgATP$^2$-specific Förster resonance energy transfer (FRET)-based sensor (AT1.03)\textsuperscript{27}, a NADPH sensor (iNAP4)\textsuperscript{28} and a pH sensor (cpYFP)\textsuperscript{29} stimulated ATP production, NADPH production and a pH response in mesophyll chloroplasts but not in GCCs (Fig. 1a-f, Extended data Fig. 1), suggesting that GC electron transport is notoriously limited or neglectable. As a control, we tested rosette leaves of two different developmental stages rendering reproducible results (Extended data Fig. 2).

By applying the ATP sensor AT1.03, we recently showed that the import of cytosolic ATP to mature mesophyll chloroplasts of Arabidopsis was absent due to the downregulation of the ATP/ADP antiporter nucleotide transporter 1 and 2 (NTTs)\textsuperscript{30}. 

---

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Illumination induced detectable ATP and NADPH syntheses in mesophyll chloroplasts but not in GCCs. The 3$^{rd}$ and 4$^{th}$ leaves of 20-22-days-old wild-type plants expressing TKTP-AT1.03, TKTP-iNAP4 and TKTP-cpYFP sensors were collected at three different time points (EoN, 2 h and 8 h of the day). a-b, plastid stromal AT1.03, c-d, plastid stromal iNAP4, and e-f, plastid stromal cpYFP of GCCs and mesophyll cells in response to illumination at 216 μmol m$^{-2}$ s$^{-1}$ for 180 s. The iNAP results presented were normalized with TKTP-iNAPc. EoN, end of night. Asterisks indicate significant statistical differences (*$P < 0.05$, **$P < 0.01$) before and after 180 s of illumination as determined by paired t-test ($n = 5$; means ± SEM).
We further observed that illumination instantly increased stromal ATP in mesophyll chloroplasts but when the light was withdrawn, stromal ATP quickly dropped to its initial level, indicating that mesophyll chloroplasts consumed ATP very rapidly. Hence, in mesophyll cells the concentration of stromal ATP is much lower than that of the cytosol and mesophyll chloroplasts are unable to import ATP from the cytosol.

Given that GCCs do not produce sufficient amounts of ATP to fuel the energy-consuming processes in the chloroplasts, like the synthesis of starch, we wondered if GCCs are capable of taking up ATP from the cytosol.

**Figure 2.** Guard cell chloroplasts import ATP. **a-b,** Incubation of GC and mesophyll cell chloroplasts expressing TKTP-AT1.03 in a buffer with and without 5 mM ATP for 5 min (n = 23, 29, 83 and 51, respectively). Scale bar, 5 µm. Asterisks indicate significant statistical differences (***P < 0.001) in FRET ratios with and without exogenous ATP as determined by non-paired t-test. **c,** mRNA expression levels of the ATP/ADP antiporter nucleotide transporter 1 (AtNTT1) and 2 (AtNTT2) genes in GC-enriched epidermal peels compared to leaves from wild-type plants. Actin (ACT2) was used as a housekeeping gene for normalization. Values are means of two independent experiments ± fold change range. Primer sequences and efficiencies are given in extended data Table 1. **d,** Diameter of guard cell and mesophyll chloroplasts isolated from 20-22-days-old wild-type plants (n = 68 and 163). Asterisks indicate significant statistical differences (***P < 0.001) for the diameters (µm) of guard cell and mesophyll chloroplasts as determined by non-paired t-test. **e,** Basal MgATP₂⁻ FRET ratio in cytosolic and plastid stroma of guard cells and mesophyll cells of leaves of 20-22-days-old plants collected at the end of night (0 h), and after 2 h or 8 h of illumination. Different letters indicate significant statistical differences analyzed by one-way ANOVA and Tukey’s HSD test (P < 0.05) (n = 5; means ± SEM).
To examine if GCCs can import exogenous ATP, we isolated GCCs expressing the ATP sensor in the chloroplast stroma from 3-week-old plants. The addition of exogenous ATP significantly increased the ATP levels in GCCs but not in mesophyll chloroplasts, demonstrating that GCCs are able to import ATP (Fig. 2a-b). The integrity of chloroplasts was verified by SYTOX™ orange staining (Extended data Fig. 3b)\textsuperscript{27}. qRT-PCR on GC-enriched epidermal peels further revealed that both nucleotide transporters *NTT1* and *NTT2* are preferentially expressed in GCs. Whereby, *NTT1* gene expression was 36x higher in GCs compared to the whole leaves, supporting the idea of ATP import to GCCs predominantly through NTT1 (Fig. 2c). We further noticed that the GCCs and GC protoplasts are significantly smaller compared to mesophyll chloroplasts and protoplasts (Fig. 2d and Extended Fig 3a). Given that the number of chloroplasts, and the ratio of chloroplasts to mitochondria are much lower in GCs compared to mesophyll cells\textsuperscript{5}, GC mitochondria are likely the main supplier of cytosolic ATP. To test this, we applied several inhibitors of the mitochondrial electron transport to the ATP sensor lines AT1.03 at the end of the night. Our data revealed that treatment with oligomycin, an inhibitor of the mitochondrial ATP-synthase, significantly lowered the cytosolic ATP levels in both mesophyll cells and GCs (Extended data Fig. 4a and 4b), whereas inhibitors of complex I (rotenone) and complex II (TTFA) of the mitochondrial transport chain partially decreased the cytosolic ATP levels in mesophyll cells but did not affect the ATP levels in GCs. This could be explained by the larger cytosolic volume of mesophyll cells and therefore the effects of rotenone and TTFA might be more prominent in GCs as they might be more concentrated (Extended data Fig. 4a and 4b). To confirm that the NADPH sensor is responsive in GCCs, plants expressing TKTP iNAP4 were infiltrated with the oxidizing agents $\text{H}_2\text{O}_2$ and menadione for 5 min. Both $\text{H}_2\text{O}_2$ and menadione suppressed the stromal NADPH sensor ratio in GCs and mesophyll cells, suggesting that chloroplasts in both cell types contain substantial amounts of NADPH in the dark (Extended data Fig. 4c and 4d).

We then compared the ATP levels in chloroplasts and cytosol in both GCs and mesophyll cells across 3 time points (Fig. 2e). While the cytosolic ATP levels remained significantly higher than the stromal ATP levels in both mesophyll cells and GCs and the stromal ATP in mesophyll chloroplasts was stably low across all time points, the stromal ATP levels in GC varied significantly among the 3 time points (Fig. 2e). Furthermore, GC stromal ATP levels were correlated with GC starch contents, as the ATP level was the lowest at 2h after the onset of illumination, when GC starch was scarce \textsuperscript{4} (Fig. 2e and Fig. 3a-3b). Assuming that protein levels of the NTTs do not change within this short amount of time \textsuperscript{31}, the change in stromal ATP levels might be explained by a higher rate of ATP consumption in GCCs at this time, when GC starch...
is almost completely hydrolyzed and starch synthesis has begun. We further examined the starch contents in GCs of ntt1 and ntt2 T-DNA lines, and found that the starch levels were significantly lower in the T-DNA lines compared to wild type, supporting that the entry of ATP from the cytosol through the NTT transporter is important for starch synthesis in GCs (Fig. 3a and 3b). The stomatal aperture size in different lines were also measured in the ntt1 and ntt2 T-DNA lines (Fig. 3c and 3d). The stomata of the T-DNA lines did not open as wide as that of the WT in the first 2 hours, possibly due to a lower starch level in GCCs of these lines at the end of night.

Figure 3. Stomatal ATP measurements and starch contents in GCCs. a-b, GC starch contents of wild-type, ntt1 and ntt2 plants. Starch granules are visualized by pseudo colors. Scale bar, 10 µm. Starch granule area is given in µm². Each GC starch value represents means ± SEM of five biological replicates of more than 30 individual pairs of GCs for each group. c-d, Stomatal aperture of 20-22-days-old wild-type, ntt1 and ntt2 plants after 0 h, 2 h and 8 h of illumination. Stomatal aperture data represent means ± SEM of more than 120 individual pairs of GCs of each group. e-f, Starch contents of isolated GCs of wild-type and stp1stp4 plants illuminated for 1 h, 2 h, 3 h and 6 h with 300 µmol m⁻² s⁻¹ RL with or without 10 µM DCMU treatment at EoN. The isolated GCs were dark-adapted for one hour before illumination and treatment. Each value represents mean ± SEM of four biological replicates of more than 110 individual GCs obtained from 3 (control) and 2 (DCMU treatment) independent experiments. Different letters indicate statistically significant differences among time points for the given genotype for P < 0.05 determined by one-way ANOVA with post hoc Tukey’s test. g-h, Illumination caused alkalization of the cytosol of mesophyll cells and GCs of 20-22-days-old plants, which was abolished by oligomycin treatment. Asterisks (*) indicate significant statistical differences (*P < 0.05, **P < 0.01, ***P < 0.001) as determined by paired t-test (n = 5; means ± SEM). EoN, end of night.
The synthesis of starch also requires the supply of carbohydrates. There are three possible sources of carbons for GC starch synthesis, photoassimilates from GC photosynthesis\(^{32}\), the import of apoplastic sugars to GCs\(^{1,26}\) or, the conversion of previously stored carbons (e.g. in the vacuole)\(^{1}\). In mesophyll cells, the coordinated actions of the photosystems and the Calvin-Benson-Bassham (CBB) cycle deliver the carbon skeletons required for starch synthesis.\(^{33}\) However, it remains unknown if GCs do photosynthetically fix carbons. Recent studies demonstrated that autonomous GC CO\(_2\) fixation is highly limited and that GCs largely rely on carbon supply from mesophyll cells for starch biosynthesis\(^{6}\). Namely, GCs import glucose (Glc) at dawn through the coordinate action of the monosaccharide-H\(^+\) symporters Sugar Transport Protein 1 and 4 (STP1 and STP4), which are essential for GC starch synthesis and stomatal opening\(^{6}\). However, it remains unknown if the observed remaining starch synthesis in both isolated guard cells and \(stp1stp4\) mutant plants derives from autonomous CO\(_2\) fixation.

To determine whether isolated GCs illuminated with red light (RL) synthesize GC starch from CBB cycle intermediates, we examined GC starch contents in response to RL in GCs isolated from wild-type and the \(stp1stp4\) mutant plants, which are devoid of GC starch at the end of the night due to its inability in taking up apoplastic hexoses\(^{6}\). As observed previously, isolated wild-type GCs accumulated starch in response to a 6 h RL treatment (Fig. 3e and 3f)\(^{26}\). This accumulation was reduced in GCs of \(stp1stp4\) (Fig. 3e and 3f), indicating that effectively isolated GCs of wild-type plants partially produced starch from carbons imported previously to the assay start, presumably hexoses, which \(stp1stp4\) GCs cannot employ. To further test if the starch accumulation observed in isolated \(stp1stp4\) GCs can be explained by GC photosynthesis, we applied 10 \(\mu\)M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II (PSII), to the isolated GCs before the start of illumination. The above described physiological assays suggest that GCs predominantly use mesophyll-derived carbons as a source for starch synthesis. However, GC-photosynthesis-dependent starch synthesis seems to occur likewise, but its exact function \textit{in planta}, where ample supply of mesophyll-derived sugars occurs, remains unknown.

GCs were reported to have a reduced amount of Rubisco (270 x lower) compared to mesophyll cells in \textit{Pisum sativum} L.\(^{35}\). Furthermore, immunostaining of Rubisco in different C3 species showed that the amount of Rubisco is much lower, or even negligible, in GCCs compared to mesophyll chloroplasts\(^{19}\). Three key enzymes of the CBB pathway were reported to be absent in the GCCs of \textit{Vicia faba}; Rubisco, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase.
Proteomic studies further revealed that Rubisco is hardly detectable in Arabidopsis GCs\textsuperscript{36}. By contrast, GCs have a high abundance of light-independent NAD-malate dehydrogenase (NAD-MDH) in chloroplasts, cytosol and mitochondria\textsuperscript{37}. In addition, substantial levels of phosphoenolpyruvate carboxylase (PEPC) are present in the cytosol, which can fix CO\textsubscript{2} into oxaloacetate (OAA)\textsuperscript{37}. When employing \textsuperscript{14}C-labeled CO\textsubscript{2}, it was observed that illumination significantly increased (12-fold higher than the fixation rate in the dark) CO\textsubscript{2} fixation in mesophyll cells of \textit{Commelina diffusa} but only slightly (20 \%) in the epidermis. In mesophyll cells of \textit{C. diffusa} and \textit{T. gesneriana} CO\textsubscript{2} was fixed into 3-phosphoglyceric acid (3PGA) and sucrose in the light and it was slowly fixed into Mal\textsuperscript{2} in the dark. By contrast, CO\textsubscript{2} in epidermal cells was fixed into Mal\textsuperscript{2} at a high rate under both light and dark conditions\textsuperscript{38,39}. Moreover, in GC protoplasts of \textit{Vicia faba} L. \textsuperscript{14}C-labeled CO\textsubscript{2} was not fixed into CBB products, but appeared in Mal\textsuperscript{2} and starch in both light and dark conditions\textsuperscript{40}. Hence, it is likely that CO\textsubscript{2} fixation in GCs is mainly mediated by the combined action of PEPC and NAD-MDH and not by Rubisco during light and dark conditions. The fixed CO\textsubscript{2} in the form of Mal\textsuperscript{2} might be rapidly converted into starch in GCs\textsuperscript{40}.

The phototropin-H\textsuperscript{+}-ATPase pathway was shown to be essential for BL-dependent degradation of GC starch at the onset of illumination\textsuperscript{4}, which in turn is required for BL-dependent stomatal opening\textsuperscript{41}. Here, we show that illumination stimulates the alkalization of cytosolic pH in both mesophyll and GCs in an ATP-dependent manner (Fig. 3g-3h, Extended data Fig. 5). This stimulation was abolished when cytosolic ATP in GCs and mesophyll cells was depleted by oligomycin treatment (Fig. 3g-3h, Extended data Fig. 5).

Finally, we propose a model describing how starch metabolism in GC integrates with stomatal function, and how GCCs obtain the resources for starch synthesis when they do not generate sufficient ATP or NADPH during illumination nor fix considerable amounts of CO\textsubscript{2} through the CBB pathway (Fig. 4). In this model, ATP and NADPH production is limited in GCCs (Fig. 1) and the presence of the NTTs enables the import of ATP into GCCs (Fig. 2). There was no detectable ATP and NADPH production in GCCs even after 2 hours of illumination (Fig. 1) and starch synthesis in GCs dependents on hexose supply from mesophyll cells\textsuperscript{5}. Hence, the main function of GCC is not photosynthesis or carbon fixation, but to serve as a reservoir of starch, which is important for the regulation of stomatal opening\textsuperscript{41}. This hypothesis is supported by the observations that stomatal opening in the first 2 hours of illumination is slower and smaller in stomata without chloroplasts than in stomata with chloroplasts in the same plants of two chloroplast mutants (\textit{arc6} and \textit{crl})\textsuperscript{43}, and GCCs lacking chlorophyll still have the ability to synthesize starch\textsuperscript{23}.\textsuperscript{23}
Figure 4: Model depicting how stomatal function is coordinated with starch and malate metabolism in GCs. a, GC starch is broken down at the initial phase of illumination. At dawn, the GC PM-associated photoreceptor kinases PHOT 1 and 2 are activated by weak BL to induce stomatal opening. In parallel, GC starch is mobilized to sustain sugar homeostasis for stomatal opening. Sugars are also an energy source for GC mitochondria to generate ATP and reducing equivalents. Through glycolysis, sugars are converted to phosphoenolpyruvate (PEP), a 3C compound, which can fix a CO$_2$ molecule and be further converted into OAA, a 4C compound, via the action of the PEPC. OAA is then further reduced to Mal$^2$ by the MDH. Mal$^2$ is important for osmoregulation and stomatal opening, and acts as a counter-ion for K$^+$. Mal$^2$ can either be directly synthesized from OAA via PEPC and MDH or be imported from the apoplast. At the same time, carbon fixation and starch/sucrose synthesis occur in mesophyll cells (Fig. 4a). and b, starch synthesis occurs in GCs after the initial phase of illumination. After complete starch degradation in GCs, stomatal opening is sustained by mesophyll-derived sugars (Fig. 4b). Hexoses, derived from sucrose from neighboring mesophyll cells through the action of cell wall invertase, are imported to GCs via the monosaccharide transporters STP1 and STP4. In GCs, hexoses are converted into glucose-6-phosphate (Glc6P), which is then transported into the chloroplast through the Glc-6-P/Pi translocators (GPTs) and can be utilized for starch synthesis. The imported sugars can also get metabolized through glycolysis and mitochondrial respiration to generate cytosolic ATP that enters the GCCs to deliver ATP for starch synthesis. Chloroplasts and mitochondria are represented by green and brown circles, respectively. Red arrows represent the energy source flow. ADP, adenosine diphosphate; AHA, H$^+$/ATPase; AKT, inward-rectifying K$^+$ channel; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CHL1, dual-affinity nitrate transporter; CLC, chloride channel of the CLC gene family; CwINV, cell wall invertase; G6P, glucose-6-phosphate; GPT, glucose-6-phosphate/Pi translocator; H$, proton; K$, potassium; KAT, inward-rectifying K$^+$ channel; MDH, malate dehydrogenase; NTT, nucleotide transport protein; OAA, oxaloacetate; PEP, phosphoenolpyruvate carboxylase; Phot, plasma membrane-associated photoreceptor kinase phototropin; STP, Sugar Transport Protein; SUC, sucrose/H$^+$ cotransporter.
MATERIAL AND METHODS

Plant Materials
Wild-type (Col-0) seedlings carrying pH2GW7-C-cpYFP (cytosolic pH sensor), pH2GW7-TKTP-cpYFP (plastid stromal pH sensor), pH2GW7-C-AT1.03 (cytosolic MgATP$^{2-}$ sensor) pEarleyGate100-TKTP-AT1.03 (plastid stromal MgATP$^{2-}$ sensor), pEarleyGate100-TKTP-iNAP4 (plastid stromal NADPH sensor) and pEarleyGate100-TKTP-iNAPc (control sensor for iNAP4) were obtained as previously described$^{27,28,47}$. The transfer DNA (T-DNA) insertion lines SALK_083518c ($ntt1$) and SALK_031126c ($ntt2$) were obtained from Arabidopsis Biological Resource Center. The stp1stp4 mutant was described previously$^6$. Arabidopsis plants were grown in soil in growth chamber with a photoperiod of 12 h at 150 µmol photon m$^{-2}$ s$^{-1}$ at 22ºC and 12 h in darkness at 18ºC. All experiments were performed with 20-22-days-old plants if not otherwise stated.

RNA Isolation and Quantitative PCR Analysis
RNA was extracted as reported in Flütsch, et al.$^{26}$ In brief, RNA from leaves was extracted from 3 entire rosettes (3 biological replicates) and frozen in liquid nitrogen. For RNA from guard cell-enriched epidermal peels, the middle veins of 12 rosettes were excised for 1 biological replicate, the leaf material was blended (Philips, ProBlend Avance) and subsequently passed through a 200 µm nylon mesh (Sefar). Guard cell-enriched epidermal peels were dried, collected and frozen in liquid nitrogen. 3 biological replicates were used in each experiment. Subsequently, total RNA was extracted using the SV Total RNA Isolation Kit (Promega) following the manufacturer’s instructions. A total of 1 µg RNA was used for cDNA first strand synthesis using the M-MLV Reverse Transcriptase RNase H Minus Point Mutant and oligo(dT)$_{15}$ primer (Promega). Transcript levels were examined by qRT-PCR using the SYBR green master mix (Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems). qRT-PCR was performed in triplicates. Transcript levels were calculated according to the comparative C$_T$ method$^{55}$ and were normalized against the expression of the Actin2 gene (ACT2; At3g18780). Error calculations were done according to Applied Biosystems guidelines (http://assets.thermofisher.com/TFSAssets/LSG/manuals/cms_042380.pdf).
Isolation of Guard Cell and Mesophyll Cell Chloroplasts

Guard cell protoplasts were isolated from 20-22-days-old plants as previously described\textsuperscript{48}. Mesophyll protoplasts were isolated using the tape Arabidopsis sandwich method\textsuperscript{49}. The chloroplasts were then isolated using a syringe barrel filled with protoplasts and pressed through a piece of 1-μm and 5-μm nylon mesh for guard cell protoplasts and mesophyll protoplasts, respectively. After isolation, chloroplasts were washed and resuspended in a buffer (300 mM sucrose, 50 mM Hepes-KOH (pH 7.5), 10 mM KCl, 10 mM NaCl, 5 mM MgCl\textsubscript{2}, and 0.1\% (w/v) BSA). The chloroplasts were incubated in buffer with or without 5 mM ATP for 5 min in room temperature before imaging\textsuperscript{27}. 25 nM SYTOX\textsuperscript{TM} orange nucleic acid stain was used to detect the non-viable cells. The stain was excited at 543 nm and emission was collected from 565 nm to 604 nm. The presence of SYTOX\textsuperscript{TM} orange will lower the AT1.03 FRET ratio as it absorbs at the emission of AT1.03 at 526-545 nm.

Confocal Imaging and Image Processing

Confocal imaging of the abaxial layer of the leaves was set up as previously described\textsuperscript{50}. Leaves were collected from 20 to 22-days-old plants at the end-of-night (EoN), 2h or 8h of the day. For inhibitor treatment, the leaves were infiltrated for 5 min by submerging in half-strength Murashige and Skoog medium with 0.01 mM oligomycin A or antimycin A. Imaging was performed with 40 × oil immersion lenses in multitrack mode using a Zeiss LSM710 NLO confocal microscope (Carl Zeiss Microscopy). After the first image was obtained, the leaves were further illuminated for 3 min at 216 μmol m\textsuperscript{−2} s\textsuperscript{−1} by a halogen lamp (HAL 100 W; Philips) of the confocal microscope before the second image was captured. Plants expressing cpYFP, iNAP4, and iNAPc were excited sequentially at 405 nm and 488 nm, and the emission signals were collected at 520 ± 16 nm. Autofluorescence was recorded at 431–469 nm. The normalized iNAP4 R\textsubscript{405/488} was corrected with iNAPc and calculated as previously described\textsuperscript{28}. AT1.03 was excited at 458 nm and the emission signals were detected at 470–507 nm (Em470–507, mseCFP image) and 526–545 nm (Em526–545, FRET image). Plants expressing AT1.03 were also excited at 515 nm (0.18 % of maximal power for all samples) to excite Venus and emission was detected at 526–545 nm (cp173Venus image). Raw FRET ratio was calculated by dividing the fluorescent intensity of mseCFP image with FRET image. All of the images were also collected for the chlorophyll fluorescence at 629–700 nm. Ratiometric images were analyzed on a pixel-by-pixel basis using x, y noise filtering. The fluorescence background subtraction was conducted based on the intensity from the dark side of the images. Confocal
images were processed with a custom MATLAB-based analysis suite\textsuperscript{51}. All the ratio representative profiles are presented in pseudo colors.

**Visualization of Starch in GCCs**

The method of visualizing starch in GCCs was adopted from Flütsch, et al.\textsuperscript{52}. After epidermal peels were harvested using precision tweezer from the abaxial side of the 3\textsuperscript{rd} or 4\textsuperscript{th} leaf of 20-22-days-old wild-type Arabidopsis plants after 0 h, 2 h and 8 h of illumination, the peels were fixed in fixative solution (50 % (v/v) methanol, 10 % (v/v) acetic acid) for at least 12 h at 4 °C in the dark. After fixation, the fixative solution was removed, and the peels were washed with 1 ml dH\textsubscript{2}O by shaking the plate slowly in circular movements. The epidermal peels were destained with 1 ml 80 % (v/v) ethanol and incubated for 15 min. Peels were washed with dH\textsubscript{2}O and incubated in 1 ml of fixative solution for 1 h at room temperature. After an hour of fixation, peels were washed with dH\textsubscript{2}O and fully covered in 1 % (v/v) periodic acid solution and incubated for 30 min at room temperature before washing with dH\textsubscript{2}O. Next, peels were stained with 500 µl Schiff reagent (1.9 g sodium metabisulfite, 3 ml 5 N HCl and 97 ml dH\textsubscript{2}O) and 50 µl of 1 mg ml\textsuperscript{-1} propidium iodide solution for 30 min at room temperature. At this stage, peels appeared pinkish. Samples were destained in dH\textsubscript{2}O for 30 min at room temperature. Choral hydrate solution (40 g chloral hydrate, 10 ml glycerol and 20 ml dH\textsubscript{2}O) was added onto microscope slides and the stained peels were gently transferred onto the microscope slides and incubated in the dark overnight. The choral hydrate solution was removed using wipes before mounting. Hoyer's solution (30 g gum Arabic, 200g chloral hydrate, 20 g glycerol and 50 ml dH\textsubscript{2}O) was added onto the peels and covered with a cover slip. The samples were stored at room temperature in the dark for 3 days before visualization under a confocal microscope. The sample was excited at 488 nm and emission was collected from 610 nm to 640 nm. Starch area was quantified using ImageJ (NIH, https://imagej.nih.gov/ij/index.html).

For GC photosynthesis experiments, leaves number 5 and 6 from eight individual plants were blended using a kitchen blender (ProBlend Avance, Philips). Subsequently, the isolated guard cells were collected on a 200-µm nylon mesh (Sefar) and incubated in 1 ml of basic opening buffer (5mM MES-bistrispropane at pH 6.5, 50 mM of KCl, and 0.1 mM of CaCl\textsubscript{2}). The isolated guard cells were dark-incubated for 1 h in a reach-in climate chamber (model no. Fytoscope FS130; Photon Systems Instruments) after which 10 µM DCMU (Sigma-Aldrich) were added to the buffer of half of the peels. Subsequently, the isolated guard cells were exposed to 300 mmol m\textsuperscript{-2} s\textsuperscript{-1} RL for 6 h. After the incubation and at the indicated time points, isolated guard cells were collected and fixed for starch quantification, as described above.
Stomatal Aperture
20-22-days-old plants were used for stomatal aperture measurements. The upper epidermal surface was stabilized by affixing a strip of 3M vinyl electrical tape while the lower epidermal surface was affixed to a strip of transparent tape. The transparent tape was then carefully pulled away from the vinyl electrical tape, peeling away the upper epidermal surface cell layer. The transparent tape carrying the lower epidermal surface was then taped on to a microscopy slide and images were immediately taken under a stereomicroscope (Olympus SZX16). The stomata were measured for the width and length using image processing software ImageJ and calculated as width/length$^{53}$.

Data Analysis
All data presented are means with standard errors (mean ± SEM). The collected data were analyzed for statistical significance using analysis of variance (ANOVA) with Tukey’s HSD, paired t-test and non-paired t-test at $P < 0.001$, $P < 0.01$, and $P < 0.05$ by SPSS (Version 20).

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249


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**Extended Data**

**Extended data figure 1:** Representative pseudo color images of mesophyll cell and GC chloroplasts from 20-22 days-old plants after 0 min and 3 min of illumination. **a**, Plastid stromal AT1.03, **b**, plastid stromal iNAP4 and **c**, plastid stromal cpYFP signals were collected at different time points (EoN, 2 h and 8 h). EoN, end of night. Scale bars, 10 µm.
Extended data figure 2: Stromal ATP generation in leaves of different developmental stages. a, Photograph of a 20-22-days-old plant. Signals of the stromal ATP sensor AT1.03 in young (5th leaf) and mature leaves (4th leaf) collected after 0 h, 2 h and 8 h of illumination. 216 μmol m⁻² s⁻¹ light was applied for 3 min after the first image was captured at the indicated time points. b, plastid stroma in mesophyll cells, and c, plastid stroma in guard cells. EoN, end of night. Asterisks indicate significant statistical differences (*P < 0.05, **P < 0.01) before and after 3 min of illumination as determined by paired t-test (n = 3; error bars ± SEM).
Extended data figure 3: Mesophyll cell and GC protoplasts and chloroplasts. a-b, Representative confocal images of GC and mesophyll cell chloroplasts and difference in protoplast sizes between GCs and mesophyll cells. (n = 99 and 107, respectively; means ± SEM). White arrow head points on GC protoplast; black arrowhead points on mesophyll cell protoplast. Scale bar, 10 µm. c-d, Intact and leaky GC and mesophyll chloroplasts expressing TKTP-AT1.03. Leaky chloroplasts were stained with SYTOX™ orange and incubated with 5 mM ATP. Fluorescence images of transmitted light detector (bright field), chlorophyll fluorescence (chlorophyll), SYTOX™ orange fluorescence (SYTOX), and raw FRET ratios (FRET) of chloroplasts are presented. Asterisks indicate significant statistical differences (***P < 0.01) between intact and leaky chloroplasts as determined by non-paired t-test (n =23, 16, 83 and 29, respectively; means ± SEM). Scale bar, 5 µm. MgATP²⁻ FRET ratios are represented by pseudo color images, where higher ratios (yellow) correspond to higher MgATP²⁻ level.
Extended data figure 4: Effects of mitochondrial electron transport chain inhibitors on cytosolic ATP and NADPH at the end of the night. a-b, Changes of cytosolic ATP levels upon treatment with 0.05 mM rotenone, 0.1 mM thenoyltrifluoroacetone (TTFA) and 0.01 mM oligomycin in GCs and mesophyll cells. Scale bar, 20 µm. Different letters indicate significant statistical differences analyzed by one-way ANOVA and Tukey’s HSD test (P < 0.05) (n = 3; means ± SEM). c-d, Effect of H$_2$O$_2$ and menadione on GCs and mesophyll cells expressing the NADPH sensor TKTP iNAP4. Scale bar, 10 µm. Ratios of the FRET and iNAP4 are represented in pseudo color images, where high ratios (yellow) correspond to high MgATP$^{2-}$ and NADPH level.
Extended data figure 5: Ratiometric images of cytosolic cpYFP expressed in mesophyll and GCs after 0 min and 3 min of illumination. a-b, 20-22 days-old plants expressing cytosolic cpYFP were imaged at different time points (EoN, 2 h and 8 h) a, without or b, with oligomycin pre-treatment and were illuminated with 216 µmol m\(^{-2}\) s\(^{-1}\) white light for 3 min after the first image was captured at 0 min. The images display pseudo colors. Scale bars, 20 µm.

Extended data Table 1: Sequences of primers used for qRT-PCR

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Peeling back the layers of crassulacean acid metabolism: functional differentiation between *Kalanchoë fedtschenkoi* epidermis and mesophyll proteomes

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ABSTRACT

Crassulacean acid metabolism (CAM) is a specialized mode of photosynthesis that offers the potential to engineer improved water-use efficiency (WUE) and drought resilience in C3 plants while sustaining productivity in the hotter and drier climates that are predicted for much of the world. CAM species show an inverted pattern of stomatal opening and closing across the diel cycle, which conserves water and provides a means of maintaining growth in hot, water-limited environments. Recent genome sequencing of the constitutive model CAM species Kalanchoë fedtschenkoi provides a platform for elucidating the ensemble of proteins that link photosynthetic metabolism with stomatal movement, and that protect CAM plants from harsh environmental conditions. We describe a large-scale proteomics analysis to characterize and compare proteins, as well as diel changes in their abundance in guard cell-enriched epidermis and mesophyll cells from leaves of K. fedtschenkoi. Proteins implicated in processes that encompass respiration, the transport of water and CO₂, stomatal regulation, and CAM biochemistry are highlighted and discussed. Diel rescheduling of guard cell starch turnover in K. fedtschenkoi compared with that observed in Arabidopsis is reported and tissue-specific localization in the epidermis and mesophyll of isozymes implicated in starch and malate turnover are discussed in line with the contrasting roles for these metabolites within the CAM mesophyll and stomatal complex. These data reveal the proteins and the biological processes enriched in each layer and provide key information for studies aiming to adapt plants to hot and dry environments by modifying leaf physiology for improved plant sustainability.

Keywords crassulacean acid metabolism, proteomics, epidermis, guard cell, starch metabolism
CHAPTER VII

INTRODUCTION

The water-conserving features of crassulacean acid metabolism (CAM) have highlighted this specialized mode of photosynthesis as a model for engineering improved water-use efficiency and drought resilience into C3 crops (Borland et al., 2014; Yang et al., 2015). CAM is a syndrome of anatomical, metabolic and physiological adaptations that facilitate CO$_2$ concentration around ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), while circumventing approximately 90% of the water loss associated with stomatal transpiration at little or no cost to photosynthetic carbon assimilation (Shameer et al., 2018). CAM improves the water-use efficiency (WUE = CO$_2$ fixed per unit water lost) of plants by up to 10-fold, compared with other types of photosynthesis, by enabling CO$_2$ uptake through open stomata and through CO$_2$ storage as malate at night, when transpiration is reduced (Borland et al., 2009). The subsequent decarboxylation of malate during the day releases CO$_2$, which is re-fixed by rubisco behind closed stomata. CAM is present in over 400 distinct genera across 36 families of vascular plants that are found in diverse ecosystems, ranging from semi-arid deserts to seasonally dry forests (Yang et al., 2015). The multiple, independent evolutionary origins of CAM indicate that this water-conserving mode of photosynthesis is built on sets of genes common to plants that use conventional C3 photosynthesis. Thus, it has been argued that the synthetic engineering of CAM in C3 plants as a means of improving plant WUE is feasible by a rewiring of the core genetic and physiological regulatory circuits (Borland et al., 2014, 2015; DePaoli et al., 2014). The recent sequencing of several CAM genomes that evolved CAM independently have paved the way for identifying prospective components for engineering CAM into a C3 background (Yang et al., 2015). In particular, the availability of the genome of the constitutive species Kalanchoë fedtschenkoi (Yang et al., 2017) provides a platform for establishing the functional genomics of CAM in a model system with straightforward plant transformation procedures and a wealth of documented biochemistry and whole-plant physiology data (Dever et al., 2015; Hartwell et al., 2016; Boxall et al., 2017, 2020). Insights acquired from systems analysis of K. fedtschenkoi are expected to highlight the molecular basis of key traits that could be engineered into non-CAM plants to sustainably enhance productivity and survival in a hotter, drier world.

Central to the water-conserving traits of CAM sits a diel cycle of stomatal movement that operates in reverse to that of plants with C3 or C4 photosynthesis. Temporal reprogramming of the expression of genes implicated in the regulation of stomatal movement has been reported for CAM species such as Agave americana, K.
fedtschenkoi and Kalanchoëi laxiflora, relative to C3 Arabidopsis (Abraham et al., 2016; Yang et al., 2017; Boxall et al., 2020). Further work is required to define the diel turnover of proteins implicated in stomatal movement, in order to determine their distribution between the leaf mesophyll cells and stomatal complex, and to understand how mesophyll metabolism interacts with stomatal regulation. Current dogma suggests that CAM stomata are regulated via diel changes in the internal partial pressure of CO₂ (pCi) that accompany the diel turnover of malate in the leaf mesophyll (Borland et al., 2014; Males and Griffiths, 2017). In addition to its proposed role as a mesophyll-derived signal that controls stomatal aperture, malate also plays a key role within the guard cells of C3 plants for osmoregulation and as a counter ion for K⁺ (Santelia and Lawson, 2016). The existence of C4/CAM-like metabolism in the guard cells of C3 plants is well reported across the literature and is linked to the turnover of malate, which is generally accepted as the predominant anion during C3 stomatal opening and closing (Fernie and Martinoia, 2009). The contrasting roles for malate metabolism within the CAM mesophyll and stomatal complex present the hypothesis of tissue-specific regulation of malate turnover in the mesophyll and epidermal layer, containing the guard and subsidiary cells. Many of the proteins required for CAM are encoded by multigene families (Yang et al., 2015; Yang et al., 2017). Thus, it is possible that different isoforms responsible for nocturnal carboxylation and daytime malate decarboxylation carry out specific roles within the mesophyll and epidermis, with malate metabolism in the epidermis having implications for stomatal regulation. A better understanding of how proteins implicated in the diel metabolism of malate, as well as that of other primary metabolites such as starch, are organized between the epidermis and leaf mesophyll cells of CAM plants will provide insight on how metabolism within these spatially separated tissues has diverged from that in C3 plants.

In addition to distinctive metabolic and physiological features, CAM species typically possess anatomical traits such as thickened, succulent leaves or stems (Borland et al., 2018). Succulence facilitates the vacuolar storage of malic acid as well as water (Balsamo and Uribe, 1988; Rodrigues et al., 2013), and enhances CO₂ trapping during the daytime decarboxylation of CAM by reducing the intercellular air space of the leaf (Borland et al., 2018). Such anatomical characteristics are likely to impose specialized roles for proteins implicated in the transport of CO₂ and water within the mesophyll and epidermis. Reduced water permeability of the leaf cuticle also appears to be a key trait shared by CAM species. In addition to curtailing water loss, the composition of epicuticular wax influences heat load and photoprotection of leaves via reflectance and light dispersion (Shepherd and Griffiths, 2006; Bernard and
Joubes, 2013). The daytime closure of stomata typifying CAM implies that heat tolerance is a critical adaptive trait for CAM plants. A better understanding of cuticular wax biochemistry in CAM species offers further opportunities for engineering enhanced heat and dehydration tolerance in plants.

We sought to provide a detailed proteome resource that captures the underlying biological functions responsible for the water-conserving and heat tolerance traits that typify CAM using the model CAM species *K. fedtschenkoi*. We performed large-scale quantitative comparisons between the leaf mesophyll and the guard cell complex isolated in epidermal peels to reveal the protein functions that exist in the mesophyll and epidermal layers, and to establish the spatial separation of protein function that exists between the two layers. As the essence of CAM is founded on diel control of metabolism and stomatal conductance, we also compared the temporal dynamics of epidermal and mesophyll proteomes over the 24-h light/dark cycle to better define the cellular behaviors underpinning CAM in these functionally distinct tissues. Distinct pathways related to stomatal regulation, CAM biochemistry and guard cell starch metabolism were of particular interest and are therefore highlighted and discussed in this study.
Tissue sampling within the context of the CAM cycle

Crassulacean acid metabolism (CAM) plants exhibit a diel separation of two major metabolic phases. At night, stomatal opening permits the net uptake of CO$_2$ (in the form of HCO$_3^-$) by phosphoenolpyruvate carboxylase (PEPC) and the subsequent conversion to and storage of malic acid in the vacuole. During the day, decarboxylation of malate releases CO$_2$ for refixation by C3 photosynthesis, behind closed stomata. A typical CAM gas-exchange profile for mature leaves (leaf pair 6, LP6) of *K. fedtschenkoi* is shown in Figure 1(a) to provide physiological context for the proteomics data obtained by sampling mesophyll and epidermis tissues over the diel cycle at 4-h intervals, as indicated.

Cell types and distribution of chloroplasts and mitochondria in the epidermis

In *K. fedtschenkoi*, stomata are surrounded by three or four subsidiary cells (Figure 1c), as reported for other species of Kalanchoë (Xu *et al*., 2018). Subsidiary cells are believed to provide a reservoir of K$^+$ and Cl$^-$ ions for uptake into guard cells during stomatal opening and to act as a sink for these ions during stomatal closure (Willmer and Pallas, 1973). Confocal microscopy and mitochondrial staining indicated the distribution of chloroplasts and mitochondria within cell types found in the epidermis tissue sampled (Figure 1). Mitochondria were detected in both guard cells and subsidiary cells (Figure 1b,c), but chloroplasts were only detected within the guard cells (Figure 1b). Chloroplasts or mitochondria were not detected in the epidermal pavement cells. Epidermal peels stained with iodine showed starch deposits confined to chloroplasts in the guard cells (Figure 1d). This, together with the localization of autofluorescence in guard cells across the epidermis (Figure 1e), indicates negligible contamination of epidermal peels with chloroplasts from the underlying mesophyll.
Figure 1. (a) Net CO$_2$ uptake (purple) and photosynthetic photon flux density (PFD) (black) measured over the 24-h light/dark cycle for leaf 6 of *Kalanchoë fedtschenkoi* and indicating the time points used for sampling (with three biological replicates each) of mesophyll and epidermis: 00:00 h (dark), 04:00 h (dark), 08:00 h (dark), 12:00 h (light), 16:00 h (light), and 20:00 h (light). (b–d) Confocal microscopy images of epidermis where Rhodamine 123 staining indicates the presence of mitochondria (green staining) in both guard cells (b) and subsidiary cells (c), whereas autofluorescence (red coloring) of chlorophyll is evidence of chloroplast localization in guard cells (b, d). Iodine staining of epidermis shows the location of starch grains confined to guard cells and indicating negligible contamination from mesophyll chloroplasts (also seen in e). The epidermis was sampled from leaf pair 6 during the first 1–2 h of the photoperiod. Scale bars: 2 μm (b), 15 μm (c), 50 μm(d) and 200 μm (e).
Protein expression in *K. fedtschenkoi* epidermis and mesophyll

Some 41,504 non-redundant distinct peptide sequences were identified across the entire data set at a false discovery rate (FDR) of <1% at the peptide level, and those peptides mapped to 9,102 *K. fedtschenkoi* proteins (Tables S1, S2). The high degree of protein sequence redundancy in plant proteomes (e.g. alternative transcription, protein families, etc.) leads to several proteins being identified by the same set of tryptic peptides. To address this ambiguity, we assessed and report protein groups, which are created by clustering proteins together that are >90% similar in sequence identity. Overall, this approach resulted in an average identification of 5,002 proteins and 2,718 protein groups per epidermis sample, and an average of 3,583 proteins and 1,973 protein groups identified per mesophyll sample (Figure 2a). In general, there was an approximately 60% overlap in protein identifications between epidermis and mesophyll proteomes, with a substantially higher number of proteins unique to the epidermis when compared with the mesophyll (Figure 2b). Multivariate partial least squares (PLS) analyses found a clear distinction between the epidermis and mesophyll proteomes identified in the first component (Figure 2c) and a discrete grouping of the biological replicates. The average Pearson coefficient for biological reproducibility was 0.92 and 0.91 for the epidermis and the mesophyll, respectively. The average Pearson coefficient between the epidermis peel and the mesophyll was 0.74. Together, these general overviews indicate that the epidermis and the mesophyll have unique proteomes. Furthermore, there are underlying differences in the proteome across the diel period (PLS components 2 and 3), which has been observed in other CAM plants (Abraham et al., 2016).

Across the proteins that were quantified, the dynamic range of the relative protein abundances spanned six orders in the epidermis and mesophyll proteomes, and in both proteomes a small fraction (approx. 3–4%) of the total proteins account for 50% of the total protein biomass (Figure S1a). We observed greater coverage of low-abundance proteins in the epidermis (Figure S1c), suggesting a more functionally diverse set of proteins expressed in this tissue compared with the mesophyll, which is dominated by proteins required for photosynthesis. As low-abundance proteins are sampled more stochastically, we implemented a limit of quantitation (LOQ) threshold to remove proteins accounting for <1% of the total observed protein intensity in the epidermis and mesophyll data sets (Table S3). Enforcing this criterion removed a substantial number of quantifiable proteins from the epidermis data set and had a lesser effect on the mesophyll data set (Figure S1b,d). The filtered data set will be used for all subsequent analyses because it allows for a more robust comparison of
relative protein abundances between the epidermis and mesophyll data sets, where both have approximately the same number of quantifiable proteins.

Figure 2. (a) Average number of identified proteins (black) and protein groups (grey) for the epidermis (EP) and mesophyll (M). For two time points, we measured an upper epidermal peel (EP12U and EP00U) and lower epidermal peel (EP12L and EP00L). Error bars represent the standard deviation across biological triplicates. (b) A Venn diagram illustrates the level of overlap observed in protein and protein group (in parentheses) identifications between the epidermis and mesophyll data sets. (c) Plot illustrating results from a three-component partial least squares (PLS) analysis. (d) A heat map contrast of calculated Pearson correlation coefficients to identify the level of similarity between samples across the entire experiment. Three companion scatterplots were arbitrarily chosen to provide a more detailed illustration of correlations in relative protein abundance between EP versus EP, EP versus M and M versus M.

High-abundance proteins have different functional roles in epidermis and mesophyll

The median protein abundance distributions of the epidermis and the mesophyll proteomes were characterized to identify the cellular processes and functions that are expressed at high protein abundance levels. When logarithmically transformed (Figure S2a), distributions were similar between the epidermis and mesophyll data sets and the high-abundance proteins, 10th decile, contain 459 and 419 proteins, respectively.
For the highest-abundance proteins within the epidermis data set, the gene ontology (GO) term 'response to cadmium ion' was the most significantly enriched process observed, and other significantly enriched stress response-related terms had a higher representation of associated proteins in the epidermis compared with the mesophyll data set (Figure S2b). For the mesophyll cells, ‘photosynthesis’ was the most significantly enriched GO process and energy-related GO terms were more represented in the mesophyll 10th decile when compared with the epidermal data set. Overall, these results suggest that the top 10% most abundant proteins in the epidermis prioritize stress-response processes, a finding that supports the concept of the epidermis as the first point of contact for the perception of changes in aboveground environmental conditions.

Comparing relative protein abundances between epidermis and mesophyll

In total, 3313 proteins showed differences in abundance between the epidermis and mesophyll data sets (Figures S3 and S4; Table S4). We performed a GO enrichment test on proteins with higher relative abundances in the epidermis peel (Figure 3; Table S5) or in the mesophyll (Figure 4; Table S6), and summarize these findings in a network to highlight the major functional groups that were observed. For the epidermis, we identified 990 significantly enriched GO terms and used CLUEGO (Bindea et al., 2009) that identified 11 major functional groups enriched in the epidermis (Figure 3b). These included proteins involved in cuticle and cell-wall biosynthesis, transport, respiration, defense and signaling. At this high level, these functional modules are similar to those found expressed in the epidermis of other plants (Suh et al., 2005; Mintz-Oron et al., 2008). For proteins with significantly higher abundances on average in the mesophyll, we identified six major functional groups (Figure 4b). These included proteins involved in photosynthetic electron transport, photophosphorylation and ATP synthesis, the Calvin cycle, nitrogen metabolism and chloroplast biogenesis, and UV-related stress response proteins.
to create a functionally organized GO term network

Figure 3. (a) Leaf diagram highlighting cells present in the epidermis proteome analysis. (b) CLUEGO network of gene ontology (GO) terms that were significantly enriched in the subset of proteins that were enriched in epidermal proteome. Each node represents an enriched GO term and edges represent functionally associated GO terms based on Kappa statistics. For each large subnetwork, major representative functions are shown. (c) A representative list of proteins and functional categories for the top 25% most significantly enriched proteins in the epidermis data set.

To provide a more detailed understanding of the proteins contained within these networks we manually interrogated the functional role and cellular implication for the top 25% most significantly enriched proteins identified between epidermis and mesophyll by leveraging resources from the TAIR database (Lamesch et al., 2012) using BLAST (Mount, 2007) (Table S4). Information from the A. thaliana orthologs, and plausible functional roles for these proteins, are highlighted (Figures 3c and 4c).
In general, proteins involved in the biosynthesis of the cuticle and cell wall were found to have the highest abundance differences between the epidermis and the mesophyll proteomes (Figure 3c). The functional role of the protein with the greatest enrichment in the epidermis (GDL15, Kaladp0351s0002.1, fold change 701, -log10 P value 34.34, ortholog of AT1G29670.1), compared with the mesophyll, has been implicated in the catalysis of acyltransferase or hydrolase reactions with lipid and non-lipid substrates (Akoh et al., 2004). We also identified several proteins with established roles in cuticle and cell wall formation, as well as proteins implicated in determining the biomechanical properties of the cell wall (Figure 3c).

Also enriched in the epidermis (Figure 3c) were proteins involved in the synthesis of secondary metabolites such as triterpenoids, phenylpropanoids and flavonoids, which are known to influence biotic interactions, have important medicinal (anti-inflammatory) properties in many species of Kalancheë (Costa et al., 2008) and can attenuate UV-B irradiation and heat damage (Hahlbrock and Scheel, 1989).

In general, carbon flux through mitochondria in the epidermis of K. fedtschenkoi appeared to be enhanced relative to that in the mesophyll, as indicated by significant
enrichments in numerous mitochondrial proteins. These included diverse mitochondrial transport proteins, proteins of the tricarboxylic acid cycle (TCA) cycle and proteins required for the c-aminobutyrate (GABA) shunt pathway, which provides an alternative carbon source for the TCA cycle, particularly if pyruvate supply becomes limiting (Bouche and Fromm, 2004). Mitochondria are concentrated within the guard cells and subsidiary cells of the *K. fedtschenkoi* epidermis. Thus, like the stomata of C3 plants, CAM stomata are similarly set up to supply extra energy for guard cell metabolism and ion fluxes via increased capacity for oxidative phosphorylation (Fricker and Willmer, 1996; Araujo et al., 2014).

Enhanced respiratory activity in the epidermis relative to the mesophyll was also supported by the enrichment of proteins implicated in cell redox homeostasis in the epidermis (Figure 3c). Of these, the protein with the greatest fold change in epidermis relative to mesophyll was a glutathione S-transferase (GSTF8, Kaladp0044s0060.1.p, fold change 340, -log10*P* value 28.48, ortholog of AT2G47730). This protein also showed dynamic diel changes in abundance in the epidermis, peaking in the middle of the photoperiod (Figure 5, cluster 4). A chloroplastic location for this GST ortholog in *K. fedtschenkoi* implies a potential role in guard cell redox metabolism and/or signaling.

Several plasma membrane intrinsic proteins (PIPs) were found to be significantly enriched in the epidermis relative to the mesophyll of *K. fedtschenkoi* (e.g. PIP1:4, Kaladp0959s0007.1.p, fold change 110, -log10*P* value 9.05, ortholog of AT4G0043; PIP2:5, Kaladp0099s0104.1.p, fold change 41, -log10*P* value 13.86, ortholog of AT3G54820). PIPs are crucial for mediating the hydraulic flow necessary for stomatal opening (Oparka and Roberts, 2001) and can also enhance the permeability of CO₂ across the plasma membrane and the chloroplast envelope, thereby increasing the effectiveness of rubisco and potentially influencing transpiration efficiency (Heinen et al., 2009; Groszmann et al., 2017).

**Stomatal regulation and signaling in the epidermis proteome**

Among the proteins most enriched in the epidermis compared with the mesophyll, we identified several proteins with known roles in regulating stomatal behavior (Figure 3c). These included a number of membrane-localized transporters such as PLASMA MEMBRANE PROTON ATPases (PMA11). Of particular interest were *K. fedtschenkoi* orthologs of AHA2 and AHA8, which peaked in protein abundance towards the end of the photoperiod and over the first part of the night (Figure 5, cluster 8; Table S4). In
Arabidopsis, AHA1 and AHA2 induce the hyperpolarization of the guard cell plasma membrane and allow K⁺ uptake through inward-rectifying K⁺ channels, thereby inducing guard cell swelling and stomatal opening (Ueno et al., 2005). The diel patterns of protein abundance for the *K. fedtschenkoi* AHA orthologs described here suggest a key role for these proteins in mediating nocturnal stomatal opening, which requires future functional analysis. Proteins implicated in guard cell signaling that were enriched in the epidermis included orthologs of phospholipase D (PLD) and aspartic protease (ASPG1), which function through abscisic acid (ABA) signaling in guard cells (Yao et al., 2012). We also identified orthologs of several calcium signaling proteins that were enriched in the *K. fedtschenkoi* epidermis (Figure 3c; Table S4). In particular, an ortholog of CPK21 (AT4G04720.1, Kaladp0040s0351.1.p, fold change 8, -log10 P value 10.94) showed a diel change in abundance, peaking during the day (Figure 5, cluster 1). In Arabidopsis, CPK21 has been shown to mediate the phosphorylation and activation of the S-type anion efflux channel SLAC1, which mediates stomatal closure.
The elevated protein abundance of this ortholog during the day in the epidermis of *K. fedtschenkoi* highlights a possible role for calcium-dependent protein kinases in mediating the daytime stomatal closure that defines CAM.

An ortholog of the sucrose non-fermenting receptor kinase 2.6, also known as Open Stomata 1 (OST1), was detected in the epidermis (but not in the mesophyll) of *K. fedtschenkoi* (Table S4). OST1 is activated in the presence of ABA and promotes anion and water efflux, the inhibition of K⁺ influx and stomatal closure (Grondin *et al*., 2015). In the CAM species *A. americana* and *K. laxiflora*, OST1 exhibits a diel rescheduling of transcript abundance to the day, compared with the night in Arabidopsis (Abraham *et al*., 2016; Boxall *et al*., 2020), consistent with a putative role for this protein in stomatal closure. The OST1 protein in *K. fedtschenkoi* did not show any detectable change in diel abundance, however. This finding reinforces the view that diel changes in transcript abundance do not necessarily reflect a change in protein abundance or activity. Future interrogation of this proteomics data set alongside complementary transcriptomics data sets for the *K. fedtschenkoi* epidermis will be valuable for revealing the layers of transcriptional and post-transcriptional control of membrane transport and signaling components that underpin CAM stomatal behavior.

**Diel protein abundance profiles in epidermis and mesophyll**

Crassulacean acid metabolism (CAM) differs from C3 and C4 photosynthesis in terms of the diel rescheduling of metabolism and stomatal regulation, so we sought to provide a proteome-wide perspective on which proteins display significant changes in their diel abundance profiles. We used *k*-means clustering to capture orchestrated co-abundance responses in the epidermal and mesophyll proteomes. We identified 691 and 371 protein abundance patterns in the epidermal peel and mesophyll proteomes, respectively, with P < 0.05, that showed at least a twofold change between two time points across the diel cycle (Tables S7 and S8). Using *k*-means and the gap statistic method (Tibshirani *et al*., 2001), proteins were grouped into 10 and 14 major clusters based on the similarity of expression patterns identified for the epidermal (Figure 5) and mesophyll (Figure 6) data sets, respectively. To detect functional specialization within each cluster, we cross-referenced the top protein blast result with its corresponding entry in the TAIR gene ontology database and quantified the frequency of each GO phrase using controlled vocabularies, where frequency refers to the number of phrases in each cluster (Berardini *et al*., 2004) (Tables S9 and S10). The three most over-represented GOs for each cluster are shown in Figures 5 and 6.
For the epidermis, the largest protein cluster consisted of proteins with a significant decrease in abundance during the middle of the night (Figure 5, cluster 1). Within this cluster, ‘translation’ and ‘RNA methylation’ were the top two most represented GO groups, and we also observed many ribosomal proteins (n = 24), suggesting an enhanced protein turnover in the epidermis, which may be linked to high respiratory activity and the turnover of reactive oxygen species (ROS) in this tissue.
as discussed above. A key aim was to provide insight on cellular behavior underpinning CAM stomatal behavior. Proteins within epidermal cluster 8, which showed abundance patterns that anticipated late photoperiod and nocturnal opening of stomata (Figure 1a), were of particular interest. The most represented GO groups within cluster 8 were ‘response to salt stress’ and ‘MAP Kinase cascade’, and the most represented GO locations within this cluster were the plasma and vacuolar membranes. Proteins found within this cluster included several plasma membrane proton ATPases, including orthologs of AHA2 and AHA8, vacuolar proton pyrophosphatases (orthologs of AVP1, AT1G15690), a vacuolar proton ATPase (ortholog of VHA, AT4G23710) and a putative voltage-gated potassium channel subunit (ortholog of KAB1, AT1G04690). The diel patterns of abundance of these proteins is consistent with the hypothesis that stomatal opening towards the end of the day and into the night requires the energization of plasma membrane and tonoplast proton pumps, as well as the activation of pathways for K\(^+\) uptake at the plasma membrane and tonoplast. Also found within cluster 8 were several MAP kinases (Kaladp0046s0065.1 and Kaladp0046s0065.3, orthologs of AT4G29810). MAP kinase signaling cascades have been implicated in CO\(_2\) signal transduction for stomatal movement in Arabidopsis (Zhang et al., 2018). The proteins described above, together with others found within cluster 8, which include proteins implicated in sphingolipid metabolism, RAS-related GTP binding and mitochondrial respiratory electron transport, represent important candidates for future functional testing of signaling and metabolic pathways, as well as ion transporters important for CAM stomatal regulation.

The major temporal cluster for the mesophyll proteome contained proteins that increased in abundance during the day and decreased during the night (Figure 6, cluster 1). The most represented GO groups within this cluster were ‘response to salt stress’, ‘photosynthesis light reactions’ and ‘response to water deprivation’. In general, this cluster included proteins belonging to photosystems, light-harvesting complexes, the maintenance of transmembrane electrochemical gradients, the Calvin–Benson cycle and photorespiration, as well as heat-shock proteins. Cluster 6 contained mesophyll proteins that showed a reciprocal diel pattern of protein abundance to that of cluster 1, i.e. proteins that showed the greatest abundance during the night. The most represented GO groups within this cluster included stress-responsive proteins, proteins implicated in defense and, intriguingly, in the light reactions of photosynthesis. Of particular note were proteins implicated in redox homeostasis (e.g. glutathione peroxidase, thioredoxin and ferredoxin). This finding supports the emerging view of the need for antioxidant activity to deal with the ROS generated by high rates of
CHAPTER VII

respiratory electron transport that occur at night in CAM plants (Abraham et al., 2016; Shameer et al., 2018).

Characterization of proteins implicated in the diel carboxylation and decarboxylation processes of CAM

A key aim of our study was to explore the hypothesis of tissue-specific regulation of metabolic processes underpinning diel turnover of malate and starch within the mesophyll and the epidermis. To provide context for these analyses we measured diel changes in malate (Figure 7a), as well as starch, in both mesophyll and epidermal peels (Figure 7b). Nocturnal accumulation and daytime mobilization of malic acid was evident in the mesophyll as well as in the epidermis peel, but on a fresh-weight basis, diel turnover of malate was over eight times higher in the mesophyll cells compared with the epidermal peels.

To sustain nocturnal CO₂ uptake and malic acid accumulation, K. fedtschenkoi mobilizes starch for the nocturnal provision of PEP, and within the mesophyll starch was steadily and almost completely degraded overnight and subsequently resynthesized during the day (Figure 7b). Substantial starch deposits were observed within the guard cells of K. fedtschenkoi (Figures 1 and 7b), but diel turnover of guard cell starch was significantly dampened compared with that of the mesophyll cells (Figure 7b). Importantly, the daytime accumulation of starch within the guard cells of K. fedtschenkoi is in marked contrast to that reported for the C3 model Arabidopsis, where starch in guard cells is broken down almost completely within the first hour of the photoperiod, potentially to generate malate and/or sugars, which provide the energy and osmolytes required to open C3 stomata at the start of the day (Horrer et al., 2016). Studies have shown that gluconeogenesis-mediated starch synthesis removes malic acid from guard cells, which can promote stomatal closure (Schnabl, 1980). Although the localization of malate in the epidermal peels cannot be definitely reconciled to the guard cells, the observed reciprocal relationship between daytime starch accumulation in the guard cells and daytime depletion of malate in the epidermis (Figure 7) is consistent with this hypothesized role for starch and malate metabolism in stomatal regulation.

Distribution of proteins responsible for C4 carboxylation between epidermis and mesophyll

Many of the proteins required for CAM are encoded by multigene families, suggesting that different isoforms responsible for nocturnal carboxylation and daytime
decarboxylation might carry out specific roles within the mesophyll and epidermis, with those isoforms present in the epidermis having implications for stomatal regulation (Figure S5).

![Graph](image)

Figure 7. (a) Day/night changes in malate content in the epidermis and mesophyll; (b) quantification of starch content within the guard cells as starch granule area for epidermal peels taken from the upper leaf surfaces. Fluorophore propidium iodide and confocal laser scanning microscopy illustrate starch granules within closed (day) and opened (night) stomata. Day/night changes in starch content measured in leaf mesophyll following enzymatic digestion and colorimetric assay determination. Each point is the mean of three biological replicates ± standard deviation of the mean.

β-Carbonic anhydrase (BCA) catalyzes the hydration of CO$_2$ to HCO$_3^-$, the substrate for nocturnal carboxylation in CAM. The most abundant protein accessions of BCA in mesophyll and epidermis were Kaladp0018s0289.1.p, ortholog of Arabidopsis BCA2, and Kaladp0538s0011.1.p, ortholog of Arabidopsis BCA1. The kfBCA1, which has been previously reported as the CAM-specific BCA on the basis of increased transcript...
abundance at night (Yang et al., 2017), showed significant diel changes in protein abundance in the epidermis (Figure 5, cluster 2) but not in the mesophyll. A third kfBCA (Kaladp0081s0140, ortholog of BCA5 in Arabidopsis) was only detected in the epidermis and had a protein abundance pattern that peaked in concert with the maximal rates of nocturnal net CO$_2$ uptake and stomatal conductance (Figures 1 and S5). This kfBCA5 is predicted to localize to the chloroplast and thus the guard cells of K. fedtschenkoi. In Arabidopsis, mathematical modelling has indicated a central role for guard cell-localized BCAs in mediating intracellular HCO$_3^-$ concentration change, which is a key mechanism in mediating CO$_2$-regulated stomatal movement (Hu et al., 2010; Hu et al., 2015). Further functional testing is required to establish whether the epidermal- enriched kfBCA5 isoform has a role in mediating the diel changes in stomatal movement that accompany the substantial changes in pCi across the day/night phases of CAM.

Phosphoenolpyruvate carboxylase catalyzes the carboxylation of PEP, which leads to nocturnal CO$_2$ uptake and malate accumulation. The PEPC protein accession with the highest relative abundance in both tissues (Kaladp0095s0055.1.p) matches the PEPC1 protein sequence in K. laxiflora, which is essential for CAM (Boxall et al., 2020) (Figure S5). For all the kfPEPC proteins identified, we did not observe significant differences in abundance between the epidermal and mesophyll proteomes. We identified phosphorylation modifications for the most abundant kfPEPC1 protein, which confirmed the same phosphorylation modification at the N-terminus (Ser-13) in both the epidermal and mesophyll data set (Figure 8; Table S11). Averaging the relative abundance of the phosphopeptides identified revealed that kfPEPC1 is phosphorylated during the night and dephosphorylated during the day in both the mesophyll and epidermis proteomes. This diel pattern of PEPC phosphorylation, which is the inverse of that found in C3 plants, is critical for the temporal/circadian control of carboxylation processes operating within the leaf mesophyll of CAM plants (Boxall et al., 2017). Nocturnal phosphorylation of PEPC in the CAM stomatal complex, as indicated by our data, is in marked contrast to the daytime phosphorylation of PEPC reported for C3 guard cells (Wang et al., 1994), and could protect the CAM enzyme against inhibition by malate during guard cell swelling and stomatal opening at night.

**Distribution of proteins responsible for malate decarboxylation between mesophyll and epidermis**

Daytime decarboxylation of malate in the mesophyll of K. fedtschenkoi is catalyzed by mitochondrial NAD malic enzyme (Dever et al., 2015). Protein accessions for several isoforms of NAD-ME were identified (Kaladp0033s0124.1.p, ortholog of AT2G13560.1
NAD ME1; Kaladp0037s0467.1.p ortholog of AT4G00570.1 NAD ME2): kfNAD ME1 had comparable abundance in the mesophyll and the epidermis, but kfNAD ME2 was significantly more abundant in the epidermis (Figure S5). Diel changes in kfNAD ME1 and kfNAD ME2 abundances were only evident in the mesophyll (clusters 4 and 13, Figure 6), and increased kfNAD ME1 abundance in the middle of the day corresponded with the period of maximal malate decarboxylation. Both kfNAD ME1 and kfNAD ME2 also peaked in abundance over the latter part of the dark period, which could indicate the breakdown of malate to provide pyruvate for mitochondrial respiration at night (Holtum et al., 2005).

Protein accessions for cytosolic/chloroplastic NADP ME were also identified (Kaladp0046s0046.2.p, Kaladp0102s0114.1.p and Kaladp0024s0016.1.p, orthologs of AT5G25880.1 and NADP-ME3, and Kaladp0092s0166.1.p and Kaladp0045s0427.1.p, orthologs of AT1G79750.1 and NADP-ME4; Table S4); all of these were more abundant in the epidermis, suggesting functional diversification in malate metabolism within the cells of the epidermis compared with the mesophyll. In addition to a role in CAM-related malate turnover, cytosolic NADP-MEs can control cytosolic pH (Martinoia and Rentsch, 1994; Lai et al., 2002) and turgor pressure in guard cells (Outlaw et al., 1981; Maurino et al., 1997; Laporte et al., 2002), whereas chloroplastic NADP-ME has been implicated in lipid biosynthesis (Wheeler et al., 2005). Together, our data highlight contrasting roles for specific NAD and NADP ME isoforms in the CAM mesophyll and epidermis.

Distribution of proteins responsible for pyruvate processing between mesophyll and epidermis

Pyruvate orthophosphate dikinase (PPDK) converts pyruvate generated from malate decarboxylation back to PEP for subsequent gluconeogenic processing and the production of sucrose and/or starch. We identified protein accessions for two PPDKs: Kaladp0076s0229 (ortholog of AT4G15530) and Kaladp0039s0092 (ortholog of AT4G15530), with Kaladp0076s0229 having enriched abundance within the mesophyll (Figure S5). PPDK activity in K. fedtschenkoi is subject to post-translational control via reversible phosphorylation (Dever et al., 2015). Phosphopeptide abundance in the most abundant PPDK protein indicated that the phosphorylation site was localized to a target residue, Ser-455 and/or Thr-454, similar to the target location of dephosphorylation/phosphorylation of PPDK in the C4 species Zea mays (Thr-456) (Figure 8; Table S11) (Chastain et al., 1997). The diel pattern of phosphopeptide abundance we observed is consistent with a previous report of phosphorylation/inactivation of PPDK during the dark in K. fedtschenkoi (Dever et al.,
2015), although diel changes in phosphopeptide abundance were more marked in PPDK from the mesophyll, compared with epidermal PPDK (Figure S5). PPDK phosphorylation is catalyzed by PPDK regulatory protein (PPDK RP), an ortholog of which was significantly more abundant in the mesophyll (Kaladp0060s0363.1. p, fold change 9.4, -log10 P value 4.91).

This PPDK-RP showed increased diel abundance at the end of the photoperiod and for most of the dark period (Figure 6, cluster 9), which is consistent with diel changes in phosphorylation/ inactivation of PPDK required for CAM. Together, the data suggest that the enzymatic capacity for daytime recovery of PEP from malate decarboxylation are higher in the mesophyll compared with the epidermis of *K. fedtschenkoi*.

Our data highlight other differences between the mesophyll and the epidermis in terms of pyruvate processing (Figure S6). Pyruvate kinase (PK) catalyzes the final

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**Figure 8.** Phosphorylation of phosphoenolpyruvate carboxylase (PEPC) and Pyruvate orthophosphate dikinase (PPDK). (a) For each time point, the average relative abundance of all identified phosphopeptide variants (i.e. sequence, post-translational modifications and charge state) for PEPC are illustrated for the epidermis (red) and mesophyll (blue) data sets. The error bars represent the standard variation across three biological replicates. The amino acid position on each peptide for localized phosphorylation modifications (79.97; STY) and methionine oxidation (15.99; M) are shown. (b) Homology-model structure for PEPC with the localized phosphorylation site at Ser-13 near the center of the homotetramer. (c) For each time point, the average relative abundance of all identified phosphopeptides variants (i.e. sequence, post-translational modifications, and charge state) for PPDK are illustrated for the epidermis (red) and mesophyll (blue) data sets. The error bars represent the standard variation across the three biological replicates. The amino acid position on each peptide for localized phosphorylation modifications (79.97; STY) and methionine oxidation (15.99; M) are shown. (d) Homology-model structure for PPDK with the localized phosphorylation sites at Thr-454 and Ser-455.
reaction of the glycolytic pathway, converting ADP and PEP to ATP and pyruvate; an ortholog of cytosolic PK (Kaladp0039s0147.2.p, fold change 45, -log10 P value 19.35, ortholog of AT5G56350.1) was significantly enriched in abundance in the epidermis. Elevated PK activity in the epidermis implies enhanced pyruvate supply to mitochondria for ATP production in the subsidiary and guard cells, compared with that in the mesophyll, whereas reduced activity of PK in the CAM mesophyll at night would be important for directing glycolytic flux towards PEP as a substrate for PEPC (Holtum et al., 2005). Further indication of the importance of pyruvate metabolism in the epidermis of *K. fedtschenkoi* is indicated by marked diel abundance changes in pyruvate decarboxylase (PDC2, Kaladp0055s0499.1.p, ortholog of AT5G54960), a protein heavily enriched in the epidermis (Figure S6). In C3 plants, PDC has been implicated in drought tolerance by stimulating the jasmonate (JA) signaling pathway (Kim et al., 2017). The diel abundance pattern of PDC in *K. fedtschenkoi* is similar to that observed for the PDC2 protein ortholog in the CAM species *A. americana* (Abraham et al., 2016), suggesting an important role for PDC in CAM that requires further testing.

*Distribution of proteins implicated in diel starch turnover between mesophyll and epidermis*

We also probed whether contrasting functions for starch turnover in the mesophyll and the guard cells of *K. fedtschenkoi* would be reflected in the profiles of proteins implicated in starch metabolism within the two proteomes (Figure S7). The most abundant starch degrading protein in both mesophyll and epidermis proteomes was an ortholog of plastidic α-glucan phosphorylase (PHS1). This finding is consistent with suggestions that CAM shows a re-routing of chloroplastic starch degradation from the C3 amyloolytic route to the phosphorolytic route catalyzed by PHS1, which can result in the production and export of glc-6-P from the CAM chloroplast (Borland et al., 2016; Shameer et al., 2018; Ceusters et al., 2019). Orthologs of α-amylases 1 and 3 (AMY1 and AMY3) were also found with comparable abundances in the mesophyll and the epidermis. Studies on Arabidopsis have indicated that although AMYs are not essential for starch degradation in the leaf mesophyll, in the guard cells starch degradation is mediated by AMY3 acting in conjunction with β-amylase 1 (BAM1) (Horrer et al., 2016). Two BAM orthologs, BAM2 and BAM9, were detected in the *K. fedtschenkoi* proteome. BAM9 was the most abundant BAM in both the epidermis and the mesophyll and showed a distinctive diel change in abundance in both the mesophyll and the epidermis, with the peak abundance noted in the middle of the night. Evidence for a catalytic role for BAM9 is currently lacking but a regulatory role
in starch degradation has been suggested (Monroe and Storm, 2018). BAM2 was only
detected in the epidermal proteome and showed a strong diel change in abundance,
peaking towards the end of the photoperiod coincident with the timing of guard cell
starch degradation (Figure 7b). BAM2 was recently shown to be catalytically active in
the presence of physiological concentrations of KCl in Arabidopsis (Monroe et al.,
2018), suggesting a possible role in guard cell starch degradation. Two proteins
implicated in the cytosolic processing of starch degradation products showed
significantly higher abundance in the K. fedtschenkoi epidermis compared with the
mesophyll (Figure S7). In Arabidopsis mesophyll cells, the amylolytic breakdown of
starch in the chloroplast produces maltose, which is exported and metabolized via a
transglucosylation reaction catalyzed by disproportionating enzyme 2 (DPE2). This
reaction produces a cytosolic heteroglycan intermediate, which is acted on by cytosolic
α-glucan phosphorylase (PHS2) to produce glc-1-P (Malinova and Fettke, 2017). The
elevated abundance of the K. fedtschenkoi orthologs of DPE2 and PHS2 in the
epidermis provides further indication of a possible divergence in the route and
regulation of starch breakdown in the guard cells and mesophyll of this CAM species.
Sustained accumulation of guard cell starch in K. fedtschenkoi over the first part of the
photoperiod is in marked contrast to the almost complete mobilization of starch in
Arabidopsis guard cells at the start of the day. The substantial starch deposits found
in the CAM guard cells imply a critical role for starch synthesis in CAM stomatal
function. An analogy can be found in the suggestion that in Arabidopsis, guard cell
starch synthesis plays an essential role in CO₂-induced stomatal closure by acting as
a sink for C-skeletons coming from malate degradation via gluconeogenesis during
guard cell osmotic adjustment (Azoulay-Shemer et al., 2016). We compared proteins
implicated in gluconeogenesis and starch synthesis between epidermal and mesophyll
proteomes and identified an ortholog of pyrophosphate-dependent
phosphofructokinase (PFP), which showed an above-average relative abundance in
the epidermal proteome yet was not detected in the mesophyll (Figure S6). Acting in
the direction of gluconeogenesis, PFP provides fru-6-P for conversion to glc-6-P,
which could be imported to the guard cell chloroplast via the plastidic glc- 6-P
translocator (GPT) for subsequent starch synthesis via ADP glucose
pyrophosphorylase (AGPase). Further work is required to establish the functional
significance of the proteins identified above in terms of guard cell starch metabolism
and CAM stomatal function.
CONCLUSION

This study provides a quantitative understanding of biological phenomena occurring at the protein level in guard cell-enriched epidermis and mesophyll from leaves of the constitutive model CAM plant *K. fedtschenkoi*. Our data indicate tissue-specific specialization of isozymes implicated in malate and pyruvate processing and in starch turnover. These findings are discussed in line with contrasting roles for these metabolites within the CAM mesophyll and stomatal complex. A key finding to emerge was the diel rescheduling of guard cell starch turnover in *K. fedtschenkoi* compared with that observed in Arabidopsis. Growing recognition of the importance of primary carbon metabolism in regulating stomatal movements in C3 plants (Santelia and Lawson, 2016) indicates a pressing need for a better understanding of metabolism within CAM guard cells. In turn, such information will be essential for establishing whether the bioengineering of CAM into non-CAM hosts will require a re-wiring of guard cell metabolism.
EXPERIMENTAL PROCEDURES

Plant materials
*Kalanchoë fedtschenkoi* wild-type (WT) plants were grown in a growth chamber with a 12-h photoperiod, with a photosynthetic photon flux density (PPFD) of 250 μmol m⁻² s⁻¹ at plant height and with day/night temperatures of 25°C/18°C. Leaf pair 6 (where pair 1 are the youngest leaves growing at the apical zone of the plant) of 12-week-old plants were selected for analysis. The leaf was divided into epidermis tissue and ground mesophyll (i.e. samples ‘EP’ and ‘M’, respectively). For sampling purposes, the leaf tip was bent over, which allowed the epidermis to be rapidly and cleanly peeled away from the mesophyll before being snap frozen in liquid nitrogen. Each biological replicate consisted of epidermal peels taken from the abaxial and adaxial surfaces of leaf pair 6 from one plant. The epidermis and mesophyll were sampled over a 24-h day/night cycle using three biological replicates for each time point (i.e. 08:00 h, dark; 12:00 h, light; 16:00 h, light; 20:00 h, light; 00:00 h, dark; 04:00 h, dark; within the growth chamber, lights came on at 08:30 h and went off at 20:30 h). All samples were immediately snap frozen in liquid nitrogen and stored at -80°C until their evaluation.

Structural characteristics of epidermal peels
The presence and distribution of chloroplasts and mitochondria within cell types present in the epidermal peels was assessed using confocal microscopy and mitochondrial staining. Epidermal peels were incubated in 0.1 M HEPES buffer, pH 7, for 5 min, followed by staining with 10 μg ml⁻¹ of Rhodamine 123 (cat. no. R8004; Sigma-Aldrich, https://www.sigmaaldrich.com) in the dark at room temperature (20–22°C) for 30 min. Peels were subsequently washed with two changes of 0.1 M HEPES buffer, pH 7, transferred to microscope slides and observed on a LEICA SP8 STED 3X microscope with excitation λ of 511 nm. Chloroplast localization was based on autofluorescence of chlorophyll at an excitation λ of 598 nm.

Malate and starch content
Malate was extracted from leaf mesophyll and epidermal peels as described by Haider *et al.* (2012) and measured using the enzymatic method described by Hohorst (1970). The starch content of the leaf mesophyll was determined as described by Haider *et al.* (2012). Guard cell starch content was determined by fixing epidermal peels in 50% v/v methanol, 10% v/v acetic acid. Starch granules were stained with a pseudo-schiff propidium iodide and subsequently analyzed using confocal laser scanning microscopy, as described by Flütsch *et al.* (2018).
Gas-exchange analysis
Net CO₂ uptake was determined for leaf number 6 (three biological replicates) over a 24-hr day/night cycle, using the LI-6400XT Portable Photosynthesis System (LI-COR Environmental, https://www.licor.com). Light and temperature within the leaf chamber were set to track the conditions established in the growth chamber, where all measurements were conducted (25°C/19°C and a diurnal PPFD of 250 µmol m⁻² s⁻¹ at plant height). The ambient CO₂ concentration was set at 400 µmol CO₂ mol⁻¹ and the relative humidity was maintained between 50 and 60%. Data were recorded every 15 min and plotted against time.

Protein extraction and digestion
Harvested epidermis and mesophyll tissues were suspended in sodium deoxycholate (SDC) lysis buffer (4% in 100 mM of NH₄HCO₃, 10mM dithiothreitol, DTT). Samples were physically disrupted by bead beating before heating at 90°C for 5 min and then centrifuged to remove cellular debris. Cysteines were blocked by adjusting each sample to 30 mM indole-3-acetic acid (IAA) and incubated in the dark for 15 min at room temperature. Samples were transferred to a 10-kDa molecular weight spin column (Vivaspin 2; GE Healthcare, https://www.gehealthcare.com) and filtered. Proteins retained on top of the filter were washed with 100 mM of NH₄HCO₃ and then resuspended in 100 mM of NH₄HCO₃ to adjust samples to 2% SDC. Protein concentrations were estimated by performing a BCA assay (Pierce Biotechnology, now ThermoFisher Scientific, https://www.thermofisher.com). Each sample was digested via two aliquots of sequencing-grade trypsin (1:75, w:w; Promega, https://www.promega.com) at two different sample dilutions (overnight), and for a subsequent 3 h at 37°C. The peptide mixture was collected by centrifugation and then adjusted to 1% formic acid (FA) to precipitate SDC. Hydrated ethyl acetate was added to each sample at a 1:1 (v:v) ratio three times to effectively remove SDC. Samples were then placed in a SpeedVac Concentrator (ThermoFisher Scientific, https://www.thermofisher.com) to remove ethyl acetate and further concentrate the sample. The peptide-enriched flow was quantified by BCA assay, desalted on RP-C18 stage tips (Pierce Biotechnology, now ThermoFisher Scientific) and then stored at -80°C until analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

LC–MS/MS
All samples were analyzed on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) coupled with a Proxeon EASY-nLC 1200 liquid chromatography pump
(ThermoFisher Scientific). Peptides were separated on a 75-μm inner diameter microcapillary column packed with 25 cm of Kinetex C18 resin (1.7 μm, 100 Å; Phenomenex, https://www.phenomenex.com). For each sample, a 2-μg aliquot was loaded in buffer A (0.1% FA, 2% acetonitrile) and eluted with a linear 150-min gradient of 2–20% buffer B (0.1% FA, 80% acetonitrile), followed by an increase in buffer B to 30% for 10 min, another increase to 50% buffer B for 10 min and concluding with a 10 min wash with 98% buffer A. The flow rate was kept at 200 nl min⁻¹. MS data were acquired with XCALIBUR™ 4.27.19 (ThermoFisher Scientific), using the top-N method where N could be up to 15. Target values for the full scan MS spectra were 1 x 10⁶ charges in the 300–1500 m/z range, with a maximum injection time of 25 ms. Transient times corresponding to a resolution of 70 000 at m/z 200 were chosen. A 1.6 m/z isolation window and fragmentation of precursor ions was performed by higher energy C-trap dissociation (HCD), with a normalized collision energy of 30 eV. MS/MS scans were performed at a resolution of 17 500 at m/z 200 with an ion target value of 1 x 10⁶ and a maximum injection time of 50 ms. Dynamic exclusion was set to 45 s to avoid the repeated sequencing of peptides.

Peptide identification and protein inference

Raw MS data files were searched against the K. fedtschenkoi 1.1 proteome FASTA database appended with the predicted chloroplast and mitochondrial proteins, as well as common contaminates (e.g. trypsin, human keratin, etc.). A decoy database, consisting of the reversed sequences of the target database, was appended to discern the FDR at the spectral level (Elias and Gygi, 2007). For standard database searching, the peptide fragmentation spectra (MS/MS) were analyzed by the CRUX PIPELINE 3.0 (McIlwain et al., 2014). The MS/MS were searched using the Tide algorithm (Diament and Noble, 2011) and configured to derive fully tryptic peptides using default settings except for the following parameters: allowed n-term methionine, a precursor mass tolerance of 10 parts per million (ppm), a static modification on cysteines (iodoacetamide; +57.0214 Da) and dynamic modifications on methionine (oxidation; +15.9949). Results were processed by PERCOLATOR (Kall et al., 2007) to estimate the q values. Peptide spectrum matches and peptides were considered identified at a q value of <0.01. Across the entire experimental data set, proteins were required to have at least two distinct peptide sequences and two minimum spectra per protein. To deal with the redundancy associated with the K. fedtschenkoi proteome database, all proteins in the FASTA database were grouped by sequence similarity (≥90%) using the UCLUST component of the USEARCH 5.0 software platform (Edgar, 2010). As described previously (Abraham et al., 2012), grouping proteins by this
threshold of sequence identity serves to: (i) maintain biologically relevant peptide information that would have otherwise been lost through protein sequence redundancy; and (ii) eliminate ambiguity in peptide assignments.

**Label-free quantification**

The MS1-level precursor intensities were derived from modest feature finder (moFF, Argentini *et al.*, 2016) using the following parameters: 10 ppm mass tolerance; a retention time window for extracted ion chromatogram of 3 min; and a time window to get the apex for MS/MS precursor of 30 sec. Protein intensity-based values, which were calculated by summing together quantified peptides, were normalized by dividing by protein length and total ion intensities and then LOESS (locally estimated scatterplot smoothing) and median central tendency procedures were performed on log_{2}-transformed values by INFERNORDN (Polpitiya *et al.*, 2008).

**Statistical analysis for differential abundances**

We performed a pair-wise comparison between the collective epidermis (EP08, EP12, EP16, EP20, EP00, EP04) proteome and the collective mesophyll proteome (M08, M12, M16, M20, M00, M04). To improve the robustness of this quantitative analysis, we removed low-abundance and randomly sampled proteins by summing protein intensities across the collective epidermis proteome or mesophyll proteome and calculating a running sum from the most abundant protein to the least abundant protein. The cumulative percentage of each protein in each collective proteome was then used to identify a limit of quantitation (LOQ), which represents the bottom 1%. After filtering the data, a Student’s *t*-test was used to identify differences between average protein abundances in each collective proteome. Using PERSEUS (http://www.perseus-framework.org) (Tyanova *et al.*, 2016), missing values were replaced by random numbers drawn from a normal distribution (width = 0.3 and downshift = 2.5). A protein was categorized as having a significant abundance difference between the epidermis peel and mesophyll proteomes if it passed a significance threshold requiring a *P* value of ≤0.05 and absolute value of log_{2} fold-change difference of >1. Diel changes in protein abundances were tested by an analysis of variance (ANOVA) with post-hoc Tukey test and categorized as a significant change in abundance for *P* values of <0.05 and at least one absolute value of log_{2} fold-change difference of >1.


**Gene ontology enrichment**

Whole-genome GO term annotation was performed using BLAST2GO (Conesa et al., 2005) with a blastp E-value hit filter of $1 \times 10^{-5}$, an annotation cut-off value of 55 and a GO weight of 5. Using CLUEGO (Bindea et al., 2009), observed GO biological processes were subjected to the right-sided hypergeometric enrichment test at medium network specificity selection and $P$-value correction was performed using the Holm–Bonferroni step-down method (Holm, 1979). There was a minimum of three and a maximum of eight selected GO tree levels, and each cluster was set to include a minimum of between 3 and 4% of genes associated with each term. Minimal reporting of functional groups was achieved by implementing CLUEGO GO term fusion and grouping settings were selected to reduce GO term redundancy. The term enriched at the highest level of significance was used as the representative term for each functional cluster. The GO terms at adjusted $P \leq 0.05$ were considered significantly enriched.

**DATA AVAILABILITY STATEMENT**

The protein data and all supplementary tables have been deposited at ProteomeXchange (Vizcaino et al., 2014) and the accession is PXD010837.

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AUTHOR CONTRIBUTIONS

AMB, PEA, NHC and JB contributed to the conception and design of the experiments, PEA, NHC and SF contributed to the acquisition of data, PEA, NHC, AMB, DCY, SP, SF and DS contributed to data analysis and interpretation, AMB and PEA drafted the manuscript, and all authors critically revised and approved the final version of the manuscript for publication.
REFERENCES


Supplemental Figure 1. a) The distribution of cumulative protein intensities (i.e., running sum) across the entire epidermal peel and mesophyll illustrates the relative contribution of each protein from the most abundant to least abundant protein. b) The distribution of cumulative protein intensities (i.e., running sum) across the entire epidermal peel and mesophyll after removing the bottom 1% of quantitative information for both the epidermal peel and mesophyll total proteomes. The inset bar graph highlights the number of proteins available for differential analyses following this operation (yellow bar). c) Two-way hierarchical cluster (Fast Ward) of Log2 normalized protein abundances for the unfiltered data and d) filtered data in which the bottom 1% was removed.
Supplemental Figure 2: a) Distribution of median protein abundances across mesophyll or epidermal data was illustrated by a violin plot. The deciles are marked for each plot. A functional enrichment test for the most abundant proteins (10th decile; green) found in the mesophyll or epidermal layers highlights investments into two major GO functional processes. b) The 10% most abundant proteins cover more enriched GO terms with highest coverage in plant defense and cell fate signaling pathways for epidermal peels and photosynthesis and carbohydrate metabolism in mesophyll.

Supplemental Figure 3. Volcano plot for pairwise comparison between the epidermal (upper peel only) and mesophyll. Proteins that passed the required thresholds for significance are plotted. The proteins that were exclusively identified in the epidermal (red) and mesophyll (blue) are highlighted.
**Supplemental Figure 4.** Proteins that passed the ANOVA test were evaluated using the Gap statistic method for \( k \)-means to identify the optimal number of clusters for the a) epidermal peel dataset (red; dotted line \( n=10 \)) and b) mesophyll dataset (blue; dotted line \( n=14 \)). c) Number of proteins and % of total proteins from ANOVA tests is reported for each epidermal peel cluster and d) mesophyll cluster.
Supplemental Figure 5. C4 metabolism. The relative abundance of each protein across the diel cycle is shown for the epidermal (red) and mesophyll datasets as a line plot. ** represents proteins that passed the significance threshold from the ANOVA test. For each box plot, a protein’s average abundance in the epidermal and mesophyll is represented and "**" represents proteins that passed the maximum versus minimum t-test threshold.
Supplemental Figure 6. Glycolysis/gluconeogenesis. The relative abundance of each protein across the diel cycle is shown for the epidermal (red) and mesophyll datasets as a line plot. "**" represents proteins that passed the significance threshold from the ANOVA test. For each box plot, a protein's average abundance in the epidermal and mesophyll is represented and "*" represents proteins that passed the maximum versus minimum t-test threshold.
Supplemental Figure 7. Starch metabolism. The relative abundance of each protein across the diel cycle is shown for the epidermal (red) and mesophyll datasets as a line plot. "***" represents proteins that passed the significance threshold from the ANOVA test. For each box plot, a protein's average abundance in the epidermal and mesophyll is represented and "**" represents proteins that passed the maximum versus minimum t-test threshold.
Supplementary Tables are available online

**Supplemental table 1:** Verbose listing of peptides identified with a q-value < 0.01 that map to proteins having at least two distinct peptides. The quantitative value (i.e., moFF intensity) is provided per peptide per sample.

**Supplemental table 2:** Verbose listing of proteins identified. The quantitative value (i.e., normalized moFF intensity) is provided per protein per sample.

**Supplemental table 3:** Data matrix of proteins that passed the limit of quantification (LOQ). The quantitative value (i.e., moFF intensity) is provided per protein per sample. Null values were replaced with an imputed value drawn from a random distribution using the Perseus software (width = 0.3, downshift = 2.5).

**Supplemental table 4:** Student's t-test results for epidermal peel versus mesophyll.

**Supplemental table 5:** Gene ontology enrichment results (ClueGO) for epidermal peel enriched protein abundances.

**Supplemental table 6:** Gene ontology enrichment results (ClueGO) for mesophyll-enriched protein abundances.

**Supplemental table 7:** Epidermal peel proteins that have significant variation in relative abundance across the measured diel period. For each protein, the k-mean cluster is provided.

**Supplemental table 8:** Mesophyll proteins that have significant variation in relative abundance across the measured diel period. For each protein, the k-mean cluster is provided.

**Supplemental table 9:** Frequency of gene ontology (GO) phrases using controlled vocabularies for epidermal peel proteins that have significant variation in relative abundance across the measured diel period. For each protein, the k-mean cluster is provided.

**Supplemental table 10:** Frequency of gene ontology (GO) phrases using controlled vocabularies for mesophyll proteins that have significant variation in relative abundance across the measured diel period. For each protein, the k-mean cluster is provided.
Quantification of Starch in Guard Cells of *Arabidopsis thaliana*

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Abstract

In this protocol, we describe how to quantify starch in guard cells of *Arabidopsis thaliana* using the fluorophore propidium iodide and confocal laser scanning microscopy. This simple method enables monitoring, with unprecedented resolution, the dynamics of starch in guard cells.

**Keywords**: Starch, Guard cells, Arabidopsis, Stomatal opening, Propidium iodide
Background

Starch is a complex polymer of glucose and represents the most abundant form in which plants store carbohydrate. Starch serves different functions, according to the cell types from which it is derived, and the external environmental conditions. In guard cells, which border the stomatal pores that control water and carbon dioxide exchange with the environment, starch can be mobilized within minutes upon transition to light, helping to generate organic acids and sugars to increase guard cell turgor and promote stomatal opening. In mesophyll cells, starch typically accumulates gradually during the day and is degraded at night to support metabolism (Santelia & Lunn, 2017).

Because guard cells comprise only a minor fraction of the total leaf, it is difficult to measure starch quantitatively using conventional methods. Up until now, starch accumulation in guard cells has been mostly visualized by iodine staining. This technique can determine the presence/absence of starch but does not provide accurate, quantitative information.

Here, we describe a fluorescence-based imaging method to quantify starch in guard cells of *Arabidopsis thaliana*. This technique is based on the covalent labeling of cell wall material and other glucan substrates, including starch, with the fluorescent pseudo-Schiff reagent propidium iodide (PS-PI). Isolated epidermal peels are treated with periodic acid to oxidize the hydroxyl groups of the glucose units to aldehyde and ketone groups. The aldehyde groups (−CHO) can then react covalently with propidium iodide, resulting in samples with highly fluorescent glucans that are well suited for confocal laser scanning microscopy. The area of single starch granules within guard cell chloroplasts can be determined using digital imaging. We applied this method to assess the dynamics of starch content in guard cells of intact Arabidopsis leaves over the diurnal cycle, and to determine the impact of fusicoccin (a chemical activator of the proton pump) on starch amounts in guard cells of isolated epidermal peels fragments floating in stomatal opening buffer (Horrer et al., 2016).

Our protocol is an adaptation of a previous mPS-PI staining technique (Truernit et al., 2008). The original method was developed to image entire plant organs for three-dimensional reconstruction of their cellular organization. The main difference between the two methods is the incubation time with the propidium iodide solution, which is 1-2 h for entire plant organs, and only about 20-40 min for epidermal peels.

The technique described here is simple, accurate and highly reproducible. By facilitating the detailed quantification of starch amounts in guard cells, this method will increase the number of questions we will be able to answer about any aspect of guard cell starch metabolism.
**Materials and reagents**

1. Pipette tips (SARSTEDT)
2. 2-well plate (Greiner Bio One International, catalog number: 665180)
3. Falcon tubes
4. Parafilm M (Bemis, catalog number: PM996)
5. Microscope slides (Thermo Fisher Scientific, Menzel-Gläser)
7. Kimtech Science precision wipes (KCWW, Kimberly-Clark, catalog number: 75512)
8. Square Petri dish (Greiner Bio One International, catalog number: 688102)
9. *Arabidopsis thaliana* ecotype Col-0
10. Methanol (Carl Roth, catalog number: 8388.4)
11. Ethanol (Reuss Chemie, catalog number: RC-A15-A)
12. Acetic acid
13. Periodic acid (Sigma-Aldrich, catalog number: P7875)
14. Sodium metabisulfite (Sigma-Aldrich, catalog number: S9000)
15. Hydrochloric acid (Carl Roth, catalog number: 4625.1)
16. Propidium iodide (Sigma-Aldrich, catalog number: 81845)
17. Chloralhydrate (Sigma-Aldrich, catalog number: 15307-R)
18. Glycerol (Carl Roth, catalog number: 3783.1)
19. Gum arabic (Carl Roth, catalog number: 4159.3)
20. Fixative solution (see Recipes)
21. Schiff Reagent (see Recipes)
22. Chloral hydrate solution (see Recipes)
23. Hoyer’s solution (see Recipes)

**Equipment**

1. Glass beaker
2. Precision tweezers (RubisTech, catalog number: 5-SA RT)
3. Pipettes (Gilson)
4. Fume hood (Renggli AG)
5. Refrigerator (Liebherr)
6. Oven (Ehret)
7. Green light LED lamp (In-house built)
8. Confocal laser scanning microscope (Leica Microsystems, model: Leica TCS SP5)
   Note: This product has been discontinued. Any confocal laser scanning microscope can be used.

9. Computer

Software

1. ImageJ (NIH USA, version 1.8, https://imagej.nih.gov/ij/)

Procedure

A. Epidermal peel harvest from Arabidopsis thaliana

1. Label a 12-well plate according to the defined time points and genotypes, and add 1 ml of fixative solution (see Recipes) into each labeled well.

2. Collect epidermal peels from the abaxial side of the 5th or 6th leaf using precision tweezers (Figure 1A; Video 1 – available online: https://bioprotocol.org/e2920). Usually, 4 replicates from 4 individual plants are harvested per time point and genotype. One well contains 4 epidermal peels. See Notes 1-3 for further details.

3. Incubate the 12-well plate containing the collected epidermal peels for at least 12 h at 4 °C in the dark. The samples can be stored in fixative solution up to 4 weeks at 4 °C. The epidermal peels are fragile and require careful handling from now onwards. See Note 4.

B. mPS-PI staining

1. Perform Steps B2-B8 in a fume hood.

2. Remove the fixative solution from the wells using a P1000 pipette (Figure 1B). Collect the waste in a glass beaker and dispose it into the halogenated liquid waste at the end of the protocol.

3. Wash the samples by adding 1 ml of dH₂O and slowly shaking the plate on the bench with circular movements. Remove the dH₂O.

4. Destain the epidermal peels by adding 1 ml of 80% ethanol and incubating at 65 °C for 5-15 min (Figure 1C). Remove the ethanol and repeat washing step (Step B3).
5. Incubate the samples at room temperature (20 °C, RT) for 1 h in 1 ml of fixative solution. Remove the fixative solution and repeat washing step (Step B3).

6. Add 1 ml of 1% periodic acid solution to the epidermal peels and incubate the plate for 40 min at RT. Make sure to cover the peels fully with the solution. See Note 5. Remove the periodic acid solution and repeat washing step (Step B3). Proceed carefully as peels are extremely fragile after this step.

7. Stain the epidermal peels by adding 500 µl Schiff reagent (see Recipes) and 50 µl propidium iodide solution (1 mg·ml⁻¹) for 20-40 min at RT. Make sure to cover the plant tissues fully with the solution. The samples should appear pinkish after this step (Figure 1D). Remove the propidium iodide solution and collect it separately in a Falcon tube. Propidium iodide waste should be treated like ethidium bromide waste.

8. Destain the samples in 1 ml of dH₂O for 20-30 min at RT.

C. Microscope slide preparation

1. Label microscope slides according to the defined time points and genotypes.
2. Perform Steps C3-C4 and C6-C7 in a fume hood.
3. Add 70 µl of chloral hydrate solution (see Recipes) onto every microscope slide. Be careful to distribute the solution at the center of the slide to avoid leaking from the borders of the slide.
4. Carefully transfer the stained epidermal peels onto the microscope slide containing the chloral hydrate solution using precision tweezers. Place all four replicates next to each other (as shown in Figure 1E). Peels should be as flat as possible on the slide. Be careful; the peels will rupture easily.
5. Without placing a coverslip, transfer the microscope slides into square Petri dishes and incubate them at RT in the dark for 24 h.
6. Remove as much chloral hydrate solution as possible from the borders of the samples using Kimtech wipes (Figure 1F). Do not touch the samples with the wipes.
7. Place 2-3 drops of Hoyer’s solution (see Recipes) onto the epidermal peels and add a coverslip. Apply gentle pressure on the coverslip to assure that the peels are flat and even on the microscope slide.
8. Transfer the slides back to the square Petri dishes and store them at RT in the dark for at least 3 days to allow the mountant to set (Figure 1G). See Note 6. The slides can be stored for several months in the dark.

Figure 1. Harvest and staining of epidermal peels. A. Epidermal peel from the abaxial side of the leaf; B. Removal of solutions; C. Destaining of epidermal peels; D. Pinkish plant tissue; E. Microscope slide containing chloral hydrate solution and epidermal peels; F. Removal of chloral hydrate solution; G. Final slide.

D. Imaging

1. Image acquisition is achieved with a Confocal Laser Scanning Microscope.
2. Set microscope as shown in Table 1.

Table 1. Standard microscope settings for guard cell starch imaging

<table>
<thead>
<tr>
<th>Microscope settings</th>
<th>Standard values</th>
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<tr>
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<tr>
<td>Zoom</td>
<td>6 × (Figure 2A)</td>
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</table>

3. Acquire 20 images of individual stomata (Figure 2A; 5 images per replicate) per microscope slide. Acquire pictures of stomata only from mesophyll-free parts (Figures 2B and 2C) of the epidermal peels. Image stomata always with the same zoom factor (6x, Figure 2A) for data comparison. See Note 7.
Data analysis

Starch granule area (in μm²) is determined using ImageJ version 1.8 (NIH USA, http://rsbweb.nih.gov/ij/) by encircling the granule area (Figure 3). A suitable scale is drawn onto the first picture using the software provided by the microscope manufacturer. Guard cell starch granule area is measured for each guard cell individually, totally collecting at least 40 values per time point and genotype. Starch content can then be expressed as an average of these values. Repeat the same experimental setup at least 3 times on independent plant material to obtain a final data set.

Notes

1. The harvest time of epidermal peels per time point and genotype should not be longer than 1-2 min. In case of harvest of multiple genotypes, harvest one replicate per genotype. Harvest the additional replicates following the same sequence to ensure similar treatment of all genotypes.

2. In case of harvest in the dark (e.g., during the night), any green light source can be used to collect the epidermal peels.
3. To obtain a suitable epidermal peel for guard cell starch staining, it is important that a large fraction of the epidermal peel is mesophyll-free.

4. Make sure that the peels do not dry out while they are stored after the harvest and during the staining procedure.

5. Be careful in handling periodic acid as this compound is toxic.

6. The microscope slides are ready for imaging as soon as the coverslip is not movable anymore.

7. Acquire images only for fully developed stomata (Figure 2D).

**Recipes**

1. Fixative solution 50% methanol
   10% acetic acid
   Make up to 500 ml with dH₂O
   *Note: Fixative solution can be stored for long-term usage at 4 °C.*

2. Schiff reagent
   1.9 g sodium metabisulfite
   3 ml 5 N HCl
   97 ml dH₂O
   *Note: Store the Schiff reagent at 4 °C for up to 2 months.*

3. Chloral hydrate solution 40 g chloral hydrate
   10 ml glycerol
   20 ml dH₂O
   *Note: Store the chloral hydrate solution at 4 °C for long-term usage.*

4. Hoyer’s solution
   30 g gum arabic
   200 g chloral hydrate
   20 g glycerol
   50 ml dH₂O
   *Note: Gum arabic is added to the water in a beaker with a magnet bar, which is placed on a shaker in a fume hood overnight to dissolve it. Chloral hydrate is then added in tiny amounts into the beaker using a funnel in a fume hood. Let it dissolve with shaking. Lastly, the glycerol is added. Allow the solution to set for a couple of days before use. Store the solution at RT in the dark. The solution stays turbid. Do not shake it.*
Acknowledgments
This work was supported by the Swiss National Science Foundation (SNSF-Grant 31003A_166539) and the ETH Zürich. The authors would like to acknowledge Mario Coiro for initial help in setting up the pseudo-Schiff propidium iodide staining method; Daniel Horrer and Diana Pazmino for adapting the method to epidermal peels. This protocol is adapted from Truernit et al. (2008). The authors declare no conflicts of interests.

References
In this thesis, we aimed at unraveling the significance of guard cell starch metabolism for stomatal movement regulation and its impact on whole plant physiology (Chapters I-IV). Given that guard cells show characteristics of both autotrophic and heterotrophic tissues, we put a special focus on how stomatal starch is synthesized (Chapters II-V). Due to the heterotrophic traits of guard cells, we also became interested in unraveling the energetic sources for metabolic processes in the guard cell chloroplast (Chapter VI). Finally, we started to translate our findings from Arabidopsis guard cell starch research to other species, such as the CAM plant Kalanchoe fedtschenkoi (Chapter VII). To address these aims, we employed a range of physiological and molecular analyses along with high-resolution microscopy and phenotyping technologies. Not only we provided solid evidence for the majority of the hypotheses made in this thesis, we also opened new fundamental questions, which will be addressed in future research.

Malate and the unresolved question about its origin in guard cells

Membrane ion transport has long been recognized to drive solute accumulation in guard cells for stomatal opening (Blatt, 2016). In parallel to this process, guard cell starch is almost completely degraded within one hour after dawn through the combined activity of the starch-degrading enzymes β-amylase 1 (BAM1) and α-amylase 3 (AMY3) supporting stomatal opening. The simultaneous loss of AMY3 and BAM1 causes a guard cell-specific starch-excess phenotype, delaying and restricting stomatal opening in amy3bam1 plants (Horrrer et al., 2016). A handful of early studies suggested that guard cell starch mobilization would result in the accumulation of malate (Allaway, 1973; Dittrich & Raschke, 1977; Raschke & Schnabl, 1978; Schnabl, 1980; Schnabl & Raschke, 1980). Malate is known to fulfil an array of roles in guard cells, most importantly it acts as a counter-ion for the positive charge imposed by the massive uptake of potassium (K⁺) during stomatal opening (Raschke & Schnabl, 1978; Schnabl, 1980). Malate also acts as a stabilizer of cytosolic pH at times of high H⁺-ATPase activity (Robinson & Preiss, 1985), as an allosteric activator of the tonoplast chloride (Cl⁻) importer Aluminum-activated Malate Transporter 9 (ALMT9) for Cl⁻ accumulation in the guard cell vacuole (De Angeli et al., 2013), as an energy source (Daloso et al., 2017; Robaina-Estévez et al., 2017; Medeiros et al., 2018a), and finally

For a long time, malate was believed to derive from blue light-induced guard cell starch degradation. However, in Chapter I of this thesis, we showed that starch degradation does not actually result in malate accumulation. Isolated guard cells from \textit{amy3bam1} mutants had malate contents similar to those of wild type at the end of the night (EoN) and after exposure to blue light for 30 min (Chapter I; Fig. 2a; Flütsch \textit{et al.}, 2020a). Moreover, we found malate contents to decrease upon blue light treatment in isolated guard cells, but not in the dark (Chapter I; Fig. 2a; Flütsch \textit{et al.}, 2020a), corroborating recent reports showing malate to be a respiratory energy source during light-induced stomatal opening (Robaina-Estévez \textit{et al.}, 2017; Medeiros \textit{et al.}, 2018).

This unexpected finding raises questions about the sources of malate in guard cells. Besides the glycolytic generation of malate from starch, a number of reports suggest that a large bulk of the guard cell malate originates from anaplerotic CO$_2$ fixation catalyzed by Phosphoenolpyruvate Carboxylase (PEPC) (Willmer & Dittrich, 1974; Schnabl, 1980; Gotow \textit{et al.}, 1988; Asai \textit{et al.}, 2000; Daloso \textit{et al.}, 2015a; Medeiros \textit{et al.}, 2017; Robaina-Estévez \textit{et al.}, 2017; Tan & Cheung, 2020). Particularly, PEPC catalyzes the carboxylation of PEP at the expense of HCO$_3^-$ to produce oxaloacetate (OAA) in the cytosol, which is then further metabolized into malate via the action of NADP-dependent malate dehydrogenase (NADP-MDH) (Tcherkez \textit{et al.}, 2005). Interestingly, PEPC was shown to be activated through phosphorylation in a H$^+$-ATPase-dependent manner by a PEPC kinase in guard cells (Outlaw \textit{et al.}, 2002). Hence, guard cell malate production is directly regulated by the blue light-triggered activation of the proton pump. One other source of malate could be the conversion of peroxisomal acetate in guard cells. Mutants affected in a peroxisomal acetyl-CoA synthetase (BZU1/ACN1), which converts acetate into acetyl-CoA, resulted in reduced guard cell malate accumulation and stomatal aperture. Furthermore, the authors highlighted that the acetyl-CoA produced in peroxisomes is independent of the TCA cycle, as acetyl-CoA cannot get translocated across the peroxisomal membrane (Dong \textit{et al.}, 2018). Lastly, guard cells possess a malate importer protein; ATP-binding Cassette transporter B 14 (ABCB14), delivering apoplastic malate to the guard cells (Lee \textit{et al.}, 2008). We have gained preliminary data showing that malate contents were sustained if malate was quantified from guard cells of intact leaves after plants were exposed to white light for 2 h (Fig. 1). In contrast, malate contents declined in isolated guard cells of wild-type and \textit{amy3bam1} plants illuminated with blue light for 30 min (Chapter I; Fig. 2a; Flütsch \textit{et al.}, 2020a). These data indicate that, despite the ongoing malate metabolization for energetic purposes,
malate was constantly replenished during the early morning in guard cells of intact leaves (Fig. 1). Given that anaplerotic CO$_2$ fixation as well as the conversion of acetate to malate could also occur under blue light in isolated guard cells and still malate contents decreased, it is likely that a large proportion of the morning malate contents derives from the mesophyll cells and import via ABCB14.

To decipher the relative contribution of each of the discussed pathways of malate accumulation in guard cells further experimental investigations will be needed, such as enzymatic malate measurements in guard cells of mutants defective in ABCB14, BZU1/ACN1 or enzymes of the anaplerotic CO$_2$ fixation pathway. However, our enzymatic quantification of malate is limited in terms of temporal resolution and therefore depends entirely on the selection of time points. To fully understand the origin of malate in guard cells and maybe more importantly the fluxes through malate, $^{13}$C-labelling experiments in mutant backgrounds could be employed. This would be possible in collaboration with Prof. Mark Stitt and Prof. John Lunn from the Max Planck Institute in Golm (Germany), where this technology is well-established. Unfortunately, Förster Resonance Energy Transfer (FRET)-based sensors are not yet available for malate, but should one become available, it would certainly open novel possibilities to study the origin and fate of this central metabolite in guard cells.

**Export of guard cell starch degradation products from the chloroplast**

In Chapter I of this thesis, we demonstrated that blue light-induced starch degradation yields glucose (Glc) to promote rapid stomatal opening (Chapter I; Fig. 2b; Flütsch et al., 2020a). Besides being a surprising finding for the fact that starch degradation was believed to yield malate, and sugars were considered to play a secondary role during stomatal opening, our discovery also evokes the question about the export of starch degradation products from the guard cell chloroplast. Breakdown products from
stomatal starch might get exported to the cytosol in the form of maltose, Glc or triose-phosphates (triose-P).

Several studies have indicated that triose-P is the major export product of guard cell chloroplasts (Ritte & Raschke, 2003; Shimazaki et al., 2007; Lawson, 2009). In such scenario, guard cell starch degradation would involve the \( \alpha \)-glucan phosphorylase (PHS1), which yields glucose-1-phosphate (G1P) through the phosphorolytic cleavage of \( \alpha \)-1,4-glycosidic bonds (Zeeman et al., 2004). G1P would then be converted into triose-P within the chloroplast and subsequently translocated to the cytosol by the Triose-phosphate/Phosphate Translocator (TPT) (Schneider et al., 2002). PHS1 was shown to not be involved in nighttime starch degradation in the leaves (Zeeman et al., 2004) and therefore represents an interesting candidate for an involvement in guard cell starch degradation. In Chapter V, we have investigated guard cell starch dynamics in \( \text{phs}1 \) mutants during the 12 h light period (Chapter V; Fig. S4; Flütsch et al., unpublished). Whereas guard cell starch was almost entirely degraded within the first hour after dawn and resynthesis of starch occurred from 1 h onwards in wild-type guard cells, starch degradation was delayed in \( \text{phs}1 \) guard cells and continued until 2 h after the EoN, reaching wild-type starch levels (e.g. at 1 h; Chapter V; Fig. S4; Flütsch et al., unpublished). However, PHS1 seems to play only a minor role in starch breakdown as \( \text{phs}1 \) mutants did not display a guard cell-specific starch excess phenotype (Chapter V; Fig. S4; Flütsch et al., unpublished) as we observed it previously for \( \text{bam}1 \) and \( \text{amy}3\text{bam}1 \) mutants (Horrer et al., 2016). The lack of PHS1 resulted in a slower degradation of starch suggesting that PHS1 is required for the complete and efficient hydrolysis of starch. Additional experimental work should include the investigation of \( \text{tpt} \) mutants to examine the contribution of triose-P export in guard cells. Interestingly the proteomic study presented in Chapter VII revealed a different situation for the CAM plant \( \text{Kalanchoe fedtschenkoi} \). The most abundant starch degrading enzyme found in both guard cell and mesophyll cell proteomes was a PHS1 ortholog (Fig. S7; Chapter VII; Abraham et al., 2020). This finding agrees with previous studies suggesting that the phosphorolytic pathway of starch degradation is the dominant one in CAM plants (Borland et al., 2016; Ceusters et al., 2019). Hence, it seems likely that guard cells of C3 and CAM plants use different routes of starch degradation and this deserves further investigations.

Since stomatal starch is mainly degraded by BAM1 and AMY3 (Horrer et al., 2016), maltose should be the predominant end product of starch breakdown in Arabidopsis. Maltose is exported from the chloroplasts by the Maltose Excess 1 (MEX1) protein during the night and represents the main route of carbon export from the chloroplasts in the mesophyll (Niittylä et al., 2004). In the cytosol, maltose is
processed by the Disproportionating Enzyme 2 (DPE2), which supplies one free molecule of Glc to elongate a cytosolic heteroglycan with another Glc moiety (Lu & Sharkey, 2004; Fettke et al., 2006). Through the action of PHS2, G1P is subsequently released from the heteroglycan (Lu et al., 2006). However, a role of MEX1 in daytime guard cell export of maltose has not yet been defined.

We therefore examined guard cell starch contents in wild-type and the ethyl methanesulfonate (EMS) mex1 (mex1-1; Niittylä et al., 2004) mutants during the first hour after the EoN (Fig. 2). Unlike in mesophyll cells, where the loss of MEX1 results in a severe starch excess phenotype (Niittylä et al., 2004), guard cell starch contents were comparable between wild type and mex1 plants at the EoN (Fig. 2). However, upon illumination, mex1 guard cells did not degrade starch, resulting in 2-fold higher starch amounts after one hour of light compared to wild type (Fig. 2).

These results suggest that MEX1 is involved in the export of starch-derived maltose, but it is not the only exporter of starch degradation products in guard cells. The wild type-like amounts of starch at the EoN in mex1 guard cells might result from reduced uptake of mesophyll-derived carbons due to the absence of maltose export from the chloroplasts at night in mesophyll cells in this mutant. Arabidopsis mutants impaired in MEX1 activity show deregulated carbohydrate and protein metabolism in mesophyll cells, as well as severe reductions in the number of chloroplasts caused by autophagy-like chloroplast degradation (Stettler et al., 2009). Hence, to investigate if the lack of MEX1 in guard cells would cause a guard cell starch-excess phenotype and to examine the full impact of its loss without the costs of the severe impairments in the beneath mesophyll, the use of guard cell-specific silencing lines of MEX1 would be preferable for experimental work. We generated microRNA-induced gene silencing (MIGS; De Felippes et al., 2012) constructs against MEX1. In particular, the 22 nt-long microRNA 173 target site was fused upstream of a 215 bp fragment of MEX1 coding.

![Figure 2. Guard cell starch contents in mex1 mutants.](image-url)
sequence (Primers are listed in Table 1). The resulting construct SF28 was expressed in the plant destination vector pART27 under the guard cell-specific KST1 promoter (Kelly et al., 2013). Subsequently, pSF28 was transformed into wild-type Arabidopsis and T1 plants carrying the transgene were selected based on their resistance against Kanamycin conferred by pART27. Putative positive plants were verified by PCR to confirm the presence of the transgene. Selection of independent stable lines will be needed prior to examination of stomatal phenotypes. Nevertheless, these lines represent a valuable tool to study the preferred form of carbon exported from the guard cell chloroplasts and the relative contribution of MEX1 to this process. Additional experimental work should also include the investigation of Arabidopsis mutants defective in DPE2 and PHS2 to shed light onto the role of these enzymes in processing maltose exported from guard cell chloroplasts. Strikingly, the two cytosolic proteins DPE2 and PHS2 were more abundant in guard cells compared to mesophyll cells in K. fedtschenkoii (Fig. S7; Chapter VII; Abraham et al., 2020). Hence, also in CAM plants different starch degradation products might be exported from the chloroplasts, at least triose-P and maltose.

<table>
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<th>ID</th>
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<th>Cloning primers</th>
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<td>MIGS-MEX1+miR173</td>
<td>AT5G17520</td>
<td>FWD - GTGATTTTTCTCTACAAGCGAACTCTCGGTGGTGATCGTGTA</td>
</tr>
<tr>
<td>MIGS-MEX1 Rev</td>
<td>AT5G17520</td>
<td>REV - GCTGATTCTCGTGAGGGAAG</td>
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Table 1. Oligonucleotides used for MIGS-MEX1 cloning.

β-amylases are unable to fully hydrolyze starch. Their action yields maltose and to a lesser extent the tri-saccharide maltotriose. The plastid localized DPE1 was shown to process maltotriose into Glc and a longer oligosaccharide (Critchley et al., 2001). Hence, the combined activity of β-amylases and DPE1 results in the accumulation of maltose and Glc. Double mutant plants of dpe1mex1 exhibit an even more severe phenotype than mex1 single mutants, suggesting that indeed both Glc and maltose are exported from the leaf chloroplasts (Niittylä et al., 2004). Concomitant with these findings, Glc transport activity at the chloroplast was observed (Schäfer & Heber, 1977; Häusler et al., 1998) and a plastidic Glc Transporter (pGlcT) was discovered (Weber et al., 2000; Cho et al., 2011). The concurrent lack of pGlcT and MEX1 caused a severe growth inhibition along with strong reductions in starch and sucrose (Suc) contents, revealing that both transport systems contribute to the export of starch degradation products in the mesophyll cells (Cho et al., 2011). Investigating guard cell starch contents in pglct and dpe1 mutants will help to understand which
starch degradation product is exported from the guard cell chloroplasts and if Glc is one of them.

**The dominant role of Glc in guard cells**

In Chapter I, we showed that Glc is the major starch degradation product in guard cells needed to accelerate stomatal opening at dawn (Fig. 2b; Flütsch et al., 2020a). In Chapter II, we revealed that guard cell starch synthesis and light-induced stomatal opening largely depend on the import of mesophyll-derived Glc at dawn via the combined action of the Sugar Transport Proteins 1 and 4 (STPs; Chapter II; Flütsch et al., 2020b). As shown in Chapter IV, Sugars Will Eventually be Exported Transporters 1, 4 and 5 (SWEETs) are also contributing to the supply of Glc to guard cells for starch synthesis and stomatal movement regulation (Flütsch et al., unpublished). Although Glc plays this surprisingly dominant role in stomatal functioning, its fate within the guard cells remains largely unknown.

In Chapter III, we have discussed the possibility that Glc acts as an energetic source (and will not be discussed again) and/or as an osmolyte during stomatal opening and would therefore be taken up into the vacuole (Review - Flütsch & Santelia, under revision). Future experimental work will be required to elucidate which tonoplast Glc importers are expressed in guard cells. To examine Glc sequestration in guard cells, investigating vacuolar dynamics during stomatal opening in mutants impaired in guard cell Glc homeostasis could help. Gao et al., (2005) demonstrated through staining with the pH probe acridine orange (AO) that guard cell vacuolar membranes of *Vicia faba* undergo dynamic changes during stomatal movements. Whereas guard cells contained various small vacuoles when stomata were closed, the small vacuoles fused to generate one large central vacuole during stomatal opening (Gao et al., 2005, 2009). We gained similar preliminary results after applying 10 µM AO to isolated guard cells (for the protocol of guard cell isolation see Material and Methods of Chapter I) of wild-type and *amy3bam1* plants before the EoN (dark-adapted) or after 1 h of light and an additional 1 h of treatment with either 10 µM fusicoccin or abscisic acid (ABA; Fig.3) to induce opening and closure, respectively. While various small vacuoles were observed in both wild-type and *amy3bam1* dark-adapted and ABA-treated guard cells, one large vacuole was stained in each of the wild-type guard cells after application of fusicoccin (Fig. 3). *amy3bam1* guard cells had one visible vacuole per guard cell, which however, did not occupy the entire volume of the cell like in the wild type (Fig. 3). These preliminary data suggest that vacuolar dynamics in wild-type and the starch degrading mutant differ during stomatal opening, with *amy3bam1* guard cells exhibiting reduced volumes of central vacuoles compared to wild type (Fig. 3).
Figure 3. Vacuolar dynamics during stomatal opening and closure. Representative images of guard cell vacuoles stained with 10 µM acridine orange (AO) in wild-type and amy3bam1 plants during darkness, treatment with 10 µM fusicoccin or 10 µM abscisic acid (ABA). Scale bar = 10 µm. WT = wild-type. For protocol of staining see Gao et al., (2005).

However, treatment with AO does not always result in the staining of vacuoles, but suffers from manual loading, variability and can be unspecific. We therefore employed the help of genetically encoded Ca²⁺ indicators, which belong to the ratiometric Ca²⁺ reporter genes that are based on combinations of GFP-related proteins (Cameleons). Usually, the FRET-based Cameleon sensors are used for the detection of plant Ca²⁺ signaling (Costa et al., 2018). However, Prof. Alex Costa from the University of Milan (Italy) kindly gifted us a Ca²⁺ sensor construct which expression is restricted to the tonoplast (Krebs et al., 2012). We plan to use this construct to detect different vacuolar dynamics during stomatal opening in mutants such as the amy3bam1 and stp1stp4, which we know are impaired in stomatal Glc metabolism. Both double mutants were transformed with the sensor and several stp1stp4 T1 plants carrying the transgene were selected. Unfortunately, the transformation did not yield any transformants for the amy3bam1 genetic background and needs to be repeated. After successful isolation of transformed plants, the transgenic lines will be used to study changes in vacuolar dynamics after fusicoccin or ABA treatment. These
experiments will certainly shed light on the role of Glc as an osmotic species during stomatal movements.

Research from recent years supports the idea that dynamic rearrangements of the stomatal cell wall are a vital part of stomatal movement control. To allow reversible stomatal movements and high turgor pressure, guard cell walls have been hypothesized to be uniquely strong and elastic. Parts of the guard cell glucose pool might be converted into UDP-glucose, the substrate of cellulose biosynthesis and most other nucleotide-sugar precursors required for the synthesis of cell wall polysaccharides (Rui et al., 2018). Confocal micrographs of propidium iodide-stained guard cells of stp1stp4 indeed hint into such a direction, as guard cells of this mutant likely have cell wall abnormalities (Chapter II; Flütsch et al., 2020b). This observation is an interesting starting point for future research that should be directed towards the investigation of the composition of the stomatal cell wall in stp1stp4 mutants to confirm the importance of apoplastic hexoses for guard cell wall integrity.

In this thesis we have discovered several double mutants with impaired guard cell Glc metabolism; e.g. amy3bam1 (Chapter I; Flütsch et al., 2020a), stp1stp4 (Chapter II; Flütsch et al., 2020b) and putatively sweet1sweet5, sweet1sweet4 and sweet4sweet5 (Chapter IV; Flütsch et al., unpublished), which are valuable tools for future investigations of the fate of Glc within guard cells. One general approach to monitor carbon fluxes in guard cells could be the use of $^{13}$C kinetic isotope labelling. In recent years, different studies have proven this method powerful even for Arabidopsis (Robaina-Estévez et al., 2017; Medeiros et al., 2018). Guard cell-enriched epidermal peels are isolated using similar protocols as the one we use for RNA isolation from guard cells (see Material and Methods of Chapter I; Daloso et al., 2016; Jalakas et al., 2017). Peels are isolated after the plants have been exposed to $^{13}$C-containing air or prior to floating isolated guard cells in a buffer containing $^{13}$C-substrates. The samples are then analyzed by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS; Daloso et al., 2015b; Robaina-Estévez et al., 2017; Medeiros et al., 2018). To follow the fluxes of Glc in guard cells the above described method could be employed using wild-type and our double mutant plants. These experiments could be done in collaboration with Prof. Mark Stitt and Prof. John Lunn from the Max Planck Institute in Golm (Germany). Alternatively, the concentration of metabolites in subcellular compartments could be estimated by the use of genetically encoded fluorescent sensors. Such biosensors exist for cytosolic Glc, the FLIPgLuΔ13 with affinities ranging from $K_d = 170$ nM to $K_d = 3.2$ mM or the similar sensor FLIPgLu600µΔ13 with a slightly higher $K_d$ (Deuschle et al., 2006; Chen et al., 2010). Targeting the expression of these sensors to other cellular compartments such as the...
vacuole or the mitochondria, as it has been done for Ca\textsuperscript{2+} sensors (Costa et al., 2018), could help to decipher the fate of Glc in guard cells.

**Coordinated action of STP1 and STP4**

STP1 and STP4 work together to provide sufficient amounts of Glc for light-induced stomatal opening and guard cell starch accumulation at dawn (Chapter II; Flütsch et al., 2020b). The observation that only the lack of both transporters caused a severe stomatal phenotype, while the single mutants show distinct phenotypes, led to the hypothesis that they might form a complex or interact with each other at the guard cell plasma membrane. Indeed, bimolecular fluorescence complementation (BiFC) assays in tobacco demonstrated that LR67 proteins from wheat, which belong to the STP13 family (Milne et al., 2019), form both homo- and heterodimers (Moore et al., 2015). To investigate the potential interaction between STP1 and STP4, one could use a range of *in vitro* assays such as the abovementioned BiFC assay, split luciferase protein-protein interaction assays or *in vitro* protein immunoprecipitation (IP). Additionally, the investigation of protein interactions in living plant tissues could be achieved through transient expression of tagged proteins in tobacco leaves and subsequent IP. A probably even more meaningful and reliable approach would be the stable co-expression of differently tagged STP1 and STP4 proteins in Arabidopsis and the investigation of *in vivo* protein interactions through co-immunoprecipitation (co-IP) or by FRET measurements.

**Altered sugar homeostasis of stp1stp4 guard cells**

Suc accumulates to higher levels in guard cells of *stp1stp4* plants compared to wild type during the early morning (Chapter II; Fig. 2a; Flütsch et al., 2020b). In Chapter II and III, we speculated about the reasons for this increased amounts of Suc (Chapter II; Flütsch et al., 2020b; Chapter III; Review - Flütsch & Santelia, *under revision*). Likely explanations are the existence of a cytosolic futile cycle of Suc (discussed in Chapter II and III and will not be discussed again) or the upregulation of plasma membrane Suc transporters in response to the lack of STP1 and STP4. The latter scenario could be easily tested using guard cell-enriched epidermal peel material from wild-type and *stp1stp4* plants and subsequent quantification of mRNA levels of putative Suc transporter genes such as *SUC1, SUC2, SUC3, SWEET11 and SWEET12* (Chapter II; Fig. EV1a; Flütsch et al., 2020b). Interestingly, the accumulated Suc is not used for guard cell starch synthesis as demonstrated by the fact that *stp1stp4* guard cells are starch-free during the first half of the day (Chapter II; Fig. 2b and 2c; Flütsch et al., 2020b). This finding hints towards sequestration of Suc in the vacuole, rendering it
unavailable for metabolic processes. An explanation for the blockage of vacuolar Suc could be a general imbalance of cytosolic proton concentration and pH homeostasis in stp1stp4 plants. More so, the energetic status in stp1stp4 guard cells might be altered, affecting transport of Suc and likely other metabolites across the plasma membrane and tonoplast.

Firstly, the proton-coupled uptake of Glc via the STPs should help replenish the H⁺ pool for the H⁺-ATPase. Furthermore, a fraction of the imported Glc likely serves as respiratory substrate for ATP production, which in turn can be utilized by the proton pump. Given that Glc is taken up into guard cells already during the dark, the activity of the proton pump might partially depend on the STP-mediated supply of Glc before and during the early phase of stomatal opening. Ion transport processes across the plasma membrane are directly coordinated with transport mechanisms at the tonoplast. Similarly to the plasma membrane, the activity of many tonoplast transporters, including the Suc-H⁺ exporter SUC4 (Chapter IV; Flütsch et al., unpublished), depend on a proton gradient (Cubero-Font & De Angeli, 2020; Horaruang et al., 2020). Possibly altered H⁺-ATPase activity in stp1stp4 double mutants might directly affect other transporters. To test such a hypothesis, H⁺-pumping experiments could be employed as we described them in Chapter I in collaboration with Prof. Atsushi Takemiya from the Yamaguchi University (Japan) using our stp1stp4 double mutant. Furthermore, to examine cytosolic ATP concentrations in stp1stp4 plants during the transition from dark to light, the cytosolic ATP sensor, pH2GWT-C-AT1.03, described in Chapter VI (Voon et al., 2018; Chapter VI, Lim & Flütsch et al., unpublished) could be transformed into the double mutants and used for subsequent estimation of cytosolic ATP levels.

Secondly, the lack of functional STP1 and 4 might directly translate into changes of cytosolic pH in guard cells. Not only through the lack of H⁺ import, but also through potential alterations in the activities of other transporters. It is becoming increasingly evident that the coordination between proton pumps and secondary H⁺ antiporter/symporters determines intracellular pH homeostasis (Pittman, 2012). Transport at the tonoplast is known to be coordinated with ion fluxes along the plasma membrane, partially because translocation of solutes across both membranes depends on the conditions of the shared cytosolic compartment (Eisenach & De Angeli, 2017; Jezek & Blatt, 2017; Cubero-Font & De Angeli, 2020). Many transport systems in guard cells are known to be directly regulated by cytosolic pH, such as the plasma membrane K⁺ in (Blatt, 1992) and K⁺ out channels (Roelfsema & Prins, 1997) and the Cl⁻ exporter GCAC1 (Schulz-Lessdorf et al., 1996). Indeed, recent work made use of a cytosolic pH biosensor and demonstrated the regulation of cytosolic pH by the
activity of an tonoplast Cl\(^{-}/\)NO\(_3^{-}\) transporter, CLCa (Demesa et al., 2020). In Chapter VI, we monitored pH changes in both the cytosol and the chloroplasts of guard cells using the pH sensors pH2GW7-C-cpYFP and pH2GW7-TKTP-cpYFP (Chapter VI; Fig. 3g and 3h; Lim & Flütsch et al., unpublished), which are modified versions of the previously used mitochondrial pH sensor pH2GW7-MT-cpYFP (Voon et al., 2018; Lim et al., 2020). To elucidate whether the lack of STP1 and 4 affects cytosolic pH homeostasis, the cytosolic pH sensor could be transformed into our stp1stp4 double mutants and FRET imaging acquired during the transition from dark-to-light.

Thirdly, the high Suc amounts in guard cells of stp1stp4 might be explained by altered activity of Suc transporters at the tonoplast. To date, the guard cell tonoplast transport systems involved in Suc translocation are largely unknown. Tonoplast Sugar Transporter (TST)-type carriers such as the Tonoplast Monosaccharide Transporters 1 and 2 (TMTs; Wormit et al., 2006; Schulz et al., 2011) have been shown to efficiently translocate Suc from the cytosol into the vacuoles (Jung et al., 2015). However, it remains unknown if these transporters are expressed and active in guard cells. Interestingly, the Schulz et al., (2011) speculated that TMTs might be coregulated with the tonoplast Suc exporter SUC4. They proposed that to prevent a futile transport of Suc caused by the parallel operation of the TMT antiport and the SUC symport, one carrier should be turned off while the other one is active (Schulz et al., 2011). Hence, constitutive activation of the TMTs in stp1stp4 might cause a blockage of Suc inside the guard cell vacuole. Alternatively, both carrier types might be active in parallel resulting in the beforementioned futile cycle of Suc. Investigating guard cell transcript levels of TMTs and SUC4 in guard cells of stp1stp4 could help to infer whether the above proposed mechanism applies to guard cells of our double mutant.

**Malate metabolism during stomatal closure**

The tonoplast malate exporter protein ALMT4 was previously shown to be required for ABA-induced stomatal closure to rapidly remove malate from the vacuole (Eisenach et al., 2017b). Although malate export from guard cells via R- and S-type anion channels has been shown (Schroeder & Keller, 1992), it remained unclear if parts of the malate are also metabolized after export from the vacuole. In Chapter IV of this thesis, we examined the contribution of vacuolar-derived metabolites to guard cell starch accumulation (Chapter IV; Fig. 4a; Flütsch et al., unpublished), as guard cell starch was proposed to act as a sink for vacuolar carbons during stomatal closure (Raschke & Dittrich, 1977; Schnabl & Raschke, 1980; Azoulay-Shemer et al., 2016; Santelia & Lunn, 2017). Interestingly, we showed that almt4 mutants accumulated
significantly less starch in guard cells during the day compared to wild type (Chapter IV; Fig. 4a; Flütsch et al., unpublished). *almt4* plants also exhibited impaired stomatal closure in response to treatment with high CO$_2$ concentrations (Chapter IV; Fig. 5a and Fig. S4b; Flütsch et al., unpublished). Thus, our data indicate that starch is partially formed from malate that has been previously stored in the vacuole and that the ALMT4-mediated release of malate from the vacuole is also required for high CO$_2$-induced stomatal closure. Support for our finding that malate is further metabolized comes from a recent study on Phosphoenolpyruvate Carboxykinase (PEPCK), which converts oxaloacetate (OAA) into phosphoenolpyruvate (PEP) during gluconeogenesis. PEPCK1 (PCK1) was found to be highly expressed in guard cells and *pck1* mutants showed impaired stomatal closure in response to darkness, but not ABA. The authors proposed that malate metabolization is important during dark phases, whereas malate export from guard cells is more involved in the response to ABA (Penfield et al., 2012). The response evoked by ABA might be too rapid to allow the gluconeogenic conversion of malate into starch. This is an interesting hypothesis and deserves further investigations. Guard cell starch quantifications in *pck1* either during the 24 h diel cycle or after exposure to darkness, ABA and high CO$_2$ concentrations could help to reveal whether the metabolic rearrangements are different depending on the closure stimuli. Interestingly, Eisenach et al. (2017) reported the opposite for ALMT4, being required for ABA-mediated closure but not during darkness. We have not yet investigated the phenotype of *almt4* mutants in response to darkness or ABA, but these experiments would certainly help to bring light into the observed activity of ALMT4 to different closure stimuli. As highlighted in the “General Introduction”, there is still a lot of uncertainty concerning the pathways of ABA and CO$_2$-induced stomatal closure therefore it seems plausible that ABA induces the rapid release of malate from the guard cells, whereas the responses to elevated CO$_2$ concentrations and darkness might involve the gluconeogenic conversion of malate to starch. In addition, Open Stomata 1 (OST1), a critical kinase in the ABA pathway does not regulate ALMT4 function. But a known component of the CO$_2$-signaling pathway Mitogen Activated Protein Kinase 4 (MPK4) together with MPK6, which was shown to be involved in the ABA-mediated immunity response of stomata, phosphorylate ALMT4 *in vitro* leading to its deactivation (Eisenach et al. 2017; Zhang et al., 2018; Montillet et al., 2013). However, the role of MPK4 and 6 in regulating the activity of ALMT4 *in planta* remains to be elucidated. An explanation for the deactivation of ALMT4 upon phosphorylation could be that the abovementioned kinases activate plasma membrane anion channels through phosphorylation and...
simultaneously deactivate vacuolar anion exporter proteins as a means of co-regulation.

**Regulation of starch synthesis in guard cells**

Whereas starch is synthesized during the day and degraded during the subsequent night in photosynthesizing leaves, the pattern of starch synthesis differs markedly in guard cells, where starch accumulates even at night (Horrer *et al.*, 2016; Santelia & Lawson, 2016; Santelia & Lunn, 2017). The unusual dynamics of starch formation in guard cells presumes that the regulation of starch metabolism in this cell type is independent from the transition between light and darkness. Thus, starch biosynthetic processes must be regulated differently in mesophyll cells and guard cells. Mesophyll cell starch synthesis is mainly regulated at the level of the AGPase, which is subject of multilevel regulation (Geigenberger, 2011). There exists ample of evidence that the AGPase is regulated allosterically and through redox homeostasis as well as transcriptionally through the spatial and temporal expression of its subunits (Crevillén *et al.*, 2003, 2005; Hendriks *et al.*, 2003). In Chapter V, we have discussed and investigated the putative regulation of the AGPase by the composition of its subunits in guard cells. We provide evidence for a role of the large subunits APL3 and APL4 in the regulation of the guard cell AGPase, which are usually not expressed in leaf tissues (Chapter V; Fig. 5; Flütsch *et al.*, unpublished).

However, as guard cell starch synthesis seems to directly integrate with the response of guard cells to stomatal closure-inducing stimuli, such as ABA and high CO₂, such factors represent interesting candidates for triggering starch synthesis in guard cells. Stomatal closure is associated with the removal of large quantities of osmotic species from the vacuole, which accumulated earlier during the day (Raschke & Dittrich, 1977; Azoulay-Shemer *et al.*, 2016; Santelia & Lunn, 2017). Therefore, it is likely that starch acts a sink for such solutes. One of these environmental factors, ABA, has been implicated with guard cell starch synthesis already in the 1970s. When detached epidermis of *Commelina communis* was treated with 100 µM ABA, guard cells contained more starch compared to untreated ones (Mansfield & Jones, 1971). Moreover, a recent metabolomic study in *Brassica napus* further highlighted that guard cell starch metabolism is involved in the response to ABA treatment, as metabolites of the pathways related to starch and Suc were found to be enriched upon exposure to ABA. Interestingly, Suc concentrations increased more than 100-fold after 15 min of ABA treatment in *B. napus* guard cells (Zhu & Assmann, 2017). At the transcriptional level, the only starch-related genes known to be induced by ABA in guard cells are Starch Excess 1 (SEX1) and BAM1 (Wang *et al.*, 2011), which are required for starch degradation (Yu *et al.*, 2001; Horrer *et al.*, 2016; Thalmann *et al.*, 2016). To gain
insights into a possible regulation of starch metabolism by ABA in guard cells, we measured starch in wild-type guard cells in the presence or absence of 100 µM ABA (Fig. 4).

As expected, starch was almost entirely degraded after 1 h of light in guard cells of control plants. In contrast, guard cells of ABA-treated plants retained their starch, with slightly increased amounts compared to the EoN (Fig. 4). Starch accumulated continuously during the experiment in guard cells of control plants, but not in guard cells of ABA-treated plants (Fig. 4). The observed starch dynamics in the ABA-treated guard cells hint towards an inhibition of starch degradation rather than active accumulation of starch. Furthermore, these data imply that during ABA-mediated stomatal closure, vacuolar metabolites might rather get exported from the guard cells than converted into starch. This could be probably explained by the fact the gluconeogenic conversion of malate and/or sugars might not be rapid enough for the response evoked by ABA. In line with these data, Hayashi and Kinoshita (2011) reported that the H^+-ATPase gets rapidly dephosphorylated and therefore inactivated upon treatment with 20 µM ABA. Given that the activation of guard cell starch degradation is directly linked the activity of the H^+-ATPase by a yet unknown signal (Horrer et al., 2016), the inhibition of the pump should also directly affect the activity of starch degrading enzymes. These data are in contrast to the transcriptomic study suggesting an induction of the starch degradation enzymes BAM1 and SEX1 upon ABA treatment (Wang et al., 2011). Hence, future investigations will be necessary to disentangle if ABA treatment blocks the degradation of starch or if starch degradation and starch synthesis occur in parallel, which could explain the mild net increase in starch amounts observed throughout the experiment after ABA treatment (Fig. 4). Furthermore, the quantification of guard cell starch after treatment with ABA in mutants affected in the vacuolar exporters ALMT4, SUC4 and Early Response to Dehydration.
Like 6 (ERDL6) investigated in Chapter IV (Chapter IV; Flütsch et al., unpublished) could help to address the question of whether or not starch is synthesized from vacuolar carbons upon ABA signaling. Finally, the interesting finding of Suc accumulation upon ABA application in *B. napus* (Zhu & Assmann, 2017) should be investigated in Arabidopsis. Suc could be synthesized from vacuolar carbons upon ABA exposure to protect the cytosolic compartment from reactive metabolites, such as free hexoses and malate, released from the vacuole or to get rapidly exported from the guard cells via some unknown plasma membrane Suc exporter.

Slightly different results were gained from initial guard cell starch experiments with high CO₂ concentrations (Fig. 5). Starch contents remained at the same levels as at the EoN after 1 h of exposure to 1000 µL⁻¹ CO₂. However, in contrast to the ABA-treated plants, guard cells accumulated high levels of starch after exposure to elevated CO₂ concentrations during the course of the experiment (Fig. 5). The most likely explanation for the starch accumulation would be enhanced photosynthetic activity imposed by the elevated amounts of available CO₂. However, to examine whether guard cells synthesize starch from carbons that were previously stored in the vacuole and retrieved for stomatal closure, exposing mutants of the vacuolar exporters investigated in Chapter IV (Chapter IV; Flütsch et al., unpublished), and subsequently determine their stomatal starch amounts should help. In contrast to ABA signaling in guard cells, CO₂ signaling is not known to involve the proton pump. Therefore, different mechanisms might apply to ABA-induced and CO₂-induced stomatal closure. As stated in the “General Introduction” of this thesis, the cascades for ABA and CO₂ signaling in guard cells are likely to converge downstream of the Ser/Thr kinase Open Stomata 1 (OST1) (Zhang et al., 2018). Investigating stomatal starch contents in mutants of both components located upstream and downstream of OST1 could help to understand to which signaling components guard cell starch metabolism responds and if this response is a shared trait of both pathways.

![Figure 5. Guard cell starch contents in plants treated with high CO₂ concentrations.](image)

Guard cell starch contents of wild-type plants at end of night (EoN) and after 1, 3 and 6 h of exposure to 1000 µL⁻¹ CO₂. High CO₂ concentrations were applied as described in Flütsch et al., 2020b. Plants were illuminated with 150 µmol m⁻² s⁻¹ of white light. Data for one experiment are shown; means ± SEM; n ≥ 36. WT = wild type. For experimental procedure see Material and Methods of Chapter I.
Mesophyll-derived Suc has been proposed as another stimulus evoking stomatal closure mediated by the cytosolic Hexokinase 1 (HXK1) in guard cells (Kelly et al., 2013, 2019; Granot et al., 2014; Granot & Kelly, 2019; Lugassi et al., 2019). HXK is a dual-function protein: it phosphorylates Glc, generating Glc6P, and it is a sugar sensor (Granot, 2008). HXK is known to directly modulate transcript levels of ABA-related genes on a whole plant level (Rolland et al., 2006). Hence, the fluctuating levels of guard cell apoplastic Suc could provide a fine-tuning mechanism to balance photosynthetic demands for CO$_2$ and prevent excessive water loss through evapotranspiration. This theory agrees with the observation that Suc accumulates to high levels in the guard cell apoplast around midafternoon when stomatal aperture decreases (Lu et al., 1995, 1997). For HXK to sense Suc it requires it to be metabolized into the hexoses Glc and fructose (Fru) either by cell wall Invertases (cwINV) or by Suc cleaving enzymes in the cytosol, such as the cytosolic Invertases (cINV) (Santelia & Lawson, 2016). To date, invertase activity in guard cells remains largely uninvestigated and should be considered in future research. In Fig. 4b of Chapter IV, we quantified guard cell soluble sugar contents from plant material harvested during the afternoon (e.g. 6 and 9 h of light; Chapter IV; Flütsch et al. unpublished). Our data revealed that Suc contents were almost 2-fold higher compared to the EoN in wild type guard cells (Chapter IV; Fig. 4b; Flütsch et al., unpublished) and therefore our observation matches with the above proposed mechanism. Interestingly, when comparing guard cell starch contents in wild-type and the guard cell-specific HXK1 overexpressor line GCHXK2 (Kelly et al., 2013), we detected strongly reduced amounts of starch at the EoN in the transgenic plants (Fig. 6).

![Figure 6. Guard cell starch contents in guard cell-specific HXK1 overexpressing lines.](image-url)

Guard cell starch contents of wild-type and GCHXK2 plants at end of night (EoN), and after 1 and 3 h of light. Plants were illuminated with 150 µmol m$^{-2}$ s$^{-1}$ of white light. Data from three experiments are shown; means ± SEM; n ≥ 110. WT = wild type. Letters indicate significant statistical differences between time points for the given genotype. Asterisk (*) indicate significant statistical differences between genotypes for a given time point for $P < 0.05$ determined by one-way ANOVA with post hoc Tukey’s test. For experimental procedure see Material and Methods of Chapter I.
Furthermore, GCHXK2 guard cells failed to accumulate starch after it was almost completely degraded at 1 h into the day, whereas wild-type guard cells began to accumulate it as expected (Fig. 6). Our findings are likely explained by the prior observed reduced stomatal conductance ($g_s$) of GCHXK2 plants that should restrict the uptake of CO$_2$ (Kelly et al., 2013). However, one could also argue that reduced starch degradation in GCHXK2 results in the lower $g_s$ amplitude and therefore affects carbon assimilation at the whole plant level. Alternatively, the overexpression of HXK1 might directly modulate the expression of genes involved in either carbon or energy metabolism affecting the accumulation of starch. Indeed, HXKs from a variety of plant species and tissues have been shown to repress the expression of photosynthetic genes as a feedback regulation of photosynthesis (Sheen, 1990, 1994; Jang et al., 1997; Cho et al., 2006). Moreover, HXK sugar sensing activity also directly repress genes of the glyoxylate cycle (Graham et al., 1994), which was shown to provide carbons and ATP in guard cells for metabolic processes (McLachlan et al., 2016; Dong et al., 2018). Thus, quantifying guard cell mRNA levels of photosynthetic, sugar transporter or energy metabolism genes could help to establish whether the effect of the HXK1 is signaling-related.

All of the above presented data demonstrate that stimuli that promote stomatal closure also directly trigger processes affecting guard cell starch metabolism (Fig. 4-6). However, it remains elusive whether starch is actively synthesized in response to ABA, high CO$_2$, darkness or even sugar signaling through HXK. The method of starch quantification developed in our lab, using the fluorophore propidium iodide, allows us to quantify net changes in guard cell starch contents (Flütsch et al., 2018). However, we are currently limited in the detection of processes occurring simultaneously, which might be the case for starch synthesis and starch degradation. Hence, other methods need to be applied, which will allow us to distinguish among these processes. One such method has been successfully used to follow carbon fluxes in mesophyll cell chloroplasts by our collaborators from Prof. Zeeman’s group (ETHZ) and Prof. Meibom’s group (EPFL), the Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS). NanoSIMS is a variant of chemical imaging, which can detect certain elements with 50 – 100 nm resolution and therefore can reveal very low concentrations down to $\mu$g g$^{-1}$ of nearly every element of the periodic table (Moore et al., 2012). Together with Léo Bürgy from the Zeeman lab, we set up a pilot experiment for $^{13}$C labelling of guard cell starch and the subsequent analysis by transmission electron microscopy (TEM) and NanoSIMS.
Fig. 7 shows the successful detection of newly incorporated $^{13}$C in chloroplasts of guard cells (Fig. 7). We, therefore labelled entire Arabidopsis plants with $^{13}$C-containing air for 15 min after the plants have been exposed to light for 4 h, when guard cells accumulate substantial amounts of starch (Horrer et al., 2016). After the labelling, the plants were moved back to normal air for a chase period of 3 h. Hence, after approximately 7 h into the day, we harvested plant samples for the subsequent imaging by TEM and NanoSIMS. The detection of $^{13}$C in guard cell chloroplast by chemical imaging represents certainly a major technological advance. However, from the $\delta_{-13}$C map in Fig. 7 we can also see that the signal is weak compared to the highlighted yellow dot at the border, which is a part of a mesophyll starch granule (Fig. 7). Thus, the method needs further optimization to be applied in guard cells, nevertheless our pilot experiment delivered promising results. In any case, following carbon incorporation into guard cell starch after the application of ABA or high CO$_2$ concentrations should be possible using $^{13}$C-labelling coupled with TEM/NanoSIMS and hence, can be used to address the above made hypotheses.

Guard cell starch metabolism in other species

Stomata evolved more than 400 years ago to promote water loss for spore desiccation in bryophytes (Puttick et al., 2018). In vascular plants stomata are intimately linked to photosynthetic gas exchange. This shift in stomatal function with the emergence of vascular plants was further accompanied by a diversification of stomatal morphologies into the dumb-bell-shaped stomata of the grasses and the kidney-shaped stomata found in other species (Fig. 8) as well as different types of photosynthesis (C3, C4 and CAM; Willmer & Fricker, 1996). Plants with C3 photosynthesis and kidney-shaped stomata have poor water use efficiencies (WUE), negatively affecting their biomass production. In such species, including Arabidopsis, a temporal disconnection between...
stomatal movements (measured as stomatal conductance = \( g_s \)) and photosynthetic assimilation (\( A \)) is observed resulting from \( g_s \) responding an order of magnitude slower to changes in environmental cues than \( A \). This is in harsh contrast to the grasses, which have particularly high WUE due to much faster stomatal movement responses (Lawson et al., 2014; Lawson & Vialet-Chabrand, 2019).

Blue light-induced starch degradation in guard cells helps to accelerate stomatal opening and therefore directly affects the temporal interplay of \( g_s \) and \( A \) in species with kidney-shaped stomata (Chapter I; Flütsch et al., 2020a). The investigation of the temporal dynamics of guard cell starch turnover got possible only very recently with the development of a staining-based guard cell starch quantification technique using high-resolution confocal microscopy to visualize the starch granules (Horner et al., 2016; Flütsch et al., 2018). Guard cell starch dynamics have been described for Arabidopsis and the CAM plant Kalanchoë fedtschenkoi, revealing significant differences among the two species. Whereas guard cell starch is degraded during the early day in Arabidopsis correlating with stomatal opening (Horner et al., 2016), starch levels peak during daytime in Kalanchoë fedtschenkoi and lower starch contents are observed during the night (Chapter VII; Abraham et al., 2020b). It remains yet to elucidate whether the net decrease at night of guard cell starch in Kalanchoë fedtschenkoi is required to open the stomata during nighttime. More so, in a pilot experiment, we used our guard cell starch staining on land plant species across the evolutionary scale (Fig. 8).

Our preliminary data revealed interesting aspects of guard cell starch metabolism. Firstly, guard cell starch granules form virtually every plant can be visualized with our method (Fig. 8). Secondly, starch is present in guard cells of the bryophyte Anthoceros agrestis only in young sporophytes, but not in mature ones which have already released the spores. Therefore, starch in guard cells appeared simultaneously with stomata (Fig. 8) and might play a role during the desiccation of reproductive tissues. Thirdly, our guard cell starch staining revealed the presence of significant amounts of starch in guard cells of grass species, including barley, rice and maize, independent from the type of photosynthesis (Fig. 8).
Hence, the ability to analyze guard cell starch contents in ancient and modern land plants opens the possibility to assess some of the following interesting questions: i) what is the function of guard cell starch in the bryophyte stomata of young sporophytes?, ii) why do guard cells of the hornwort Marchantia polymorpha not contain starch or is this an artifact of the time of sampling?, iii) is starch degradation occurring and important in lycophytes and ferns?, iv) what are the differences in guard cell starch metabolism between C3 and C4 species and how does the morphology of guard cells influence the starch metabolism of C3 and C4 species?, v) is starch degradation also required in grasses and does it relate to the speed of stomatal responses? and finally vi) are the functions of starch-related enzymes conserved among land plants?
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GENERAL DISCUSSION AND OUTLOOK


This thesis marks the end of four years of PhD work in the starch group. I look back on happy times full of motivation and fun, but also difficult times including our move to ETH Zurich, setting up a molecular biology lab from scratch and of course the omnipresent Corona virus. However, especially during these difficult times I learned that everything can be managed by team efforts.

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