HELLEN OLIVEIRA DE OLIVEIRA

UNDERSTANDING THE FUNCTIONAL ROLE OF THE ORGANIC ACIDS DURING STOMATAL MOVEMENTS AND STRESS RESPONSES IN *Arabidopsis thaliana*

Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Wagner L. Araújo

Co-Adviser: David Barbosa Medeiros

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BIOGRAPHY

Hellen Oliveira de Oliveira, daughter of Wany Socorro Oliveira Silva, was born in Belém, Pará state, Brazil, on January 15th, 1992. In 2010, she started the undergraduate course in Agronomy at the Universidade Federal Rural da Amazônia (UFRA), Belém, Pará state, Brazil, and achieved her bachelor’s degree in January 2015. In March 2015, she started her Master's course at the same university, achieving a Master's degree in Applied Biotechnology for Agriculture in February 2017. In August of the same year, she started her doctoral studies in Plant Physiology at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais state, Brazil under the supervision of Prof. Wagner L. Araújo.

ABSTRACT

OLIVEIRA, Hellen Oliveira, D.Sc., Universidade Federal de Viçosa, December, 2022. **Understanding the functional role of the organic acids for stomatal movements and stress responses in *Arabidopsis thaliana*.** Advisor: Wagner Luiz Araújo. Co-advisor: David Medeiros Barbosa.

Organic acids (OAs) are central to cellular metabolism performing several functions: beyond their role as intermediates of the tricarboxylic acid cycle and precursors for the biosynthesis of other molecules, they are also a predominant part of root exudates, modulators of redox states between cellular compartments, and key regulators in the response of guard cells to environmental stimuli. In this thesis, we present distinct aspects of the complex regulation of OAs and how their transport mediates stomatal movements in guard cells. We initially summarize the current molecular advances underlying the complex regulation of stomatal development and movements to dynamic environmental conditions. In doing so, we were able to showcase that (*i*) stomatal closure in response to abscisic acid (ABA) depends on the disassembly of microtubules by the degradation of a stabilizing protein during the stomatal opening, (*ii*) sucrose supply in short days results in the accumulation of an conserved energy sensor kinase that promotes stomatal development, and (*iii*) that production of a non-protein amino acid gamma-aminobutyric acid (GABA) is necessary to reduce stomatal opening and transpirational loss through down-regulation of a vacuolar anion transporter (ALMT9) of stomatal cells in response to drought. In chapter 3, we addressed the importance of OAs for plant performance in the context of mitochondrial and guard cell metabolism under abiotic stress conditions. Accordingly, we reviewed the role of OAs as root chelating agent, in biosynthesis of stress signaling and osmoregulatory solutes during stomatal movements. We conclude that plants use different mechanisms to regulate and accumulate OAs, depending on the cell organ, cell compartment, and stress condition, allowing the proper functioning of physiological and biochemical responses in plants following stress conditions. In chapter 4, to obtain a more comprehensive picture of the vacuolar transport of OAs in guard cells, we analyzed how the impaired malate accumulation impacts effects on stomatal behavior, photosynthetic capacity and primary metabolism in leaves of plants with individual and combined repression of tonoplast dicarboxylate transporter (tDT), ALMT6 and ALMT4 channels. Briefly, the results presented here provide evidence on (*i*) the inefficiency of stomatal opening caused by ALMT6 repression in *almt6*, *almt6 tdt-1* and *almt6 almt4* lines suggesting that, ALMT6 compensates for vacuolar malate transport in guard cells upon tDT repression with respect to the stomatal opening. However, tDT and ALMT4 are important in the proper storage of dicarboxylates in the vacuole of mesophyll cells. In addition, (*ii*) we also observed that *almt6 almt4* plants maintained growth by increasing dark respiration and sugar accumulation, whereas in *almt6* and *almt4* plants this accumulation maintained respiration rates, with unchanged and impaired growth, respectively; (*iii*) repression of ALMT6 and ALMT4 channels led to slower stomatal kinetics and lower stomatal conductance, highlighting the importance of ALMT6 for stomatal opening, and that non-functional ALMT4 likely downregulates the activity of other ion and solute transport channels in guard cells during stomatal opening. Despite that, we did not observe effects on stomatal behavior under high CO_2, and, as a result, all mutants were responsive to ABA during stomatal closure, indicating the possible activity of OAs and other ion channels in the guard cell plasma membrane. Future analyses are required to determine if alternative ALMT family members and/or others ions transporters are functioning at guard cell transcriptional levels. This input knowledge will be necessary for a better understanding of the mechanisms used to bypass the impaired accumulation of organic acids in these plants.

Keywords: Environmental stress. Mitochondrial metabolism. Vacuolar transport. Organic acids. Respiration. Stomata.

RESUMO

OLIVEIRA, Hellen Oliveira, D.Sc., Universidade Federal de Viçosa, dezembro de 2022. **Entendendo o papel funcional dos ácidos orgânicos para os movimentos estomáticos e respostas ao estresse em *Arabidopsis thaliana*.** Orientador: Wagner Luiz Araújo. Coorientador: David Medeiros Barbosa.

Os ácidos orgânicos (AOs) são fundamentais para o metabolismo celular desempenhando diversas funções: além de seu papel como intermediários do ciclo dos ácidos tricarboxílicos na produção de poder redutor e precursores para a biossíntese de outras moléculas, eles também são parte predominante dos exsudados radiculares, capazes de modular os estados redox entre os compartimentos celulares e estão envolvidos como reguladores chaves na resposta das células guarda aos estímulos ambientais. Nesta tese, apresentamos aspectos distintos da complexa regulamentação dos AOs e como o seu transporte medeia os movimentos estomáticos em células guarda. Inicialmente reunimos os avanços moleculares subjacentes à complexa regulamentação do desenvolvimento e movimentos estomáticos para condições ambientais dinâmicas. Ao fazer isso, conseguimos demonstrarque (*i*) o fechamento estomático em resposta ao ácido abscísico (ABA) depende da desmontagem dos microtúbulos pela degradação de uma proteína estabilizadora durante a abertura estomática, (*ii*) o fornecimento de sacarose em dias curtos resulta no acúmulo de uma quinase sensorial conservada de energia que promove o desenvolvimento estomático, e (*iii*) que a produção de um aminoácido não proteico ácido-aminobutírico (GABA) é necessária e suficiente para reduzir a abertura estomática e perda transpiracional através da desregulamentação de um transportador vacuolar de ânions (ALMT9) de células estomáticas em resposta à seca. Além disso, fornecemos novos conhecimentos sobre a sinalização energética e formação dos estômatos, bem como a regulamentação dos movimentos estomáticos e mecanismos moleculares para o estresse ambiental nas plantas. No capítulo 3, abordamos a importância dos AOs para o desempenho das plantas em um contexto de metabolismo mitocondrial e de célula guarda sob condições de estresse abiótico. Dessa forma, revisamos o papel dos AOs na tolerância ao alumínio como agentes quelantes radiculares, na biossíntese da sinalização de estresse e solutos osmorregulatórios durante os movimentos estomáticos. Concluímos que as plantas utilizam de diferentes mecanismos para regular e acumular AOs, dependendo do órgão celular, do compartimento celular e da condição de estresse, permitindo o funcionamento adequado das respostas fisiológicas e bioquímicas nas plantas após condições de estresse. No capítulo 4, para obter um quadro mais abrangente do transporte vacuolar de AOs em células guarda, analisamos como o acúmulo prejudicado de malato impacta nos efeitos sobre o comportamento estomático, a capacidade fotossintética e metabolismo primário em folhas de plantas com repressão individual e combinada do transportador de dicarboxilatos do tonoplasto (tDT), ALMT6 e ALMT4. Resumidamente, os resultados aqui apresentados fornecem evidências sobre (*i*) a ineficiência da abertura estomática causadas pela repressão de ALMT6 em linhas *almt6*, *almt6 tdt-1* e *almt6 almt4*, sugerindo que, ALMT6 compensa o transporte de malato vacuolar em células guarda em relação a repressão de tDT no que respeita a abertura estomática. Entretanto, tDT e ALMT4 são importantes no armazenamento adequado de dicarboxilatos no vacúolo das células mesofílicas; (*ii*) observamos também que plantas *almt6 almt4* mantiveram o crescimento aumentando a respiração noturna e acúmulo de açucares, enquanto que em plantas *almt6* e *almt4* este acúmulo manteve as taxas respiratórias, com crescimento inalterado e prejudicado, respectivamente; (*iii*) a repressão dos canais ALMT6 e ALMT4 levou a uma cinética estomática mais baixa, destacando a importância de ALMT6 para a abertura estomática, e que ALMT4 não funcional desregula a atividade de outros canais de transporte de íons e solutos em células guarda durante a abertura estomática. Apesar disso, não observamos efeitos sobre o comportamento estomático sob alto nível de CO_2 e, como resultado, todos os mutantes foram responsivos ao ABA durante o fechamento estomático, indicando a possível atividade dos AOs e outros canais de íons na membrana plasmática da célula guarda. Análises futuras são necessárias para determinar se os membros alternativos da família ALMT e/ou outros transportadores de íons estão funcionando em níveis de células guarda e de transcrição. Esse conhecimento de entrada será necessário para uma melhor compreensão dos mecanismos usados para contornar o acúmulo prejudicado de ácidos orgânicos nessas plantas.

Palavras-chave: Estresse ambiental. Metabolismo mitocondrial. Transporte vacuolar. Ácidos orgânicos. Respiração. Estômato.

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Chapter 1

General introduction

1. GENERAL INTRODUCTION

Stomata, microscopic structures located in the leaf epidermis, are formed by two specialized guard cells and, a sub-stomatal intercellular space (called pore) that is continuous to the mesophyll tissue and controls the opening and closing of the stomas through which land plants exchange gases with the atmosphere. Accordingly, stomata are the predominant sites of CO_2 uptake during photosynthesis and water loss through transpiration, also supporting leaf cooling and overall metabolic flux (Lawson, 2009).

Phylogenetic studies revealed that stomata are ancient structures present in the earliest land plants and predate the divergence of bryophytes and tracheophytes (Clark et al., 2022). In response to environmental variations, carbon uptake and water use efficiency were optimized through the formation of a diversity of stomata sizes, densities, and morphologies (Franks and Beerling, 2009; Franks and Farquhar, 2007). The formation of stomata is well understood in dicotyledons, such as Arabidopsis, which briefly involves key processes such as asymmetric divisions from a committed protodermal cell, called a meristemoid mother cell (MMC) that divides asymmetrically to form a meristemoid (Zhao and Sack, 1999). This meristemoid is regulated by the transcription factor SPEECHLESS (SPCH) and undergoes consecutive asymmetric cell divisions until it differentiates into a guard mother cell (CMG) promoted by MUTE, which in turn lead to two symmetric guard cells produced by further regulating FAMA, thus completing the development of stomata (Casson and Hetherington, 2010; Zhao and Sack, 1999).

The mechanisms that determine the degree of stomatal opening and closing are associated with the adjustment of guard cell turgor and are controlled by various environmental stimuli such as light, air temperature, CO_2 concentration, relative humidity, and plant hormones such as abscisic acid (ABA) (Assmann and Jegla, 2016; Hsu et al., 2021). One of the signals perceived by plants is very well elucidated by their effects triggered by blue or red light detected by the chloroplast and photoreceptors (Shimazaki et al., 2007). Phototropins drive H^+ATPase activation and proton extrusion that hyperpolarize the guard cell plasma membrane (Kinoshita and Hayashi, 2011) and provide the driving force for potassium (K^+) uptake by activation of KAT1/2 K^+ channels that, together with their counter chloride (Cl^-) ion, malate, and sugars promote osmolyte accumulation in the vacuole, changing guard cell turgor and leading to stomata opening (Assmann and Jegla, 2016). Regarding red light, it remains unknown whether its contribution is first perceived by the mesophyll cells and then transmitted as a signal to the guard cells or whether this signal is directly associated with the guard cells (Busch, 2014). In contrast, ABA inhibits proton extrusion and activates other calcium (Ca^{2+}) uptake channels as well as slow (S-Type) anion channels and fast (R-type) anion efflux channels (Dreyer et al., 2012) leading to membrane depolarization and activation of an external GORK voltage-dependent K^+ efflux channel (Hosy et al., 2003). Together, this combined regulation drives the decrease in guard cell vacuolar volume, and consequent loss of water from the apoplastic space causing stomatal closure (Hedrich, 2012).

Guard cell metabolism is likely affected by multiple endogenous signals and environmental stimuli that regulate the concentrations of metabolites and ions that are fundamental to the mechanism of stomatal opening and closing (Pandey et al., 2007; Roux and Leonhardt, 2018; Yu and Assmann, 2014). Among these factors, the accumulation of organic acids (OAs) is a key point that links gas exchange and photosynthetic activity to the mitochondrial metabolism (Daloso et al., 2017a; Fernie and Martinoia, 2009). Briefly, OAs act as intermediates and enzymatic regulators of the tricarboxylic acid (TCA) cycle (Araújo et al., 2012; Nunes-Nesi et al., 2013) and serve as a carbon skeleton for the biosynthesis of amino acids, sugars, and nucleotides (Fernie and Martinoia, 2009). Furthermore, OAs perform multiple functions such in the photosynthesis of C4 and Cam plants (Maier et al., 2011) and also work as chelating agents in nutrient and heavy metal uptake (Weisskopf et al., 2006) . Among the OAs, malate is considered the most abundant being present in all cell types, yet its levels are rather variable to species, cultivar or even among individual leaves of a single plant (Fernie and Martinoia, 2009). During the night, C_3 plants can reach concentrations above 350mM of malate, and during the day, decarboxylation of malate can decrease its levels to 200mM in leaves (Martinoia and Rentsch, 1994). Furthermore, malate has pKa values of approximately 3.1 and 5.1 depending on the salt concentration in the medium, giving the possibility that, at physiological pH, malic acid is found in the form of Mal^{-2}, but it may also be present in the vacuoles of plants at acid pH as $Hmal^-$ or H_2mal (Martinoia and Rentsch, 1994; Raven, 1990). These characteristics make it a crucial element in cellular pH regulation (Hurth et al., 2005), osmoregulation, and stomatal movement signaling (Daloso et al., 2017a), and it is also involved in responses to both biotic (Bolwell et al., 2002) and abiotic stresses (Finkemeier and Sweetlove, 2009; Panchal et al., 2021).

The regulation of malate levels in the cell is closely tied to a refined control of its concentrations within the various cellular compartments. Thus, the malate produced by the TCA cycle, or coming from starch degradation and ß-oxidation (Daloso et al., 2017a) can be transported to the vacuole where it will be stored, having its concentrations increased or decreased to maintain optimal cytosolic conditions (Hurth et al., 2005). In this context, the exchange of molecules through the inner membranes of the mitochondria, chloroplast and vacuole is impermeable to the most metabolites and, therefore, it is necessary to use specific proteins to import and export these molecules and compounds. In the plasma and vacuole membranes malate transport can be mediated by carrier proteins and specific transporters extensively investigated at the guard cell level (Imes et al., 2013; Luu et al., 2019; Medeiros et al., 2018; Sasaki et al., 2022).

It has been recently shown that cytosolic malate concentrations influence the activity of "S-type" anion channels which are those dependent on a slower depolarization voltage (Wang et al., 2018). Accordingly, the SLAC and SLAH genes in *Arabidopsis thaliana* are characterized by encoding channels responsible for chloride and nitrate (NO_3^-) transport in the plasma membrane of guard cells during stomatal movement (Geiger et al., 2011; Vahisalu et al., 2008). These genes dramatically inhibit K^+ channel (KAT1) activation during stomatal closure, acting as regulators and signals for stomatal movement by SLAC1/SLAH3 KAT1 protein-protein interaction in Arabidopsis (Zhang et al., 2016). Wang et al. (2018) further observed that malate at 1mM concentration indirectly activates S-type channels in guard cells. During stomatal closure, malate, temporarily accumulated in the vacuole, metabolized or directed to the apoplast by activation of channels and transporters (Daloso et al., 2017a). Therefore, it is possible that malate concentrations in the cytosol reach low values and that high cytosolic malate concentrations (>10mM), derived from starch degradation, inhibit the activation of these channels, consequently leading to stomatal opening (Wang and Blatt, 2011). The malate channel AtABCB14 is a positive regulator of stomatal opening involved in apoplastic malate transport when cytosolic malate concentrations are reduced and under high CO_2 concentrations (Lee et al., 2008). Similarly, during ABA-mediated stomatal closure, darkness and high CO_2, fast "R-type" channels of the ALMT (Aluminum Activated malate Transporter) family AtALMT12/ AtQUAC1 (Quick Anion Channel) mediate malate export into the apoplastic space (Meyer et al., 2010; Sasaki et al., 2010). Malate transport does not only occur at the plasma membrane but it is also directed into the guard cell vacuole, mainly by channels of the ALMT family and by the Tonoplast Dicarboxylate transporter (tDT) (Kovermann et al., 2007a; Liu and Zhou, 2018). In *Arabidopsis,* ALMT9 was the first member of the ALMT family to be shown to mediate malate and fumarate currents directed into the vacuole of mesophyll cells (Kovermann et al., 2007a). Subsequently, it was found that ALMT9 is a Cl^- channel and that its activity is governed by cytosolic malate concentrations, which stimulates Cl^- influx currents from the cytosol into the vacuole by AtALMT9 (Zhang et al., 2013). It should be noted that malate also regulates channel activity, in addition to serving as a signaling molecule. AtALMT9 is expressed in various cell types, including guard cells, and has also been shown to be important during the stomatal opening in response to light thus, knockout mutants of this channel, exhibit reduced stomatal opening (De Angeli et al., 2013a) and are negatively regulated by γ-aminobutyric acid (GABA) to control stomatal closure mainly under water stress (Xu et al., 2021). Another member of the ALMT family, specific to guard cells, ALMT6 mediated malate inward currents dependent on micromolar Ca^{2+} concentrations, and vacuolar pH and cytosolic malate regulate the activation threshold of AtALMT6-mediated currents, showing to be involved in both malate influx and efflux, depending on the tonoplast potential. These stimuli will induce ALMT6 regulation during stomatal opening and closing with the combined action of other channels and transporters (Meyer et al., 2011a). Although its activity is bidirectional on malate currents in the tonoplast of guard cells, *almt6* mutant plants are characterized by a more impaired response during the opening movement, presenting reduced stomatal conductance when compared to plants with the functional gene in *A. thaliana* (Meyer et al., 2011a). Stomatal movements also utilize ABA-stimulated phosphorylative pathways to regulate the activity of anion channels such as ALMT4, characterized mainly by mediating malate currents from the vacuole towards the cytosol during stomatal closure (Eisenach et al., 2017a). This channel seems to exert a strong influence on stomatal movements, as plants with functional loss of this gene show a much slower stomatal closure when exogenous ABA is applied (Eisenach et al., 2017a). Additionally, tDT was the first transporter identified and characterized to be involved in malate transport in the tonoplast of guard cells (Emmerlich et al., 2003a), being an antiport in the entry of malate to the vacuole in exchange for fumarate and/or citrate to the cytosol, with its affinity for malate being reduced at declining cytosolic pH (Frei et al., 2018a). Although its functionality does not directly impact stomatal movement even under high CO_2, light intensity, concentrations of ABA and organic acids (Medeiros et al., 2017b), this transporter exhibits a significant impact on mitochondrial metabolism by the accumulation of malate and fumarate in the vacuole of mesophyll cells (Medeiros et al., 2017b), participating in the regulation of cytosolic pH homeostasis (Hurth et al., 2005), and during nitrate assimilation in plants (Hu et al., 2009).

Taken together, the aforementioned studies suggest that the metabolism and transport of OAs, specifically malate, plays a key role in the regulation of stomatal movements and that, probably, there is a functional redundancy of the specific vacuole transporter proteins of guard cells. Furthermore, it is seemingly that this accumulation of ions and osmoregulatory is dependent on endogenous and exogenous stimuli such as light, ABA, CO_2 concentration, pH, and apoplastic/cytosolic malate concentrations. Therefore, we posit that understanding OAs metabolism and guard cells involves a precise and refined regulation of multiple factors, and thus future studies are required to decipher their importance in responses to different environmental stimuli and stressors for the proper growth and development of resilient crops in a scenario of climate changes.

Layout chapter objectives

This thesis is largely focused on the role of vacuolar transport of organic acids in guard cells and their metabolism under environmental variations. That said, the main objectives of this work were: (*i*) to highlight advances in the mechanisms related to microtubule dynamics and energy sensing within guard cells to environmental perturbations; (*ii*) to revisited the contributions of OAs metabolism in plant responses to abiotic stresses; (*iii*) to investigate the functional redundancy of vacuolar OAs transporter, related to stomatal movements and central metabolism of *A. thaliana*. To achieve these objectives, several complementary biometric, physiological, metabolic, and molecular approaches were used in previously characterized mutant plants with distinct stomatal responses. The first two chapters are subdivided into specific sections, whereas the last chapter contain an introduction, material and methods, and a discussion of the results obtained. In the end, I further highlight the concluding remarks of this thesis, within the conclusions and perspectives on the regulation of stomatal movements in guard cells.

Chapter 2. Guard cell regulation: pulling the strings behind the scenes

Guard cell metabolism is precisely refined during stomatal opening and closing in response to environmental signals, therefore, those cells need to develop precise mechanisms of when and where stomatal cell proliferation is established in the face of the surrounding environment. Interestingly, there are several genes involved in cell proliferation that resemble those in stomatal development and movement. In this chapter, current studies involving the role of microtubules and the novel function of the non-protein amino acid γ-aminobutyric acid (GABA) in modulating stomatal closure and opening are discussed, providing new insights into the highly exquisite molecular mechanisms of guard cells in the face of environmental and stress responses.

Chapter 3. Understanding the dynamics of plant organic acids metabolism following abiotic stresses

Although several molecular and physiological approaches already demonstrate the importance of OAs for cellular metabolism, their role in responses to distinct abiotic stresses is highly dynamic and versatile. In this chapter, we address the established roles of OAs and highlight recent findings comprising the synthesis and regulation of OAs, their contribution as root exudates in stress tolerance, their supply of reductants for detoxification of reactive oxygen species (ROS), and biosynthesis of stress signaling solutes. We argued that metabolic complexity and broad pathway and networks are affected in response to changes in OAs metabolism in response to physiological and environmental factors. In summary, a detailed understanding of OAs metabolism is likely able to help us to improve the growth and performance of crops.

Chapter 4. On the significance of vacuolar transport of malate in guard cells during stomatal regulation

Vacuolar malate transport has been characterized at the molecular level and is carried out by channels of the Aluminum Activated Malate Transporter (ALMT) family and the Tonoplast Dicarboxylate Transporter (tDT). tDT knockout mutant plants (*tdt*) show no impact on stomatal function, although presented decreased malate and fumarate accumulation in leaves, mainly in the mesophyll cells. The ALMT6 channel is guard cell-specific, and acts on both malate influx and efflux during stomatal movements. Interestingly, the absence of functionalALMT6 in *Arabidopsis thaliana* plants has a greater impact on stomatal opening, which is highly impaired by the lower accumulation of malate in the vacuole. Here, we hypothesize that vacuolar malate transport in plants with non-functional tDT is supported by the ALMT6 channel, whereas tDT does not compensate for malate accumulation in guard cells in *almt6* knockout plants. Being aware that the guard cell possesses the AtALMT4 channel, involved specifically in the efflux of malate into the cytosol, *almt4* mutant plants were also used. Mutants plants show a slower and lesser degree of stomatal closure in the absence of ALMT4, highlighting it as a crucial malate channel involved in stomata regulation. It seems reasonable to suggest that although ALMT6 is present in *almt4* knockout lines and regulates malate currents into the cytosol, highlighting a greater contribution of ALMT6 in stomatal opening and ALM4 in stomatal closure. Next, we investigated the functional redundancy of vacuolar malate transport in the combined absence of these channels. To answer this, double knockout mutant plants for ALMT6 and tDT and non-functional ALMT6 and ALMT4 were investigated regarding stomatal movements, photosynthetic capacity and guard cell metabolism.

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Chapter 2

Guard cell regulation: pulling the strings behind the scenes

Trends in
Plant Science

Spotlight

Guard cell regulation: pulling the strings behind the scenes

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The precise regulation of stomata is critical to plant growth and development, especially under drought conditions. Two recent studies (Dou *et al*., Xu *et al*.) shed new light on stomata physiology in response to dynamic environmental conditions, revealing novel key mechanisms related to microtubule dynamics and energy sensing within the guard cells.

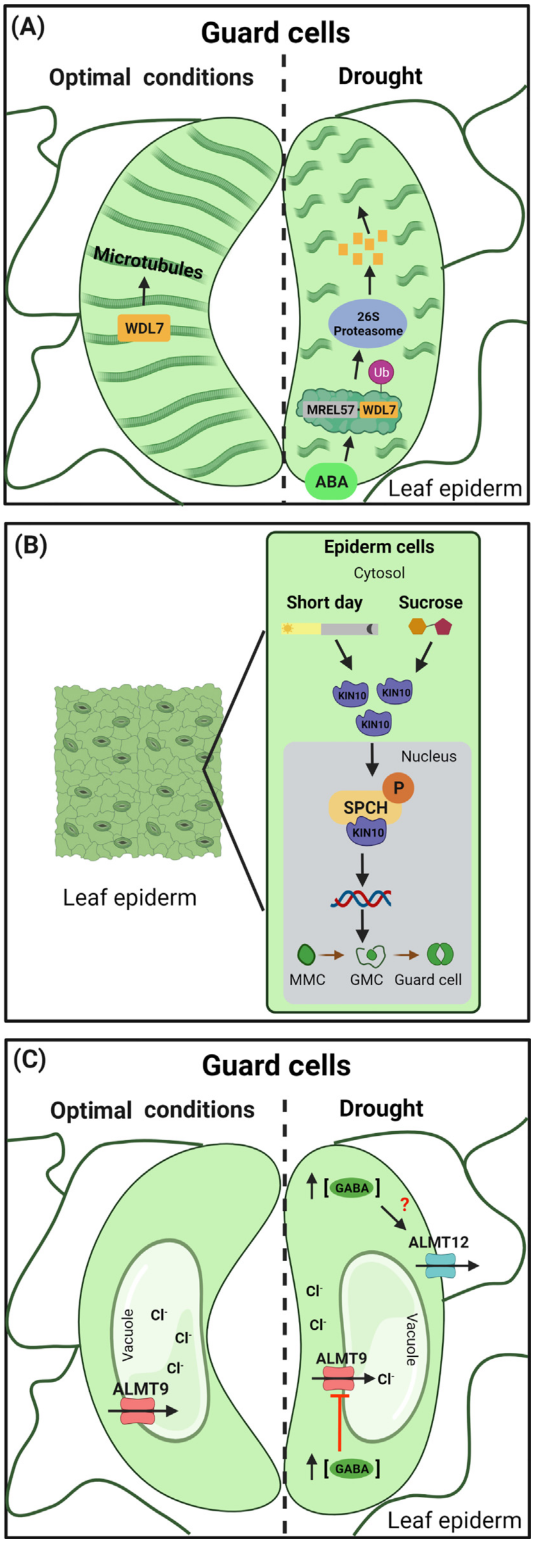
Terrestrial plants produce microscopic structures called stomata on the surface of their leaves, which are precisely regulated to facilitate their opening or closing in response to environmental conditions or stressors [1]. Stomatal cells have developed a complex system designed to recognise and respond to optimal conditions and facilitate the precise regulation of stomatal cell proliferation.

Environmental factors (e.g., light, temperature, and water availability) frequently affect stomatal development, leading to differential patterns in stomatal number and distribution across leaves [2]. Stomatal cells are characterised by a predefined set of rules that determine their origin and development. Briefly, the founder cell of stomata, the meristemoid mother cell, undergoes an asymmetric entry division to form a meristemoid. This meristemoid then experiences two distinct cell fates, undergoing subjacent asymmetric cell divisions facilitating its proliferation or the differentiation of the guard mother cell (GMC), which in turn produces parallel guard cells, thus completing the stomata [2]. The transcription factor, speechless (SPCH) coordinates the expression of hundreds of genes that trigger cell fate determination within the stomatal lineage differentiation that culminates in the establishment of the GMC [2]. Despite the singularity of stomatal development and function, several genes involved in cell proliferation have similar effects on stomatal movement. Here, we highlight: (i) the conserved role of the microtubules [3], and (ii) the function of the non-protein amino acid gamma-aminobutyric acid (GABA) in regulating stomatal movements [4].

Pulling the strings: the significance of microtubules in modulating guard cell dynamics

Research into stomatal biology has led to significant advances in our understanding of the dynamic nature of stomatal pores in response to endogenous and environmental variations. Recent evaluations of the data from 599 plant species revealed an important balance in stomatal development and highlighted the importance of dynamic stomatal regulation in plant health. Namely, the balance between photosynthetic efficiency and pathogen infection [5]. Similar stomatal densities on both the upper and lower surfaces (amphistomy) of the leaf are assumed to maximise photosynthesis while simultaneously increasing pathogen susceptibility [5]. Given this balance, the question arises: How do plants maximise photosynthesis without increasing their risk of infection with leaf pathogens? Significant advances have been made in describing the molecular shifts that culminate in actin cytoskeletal rearrangements in guard cells following bacterial infection in *Arabidopsis thaliana* [6]. Briefly, Lu *et al*. showed that calcium-dependent protein kinase 3 (CPK3) phosphorylates actin-depolymerization factor 4 (ADF4), which mediates actin filament organisation in the guard cells following infection with *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000) [6]. Furthermore, the authors demonstrated that *cpk3-2* knockout mutants display inefficient stomatal closure during Pst DC3000 infection, which leads to bacterial susceptibility [6]. Consequently, this study represents a significant advance in our understanding of the framework underlying the common machinery associated with immunity and photosynthesis.

Further progress on stomatal cytoskeletal dynamics was demonstrated by revealing that stomatal closure in response to abscisic acid (ABA) depends on microtubule disassembly [3]. Investigations using the *A. thaliana* mutant, *rpn1a-4*, which exhibits a deficiency in the 26S proteasome, revealed that ABA-induced stomatal closure is most likely the result of microtubule disassembly [3]. After 10 minutes of treatment with oryzalin, which is known for its disruption of microtubule dynamics, wild type plants were characterised by a decrease in the density of cortical microtubules, which did not occur in the guard cells of the *rpn1a-4* plants. Prolonged drug exposure culminated in the disappearance of most microtubules in the wild type guard cells [3]. In an expansion of this assay, the addition of a microtubule-stabilizing drug (Taxol), which suppresses microtubule disassembly, revealed that the temporal degradation of Wave-dampened2 (WVD2)/WVD2-Like 7 (WDL7) precedes stomatal closure. Stomatal closure based on ABA signalling is also dependent on WDL7 degradation, as revealed by a reduction in ABA responsiveness in *WDL7* overexpressing plants, which causes hypersensitivity to soil drying and reduced survival under drought [3]. Taken together, these studies revealed that ABA alters ubiquitin 26S proteasome activity, triggering WDL7 proteolysis in guard cells and promoting microtubule disassembly, leading to stomatal closure in response to ABA treatment and soil drying (Figure 1A). Proteolysis is an energetic, expensive process; thus WDL7 turnover may impact overall cell cycle progression, further affecting plant stress responses. Similarly, the protein



Trends in Plant Science

Figure 1. Strings and curtains mediating stomatal movement and development in response to environmental and stress stimuli. (A) Disassembly of the microtubules (strings) is mediated by a proteasome-dependent ubiquitin-26S pathway. Protein WDL7 promotes the stabilisation of microtubules triggering stomatal opening under optimal conditions. However, ubiquitin E3 ligase protein MREL57 ubiquitinates and targets WDL7 protein for degradation, which triggers the disassembly of guard cell microtubules, promoting stomatal closure in response to ABA and water deficits. (B) KIN10, the catalytic α-subunit of the SnRK1 energy sensor, interacts with transcription factor speechless (SPCH) to induce stomata formation. Moderate energy stress following shortened days leads to reduced sucrose supply and results in the accumulation of KIN10 in the epidermal cells (curtains). This results in KIN10 translocation to the nucleus, where it phosphorylates and stabilises SPCH, which in turn coordinates the expression of the genes that promote stomatal development. (C) GABA appears to regulate ALMT activity, inducing stomatal closure under drought conditions. Water deficit enhances GABA synthesis and its accumulation in the leaves directly impacts the activity of anionic ALMT9. It remains unclear whether increased GABA synthesis in response to drought conditions occurs in the guard or epidermal cells. Water limitations and GABA concentration work together to negatively regulate ALMT9, causing the inhibition of Cl^- influx from the cytosol to the vacuole, culminating in stomatal closure. GABA is also likely to be involved in the regulation of ALMT12, which seems to affect stomatal closure, at least in epidermal cells dealing with dark-to-light transitions. This figure was created using BioRender (https://biorender.com). Abbreviations: ABA, abscisic acid; ALMT, ALUMINUM-ACTIVATED MALATE TRANSPORTER; GABA, γ-aminobutyric acid; GMC, guard mother cell; MMC, meristemoid mother cell; MREL57, MICROTUBULE-RELATED E3 LIGASE57; WDL7, WAVE-DAMPENED2-LIKE7.

JAV1-ASSOCIATED UBIQUITIN LIGASE1 (JUL1), which was previously associated with the jasmonate signalling pathway, also plays a positive role in stomatal closure [7]. In addition, JUL1 is involved in ABA-mediated microtubule disorganisation and stomatal closure [7]. It seems reasonable that microtubule dynamics may fine-tune guard cell responses to both biotic (herbivores) and abiotic (drought, atmospheric CO_2, and temperature) environmental cues. Nevertheless, it remains contentious whether microtubule dynamics and the jasmonate signalling pathway are connected and can further modify stomatal responses. Further investigations into how these key pathways modulate plant growth and stress response should expand our understanding of stomatal responses and their benefit to plants as a whole.

New players in the metabolic regulation of guard cells

Sugars provide a connection between energy metabolism and stomatal regulation, but the mechanisms underlying energy sensing and stomatal development remain unclear. A recent study by Han *et al*. demonstrated that during shorter days, when energy is a limiting factor, the stomatal index is largely reduced [8]. Interestingly, under these conditions, KIN10, the catalytic α-subunit of the sucrose non-fermenting-1 (SNF1)-related kinase 1 (SnRK1) complex, moves to the nucleus of stomatal lineage cells [8]. In these cells, SnRK1 phosphorylates and stabilises SPCH, ultimately boosting stomatal development [8] (Figure 1B). The same study also demonstrated that, among the sugars, including glucose, fructose, glucose-6-phosphate, and fructose bisphosphate, sucrose exhibits the most influence on stomatal development because it increases stomatal cell proliferation under both low-light and short-day conditions. Higher stomatal proliferation was shown to be directly related to the *in vivo* interaction between KIN10 and SPCH [8] (Figure 1B). In fact, sucrose breakdown in the guard cells was demonstrated to regulate stomatal dynamics during transitions from dark to light, elevating the levels of tricarboxylic acid cycle intermediaries [9]. The metabolic signature associated with stomatal aperture and closure under light/dark cycles is substantially different for angiosperms and ferns, revealing faster light stomatal responses in angiosperms [10]. Remarkably, the slower light stomatal responses in ferns (*Microsorum scolopendria* and *Phlebodium aureum*) are seemingly related to a differential metabolic cluster that includes the non-protein amino acid GABA [10].

Although GABA has been proposed as a signalling molecule in previous studies, it was only recently reported that the activity of aluminium-activated malate transporters (ALMTs) might be regulated by GABA [11]. Interestingly, Xu and colleagues revealed that GABA antagonises light- and dark-induced stomatal movements [4]. By using a multispecies assay, they further demonstrated that GABA is likely the master metabolite modulating stomatal closure [4]. Interestingly, GABA accumulates in guard cells in response to water limitation, helping to regulate water loss by arresting stomatal opening via ALMT9, which improves drought resilience in *A. thaliana* [4]. It was also verified that both ALMT9 and ALMT12 are involved in guard cell GABA signalling, revealing that GABA acts on stomatal opening via ALMT9 and on stomatal closure via ALMT12 [4]. Taken together, these findings clearly show that GABA is an *in planta* signalling molecule that modulates stomatal movement (Figure 1C). Notably, disruption of GABA biosynthesis was shown to severally inhibit the organisation of actin filament dynamics and impair pollen tube growth, emphasizing the importance of GABA signalling to cytoskeleton organisation [12]. This means that it might be very interesting to investigate the connections between GABA signalling and the cytoskeleton in guard cells. This may provide valuable insights into stomatal regulation in response to changing environmental conditions, helping in the development of resilient crops for (a)biotic stresses.

These aforementioned findings provide novel insights into the regulation of stomatal movement and deepen our understanding of the molecular mechanisms underlying plant responses to environmental stress. It is now necessary to elucidate how and whether GABA and the cytoskeleton are interconnected and whether these interactions affect only the stomata or the whole-plant response to drought.

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Declaration of interests

The authors declare that there is no conflict of interest.

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Chapter 3

Understanding the dynamics of plant organic acids metabolism following abiotic stresses

For submission

plants

MDPI

Review

Understanding the dynamics of plant organic acids metabolism following abiotic stresses

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Abstract: Plants experience many adverse environmental stressful conditions during their development (e.g. shade, high light, drought, hypoxia, temperature, and metal toxicity), which impact negatively their growth and productivity. In addition to their roles as intermediates of the tricarboxylic acid (TCA) cycle, organic acids (OAs), have been suggested to perform several functions in overall plant metabolism. It has been shown that OAs assist in stress tolerance as root-chelating agents, provide reducers to contribute to reactive oxygen species (ROS) detoxification, and operate in the biosynthesis of stress-signaling and osmoregulatory solutes during stomatal movements. Here, we revisited the mechanisms by which OAs regulate the metabolism of plants in response to specific abiotic stresses. Within such a multi-layered understanding scenario, the wide range of pathways and networks that are affected in response to changes in OAs metabolism offers interesting and not yet fully explored applications in agriculture following abiotic stresses. Finally, we consider how understanding stress response may benefit strategies for designing new resilient crops by targeting OAs metabolism.

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1. Introduction

Plants are constantly facing adverse environmental conditions, including abiotic stress such as drought, salinity, heat, heavy metals etc. To cope with such different abiotic stimuli, plants have developed diverse mechanisms that are highly distinct according to the species, developmental stages, tissue, and cell type [1]. Among a wide variety of strategies, metabolic adjustment is a common mechanism of plant response to abiotic stress conditions [2]. More specifically, the biosynthesis, accumulation, transport, and exudation of organic acids (OAs) have been associated to plant adaptation to environmental stimuli [3,4]. Organic acids are mainly produced in the mitochondria through tricarboxylic acid (TCA) cycle, and they have recognized roles in primary processes such as reduced coenzymes production and energy conversion, and supply of carbon precursors for several biosynthetic pathways [5]. Organic acids can form active cytosolic and storage vacuolar pools that can maintain the redox balance, ATP production, acidification of extracellular spaces, and ionic balance in plant cells [6]. It has been proposed that the coordinated accumulation of OAs in certain plant tissues or compartments, as well as their exudation, represent a pivotal strategy to meet the metabolic and physiological cell demands under different environmental conditions [4,5,7]. In this mini-review, we cover the current understanding of the contribution of OAs metabolism in plant responses to abiotic stress conditions. We additionally discuss the importance of OAs for plant performance with a particular emphasis on the mitochondrial and guard cell contexts. Finally, we discuss how and to which extent enhancing our understanding of stress response from a metabolic perspective may be of significance for designing resilient crops by specifically targeting OAs metabolism.

2. Organic acids metabolism in plants

For a better understanding of OAs' roles in plant metabolism, we first described how they are synthesized and their main regulation points within plants. Organic acids can be formed within distinct cycles, metabolic pathways, and cellular compartments [8–11]. The main production site of OAs is well elucidated through the TCA cycle reactions that occur in the mitochondrial matrix. This pathway is composed of a set of eight enzymes that act after decarboxylation of pyruvate to Acetyl-CoA by pyruvate dehydrogenase, releasing CO$_2$, producing flavin adenine dinucleotide (FADH$_2$) and nicotinamide adenine dinucleotide (NADH) that will be used in the mitochondrial electron transport chain (mETC) [9]. In the "cyclic" mode of TCA cycle, Acetyl-CoA is converted to citrate by citrate synthase, which is followed by the isocitrate production pathway by aconitase. From that moment, two successive oxidative decarboxylation reactions occur, one by the conversion of isocitrate to 2-oxoglutarate (2-OG) by isocitrate dehydrogenase, and the other by the action of 2-OG dehydrogenase (2-OGDH) that converts 2-oxoglutarate to Succinyl-CoA, wherein each of the decarboxylation reactions producing a molecule of NADH and CO$_2$ [12,13]. Subsequently, the enzyme Succinyl-CoA ligase combined ATP synthesis from ADP and Pi converting Succinyl-CoA to succinate, which in turn is oxidized to fumarate by the succinate dehydrogenase and undergoes a reversible reduction to FADH$_2$, revealing the unique enzyme to function both within the TCA cycle and the mETC [12,13]. Fumarase catalyzes the reaction of fumarate to malate [14,15], and then the NAD$^+$ dependent-malate dehydrogenase (MDH) mediates the reversible oxidation of malate to oxaloacetate and production of another NADH molecule. Finally, oxaloacetate react again with Acetyl-CoA to replenish the TCA cycle [12,13].

Tricarboxylic acid cycle intermediates can act in alternative pathways as precursors in the biosynthesis of other molecules, as well as to balance the levels of OAs in plant overall metabolism. Deviations from the standard pathway ("non-cyclic" mode of TCA cycle) can also occur during stress conditions, in which plants require fine-tuned regulation to meet the constant energy changes and demand for OAs to cope with these multiple environmental stimuli. That said, certain enzymes including as 2-OGDH, fumarase, and MDH have been suggested as major checkpoints for TCA cycle regulation (Table 1) [12].

Table 1. Organization of key enzymes regulating tricarboxylic acid (TCA) cycle intermediates and alternative pathways.

Enzymes	**Conversion**	**Regulation**	**Metabolic pathways**
Citrate synthase	Acetil-CoA→Citrate	n.d	n.d
Aconitase	Citrate → Isocitrate	n.d	n.d
Isocitrate dehydrogenase	Isocitrate→2-Oxoglutarate	n.d	n.d
2-Oxoglutarate dehydrogenase	2-Oxoglutarate→ Succinyl-CoA	Ca+2, ATP, ADP, AMP, thiamine, TRX, PHY	C and N metabolism, Amino Acid biosynthesis
Succinil-CoA ligase	Succinyl-CoA→Succinate	n.d	Glyoxylate cycle
Succinate dehydrogenase	Succinate→Fumarate	n.d	n.d
Fumarase	Fumarate→Malate	Pyruvate, 2-OG, ATP, ADP, AMP, TRX, PHY	Amino Acid and N Metabolism, Glyoxylate cycle
Malate dehydrogenase	Malate→Oxaloacetate	NADH, ADP, ATP, AMP	Redox homeostasis, Photosynthesis, Photorespiration

1 n.d: not determined; TRX: thioredoxins; PHY: phytochrome

2-Oxoglutarate dehydrogenase directly links central metabolism to carbon (C) and nitrogen (N) metabolism by the biosynthesis of primary metabolites synthesized from 2-OG, such as the amino acid glutamate (Glu), from other amino acids and proteins, but also assists in the maintenance of the TCA cycle with the generation of a new 2-OG molecule

[16]. 2-OGDH complex can be allosterically regulated by Ca+2, ATP/ ADP, NADH/ NAD+, and thiamine pyrophosphate[13,17], and also by the thioredoxin (TRX) system [18] and the phytochrome system [19]. In a study using specific inhibitors of the 2-OGDH, it was possible to observe marked reductions in respiration rates, changes in the levels of TCA cycle intermediates, and amino acids that are crucial for nitrate assimilation, in potato tubers [20]. This reduction in respiration is associated with an imbalance in C-N metabolism and cellular homeostasis by altered expression of genes associated with amino acid networks, plant respiration, and sugar metabolism [21] . Furthermore, studies using transgenic and mutant lines of the E1 and E2 subunit of 2-OGDH demonstrated its importance in fruit maturation and leaf senescence [22] , as well as in plant growth and seed production [23,24]. In addition, 2-OG is linked to Glu-derived gamma-aminobutyric acid (GABA shunt) synthesis in the cytosol, which in turn can be catabolized into succinic semialdehyde (SSA) by GABA transaminase, subsequently converted to succinate by SSA dehydrogenase, which enters the mitochondria to replenish the TCA cycle [25]. GABA plays an important role in various physiological and molecular processes in plants, such as in C and N balance [26], control of ROS levels [27], reproductive function of plants under heat stress [28], osmotic adjustment [29], and acts as a signaling molecule to abiotic stresses [30]. Succinate, on the other hand, is a major rhizospheric root exudate, with a repressive effect on phosphorus (P) solubilization in N-fixing bacteria [31], in addition to acting in cell elongation, and participating in the glyoxylate cycle in glyoxysomes [32]. Although to a lesser extent, the latter is also known to produce OAs during germination of oil plant seeds. Notably, this occurs by the need for carbohydrate formation from lipids, which occurs via the synthesis of succinate by glyoxysomal succinate oxidase from acetyl-CoA of fatty acids from the breakdown of lipids, followed by the production of other OAs, such as malate and oxaloacetate in the TCA cycle.

Another branch point of TCA cycle is associated with mitochondrial fumarase (fumarate hydratase; EC 4.2.1.2), responsive both for maintaining balance of the TCA cycle, but also for conserving and diverting C to amino acid metabolism [33–35] and glyoxylate cycle, where succinate is converted to fumarate and then malate via succinate dehydrogenase and fumarase [36]. Fumarase has its regulation controlled by concentrations of pyruvate, 2-OG, and adenine nucleotides ATP, ADP, and AMP [37] and by TRXs that act to adjust its activity to modulate the redox status of TCA cycle [18,38]. This enzyme can also be regulated by phytochrome-mediated Ca^{+2} concentrations in response to light in Arabidopsis [39]. Furthermore, fumarase activity can be regulated by epigenetic mechanisms of methylation of its gene promoter in response to salinity [40], but not under anoxic conditions, where its expression is mainly affected by atmospheric N_2 and CO_2 [41]. An approach using transgenic tomato (*Solanum lycopersicum*) plants of fumarase antisense lines revealed reductions in respiration, but also their detrimental impact on photosynthetic rates marked by lower stomatal aperture, decreased fruit production, and root architecture [14,42]. This corroborates with previous studies, where fumarase activity was seen to be higher in guard cells compared to cells in the mesophyll of *Vicia faba* and *Pisum sativum* [43,44].

The enzyme malate dehydrogenase (MDH) is involved both in central metabolism by catalyzing the reversible reduction of oxaloacetate to malate, and in redox homeostasis between cellular compartments, which through different isoforms performs the indirect transfer of reducing equivalents NADH (mitochondria, cytosol, peroxisome plastids) and NAD(P)H (chloroplasts) that serve as a substrate that play important roles in all plant metabolism [45–48]. Mitochondrial MDH (mMDH) can be regulated by levels of NADH [13] , ADP, ATP, and AMP [49], and unlike the other key enzymes mentioned above, it is not regulated by TRX [18], whereas NADP-MDH is regulated by light through this ferredoxin-Trx system [50–52]. During photorespiration the mMDH is also involved in the conversion of glycine (Gly) to serine (Ser), which reduces oxaloacetate to malate and regenerates NAD^+ for glycine oxidation by the Gly decarboxylase [53]. Single and double mutants of mMDH are characterized by impacts not only in respiration rates but also photorespiration and growth of Arabidopsis plants [54]. Indeed, overexpression of different MDH isoforms (mitochondrial and cytosolic) significantly increased malate levels in leaves, roots and root exudates, that conferred higher P availability in cotton (*Gossypium* L.) [55] and enhanced Al tolerance in tobacco (*Nicotiana tabacum* L.) [56], respectively. All these examples considered; it seems rather clear that the TCA cycle function is of key importance to cellular metabolism. Notwithstanding, further studies should be devoted to identifying whether, and to what extent, plant species-specific factors and/or experimental conditions affect *in vivo* respiratory pathways, particularly the TCA cycle.

3. Interactions between CO_2 assimilation and mitochondrial metabolism

The importance of the TCA cycle and mitochondrial OAs metabolism in various metabolic processes, including energy production, reducing equivalents, biosynthesis of other molecules such as amino acids, and their relative participation in photosynthetic performance has been explored in the previous section. Within this section we will address, in more detail, the interaction of the TCA cycle with the photosynthetic process, and how OAs interact with mesophyll cells and guard cells during these processes. Although mitochondrial metabolism and photosynthesis are in two distinct subcellular compartments, both are highly correlated and regulated within the plant cell, acting as both substrate and product for their functioning [11]. The latter is responsible for assimilating atmospheric CO_2 that diffuses into the cells through the stomatal pore, for the production of carbohydrates, where part of these sugars can be catabolized into ATP and reducing power by oxidative respiration in response to cellular energy demand. Mitochondria have been proposed to provide large amounts of ATP to sustain sucrose synthesis rates during the photosynthetic process in both plants [57] and microalgae [58,59]. Its contribution is due to the regulation of the cellular redox status in the dissipation of excess reducing power, especially in leaves under high light incidence, which by the activity of the chloroplast NADP-MDH enzyme uses the excess NADPH to import malate into the mitochondria in exchange for oxaloacetate and regenerate the electron acceptor NADP, and through this transfer of reducing equivalents ATP synthesis is supported via the mETC [11,60,61]. As well as providing reducing equivalents via malate to the photorespiratory pathway in the peroxisomes [62]. Recently it was also seen that photorespiration supplies a large amount of reducing equivalents to mitochondria during whole-plant photosynthesis, which exceeds the NADH dissipation capacity of mETC [63]. Parallel to this, the activity of TCA cycle enzymes tends to be reduced in illuminated leaves compared to leaves in the dark, a fact that occurs primarily due to the inhibition of pyruvate dehydrogenase and the need for carbon skeletons derived from 2-OG.[5,11]. A range of mutants with deficient expression and activity of TCA enzymes has been well characterized demonstrating the importance of mitochondrial OAs metabolism in photosynthetic performance. For example, tomato plants with disrupted fumarase activity exhibit considerable reductions in mitochondrial activity and impaired photosynthesis triggered by reductions in stomatal conductance [14]. Similarly, a deficiency of succinate dehydrogenase expression leads to decreased malate and fumarate levels in conjunction with an increase in stomatal conductance and carbon assimilation in tomato plants [64]. Reduced expression and activity of mitochondrial malate dehydrogenase results in increased carbon assimilation and shoot growth [65], as well as altered growth and root architecture [42]. In contrast, antisense mutants for the mitochondrial citrate synthase gene showed no changes in photosynthetic rates but were characterized by altered leaf pigment levels and impaired nitrate assimilation [66]. Thus, it has been shown that photosynthesis is largely dependent on OAs produced in the mitochondria, contributing to the precise regulation of the energy balance within a plant cell.

At the level of energy conversion and reducing equivalents production, we somewhat understand the contribution of these two processes concomitantly, but at the level of CO_2 assimilation OAs have already been widely described for their crucial role in the photosynthesis of C_4 and CAM (Crassulacean Acid metabolism) plants as intermediates in CO_2 fixation [67,68]. Briefly, in CAM plants, CO_2 is captured during the night by open stomata and can be mainly incorporated into malate by the cytosolic phosphoenolpyruvate carboxylase (PEPcase) and stored in vacuoles, and during the daytime period, CO_2 decarboxylation and refixation occur by the Calvin-Benson cycle with closed stomata [69]. In the case of C_4 plants, the alternative source of malate in the light may occur by the anaplerotic fixation of CO_2, in the mesophyll cells, catalyzed by the PEPcase. This enzyme is responsible for the carboxylation of PEP that produces OAA, which in turn is rapidly reduced to malate by NADP-MDH and diffuses to the vascular bundle sheath cells where it is decarboxylated in the chloroplasts by the enzyme NADP-malic enzyme (NADP-ME) and then the CO_2 is finally refixed by Rubisco [70,71]. Malate derived from mesophyll cells has been described to directly influence stomatal conductance [64,72]. During stomatal opening the cell stores can be regulated by apoplastic malate concentration through activation of voltage-dependent properties of anion channels in the guard cell plasma membrane or as a product of star5ch breakdown and anaplerotic CO_2 fixation [73–75]. Therefore, OAs, especially malate, can be imported from the apoplastic space to support the accumulation of this OA in guard cell metabolism, which we will address in the following topic. Notably, compelling evidence has revealed that fluctuations in environmental conditions disrupt not only photosynthesis but also respiration, ultimately impacting plant growth [7,54,66,76], revealing that OAs are key to ensuring abiotic stress tolerance and the development of resistant crops.

4. Organic Acids modulate stomatal function and its responses under stressful conditions

In plants, stomatal movements are tightly controlled by changes in cell turgor pressure and OAs play an important role as osmotically active solutes and regulators of cell turgor under different environmental conditions. The vacuole plays an important role in the storage of organic compounds such as sugars and OAs and íons e.g. nitrate (NO_3^-), chloride (Cl^-) and potassium (K^+). During stomatal closure, these solutes leave the vacuole towards the guard cell cytosol and there to outside the guard cells [77,78]. This stomatal movement can be regulated by the controlled transport of osmoregulatory ions through various ion channels and pumps [79–83] (Fig. 1). Abscisic acid (ABA) synthesis and signaling, dark, high concentrations of CO_2, and ozone [84,85] are common stimuli inducing stomatal closure. Upon stomatal closure induction, the regulation of various ion channels and transporters that induce the depolarization of the plasma membrane promotes the stomatal closure [86–88]. Malate channels located either at the plasma membrane or at the tonoplast play a major role in this process. Among these channels the ABC transporter AtABCB14 was shown to control the transport of malate from the apoplast to the cytosol of guard cells [80]. Thus, AtABCB14 would play a role as a negative modulator of stomatal closure induced under high CO_2 concentrations, and that the exogenous application of malate minimizes this response [80]. In addition, members of the Aluminum Activated Malate Transporter (ALMT) family have been characterized in Arabidopsis as mediators of malate currents during stomatal movements [89,90]. The AtALMT12/ AtQUAC1 (Quick Anion Channel) is an R-type channel located at the plasma membrane of guard cells being responsible for outward malate transport during stomatal closure in response to high CO_2, darkness, and ABA [82,83]. Another member of this family is AtALMT6, which is located at the tonoplast and is regulated by cytosolic calcium, pH, light, and ABA concentrations [81]. AtALMT4 is a malate efflux channel from the vacuole to cytosol in ABA-induced stomatal closure and its activity depends on the phosphorylation [91]. Although the AtALMT9 channel is a Cl^- influx channel located also in the vacuole membrane, its regulation is controlled by cytosolic concentrations of malate mediating the stomatal opening [77]. Recently, the ALMT9 channel was shown to be negatively regulated by γ-aminobutyric acid (GABA) under water restrictions, increasing drought tolerance and water use efficiency in Arabidopsis [29]. Similarly, GABA may also be involved in the regulation of ALMT12, although, under the conditions tested, stomatal closure was not observed in intact leaves dealing with the dark-to-light transition and may be limited to epidermal cells. Therefore, further assays with epidermal cells may unravel whether ALMT12 may have an inhibitory effect of GABA on the stomatal closure [29]. GABA appears to exert multiple physiological effects by regulating the ALMT channels activity, including regulation of the pollen tube [92] and root growth with the regulation of ALMT1 activity in the malate flow in wheat roots, causing less malate exudation to chelate with Al $^{3+}$ and impair root growth [93]. Therefore, it has been proposed that the regulation by GABA of anion-activated currents is a characteristic of the ALMT family and that this relationship may have a highly important role in the C:N balance, in the regulation of cytosolic pH, and protection against oxidative stress, as well as in salinity and drought tolerance [94].

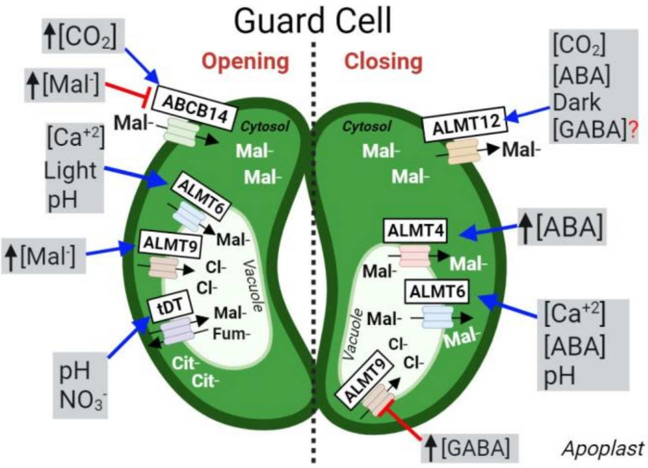

Figure 1. Transport and regulation of organic acids from the apoplastic space into the guard cells. Malate influx from apoplast to guard cell (GC) is mediated by ABCB14 located at the plasma membrane, and has its regulation controlled by malate levels and elevated CO_2. ALMT6 participates in malate influx and efflux in the GC vacuole and can be activated by Ca^{+2}, light, pH, and ABA. ALMT9 can be regulated by malate concentrations and import Cl^- ions into the GC vacuole during stomatal opening, and be negatively regulated by γ-aminobutyric acid (GABA) at stomatal closure in response to water stress. The Tonoplast Dicarboxylate Transporter (tDT) imports malate into the GC vacuole in exchange for citrate and/or fumarate, and its activity is regulated by pH and NO_3^-. ALMT4 is specific in the efflux of malate from the GC vacuole into the cytosol and is ABA-dependent. Another channel involved in stomatal closure is AMT12 responsible for exporting malate into the apoplastic space depending on CO_2, ABA, dark and possibly GABA concentrations. The arrows in blue indicate positive regulation, and the inhibitors, in red, indicate negative regulation of the channels and transporters. Created with BioRender.com.

As aforementioned, malate appears to be the major OA involved in the regulation of stomatal opening by anion channels. However, a Tonoplast Dicarboxylate Transporter (tDT) was the first vacuolar malate transporter characterized at the molecular level [79]. Although present in the guard cell vacuole, plants with functional loss of tDT did not exhibit impaired stomatal movement when exposed to different stimuli [95], yet they showed significant metabolic reprogramming due to the reduced malate and fumarate accumulation in mesophyll vacuoles, which is not observed in guard cells [95]. Subsequently, Frei et a., 2018 [###] characterized tDT as malate and citrate antiport, and that its affinity for malate decreases with decreasing pH, while the affinity for citrate increases. In addition, this transporter exhibits affinity for fumarate and succinate as substrates, but not for 2-OG [96]. It has been further demonstrated that tDT is essential for the regulation of pH homeostasis [97]. In addition, tDT may also play an important role during NO_3^- assimilation, being regulated by a calcineurin B-like gene (CLB) that interacts with a protein kinase (CIPK), CIPK8 when induced by NO_3^- [98]. During nitrate-dependent amino acid synthesis, there is a release of OH^- ions and consequently an increase in pH, and under these conditions, tDT appears to increase its expression and malate transport to bind to OH^- ion and balance the cytosolic pH [98]. That said, tDT may have a major contribution, especially under salt stress conditions associated with NO_3^- assimilation, by precisely regulating malate- and fumarate-mediated pH when NO_3^- is taken up [15,99].

5. Organic acids shape responses to environmentally stressful conditions

Plants are organisms with a sessile lifestyle that have developed complex signalling pathways to mediate survival under stress conditions. A highly intriguing and enigmatic case of plants' responses to abiotic stress is related to its metabolic adjustment, wherein thousands of metabolites may be produced in response to a specific environment. One of many classes of versatile compounds linking plant physiology to environmental responses is the OAs which are synthesized on distinct cell compartments, including mitochondria, cytosol, and chloroplasts. The coordination of energy homeostasis is widely related to ATP production and OAs are directly involved in this network, contributing to several functions in the cell metabolism [33,72]. Exportation of OAs from mitochondria to other organelles is associated with multiple functions including the biosynthesis of amino acids and osmoregulatory molecules. Therefore, diverse stress responses are directly connected to OAs metabolism in plant cells which are involved in the regulation of stomatal behaviors, redox balance, and nutritional adjustments [75,100–102]. Given the flexibility of OAs around subcellular compartments, it is of extreme importance to understand how OAs regulate metabolism and physiology in response to environmentally stressful conditions (Fig. 2). Within the next sections, we discuss the importance of OAs metabolism in response to specific stress conditions, including acid soils, hypoxia, flooding and drought.

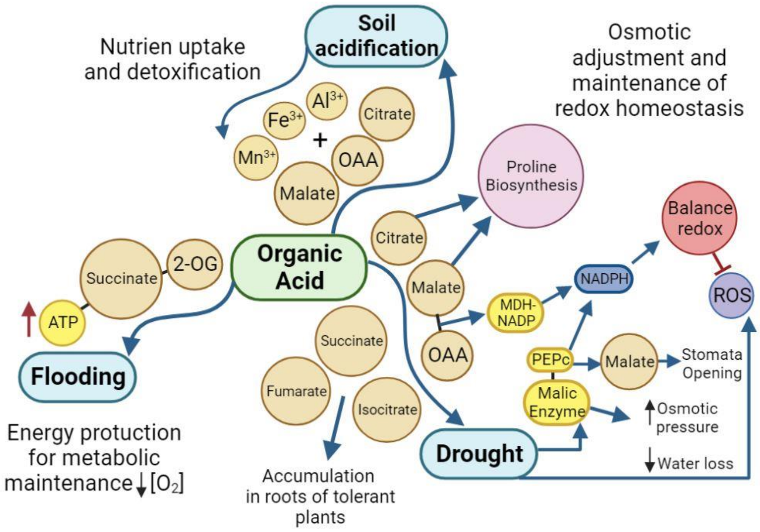

Figure 2. Reprogramming organic acids metabolism in response to abiotic stresses. Organic acids (OAs) can be synthesized in the mitochondria and follow the cyclic flow of TCA cycle or divert to alternative pathways for biosynthesis of other molecules and to maintain Oas levels under adverse environmental conditions. Organic acids can be secreted by roots and bind to toxic cations such as Al^{3+} and Mn^{3+} to alleviate toxicity in acidic soils, as well as aid in the uptake of nutrients such as Fe^{+3}. Under conditions with low O_2 concentrations, 2-oxoglutarate (2-OG) and succinate provide energy in the form of ATP for the maintenance of plant metabolism and survival. Under drought stress, the reprogramming of Oas metabolism is more complex. Citrate and malate serve as carbon skeletons for the biosynthesis of proline, an osmoregulatory, stress-signaling, and proton removal agent that prevents cell damage; isocitrate, succinate, and fumarate confer drought tolerance by accumulating predominantly in roots. Malate helps control the redox balance in chloroplasts by the action of MDH-NADP providing reducing power in the form of NADPH and thus the redistribution of reduction equivalents to prevent the excess of reactive oxygen species (ROS) in chloroplasts. The

enzymes PEPcase and NADP-dependent malic enzyme also assist in the production of NADPH to contain ROS production. In addition, PEPcase synthesizes oxaloacetate and next malate is produced by MDH and assists in stomata opening, and malic enzyme increases osmotic pressure to prevent water loss under water-restricted conditions. Created with BioRender.com.

5.1. Soil acidification

Tropical and subtropical farmlands usually experience environmental adversities that reflect from acid soils (pH≤ 5.5), culminating in the so-called "acid soil syndrome". These phenomena involve nutritional disturbances that include toxic levels of aluminium (Al), manganese (Mn), and iron (Fe) as well as limitations in phosphorus (P) and nitrogen (N) availability [3,103]. Notably, responses to Al toxicity and low P directly involve Oas homeostasis, emerging the central importance of the TCA cycle to ensure plant survival under these conditions.

Roots produce Oas in an attempt to ensure nutritional homeostasis, in which some of these Oas may bind to Al and sustain root development under P limitations [104,105]. Thus, the most accepted mechanism to improve Al tolerance is based on the metal neutralization in both intracellular spaces and around the rhizosphere [105,106]. These tolerance mechanisms involve the transport of organic acids through aluminum-activated anionic efflux channels. Al has been reported to induce expression of the ALMT1 gene of the ALMT (Aluminum-activated Malate Transporters) family and the MATE gene for malate and citrate exudation, which bind to Al while still in the rhizosphere and prevent its entry via the root, but also once in contact with the extracellular space Al can still be stored in the vacuole of the cells [107,108]

The mitochondrial metabolism reprogramming upon Al toxicity involves the non-cyclic functioning of the TCA cycle to maximize the production of citrate, malate, and oxaloacetate and reducing thus impairments on other pathways [105,106]. It is postulated that root exudates mediate Al exclusion boosting root elongation under Al toxicity [109]. Intriguingly, the overproduction of malate and citrate does not always result in higher Al tolerance, once sensitive genotypes produce and exudate a large amount of Oas [110,111]. Repression of organic acid biosynthesis enzymes, such as MDH, results in decreased root length and decreased exudate pH, associated with reduced flexibility of root respiratory rates [12,42], so this enzyme plays substantial contribution in root elongation Furthermore, the exudation of organic acids, mainly citrate, confers a dual advantage to plants from acidic soils, linking aluminium tolerance and increased insoluble Al-P [3]. Enzymes such as PEP carboxylase and citrate synthase have increased levels in response to lack of P, much of which is diverted from TCA to root exudation [112,113]. Tobacco plants overexpressing citrate synthase grow efficiently in alkaline soils with low insoluble P content (López-Bucio et al., 2000), further demonstrating their role under limiting conditions. Remarkably, crop yield impacts following the occurrence of TCA cycle non-cyclic fluxes under Al toxicity must be properly determined to minimize yield penalties.

5.1. Flooding

Humans have been selecting plants based on stem elongation, which reaches a climax during the Green Revolution of the 1960s. This selection resulted, for instance, in cultivars wheat (*Triticum aestivum*) and rice (*Oryza sativa*) with a reduced stature but extremely productive. Nevertheless, flooding events have increased from 1985 to 2010 in countries with large productions of these grains [114], wherein the shorter height of the modern varieties impairs the flood scape largely. Responses to flooding stress may limit the production beyond 30% even on flooding-resilient species such as rice [115]. In this context, metabolic shifts related to O_2 occurred in plants coping with flood stress, in which Oas improve plant survival under this condition. The transcriptional factor SUBMERGENCE 1 (SUB1) activates several genes involved in starch breakdown and sugar metabolism [116]. Following these modifications, fermentation pathways shape differentially energy metabolism across species, highlighting the early induction of Alcohol dehydrogenase (*ADH*) in flood-tolerant species [117]. Loss of function of *ADH* and Pyruvate decarboxylase (*PDC*) leads to rapid death under flooding of rice, Arabidopsis, and maize (*Zea mays*) [118–121]. Moreover, *Lotus japonicas* grown under flooding demonstrated a non-cyclic operational mode for the TCA cycle, showing a large accumulation of succinate [122]. It was assumed that channeling between 2-OG and succinate improves ATP production under flooding, enhancing overall plant survival [122]. Altogether, the fermentation metabolism imposed by flood conditions seems to reorganize the TCA cycle as well as other related pathways (e.g. photosynthesis, photorespiration, and redox metabolism), which ultimately improve plant survival under these conditions.

5.1. Drought

Drought is probably the environmental factor that limits plant growth and development the most [123,124]. It triggers physiological and metabolic changes that lead to ABA synthesis and responses, which in turn causes stomatal closure, suppression of cell growth, and reduced photosynthesis [125,126]. In response to drought plants accumulate compatible solutes and Oas from oxidative respiration that can serve as direct sources for signalling metabolites under abiotic stresses [127]. For instance, bean plants (*Vicia faba L.*) grown under field conditions exhibited high levels of Oas such as citrate, malate, and lactate which may act as carbon sources for proline biosynthesis under water deficit [128]. Under adverse conditions glutamate is synthesized through glutamate dehydrogenase and links Oas from oxidative respiration and proline synthesis, increasing the efficiency in removing excess H^+ [128]. The accumulation of malate and proline also protects young tissues from damage caused by progressive soil drought, as observed in pea plants [129]. In agreement, in sunflower (*Helianthus annuus* L.) plant organs have different regulatory mechanisms, where water stress-sensitive lines accumulated Oas such as maleate, gluconate, and malonate, as well as amino acids in shoots whereas tolerant lines were characterized by decreased levels of isocitrate, succinate, and fumarate, and sugars, predominantly in the roots [130]. These findings suggest that accumulation of OAS, together with changes in amino acids and sugars, confer high osmotic adjustment under water-restricted conditions and allow the plants to withstand water deficiency for a certain period of time. Another common response to abiotic stresses in plants is the enhanced synthesis of ROS. Reactive oxygen species production in the chloroplast has been proposed as one of the main drivers of redox signaling responses or damage in plant cells during water stress [131,132]. In this context, malate export helps to control the redox balance in chloroplasts by the action of the NADP-MDH, a key enzyme in the malate valve between the chloroplast and cytosol. This enzyme is responsible for converting oxaloacetate into malate using NADPH as electron source and facilitating the regeneration of $NADP^+$ the final electron acceptor in the light reactions of photosynthesis. Following water deficit conditions, there is a shortage of $NADP^+$ and increased activity of NADP-MDH to regulate the maintenance of redox homeostasis under adverse environmental conditions [133]. Therefore, the circulation of malate between cell compartments allows the redistribution of reducing equivalents favoring ROS detoxification in the chloroplasts [134]. It has been investigated that tobacco plants submitted to drought significantly increase the level of transcripts encoding PEPcase and the plastid isoform of the NADP-dependent malic enzyme (NADP-ME), generating higher amount of NADPH to deal with the overproduction of ROS under drought [135]. This is because during adverse abiotic stress conditions, the redox response of plants usually requires additional supply of NADPH needed by the parthways that utilize it. NADP-ME can provide the reductive coenzyme NADPH active in the antioxidant system, mainly in the metabolism of ROS by the ascorbate-glutathione parthway and NADPH-dependent thioredoxin reductase [136,137]. In addition, NADP-ME activity can increase osmotic pressure to decrease water loss [138]. The reduced expression of chloroplast NADP-ME in tobacco plants shows that malate content is reduced in guard cells, with consequent lower stomatal aperture and higher water use efficiency, early flowering and shorter life cycle under water stress [139]. Whereas Arabidopsis overexpressing cytosolic NADP-ME has it increased malate levels and great sensitivity to PEG treatment [140]. These works show the different modulations of this enzyme expression and malate accumulation in responses to environmental changes and plant homeostasis. The PEPcase synthesizes malate to contribute to osmotic maintenance in stomatal opening under either optimal conditions or under osmotic stress [141,142], but especially under condition it is enzyme that acts in the replacement of intermediates of the TCA cycle and thus provides metabolites for nitrogen assimilation and amino acid synthesis [143] ######

6. Concluding remarks and perspectives

Organic acids are of great importance in the central metabolism of plants, integrating several strategies to alleviate the tensions caused by abiotic stresses. Within this review, we provided circumstantial evidence that plants present different mechanisms to regulate and control the levels of OAs depending on the cell organ, organell, and most importantly on stressful conditions. Organic acids metabolism and its associated metabolic responses are also associated with the biosynthesis of stress signaling related molecules such as proline and GABA. Among the OAs discussed here, although they exhibit similar biological functions, malate appears to be the major connection to stomatal movements, operating as active osmolyte and regulator of influx and efflux channels, whereas fumarate has been widely described previously for exhibiting specific respondes regarding pH maintenance during nitrate assimilation and as a temporary carbon semidrain analogous to sucrose and starch. However, among the OAs discussed here, malate and its related enzymes appear to have greater prominence in the overall regulation of responses and metabolism, and it is important to emphasize that it is necessary to have a balance between OAs for better functioning of physiological and biochemical responses in plants. The significance and potential of these notions in shaping a tailored developmental program for plants facing environmental stress are of agronomical importance and may contribute to improving plant *fitness* under stressful conditions worldwide.

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Chapter 4

On the significance of vacuolar transport of malate in guard cells during stomatal regulation

4.1. **ABSTRACT**

Organic acids (OAs) play several functions in plant cell metabolism and, among them, malate has been suggested to govern the regulation and signaling of stomatal movements through specific channels and transporters located in the plasma membrane and vacuole of mesophyll and guard cells. The functional redundancy between the tonoplast dicarboxylate transporter (tDT) and ALMT (Aluminium-Activated Malate Transporter) family channels concerning stomatal movements has been previously suggested. Here we addressed the combined function of tDT, ALMT6 and ALMT4, using both single (*tdt-1*, *almt6*, *almt4*) and double mutants (*almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1,* and *almt6::4-2)*, from a biometric, physiological and metabolic perspective. The malate influx mutants *almt6*, *almt6::tdt-1-1,* and *almt6::tdt-1-2* were characterized by reduced malate levels and impaired photosynthetic rates, leading to lower growth in double mutants. Although lower photosynthetic rates were observed in *almt6*, extensive metabolic and respiratory reprogramming sustained its growth yet *almt6* plants showed a slower stomatal opening in response to dark-to-light transition. Therefore, our results suggest that ALMT6 plays a key role in malate transport in guar cells and is able to compensate for the absence of functional tDT with respect to stomatal opening. The *almt6::4-1 and almt6::4-2* mutants showed impaired stomatal opening phenotype under normal conditions, possibly due to ALMT6 repression, and slower stomatal closure kinetics in response to light/dark stimulus. In addition, *almt6 almt4* plants-maintained growth by increased dark respiration and sugar accumulation. Collectively, our results indicate that tDT and ALMT4 are important for adequate dicarboxylate storage in the vacuole of mesophyll cells, supported observed changes in growth and metabolism. Although I did not observe overt phenotypes on stomatal behavior under various stimuli, future analyses will be needed to determine whether members of the ALMT family and/or other ion transporters are functioning at the guard cell and transcript level for better understanding of the mechanisms used to compensate for impaired malate accumulation in these plants.

Keywords**:** organics acids, anion channel, stomatal movement, photosynthesis

4.2. INTRODUCTION

Stomata, structures located in the leaf epidermis, are formed by a pair of guard cells that allow the absorption of CO_2 for photosynthesis and the release of water vapor by transpiration (Lawson, 2008). The degree of stomatal opening and closing is driven by changes in the concentration of osmotically active solutes in the guard cell vacuoles. Among them, malate has been characterized as an important regulator and signaling molecule of this mechanism (Daloso et al., 2017b), playing a role in the osmotic control, activity of anionic channels, and the energy metabolismof the guard cells (Araújo et al., 2011; Nunes-Nesi et al., 2007).

The tonoplast dicarboxylate transporter (tDT) was the first characterized vacuolar malate transporter (Emmerlich et al., 2003b) being able to mediate an antiport transport of malate citrate (1:1) from the cytosol to the vacuole (Frei et al., 2018b). The tDT is expressed both in guard cells, and in mesophilic cells; however, it is more expressed in mesophyll cells compared to guard cells (Bates et al., 2012; Medeiros et al., 2017a). Plants with reduced tDT expression exhibited substantially reduced levels of malate and fumarate (Emmerlich et al., 2003b). Regarding stomatal movements, no expressive changes in tDT responses were observed even when subjected to different stimuli, such as CO_2 transition, light / dark response, and abscisic acid (ABA) (Medeiros et al., 2017a). It is important to note that knockout plants for tDT presented residual malate activity (Emmerlich et al., 2003b), suggesting that other malate carrier proteins are activated to compensate the lack of tDT.

It has been therefore suggested that the inflow and outflow of malate occurs through proteins belonging to the ALMT (Aluminum-Activated Malate Transporter) family. In *Arabidopsis thaliana* 14 members of the ALMT family are subdivided into three clades. Clade II includes AtAMLT 4, 5, 6, and 9, which are directed towards vacuole membranes (Kovermann et al., 2007b; Sharma et al., 2016). ALMT9 was the first member of the ALMT family characterized by mediating the flux of malate and fumarate directed to the vacuole of mesophyll cells (Kovermann et al., 2007b). It was further demonstrated that ALMT9 is a chloride (Cl^-) inflow channel from the cytosol to the vacuole and that its activity is regulated and signaled by cytosolic concentrations of malate during stomatal opening (De Angeli et al., 2013b). Accordingly, the *almt9* knockout mutant is characterized by a reduced stomatal opening (De Angeli et al., 2013b).

The ALMT6 channel is guard cell-specific and operates in the transport of malate between the cytosol and the vacuole. This channel has been described as a modulator of both stomatal opening and closure, being induced not only by $Ca^{2+,}$ pH, and cytosolic malate concentrations, in addition to being mediated by light and ABA (Meyer et al., 2011b). Arabidopsis mutant plants with reduced expression of ALMT6 (*almt6*) did not show changes in stomatal conductance compared to wild-type plants, suggesting a functional redundancy with other malate transport proteins in guard cells. The malate efflux required for stomatal closure can be conducted by ALMT4, which is activated by phosphorylation of the MPK4 and MPK6 kinases, and indirectly regulated by ABA(Eisenach et al., 2017b). Furthermore, Knockout plants for ALMT4 showed impaired growth and stomatal closure (Eisenach et al., 2017b). Collectively, these studies suggest that the functional redundancy of vacuolar malate transport in guard cells appears to be essential for maintaining stomatal function. However, it is believed that different malate transporters may be of relative importance in response to the various metabolic stimuli and necessary adjustments during the regulation of stomatal movements.

We focused to investigate whether changes in malate levels promote impairments in stomatal movements, photosynthetic capacity, and central metabolism in plants with functional loss of vacuolar organic acids transporters guard cells. For that, a range of physiological and biochemical factors were analyzed in both single and double mutants involved in stomatal responses, namely *almt4, almt6,tdt-1*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1-1* and *almt6::4-1-2*. All mutants were characterized by reduced leaf malate levels, except for the *almt6amt4* double mutants which display leaf malate accumulation. Plants with functional loss of ALMT6 showed reduced photosynthetic rates (A_N) and stomatal conductance (g_s), and impaired growth in the *almt6 tdt-1* double mutants. We highlight ALMT6 as essential during stomatal opening, being able to compensate the absence of functional tDT, at least with respect to stomatal regulation. Although *almt6* and *almt6 almt4* have impaired stomatal function, metabolic and respiratory reprogramming were able to kept growth unchanged, most likely related to higher sugar accumulation in the leaves of these plants. Our results suggest that repression of ALMT4 is able to promote a metabolic readjustment that impacts stomata opening by the activity of efflux channels in the plasma membrane of guard cells.

4.3. **MATERIALS AND METHODS**

Isolation of T-DNA insertion mutants and genotypic characterization

Arabidopsis T-DNA-insertion lines for tDT (At-5g47560) gene encoding the vacuolar dicarboxylate transporter (*tdt-1*; SAIL_681_C09) were obtained from the SAIL collection (Syngenta Arabidopsis Insertion Library), while the ALMT family mutants, specifically the line

ALMT6 (At2g17470) was obtained from the GABI-KAT collection (*almt6-1*; GABI_259D05) and the line ALMT4 (At1g25480) was obtained from Salk Institute Genome Analysis Laboratory - SIGn23 (*almt4-1*; SALK_086236),. For genotyping the T-DNA insertion lines, leaf samples of plant from each genotype were collected separately, and genomic DNA was extracted for PCR analysis.

The PCR resulted in a genomic fragment of the target gene using left primer (LP) and right primer (RP) and the T-DNA insertion using a T-DNA specific left border primer (LB). The primers used were: for *Attdt-1*, LP (5'- AAG AGA AAA CTC ACC AGT CAC CAC-3'); RP (5'- AAG TGA ACT CTG TGA TGG TAG CAG-3') and LB3 (5'- GAA TTT CAT AAC CAA TCT CGA TAC AC-3'); for *almt6-1*, LP (5'- GCA TTC AGG GTG TTC TTG TTG-3'), RP (5'-GAT TGA ATA GCG GAC CTG TAC C - 3') and LB08409 (5' – ATA TTG ACC ATC ATA CTC ATT GC - 3'); for *almt4-1*, LP (5'-ATA ACC ATT ATA GCA CCT CAT TTG C-3'), RP (5'- ATC TTC CGG ATT TTG ATT AAA AAT C-3'), and LB1.3 (5'- GAT TTT GCC GAT TTC GGA ACC ACC AT-3').

After initial screening, knockout lines were isolated and homozygous plants were selected for further analyses. To obtain two double mutant lines, crossings were performed using homozygous mutant *almt6-1* and *tdt1-1*, and homozygous mutant *almt6-1* and *almt4-1*, respectively. The double-mutant F1 generation was confirmed by PCR and the heterozygous lines were selected. The seeds of these plants were harvested and placed to germinate; the F2 plants were analyzed by PCR until homozygous F2 plants were confirmed by PCR and the reduced gene expression pattern was further analyzed by qRT-PCR. The primers used were: for *Attdt-1*, LP (5' – AAG AGA AAA CTC ACC AGT CAC CAC - 3'); RP (5' – AAG TGA ACT CTG TGA TGG TAG CAG – 3'); for *almt6*, LP (5' – AGC CTC CAC ATG GAC CTT ACA G – 3'); RP (5' – GAT ACA GGC AGC TCC AGA GAA ACG – 3'); for *almt4*, LP (5' - - TCT TTC GGA AAT TCA GGC AGC TC – 3'); RP (5' – AAC GTT CCC TGG GCT TAG CTT C – 3'); for actin endogenous control LP (5' – ACG GTA ACA TTG TGC TCA GTG GTG – 3'); RP (5' – CTT GGA GAT CCA CAT CTG CTG GA – 3').

Growth conditions and evaluation of biometrics parameters

All Arabidopsis plants used in this study were of the Colombia ecotype (Col-0) background. Seeds from homozygous plants were surface-sterilized and incubated for 2 days at 4 °C in the dark on agar plates containing half-strength MS medium supplemented with 1% sucrose (w/v) (Murashige and Skoog 1962). Seeds were subsequently germinated and grown under short-day conditions (8 h/16 h of light/dark) with an irradiance of 150 μmol photons m^{-2} s^{-1}, 22 °C and 20 °C in the light and dark respectively, and 60% of relative humidity. After 10 days, the seedlings were transferred to the commercial substrate (Carolina Soil, Kingston, North Carolina, EUA) and grown in a growth chamber under the same conditions.

Whole rosettes from 4-week-old plants were harvested, and the rosette fresh and dry weight, leaf area (LA), and specific leaf area (SLA) were measured. LA was measured by a digital image method using a scanner (Hewlett Packard Scanjet G2410, Palo Alto, Califórnia, EUA), and the images were processed using ImageJ software (Schindelin et al., 2015) whereas SLA was calculated as described by (Hunt et al., 2002).

Gas exchange and Chlorophyll Fluorescence Measurements

Gas exchange parameters were determined in 4-week-old plants, simultaneously with chlorophyll a (Chl *a*) fluorescence measurements using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LICOR Inc.) Instantaneous gas exchanges were measured after 1 h of illumination during the light period under 150 mmol m^{-2} s^{-1} (light of growth) PPFD (Photosynthetic Photon Flux Density) at the leaf level. The reference CO_2 concentration was set at 400 mmol CO_2 mol^{-1} air. All measurements were performed using the 2 cm^2 leaf chamber at 25°C, while the amount of blue light was set to 10% PPFD to optimize the stomatal aperture.

Dark respiration (Rd) was measured using the same gas-exchange system as described above after at least 1 h during the dark period.

Photosynthetic light-response curves (A/PPFD) were initiated at C_a of 400 μmol mol^{-1} and PPFD of 1,000 μmol m^{-2} s^{-1}. Then, the PPFD was increased to 1,200 μmol m^{-2} s^{-1} and afterward decreased stepwise to 0 μmol m^{-2} s^{-1} (12 different PPFD steps). Simultaneously, Chl *a* fluorescence parameters were obtained (Yin et al., 2009). The responses of A_N to C_i (A_N/C_i curves) were determined at saturated light of 1,000 μmol m^{-2} s^{-1} at 25°C under ambient oxygen. Briefly, the measurements started at C_a of 400 μmol m^{-2} s^{-1}, and when the steady-state was reached, C_a was decreased stepwise to 50 μmol m^{-1}. Upon completion of the measurements at low C_a, C_a was returned to 400 μmol m^{-2} s^{-1} to restore the original A_N. Next, C_a was increased stepwise to 1.200 μmol m^{-2} s^{-1} in a total of 11 different C_a values (Long and Bernacchi, 2003).

Estimation of g_m, V_{cmax}, J_{max}, and Photosynthetic Limitations

The C_c was calculated following (Harley et al., 1992) as:

$$C_c = (\Gamma^* (J_{flu} + 8 (A_N + R_L))) / (J_{flu} - 4 (A_N + R_L)$$

where the conservative value of Γ^* for Arabidopsis was taken from (Mott et al., 2008). Then, g_m was estimated as the slope of the AN versus Ci – C_c relationship
as:

$$g_m = A_N / (C_i - C_c)$$

From A_N/C_i and A_N/C_c curves, the V_{cmax} and the J_{max} were calculated by fitting the mechanistic model of CO2 assimilation (Farquhar et al., 1980) using the C_i - or C_c -based temperature dependence of kinetic parameters of Rubisco (K_c and K_o) (Mott et al., 2008). Then, V_{cmax}, J_{max}, and g_m were normalized to 25°C using the temperature-response equations from (Sharkey et al., 2007).

Determination of metabolite levels

Whole rosettes from 4-week-old were harvested in the middle of the day. Rosettes were flash-frozen in liquid nitrogen and stored at - 80°C, and subsequently lyophilized for one week until further analyses. The sample was macerated and approximately 10 mg of dry matter was subjected to methanolic extraction, as described by (Lisec et al., 2006). The levels of starch, sucrose, fructose, and glucose were determined as described previously (Fernie, 2001). Proteins and amino acids were determined as described previously (Gibon et al., 2004). Malate and fumarate were determined as detailed by (Nunes-Nesi et al., 2007). The photosynthetic pigments were determined as described (Porra et al., 1989).

Stomatal opening and closing kinetics measurements

To evaluate the kinetics of stomatal opening and closing, the g_s values were recorded at intervals of 30 seconds using an open-flow infrared gas-exchange analyzer system (LI-6400XT; LI-COR) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR). The g_s responses to dark/light and light/dark transitions were measured in plants acclimated to dark or light for at least 2 h. The light in the chamber was kept turned off/then turned on for 10/40 min and turned on/turned off 10/40 min. The CO_2 concentration in the chamber was kept at 400 μmol mol^{-1} air.

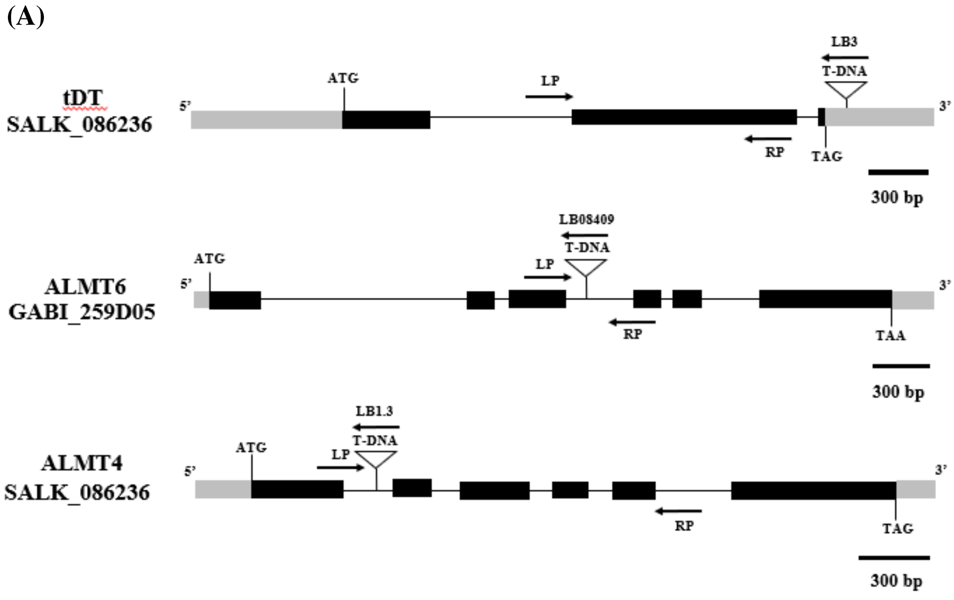
Experimental Design and Statistical Analysis

The data were obtained from the experiments using a completely randomized design using eight genotypes, with the except for the stomatal opening and closing kinetics, which were performed in a randomized block design. Additionally, the experiments were repeated at least three times with similar phenotypes observed each time. All data are expressed as means ± SE ($n = 5$). Data were tested for significant ($P < 0.05$) differences using Student's tests. All the statistical analyses were performed using the algorithm embedded into Microsoft Excel.

4.4. RESULTS

Genotyping of T-DNA insertional mutants of malate transporters

To investigate the tDT and vacuolar ALMT channels are potentially involved in stomatal movements, we characterized plants with functional loss of tDT, ALMT6 and ALMT4 in independent T-DNA insertion lines for each gene. First, tDT and ALMT4 mutants were screened. The homozygosity of each mutant line was confirmed using primer pairs designed to span the T-DNA insertion sites of each gene (Fig. 1a). The reduction of transcripts levels was further confirmed in all homozygous mutant lines by RT-qPCR(Fig. 1b,c,d). Furthermore, we generated two double mutant lines for ALMT6 and tDT genes, and two double mutant lines for the ALMT6 and ALMT4 genes, which were named *almt6::tdt-1-1*, *almt6 :: tdt -1-2*, *almt6 :: 4-1* and *almt6 :: 4-2*. Consequently, the mutant lines *almt6::tdt-1-1* and *almt6::tdt-1-2* exhibited reducedtranscripts for the ALMT6 and tDT genes when compared to the wild type (Fig. 1b and 1c). Similarly for the ALMT6 and ALMT4 genes, the two isolated lines *almt6::4-1* and *almt6 :: 4-2* showed low levels of normalized gene expression for both genes (Fig. 1c and 1d).



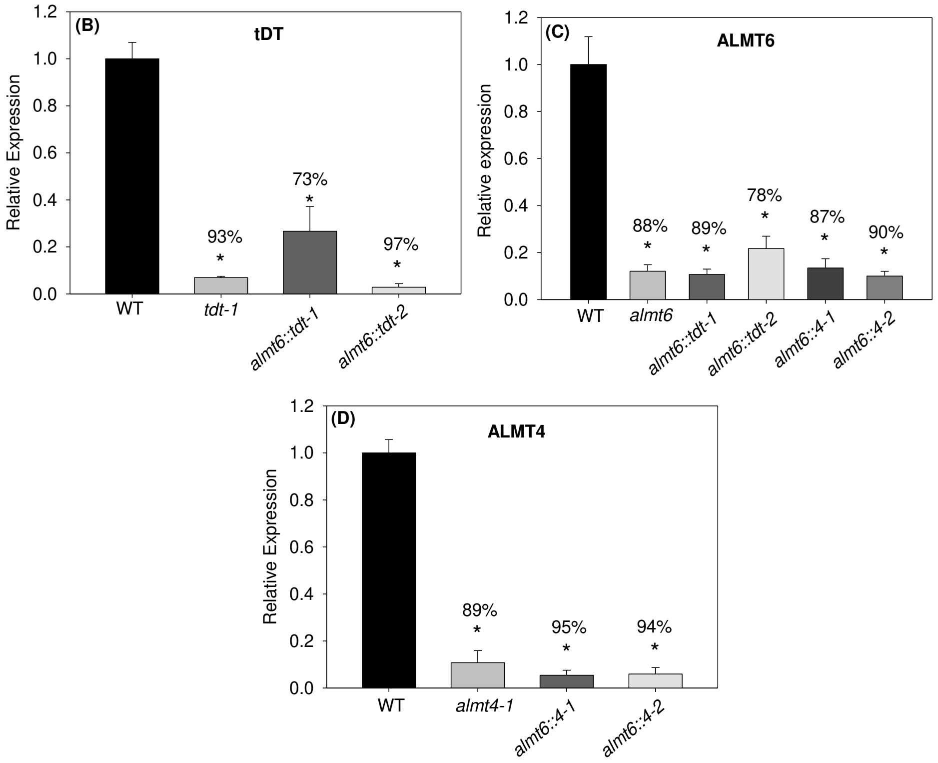


Figure 1: tDT, ALMT6 and ALMT4 expression in Arabidopsis leaves of *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1*, *almt6::tdt-2*, *almt6::4-1* and *almt6::4-2* mutant lines. **(A)** Schematic representation of the tDT, ALMT6 and ALMT4 gene structure. T-DNA insertion sites of the selected mutant lines are indicated. **(B)** qRT-PCR gene expression analysis of tDT, ALMT6 and ALMT4 in leaves of *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1*, *almt6::tdt-2*, *almt6::4-1* and *almt6::4-2* mutant lines. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 4).

The *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2* mutants show differences in vegetative growth under short-day conditions

Given that malate accumulates within the guard cell vacuole during stomatal opening and serves as a source of carbon skeletons for other molecules, we assessed the impact of mutations in tDT, ALMT6 and ALMT4 on the vegetative growth of Arabidopsis plants. We observed evident changes in 4-week-old plants growing under short-day conditions (8 h / 16 h), wherein the mutant lines related to stomatal opening, namely *tdt-1*, *almt6::tdt-1-1* and *almt6:: tdt-1-2*, were characterized by reductions of the rosette fresh weight, rosette dry weight , leaf area and number of leaves compared to wild type (Fig. 2). Concerning the to mutants related to stomatal closure, *almt4* plants exhibited reductions in fresh weight, dry weight, leaf area and the number of leaves. Surprisingly, *almt6* plants and double mutants *almt6::4-1* and *almt6::4-2* did not differ in the analyzed growth parameters (e.g. dry rosette weight, fresh weight of the rosette, leaf area and number of leaves). Despite the evident differences in terms of total leaf area between mutant and wild-type plants, the specific leaf area (SLA) was not altered by the lack of expression of tDT, ALMT6 and ALMT4 in both single and double mutant lines (Fig. 2.f).

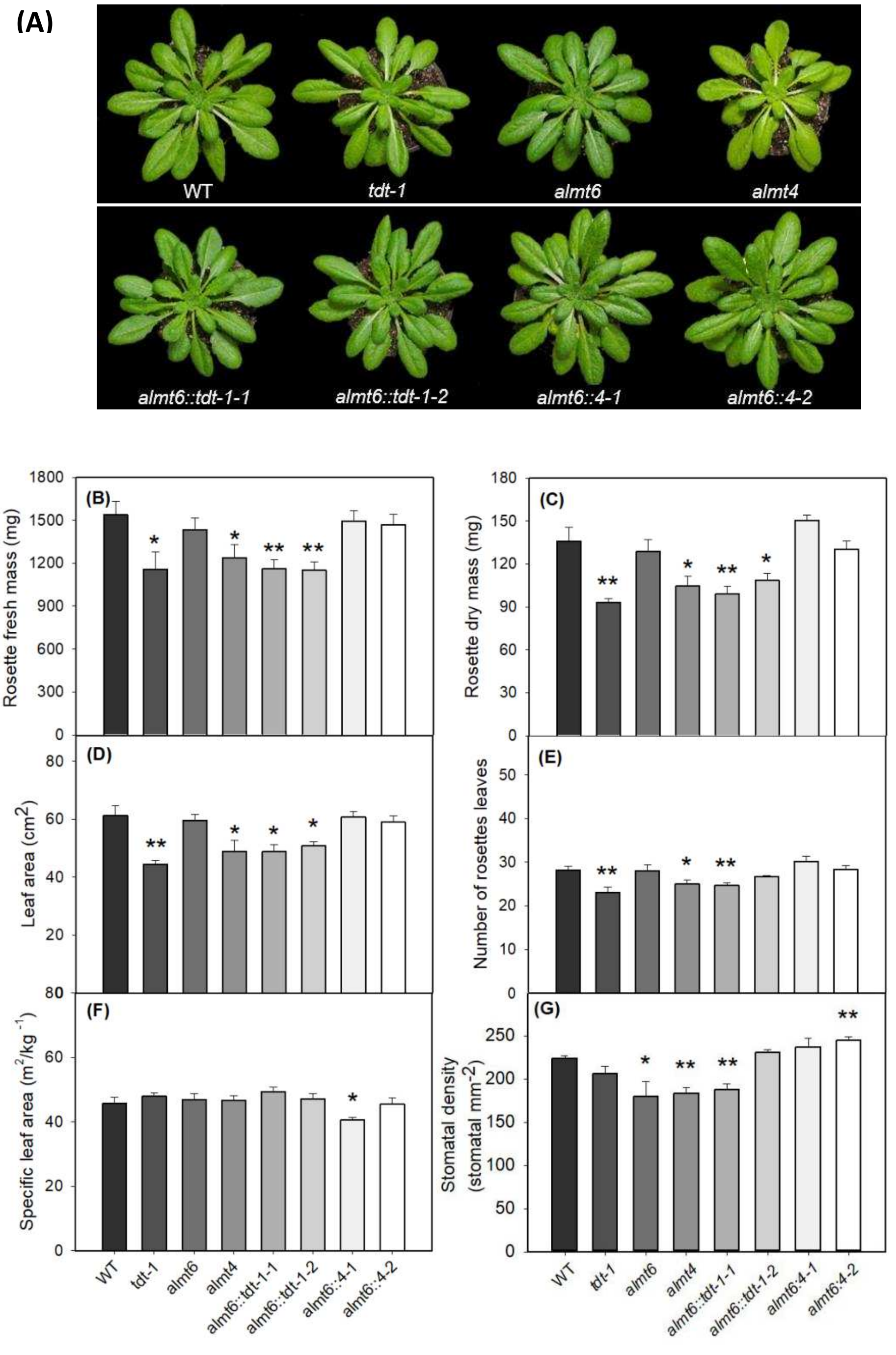


Figure 2: Growth phenotype of Arabidopsis tDT, ALMT6 and ALMT4 mutant lines. **(A)** tDT, ALMT6 and ALMT4 mutant plants after 4 weeks of cultivation. **(B)** Total rosette fresh weight, **(C)** total rosette dry weight, **(D)** rosette area, **(E)** number of leaves, **(F)** specific leaf area leaf, (G) stomata density. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 6).

We further evaluated the impact of mutations on the stomatal density, and observed that the lines *almt6*, *almt4* and *alm6::tdt-1-1* showed significantly reduced stomatal density compared towild type, whereas the almt6::4-2 line presented higher stomatal density values, , compared to wild type. Otherwise, no differences were observed in the stomatal density for *almt6::tdt-1-2* and the single mutant line *tdt-1* (Fig. 2g).

Photosynthetic capacity Is altered in *almt6* and *almt6 x tdt-1* mutants plants

We further conducted a characterization of the photosynthetic capacity by first analyzing the instantaneous gas exchange parameters under growth radiation (150 µmol m^{-2} s^{-1}). The double mutants, *almt6::tdt-1-1*, *almt6::tdt-1-2* and *almt6::4-1* showed reductions in net photosynthesis (A_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration (E) compared to the wild type, while *almt6::4-2* showed also significant reductions in A_N and g_s, with no impact on C_i and E (Fig.3). For single mutants, *almt6* showed reductions in g_s, C_i and E, however, these changes did not reflect in A_N changes, while photosynthetic parameters remained unchanged in *tdt-1* and *almt4* compared to the wild type. Regarding dark respiration rates (R_d), *tdt-1* and the double mutants *almt6 :: 4-1* and *almt6 :: 4-2* showed significant increases compared to wild type, while *almt6*, *almt4*, *almt6::tdt-1-1* and *almt6::tdt-1-2* did not present any differential response.To further characterize photosynthesis in these lines we next evaluated the response of A_N to the photosynthetically active photon flux density (PPFD) that ranged from 0 to 1200 µmol m^{-2} s^{-1}. We observed that the *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2* exhibited significant changes in A_N right at the initial irradiance points, while *tdt-1* remained unchanged regardless of irradiance (Fig. 4).

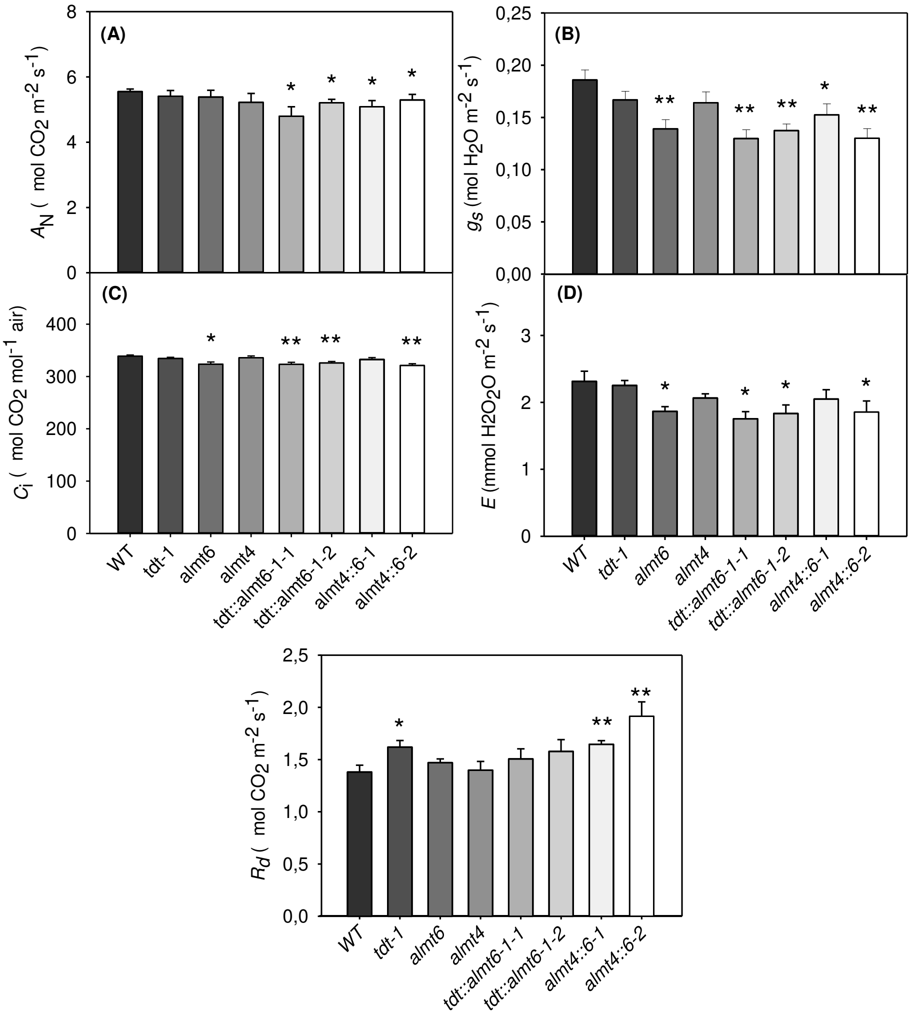


Figure 3: Gas-exchange parameters and dark respiration in wild-type and *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1* and *almt6::4-2* mutant plants measured under growth irradiance (150 µmol m^{-2} s^{-1}). (A) A_N, Net photosynthesis; (B) *gs*, stomatal conductance; (C) *C*i, intercellular CO$_2$ concentration; (D) *E*, transpiration, (E) R_d, Dark respiration. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 6).

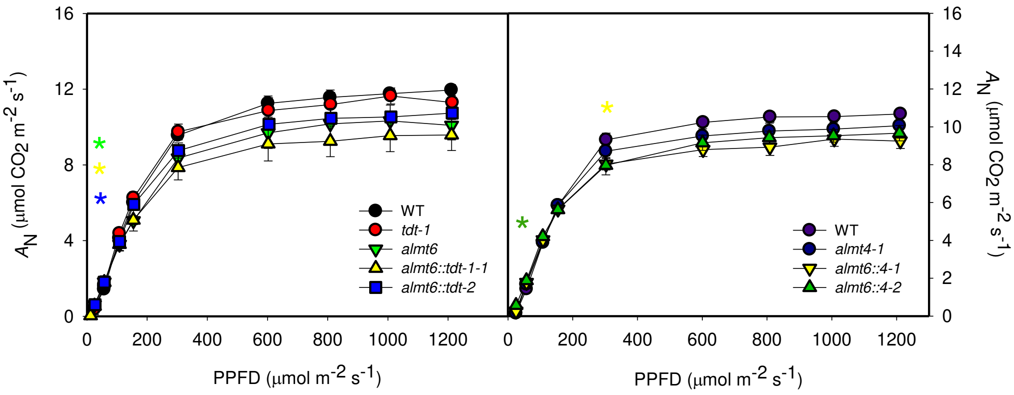


Figure 4: Photosynthetic light-response curves in deficient mutants. WT *tdt-1*, *almt6*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt4*, *almt6::4-1* and *almt6::4-2* plants. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 6).

Lower rates of light-saturated A_N (A_{PPFD}) were observed in the *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2* plants, with significant changes also for compensation irradiance (I_c) (Table 1). The *almt6* and *almt6::tdt-1-1* mutants also showed lower values for light use efficiency ($1 / \phi$) and saturation irradiance (I_s), while no changes in these parameters were observed for *almt6::tdt-1-2* (Table 1). Concerning the mutants related to stomatal closure, *almt4* plants did not show significant differences for A_N regardless of irradiance when compared to wild-type plants. On the other hand, in the double mutants *almt6::4-1* and *almt6::4-2* we observed changes in A_N depending on the irradiance as revealed with the lower values of light-saturated A_N (A_{PPFD}), $1 / \phi$ and I_c, whereas no difference was observed for I_s (Table 2).

Table 1: Photosynthetic parameters from light-response curves in WT *tdt-1*, *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2*, plants. An asterisk denotate values that were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

Parameters	WT	*tdt-1*	*almt6*	*almt6::tdt-1-1*	*almt6::tdt-1-2*
A_{PPFD} (μmol m^{-2} s^{-1})	11.86 ± 0.27	11.97 ± 0.59	**10.36 ± 0.62**	**9.50 ± 0.68**	**10.35 ± 0.30**
$1 / \phi$	15.99 ± 0.66	15.99 ± 0.99	**18.21 ± 0.72**	**19.37 ± 1.11**	16.27 ± 0.81
I_c (μmol m^{-2} s^{-1})	27.79 ± 1.83	26.35 ± 3.05	**19.64 ± 2.70**	**18.03 ± 2.51**	**22.26 ± 1.77**
I_s (μmol m^{-2} s^{-1})	293.99 ± 11.49	**259.97 ± 10.52**	**297.37 ± 24.70**	**287.76 ± 11.51**	303.44 ± 25.76

*A_{PPFD}: Net CO_2 assimilation rate saturated by light; *I*c: light compensation point; *I*s: light saturation point; $1 / \phi$: Light use efficiency.

Table 2: Photosynthetic parameters from light-response curves in WT *tdt-1*, *almt6::4-1* and *almt6::4-2*, plants. An asterisk denotate values that were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

Parameters	WT	*almt4*	*almt6::4-1*	*almt6::4-2*
A_{PPFD} (μmol m^{-2} s^{-1})	10.60 ± 0.19	9.91 ± 0.39	**9.19 ± 0.39**	**9.54 ± 0.36**
$1 / \phi$	18.40 ± 0.29	18.27 ± 0.37	**17.29 ± 0.20**	**14.81 ± 1.60**
I_c (μmol m^{-2} s^{-1})	28.11 ± 2.17	25.48 ± 2.52	**22.28 ± 0.76**	**20.34 ± 0.97**
I_s (μmol m^{-2} s^{-1})	252.65 ± 16.05	239.86 ± 11.63	234.77 ± 8.98	271.64 ± 7.70

*A_{PPFD}: Net CO_2 assimilation rate saturated by light; *I*c: light compensation point; *I*s: light saturation point; $1 / \phi$: Light use efficiency.

The A_N responses to the internal CO_2 concentration (A_N / C_i curves; Fig. 5, a and c) were obtained, which were then converted into A_N responses to the chloroplast CO_2 concentration (A_N/C_c curves; Fig. 5 b, d). Under CO_2 concentration ranging from 50 to 1200 µmol of CO_2 m^{-2} s^{-1}, the *A*/*Ci* curves showed a moderate relation with the previous data. Under these conditions, *tdt-1*, *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2* showed significant reductions in A_N, when compared to wild type. Concerning stomatal closure mutants, *almt4* and *alm6::4-1* did not show any changes when compared to the wild type, unlike *almt6::4-2* which presents decreases in A_N following C_i increases.

We additionally analyzed the diffusive, photochemical and biochemical restrictions to photosynthesis. Under the concentration of ambient CO_2 (400 µmol m^{-2} s^{-1}) stomatal opening mutants, *tdt-1*, *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2,* exhibited smaller A_N, g_s and E (Table 3). The double mutants *almt6::tdt-1-1* and *almt6::tdt-1-2* displayed lower values of photochemical efficiency of PSII (F_v '/ F_m') and electron transport rate. Although the *almt4* showed a slight increase in A_N, the double mutants *almt6::4-1* and *almt6::4-2*, did not show significant differences in the parameters represented in Table 3. No impact on the intrinsic efficiency of water use. (WUEi) were observed for genotypes involved in both stomatal opening and stomatal closure.

Overall, C_i and C_c estimates were similar for almost all genotypes and WT plants, although an increase for C_i was observed in the double mutants *almt6::tdt-1-1* and *almt6::tdt-1-2*, and for C_c in the plants *almt6* compared to the wild type (Table 4). The g_m, estimated using a combination of gas exchange and chlorophyll *a* fluorescence parameters using two independent methods, was lower in *tdt-1*, *almt6*, *almt6::tdt-1-1* and *almt6::tdt-1-2* in comparison to the wild type. In addition, we estimated the maximum carboxylation speed (V_{cmax}) and the maximum capacity for electron transport rate (J_{max}). Accordingly, when estimated on C_i basis, the V_{cmax} were lower for *tdt-1*, almt6, *almt6::tdt-1- 1* and *almt6::tdt-1-2,* while J_{max} was lower for *tdt-1* and *almt6::tdt-1-2*. On a C_c basis, V_{cmax} also decreased for these lines, while J_{max} decreased only for *tdt-1*. The J_{max}: V_{cmax} ratio in C_i base was higher for the mutants *tdt-1*, *almt6*, *almt6 :: tdt-1-1* and *almt6::tdt-1-2*, whereas in the base C_c this ratio was higher only in the double mutants *almt6 :: tdt-1-1* and *almt6::tdt-1-2*.

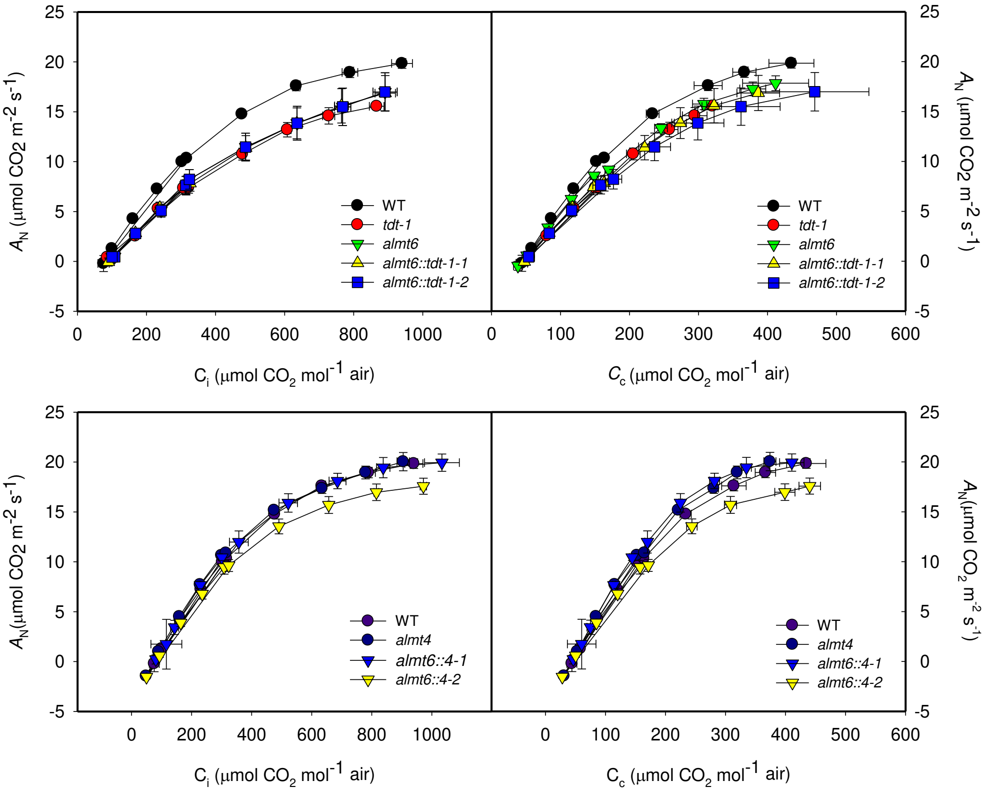


Figure 5: Net photosynthesis (A_N) curves in response to substomatal (C_i) or chloroplastic (C_c) CO_2 concentrations in WT, *tdt-1*, *almt6*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt4*, *almt6::4-1* and *almt6::4-2* plants. Data presented are mean ± SE (n = 5) obtained in independent assays (five plants in each assay).

The analyzed photosynthetic limitations were divided into stomatal (l_s), mesophilic (l_m) and biochemistry (l_b) (Table 4). Photosynthetic rates in stomatal opening-related double mutants, *almt6::tdt-1-1* and *almt6::tdt-1-2*, were mainly restricted by l_s (24% in plants similar to WT, 20% and 19% in doubles mutants, respectively). The l_m represents, on average, 44% wild type, and 39%, 40% and 38% in *almt6*, *almt4* and *almt6::4-2*, respectively. When analysing l_b, 31% was restricted in WT, whereas *almt4* and *almt6::4-2* plants showed 38% and 41%, respectively (Table 4).

Table 3: Photosynthetic characterization *of tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1* and *almt6::4-2* mutant plants. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type (P < 0.05). Values are presented as mean ± SE (n = 5).

Parameters	**WT**	***tdt-1***	***almt6***	***almt4***	***almt6::tdt-1-1***	***almt6::tdt-1-2***	***almt6::4-1***	***almt6::4-2***
A_{N} (µmol $\mathrm{CO_2}$ $\mathrm{m^{-2}\ s^{-1}}$)	9.99 ± 0.22	**7.34 ± 0.32**	**8.57 ± 0.26**	**10.64 ± 0.09**	**7.43 ± 0.77**	**7.63 ± 0.89**	10.42 ± 0.30	9.45 ± 0.68
g_s (mol $\mathrm{CO_2}$ $\mathrm{m^{-2}\ s^{-1}}$)	0.124 ± 0.00	**0.10 ± 0.00**	**0.10 ± 0.00**	0.13 ± 0.00	**0.11 ± 0.00**	**0.10 ± 0.00**	0.13 ± 0.00	0.13 ± 0.00
E (mmol H2O $\mathrm{m^{-2}\ s^{-1}}$)	3.50 ± 0.12	**2.78 ± 0.13**	**2.52 ± 0.08**	4.01 ± 0.26	**2.73 ± 0.08**	**2.73 ± 0.21**	3.59 ± 0.27	3.41 ± 0.30
WUEi (A_{N} / g_{s})	80.42 ± 1.84	77.16 ± 3.08	91.25 ± 5.48	81.92 ± 2.48	70.44 ± 4.55	70.44 ± 4.55	81.63 ± 1.29	74.92 ± 2.92
$F_{\mathrm{v}}/F_{\mathrm{m}}$	0.76 ± 0.00	0.75 ± 0.00	0.75 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.74 ± 0.01	0.80 ± 0.04	0.76 ± 0.00
F_{v}’ / F_{m}’	0.50 ± 0.00	0.48 ± 0.00	0.48 ± 0.00	0.51 ± 0.00	**0.47 ± 0.00**	**0.47 ± 0.00**	0.52 ± 0.00	0.51 ± 0.00
J_{flu}	86.42 ± 3.16	**66.40 ± 2.54**	80.47 ± 5.15	90.13 ± 2.53	**67.41 ± 6.77**	**63.26 ± 6.73**	91.68 ± 4.51	78.07 ± 6.48

A_{N}, Net photosynthesis rate; g_s, stomatal conductance; E, transpiration; WUEi, intrinsic water use efficiency; R_{d} dark respiration, $F_{\mathrm{v}}/F_{\mathrm{m}}$, maximum PSII photochemical efficiency; F_{v}’/F_{m}’, actual PSII photochemical efficiency; J_{flu}, electron transport rate estimated by chlorophyll fluorescence parameters.

Table 4: Photosynthetic characterization *of tdt-1*, *almt6*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt4*, *almt6::4-1* and *almt6::4-2* mutant plants. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type (P < 0.05). Values are presented as mean ± SE (n = 6).

Parameters	**WT**	***tdt-1***	***almt6***	***almt4***	***almt6::tdt-1-1***	***almt6::tdt-1-2***	***almt6::4-1***	***almt6::4-2***
C_{i}	302.31 ± 2.09	307.22 ± 2.95	293.27 ± 5.64	299.39 ± 2.18	**314.55 ± 4.76**	**316.99 ± 4.88**	300.88 ± 0.94	308.49 ± 3.15
C_{c}	156.85 ±5.43	160.72 ± 1.77	**142.51 ± 1.55**	158.35 ± 5.98	157.49 ± 4.75	165.81 ± 12.84	151.48 ± 4.89	171.01 ± 3.98
$g_{\mathrm{m\_Harley}}$ (mol $\mathrm{CO_2}$ $\mathrm{m^{-2}\ s^{-1}\ bar^{-1}}$)	0.07 ± 0.00	**0.05 ± 0.00**	**0.06 ± 0.00**	0.08 ± 0.00	**0.05 ± 0.00**	**0.05 ± 0.00**	0.07 ± 0.00	0.07 ± 0.00
$V_{\mathrm{cmax\_Ci}}$	34.89 ± 0.68	**24.67 ± 1.11**	**30.14 ± 1.28**	37.02 ± 0.75	**25.11 ± 2.67**	**26.73 ± 3.07**	36.62 ± 0.90	32.46 ± 1.90
$V_{\mathrm{cmax\_Cc}}$	69.66 ± 3.41	**55.24 ± 5.60**	**57.05 ± 3.10**	72.07 ± 2.35	**55.61 ± 5.69**	**51.57 ± 6.45**	77.67 ± 2.94	60.10 ± 4.79
$J_{\mathrm{max\_Ci}}$	79.22 ± 1.53	**63.04 ± 2.83**	78.16 ± 1.54	83.75 ± 1.59	**66.13 ± 6.28**	75.50 ± 8.08	81.81 ± 1.73	80.05 ± 7.51
$J_{\mathrm{max\_Cc}}$	91.76 ± 2.75	**78.68 ± 2.72**	81.59 ± 3.96	98.22 ± 2.04	81.93 ± 8.94	78.15 ± 7.50	99.24 ± 2.28	84.15 ± 3.65
$J_{\mathrm{max\_Ci:\ Vcmax\_Ci}}$	2.27 ± 0.04	**2.56 ± 0.09**	**2.75 ± 0.17**	2.27 ± 0.04	**5.65 ± 0.09**	**2.85 ± 0.18**	2.24 ± 0.02	2.48 ± 0.21
$J_{\mathrm{max\_Cc:\ Vcmax\_Cc}}$	1.33 ± 0.06	1.43 ± 0.03	1.45 ± 0.02	1.37 ± 0.05	**1.47 ± 0.03**	**1.55 ± 0.08**	1.29 ± 0.03	1.42 ± 0.07
Stomatal limitation	0.24 ± 0.01	0.23 ± 0.00	0.25 ± 0.00	0.23 ± 0.01	**0.20 ± 0.01**	**0.19 ± 0.01**	0.23 ± 0.01	**0.21 ± 0.01**
Mesophyll limitation	0.44 ± 0.02	0.44 ± 0.01	**0.39 ± 0.00**	**0.40 ± 0.02**	0.46 ± 0.01	0.41 ± 0.03	0.43 ± 0.01	**0.38 ± 0.01**
Biochemical limitation	0.31 ± 0.02	0.34 ± 0.00	0.35 ± 0.02	**0.38 ± 0.02**	0.34 ± 0.01	0.42 ± 0.02	0.34 ± 0.01	**0.41 ± 0.00**

C_{i}: sub-stomatal $\mathrm{CO_2}$ concentration; C_{c}: chloroplastic $\mathrm{CO_2}$ concentration; g_{m}: mesophyll conductance to $\mathrm{CO_2}$ estimated by Harley method; $V_{\mathrm{cmax\_}Ci\ \mathrm{ou}\ Cc}$: maximum carboxylation capacity based in C_{i} or C_{c}; $J_{\mathrm{max\_}Ci\ \mathrm{ou}\ Cc}$: the maximum capacity for electron transport rate based in C_{i} or C_{c}; stomatal limitation; mesophyll limitation and biochemical limitation.

Changes in the accumulation of malate affects stomatal responses to different stimuli

To better assess the impact of the lack of tDT, ALMT6 and ALMT4, on stomatal conductance (g_s) in Arabidopsis plants, we investigated the behavior of stomatal responses after dark/light transitions and following normal/high CO_2 concentrations changes. Additionally, we evaluated the response of intact leaves after incubation with ABA by using isolated epidermal fragments and analyzing the stomatal opening.

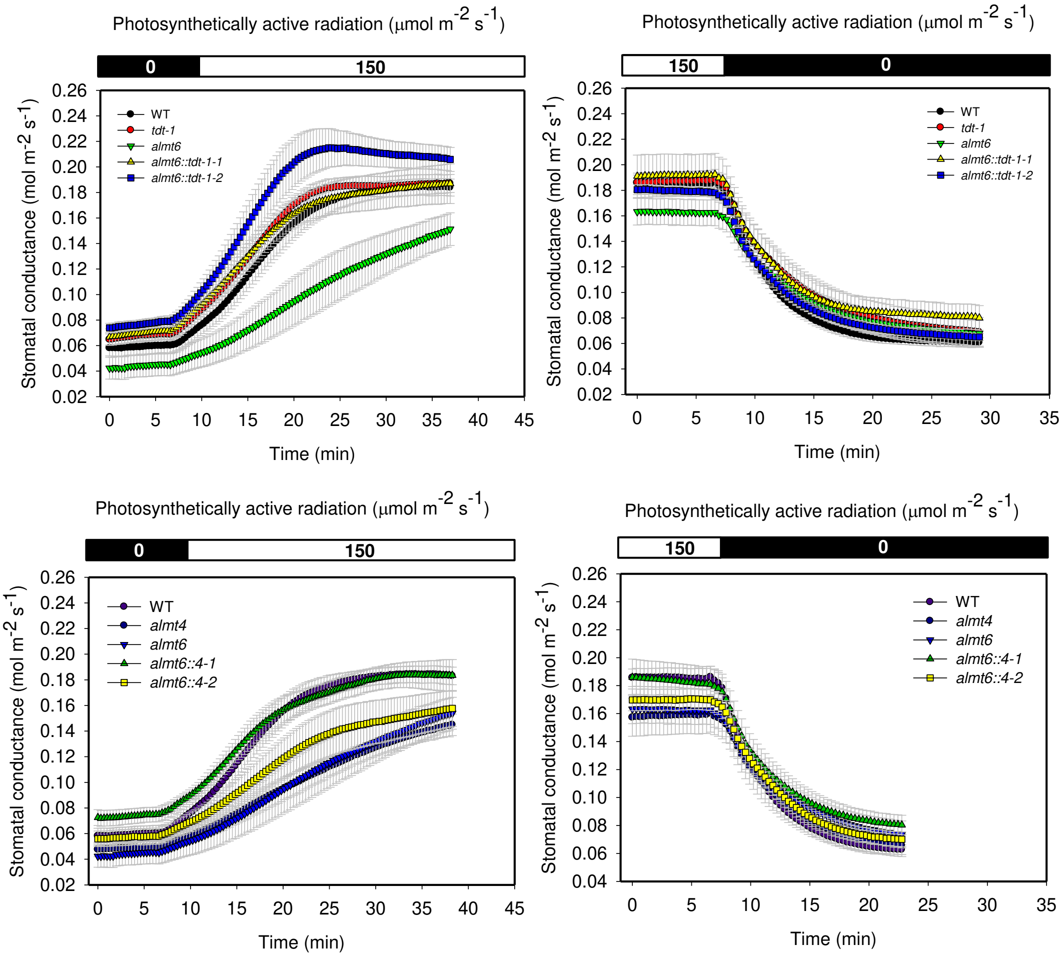


Figure 6. Stomatal opening and closing kinetics in response to light/dark/light in the plants *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1* end *almt6::4-2*. Data are means 5 SE (n = 5) obtained in two independent experiments with comparable results.

Our results confirm that the stomatal opening kinetics stimulated by light was most affected in *almt6* mutants, with a noticeably slower opening and with lower g_s values (Fig- 6). For the *tdt-1* line, we did not observe significant differences in kinetics in response to the transition from light to dark. Interestingly, the opening kinetics stimulated by light were less affected in the double mutants *almt6::tdt-1-1* and *almt6::tdt-1-2*, with a tendency for faster opening and higher g_s before gas stabilization for *almt6::tdt-1-2*. For stomatal closure induced by the transition from light to dark, the *almt4* mutant showed lower initial g_s values and reached stomatal closure with a g_s similar to the wild type. About the mutant lines, *almt6::4-1* had a lower gas during stomatal closure in response to the dark, while *almt6::4-2* displayed a tendence to have lower g_s when compared to the wild type (Fig. 6).

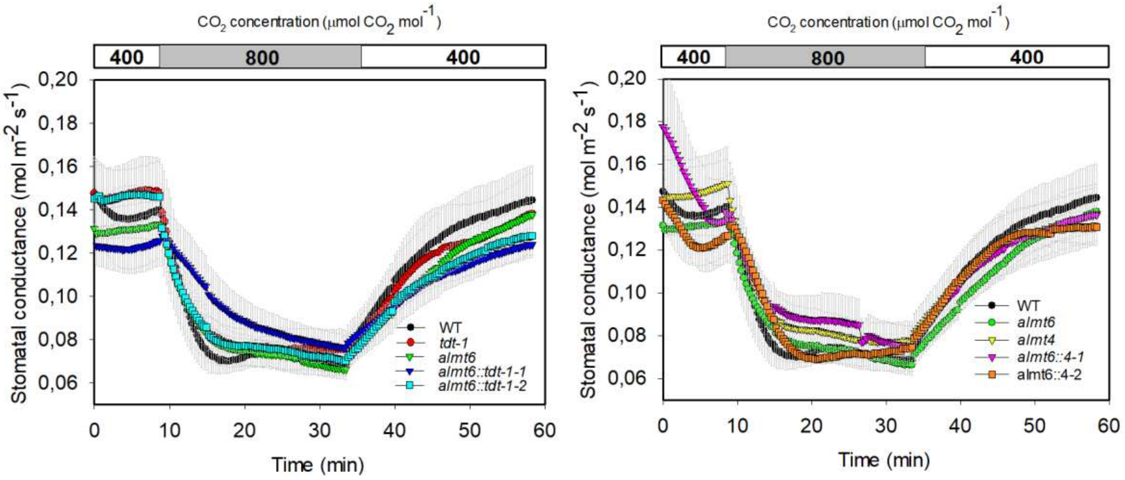


Figure 7: Stomatal opening and closing kinetics in response CO_2 concentration in the plants *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1* and *almt6::4-2*. Data are means 4 SE (n = 4) obtained in two independent experiments with comparable results.

Next, we evaluated the stomatal kinetics after changes from normal to high and high to normal CO_2 concentrations. No statistical differences ($P < 0.05$) were observed in any of the mutants involved in stomatal opening and closing compared to the control. However, it is possible to evaluate the behavior in g_s in the initial intervals (400 µmol CO_2 mol^{-1}) with lower values for *almt6* and *almt6::tdt-1-1*, as revealed by the double mutant that shows slower kinetics under high CO_2 with less pronounced reductions in g_s, and subsequently lower stomata opening during the recovery to normal CO_2 concentrations when compared to the wild type. Although contrasting g_s behavior for the *almt6::4-1* and *almt6::4-2* double mutants were observed under normal CO_2, similar response were observed in the transition from normal to elevated CO_2. Additionally, as CO_2 concentration elevated, the *almt6::4-1* double mutant close its stomata more slowly, as does the single *almt4* mutant. Upon resumption of normal CO_2 concentrations, *almt6::4-1* and *almt6::4-2* stabilizes g_s with values below wild type.

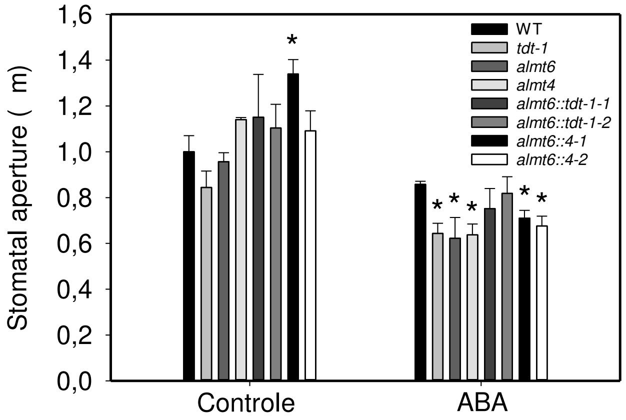


Figure 8: Kinetics of stomatal opening and closing in response to ABA in plants *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2*, *almt6::4-1* and *almt6::4-2*. ABA or ethanol (solvent control) was added to the opening buffer. After more than 2 h of incubation, the stomatal aperture was examined in the isolated epidermal fragments. Five leaves from different plants were evaluated, and the apertures of at least 15 stomata per leaf were measured, totaling at least 90 stomata per genotype. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

Regarding stomatal opening after incubation with ABA and ethanol solvent (control), we did not observe significant differences for most mutants with impaired malate and fumarate accumulation in leaves, although a trend of more closed stomata was observed for *tdt-1*, and more open stomata for the single mutant *almt4* and double mutants *almt6::tdt-1-1*, *almt6::tdt-1-2* and *almt6::4-2* in the control relative to wild type plants. Regarding the *almt6::4-1* double mutants, involved in stomatal closure, we observed higher stomatal opening when incubated in the opening solvent compared to the wild type plants. Comparing genotypes when epidermal fragments were incubated in ABA, we observed a lower degree of stomatal opening in single mutants for tDT, ALMT6 and ALMT4 and the double mutants linked to stomatal closure *almt6::4-1* and *almt6::4-2*. Interestingly, non-functional ALMT6 and tDT in the *almt6::tdt-1-1* and *almt6::tdt-1-2* lines did not confer expressive differences in the degree of stomata opening compared to wild type when exposed to ABA (Fig. 8).

Mutations affect the primary metabolism in the leaves

Given that significant changes in vegetative growth, followed by changes in photosynthetic rates and respiration were observed in single and double mutants, we further investigate to which extent this response is related to carbon metabolism. We analyzed the level of metabolites in two periods, early in the day and late in the day, to see how the impaired organic acid accumulation of these mutant lines would interfere with the metabolic changes throughout the day. When assessing photosynthetic pigment levels early in the day and at the end of the day, we observed significant differences among the genotypes (Fig. 9). The single mutants *tdt-1*, *almt6* and *almt4*, the double mutants *almt6::tdt-1-1* showed higher chlorophyll a contents early in the day (Fig. 9.a), and as it reached the end of the day, their contents did not differ from the wild type plants (Fig. 9.b). While in the *almt6::4-1* line the chlorophyll *a* content was lower during the day (Fig. 9.a), both *almt6::4-1* and *almt6::4-2* lines reach the end of the day with lower contents of this pigment compared to wild type (Fig. 9.b). Regarding chlorophyll *b*, *tdt-1* expressed no difference in both periods (Fig. 9. c and d), whereas the *almt6, almt6::tdt-1-1* and *almt6::tdt-1-2* have high pigment contents at the early in the day (Fig. 9.c), and decrease at the end of the day, with highlights also for the double *almt6::4-1* and *almt6::4-2* (Fig. 9.d). The content of carotenoids is high in most lines early in the day (Fig 9.e), and show a reduction at the end of the day most expressive for the lines *almt6::tdt-1-2*, *almt6::4-1* and *almt6::4-2* (Fig 9.f).

When it comes to total amino acid and protein levels, we observe that *tdt-1* has higher levels of these metabolites in both periods, whereas *almt6* and *almt4* further accumulate more amino acids (Fig. 10). In the case of the stomatal opening-related double mutants, *almt6::tdt-1-1* and *almt6::tdt-1-2* have a higher amino acid accumulation early in the day (Fig 10.c), and the closing-related mutants, *almt6::4-1* and *almt6::4-2* have even lower protein contents in the leaves at the end of the day (Fig. 10.b).

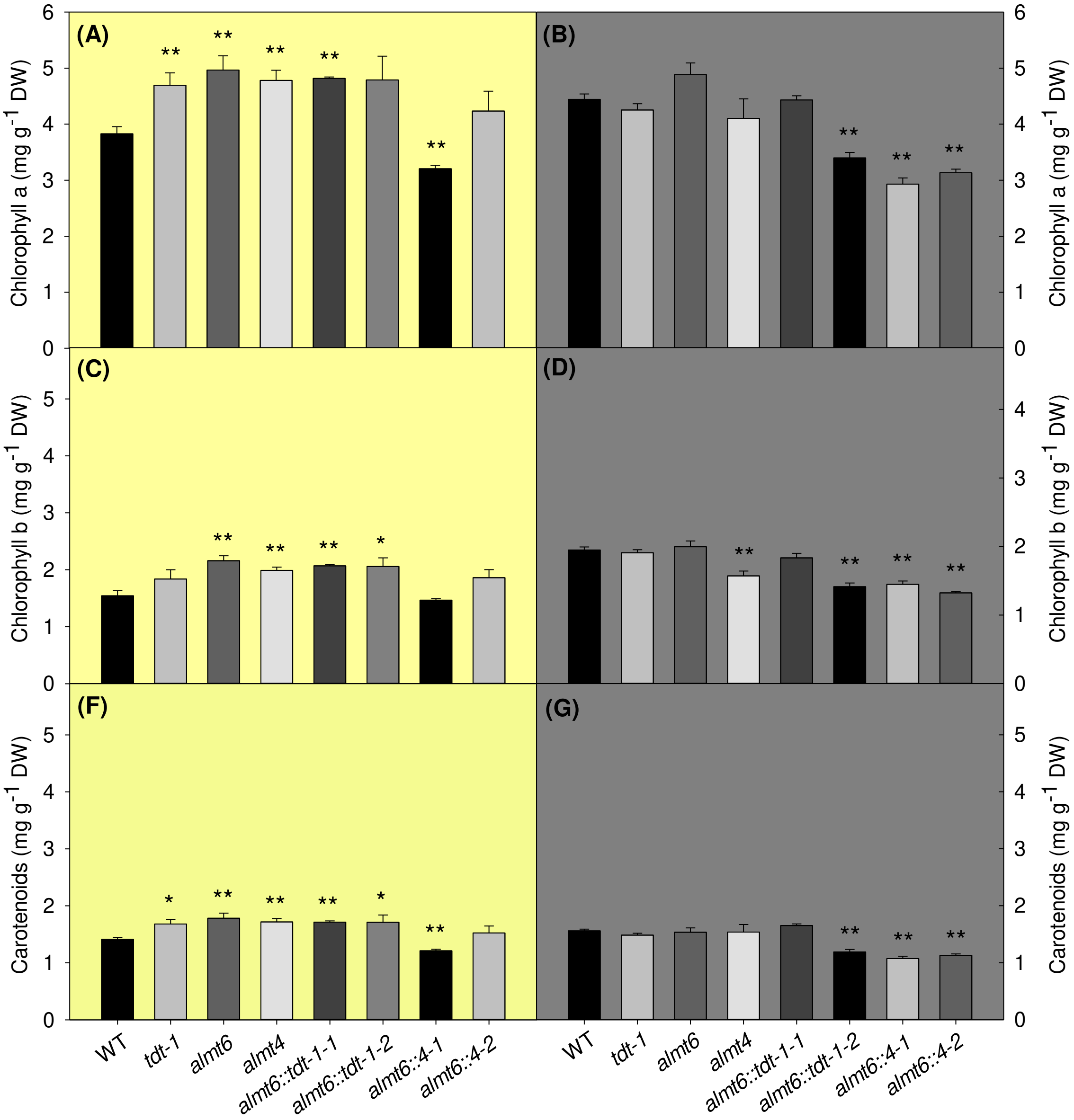


Figure 9: Effect of reduced expression *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2, almt6::4-1* and *almt6::4-2* on metabolite levels in fully expanded leaves of Arabidopsis plants collected early in the day and late in the day. **(A)** chlorophylla *a*, **(C)** chlorophylla *b*; **(E)** carotenoids early in the day; **(B)** chlorophylla *a*, **(D)** chlorophylla *b*; **(F)** carotenoids late in the day. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

We next extended our analyses to determine the carbohydrate content in the leaves the single and double mutants. The *tdt-1* mutant lines exhibited reduced levels of starch for the periods analyzed (Fig. 11). While *almt6::tdt-1* showed lower contents and a slight trend toward *almt6::tdt-2* early in the day (Fig. 11.a), both lines accumulated starch by the end of the day (Fig. 11.b). The double mutants *almt6::4-1* and *almt6::4-2* tend to accumulate more starch regardless of the period analyzed (Fig. 11.a and b).

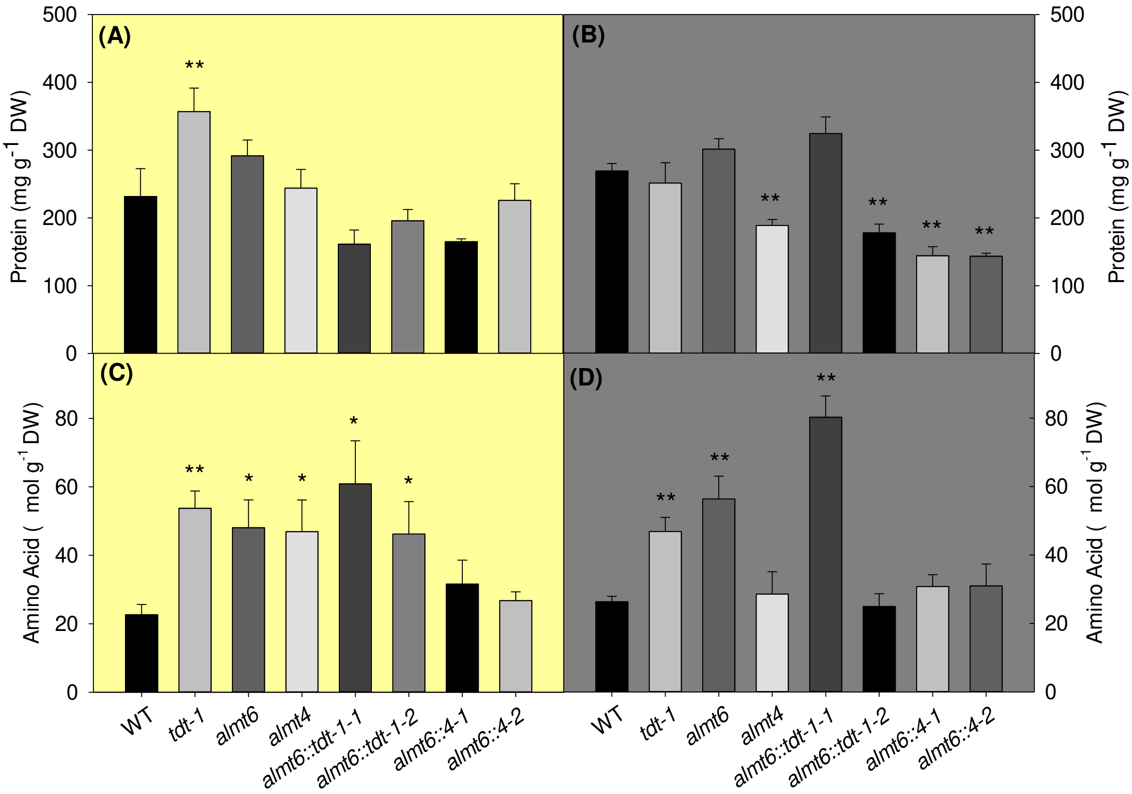


Figure 10: Effect of reduced expression *tdt-1*, *almt6*, almt4, *almt6::tdt-1-1*, *almt6::tdt-1-2, almt6::4-1* and *almt6::4-2* on metabolite levels in fully expanded leaves of Arabidopsis plants collected early in the day and late in the day. **(A)** protein **(C)** amino acid early in the day; **(B)** protein; **(D)** amino acid late in the day.
An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

In addition, glucose (Glu), fructose (Fruc) and sucrose (Suc) levels were measured in the same samples. Early in the day, these analyses revealed a significant increase in Glu levels for the single mutant *almt4* and double mutants *almt6::4-1* and a trend for *alm6::4-2* (Fig. 11.c). Fru was accumulated in leaves of non-functional ALMT6 and ALMT4, with consequent accumulation also in their double mutants *almt6::4-1* and *almt6::4-2* (Fig. 11.e). In respect of non-reducing sugar, Suc seems to be the main sugar to accumulate in *tdt-1* line (Fig. 11.g). At the end of the day, the single lines *almt6* and *almt4* and the double lines *almt6::4-1* and *almt6::4-2* represented elevated Glu and Suc levels compared to the wild type (Fig. 11.d and h). Conversely, *almt6::tdt-1-1* and *almt6::tdt-1-2* lines, did not show statistical differences in sugar accumulation (Fig. 11).

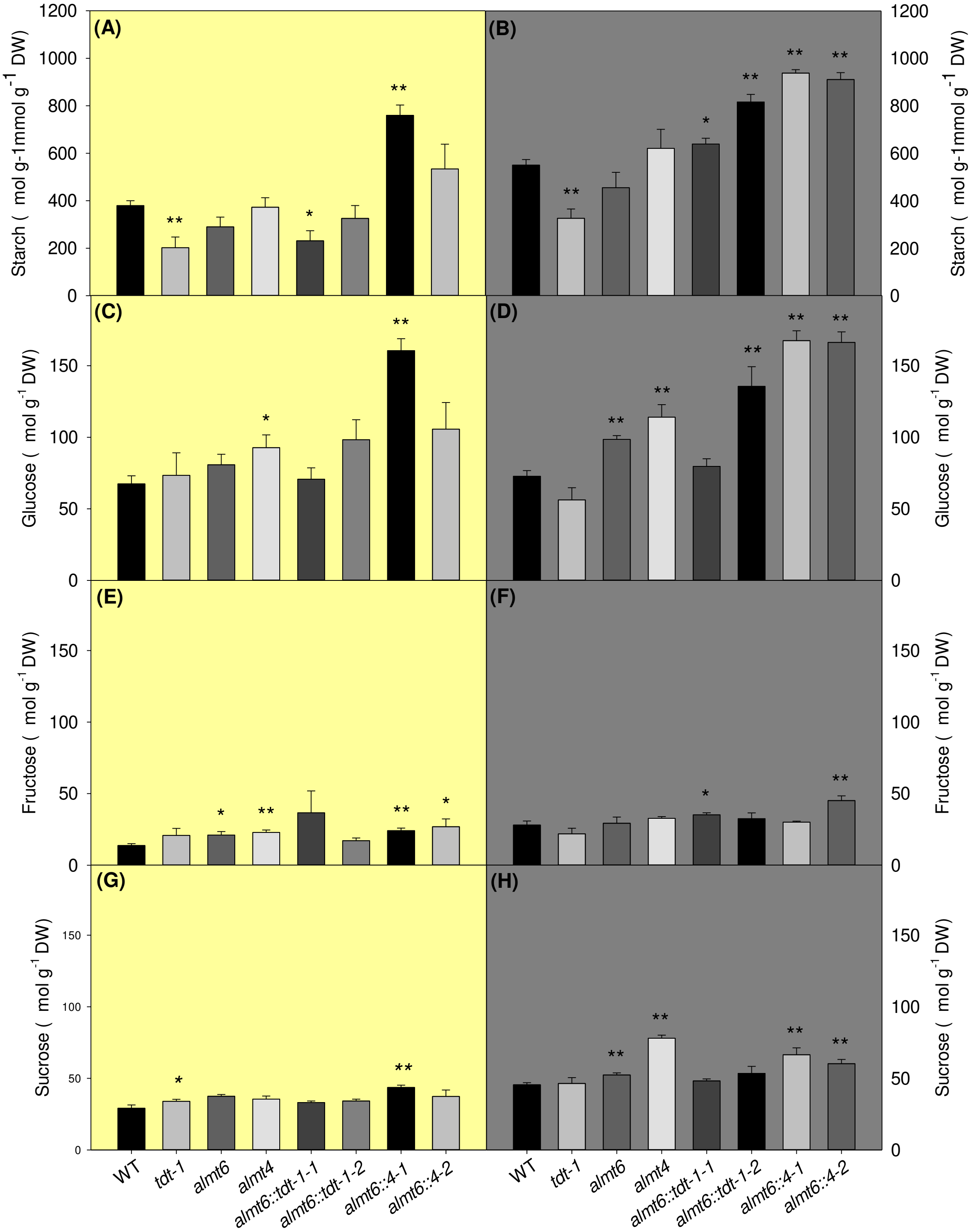


Figure 11: Effect of reduced expression *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2, almt6::4-1* and *almt6::4-2* on metabolite levels in fully expanded leaves of Arabidopsis plants collected early in the day and late in the day. **(A)** starch; **(C)** glucose; **(E)** fructose; **(G)** sucrose early in the day; **(B)** starch, **(D)** glucose; **(F)** fructose; **(H)** sucrose late in the day. An asterisk denotes values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

With respect to organic acids, malate was significantly reduced in the all single lines and double mutants *almt6::tdt-1-1* and *almt6::tdt-1-2* in leaves collected during the early part of the day, whereas *almt6::4-1* showed malate accumulation (Fig. 12.a). As it got to the end of the day, these levels remained reduced for *tdt-1* and *almt6*, but not for *almt4* (Fig. 12.b). While the double mutants had contrasting responses, where *almt6::tdt-1-1-1* and *almt6::tdt-1-2* lines continued to have their malate levels reduced, and *almt6::4-1* and *almt6::4-2* accumulated this organic acid compared to the wild type (Fig. 12.b). For fumarate, non-functional tDT plants showed reduced levels, and *almt6::tdt-1-1* had the same behavior early in the day (Fig. 12.c). The most significant responses of this organic acid are related to the end of the diurnal period, represented by a decrease of its content both in non-functional tDT, ALMT6 and ALMT4, and in the respective double mutants involved in stomatal opening and closing (Fig. 12.d).

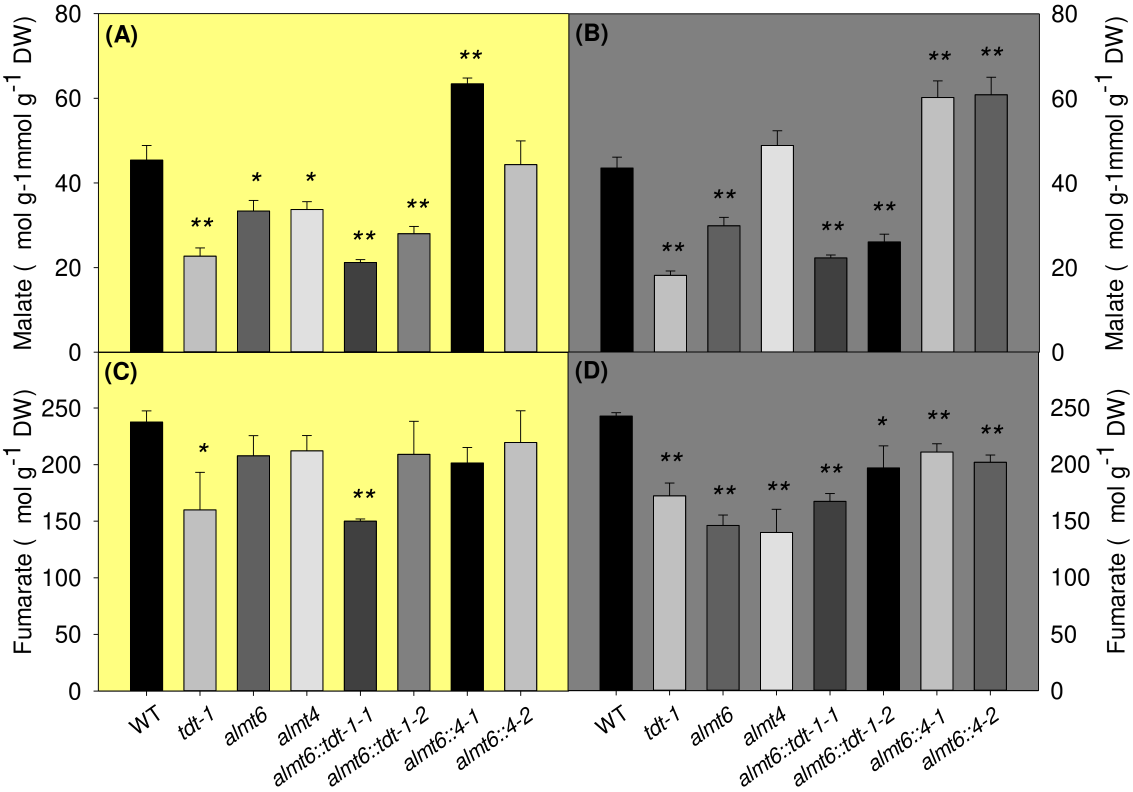


Figure 12: Effect of reduced expression *tdt-1*, *almt6*, *almt4*, *almt6::tdt-1-1*, *almt6::tdt-1-2, almt6::4-1* and *almt6::4-2* on metabolite levels in fully expanded leaves of Arabidopsis plants collected early in the day and late in the day. **(A)** malate **(C)** fumarate early in the day; **(B)** malate; **(D)** fumarate late in the day. An asterisk denotate values which were determined by the Student's t-test to be significantly different from wild-type ($P < 0.05$). Values are presented as mean ± SE (n = 5).

4.5. DISCUSSION

We evaluated the combined action of malate influx and efflux channels of guard cell vacuolar membrane involved in both stomatal opening and closing. The individual characterization at the molecular level of the Tonoplast Dicarboxylate Transporter (tDT) (Emmerlich et al., 2003; Medeiros et al., 2017; Frei et al., 2018) and the channels ALMT6 (Meyer et al., 2011) and ALMT4 (Eisenach et al, 2017), previously highlighted, substantial evidence of a functional redundancy in malate transport in guard cells. In order to investigate how the combined absence of the vacuolar transporter and malate channels affect organic acid accumulation and stomatal function, this study focused on the morphological, photosynthetic and metabolic characterization of single mutant lines *tdt-1*, *almt6* and *almt4*, but mainly focused on the response of *almt6*, *tdt-1* and *almt6::almt4* double mutants.

Functional absence of ALMT6 alters stomatal movement and photosynthetic capacity in *almt6 tdt-1* mutant lines

Malate has been proposed to exert multiple functions, besides being an intermediate of TCA in all plant species, it is an essential carbon molecule storagee (Fernie et al., 2004; Fernie and Martinoia, 2009). Here, we demonstrated that the impacts caused by impaired malate accumulation negatively affected the growth phenotype of *tdt-1*, *almt4* and *almt6 tdt-1* lines under short day conditions with respect to dry mass, leaf area and leaf number, whereas the *almt6* and *almt6 almt4* lines were unchanged (Fig. 2). Indeed, simple Arabidopsis mutants lacking tDT transporter activity, showed the same growth pattern under short days, however, they were independent of changes in stomatal density and photosynthetic capacity (Medeiros et al., 2017), as also observed in our study (Fig. 3). These results are associated with a reprogramming of guard cell metabolism to compensate for impaired vacuolar malate storage (Medeiros et al., 2017), supported by the residual malate import activity found in *tdt-1* plants (Emmerlich et al., 2003; Hurth et al., 2005). A likely explanation is that vacuolar ALMT family channels can partially compensate for the loss of tDT, by the activity of the ALMT6 channel, which is expressed almost exclusively in guard cell vacuoles and mediates malate currents activated by cytosolic Ca^{2+} and modulated by vacuolar pH (Meyer et al., 2011). Plants *almt6* showed no clear and reproducible phenotype regarding stomatal opening and conductance in epidermal strips under the conditions analyzed, still containing residual malate currents in isolated vacuoles, although they found no difference in mRNA levels of tDT and ALMT9 (Meyer et al., 2011). Differently, our results clearly denote negative effects on stomatal function caused by non-functional ALMT6 in *almt6* lines and in the double mutants *almt6 tdt-1* and *almt6 almt4*, since loss of ALMT4 function causes normal stomatal opening (Eisenach et al., 2017). Unexpectedly, the growth pattern was differential between genotypes, unchanged for *almt6* and *almt6 almt4* lines, but impaired for *almt6 tdt-1* double mutant lines under short day conditions. Medeiros et al (2017) revealed the high expression of ALMT6 in the tonoplast of guard cells, corroborating with our findings about compensation by this channel in absence of tDT, at least with respect to adequate malate and fumarate storage in these cells.

We also performed a detailed photosynthetic characterization, in order to investigate whether the differences on vegetative growth were associated with changes in the photosynthetic capacity of these mutant lines. Our results showed that net CO_2 assimilation rates were reduced in *almt6* plants and the *almt6 tdt-1* and *almt6 almt4* double mutants under saturation irradiance, compensation and light use efficiency (Table. 1). This was also observed when we estimated the photosynthetic capacity of the AN curves for C_i or C_c as well as for PPFD (Table. 4 and 5). The photosynthetic capacity of plants can be influenced by diffusive and biochemical constraints such as stomatal conductance (g_s), mesophilic conductance (g_m) and the biochemical conductance (g_b) that determine the photosynthetic performance (Flexas et al., 2012). Thus, photosynthetic limitations were estimated and revealed that g_s had the greatest contribution to A_N observed in the *almt6 tdt-1* double mutant lines, sufficiently to decrease the intercellular CO_2 concentration limiting photosynthesis, which likely reflect in reduced growth of these plants. Whereas the simple *almt6* mutants have lower A_N due to lower g_m and lower C_c values. Although the values of Rubisco carboxylase activity (V_{cmax}) and photosynthetic electron transport capacity (J_{max}) were lower for the *almt6* and *almt6 tdt-1* lines, greater differences observed in A_N in these plants are actually due to differences in g_s and g_m, therefore, the biochemical limitations caused are mainly due to the diffusive limitations observed. Importantly, CO_2 diffusion barriers can be influenced by the size of different anatomical components, such as leaf mass per unit area (LMA), wall thickness, and stomatal density, which can be potential targets for modifying gm and thus intrinsic water use efficiency (Flexas et al., 2008; Niinemets et al., 2009; Niinemets et al., 2009). Our results showed that the photosynthetic reductions observed in *almt6* and *almt6 tdt-1* are related not only to diffusive limitations, but also linked to anatomical limitations, determined by the lower stomatal density found in these plants (Fig.2).

Our results become more interesting when we further confirm that slower growth in *tdt-1* plants was accompanied by enhanced dark respiration (Fig. 3e) supported mainly by the fact that these plants have higher accumulation of malate in the cytosol and, consequently, it is used as a supplier of intermediates to supply the demand for respiratory metabolism in the TCA cycle (Medeiros et al, 2017), thus highlighting the connection between TCA cycle function and growth (Araújo et al., 2011a; Nunes-Nesi et al., 2013). Mutants of the ALMT4 channel show impaired growth and stomatal opening, caused by a reduced ability to store malate and fumarate in the vacuoles of the mesophyll, as well a reduced root growth due to impaired dicarboxylate storage or release for respiration (Eisenach et al., 2017). Thus, in mesophyll tissue, the release of malate and fumarate from the vacuole for respiration during the night may be an important function of ALMT4 (Eisenach et al., 2017). This is in agreement with the lower growth of *almt4* plants, since vacuolar malate is not efficiently exported to the mesophyll cytosol and neither tDT was sufficient to mediate this transport and maintain growth under the experimental conditions. Whereas, the higher respiration observed in *almt6 almt4* plants may have been supported by functional tDT to maintain cellular homeostasis in the mesophyll cells, alter mitochondrial metabolism and sustain growth. As observed in *almt6* plants, which maintained dark respiration, by the likely presence of the tDT transporter and its ability to accept various dicarboxylates as transport molecules involved in the TCA cycle, in addition to malate, such as fumarate, citrate, and succinate (Frei et al., 2018).

Stomatal opening and closing mutants maintain growth at the expense of metabolic reprogramming

It has been described that during stomatal opening, malate accumulation in guard cells can be derived from the mesophyll via the plasma membrane-specific transporter AtABCB14, or as a product of starch breakdown or anaplerotic CO_2 fixation (Talbott and Zeiger, 1993; Lee et al., 2008; Daloso et al., 2015). On the other hand, during stomatal closure, accumulated malate can be metabolized, partially converted to starch, or released into the apoplastic space by the ALMT family member efflux channel, AtQUAC1 (Penfield et al., 2012; Medeiros et al., 2016). Tomato (*Solanum lycopersicum*) plants with constitutively reduced expression of *Sl*SDH2-2 that encodes the iron-sulfur subunit of succinate dehydrogenase demonstrated increased stomatal conductance and photosynthesis mediated by the effect of organic acids (Araújo et al., 2011b). And that constitutive inhibition of mitochondrial fumarase decreases photosynthesis as a result of impaired stomatal function in tomato plants (Nunes-Nesi et al., 2007). These studies indicate that apoplastic malate and fumarate content can modulate stomatal function and in turn drive stomatal movements with effects on leaf metabolism. The results presented here demonstrate that impaired organic acid transport culminates in changes in gs and gm in plants with ALM6 repression, promoting changes in primary metabolism, especially carbon metabolism, under normal growth conditions. Nevertheless, *almt6 tdt-1* plants were shown to degrade more starch in the early hours of the day in order to provide carbon skeletons to modulate stomatal aperture, to which it appears to have been remobilized, at least in part, into sugars during the nighttime period to maintain respiratory rates, and in consensus impaired photosynthetic capacity (lower gs and A_N) and growth. Another fact supporting the accumulation of sugars in these lines may be linked to the efflux of malate from guard cells via the active channel ALMT4. The concomitant increase of the ^{13}C marker in some intermediates of the TCA cycle led to the conclusion that most of the accumulated malate is used as a respiratory energy source during light induction (Daloso et al., 2015; Robaina-Estévez et al., 2017). Contrasting the *almt6* plants, which had high levels of sugars, mainly, glucose and sucrose, in the very first hours of the night period, for maintenance of respiration rates and growth of these plants, which can also be explained by the maintenance of respiration in *almt4* plants. Differential sucrose accumulation in mesophyll cells, apoplast and guard cells has been proposed to be an important key point linking photosynthesis and stomatal opening processes, given the negative correlation between sucrose and g_s contents (Gago et al, 2016), Furthermore, sucrose can be produced within guard cells but also imported from mesophyll cells to maintain osmotic adjustment during stomatal opening, moreover, it can be broken down to sustain glycolysis and mitochondrial metabolism (Kang et al., 2007), thus supporting our results. In *almt6 almt4* plants the increases in hexoses and sucrose were not due to starch degradation, since this metabolite accumulated earlier in the day. This result suggests, that much of the sucrose found in these lines may be derived from mesophyll cells in order to maintain osmotic balance and stomatal opening. However, were not sufficient to bypass impaired stomatal function caused by ALMT4 and ALMT6 repression, but efficient to provide carbon skeletons for the maintenance of respiration and growth. In a kinetic isotope labeling approach experiment of [U - ^{13}C]-sucrose revealed that sucrose degraded during stomatal opening can be further enriched in glutamate and glutamine (Medeiros et al., 2018). Confirming indeed that part of sucrose was mainly allocated for energy metabolism, given the unchanged amino acid contents in *almt6 almt4* plants compared to wild-type plants.

Plants *almt6* and *almt4* exhibit impaired opening kinetics in response to the dark/light transition

The extent and speed of stomatal movements are intrinsically linked to the solute transport capacity and the speed at which transport responds to internal and external environmental stimuli (Lawson and Blatt, 2014). Here we determined the speed of stomatal responses with attention to variations in PPFD, CO_2 concentration, and ABA in lines with impaired malate storage in the guard cell vacuole. Our results confirmed impaired stomatal regulation in *almt6* plant, with slower opening at mainly the dark-light transition (Fig. 6), although in both kinetics the g_s value in the stationary phase was reduced. It has been described that faster stomatal closure and slower opening are associated with tight control of water loss which has a negative effect on leaf carbon gain, because delayed stomatal opening reduces assimilation (Vico et al., 2011). Furthermore, there is general agreement that, when considering fully induced leaves, delays in stomatal response to variable light are the most relevant factor for leaf gas exchange, with biochemical delays occurring on much shorter time scales (Weber et al., 1985; Knapp and Smith, 1988). These findings explain, at least in part, the observed changes in V_{cmax} on a C_c basis of plants with ALMT6 repression, recurring from slower increases in g_s, limiting CO_2 diffusion and reducing A. Another prevailing point is tied to the lower stomatal density found in *almt6*, as leaves with a higher number of stomata have faster stomatal responses and higher overall g_s, compared to leaves that have lower stomatal density (Faralli et al., 2019). A recent study, demonstrated convincing evidence for the importance of the ALMT6 channel during light-induced stomatal opening. Epidermal strips of the *almt6* mutant opened the stomata less efficiently in response to blue light illumination and treatment with fusicoccin, a strong activator of plasma membrane H+ -ATPase (Ye et al., 2021). Additionally, *almt6* shows expressive impairment in blue light-induced stomatal opening under low Cl^- conditions, suggesting that ALMT6, like ALMT9 also contributes to Cl^- influx into the vacuole during stomatal opening, with no compensation of ALMT9 expression in *almt6*, and it is possible that both channels function additively and/or cooperatively. Surprisingly, the *almt6 tdt-1* and *almt6 almt4* double mutants did not experience the same order impact on stomatal opening in response to PPDF variations, although the *almt6::tdt-1-2* line showed a faster opening response, gs values matched the other mutants at stationary phase (Fig. 6) .

tDT repression in Arabidopsis plants does not strongly impair either the kinetic responses to dark, light, or high CO_2, or the stomatal opening after incubation with ABA (Medeiros et al., 2017), corroborating with the response described here. Previously, it was also shown that *almt4* mutants are insensitive to dark and light stimuli (Eisenach et al., 2017). During stomatal opening ALMT4 should be inactivated (Eisenach et al., 2017), this implies that stomatal opening should be faster in *almt4* plants, however, here we observed reduced opening rates at the dark-light transition whereas stomatal closure at the light-dark transition was unaffected. Since ALMT4 mediates anion efflux, we believe that it is unlikely that impaired ALMT4 activity is directly responsible for the slow opening phenotype observed in *almt4*. Furthermore, *almt4* mutant plants still demonstrate impaired stomatal closure in response to

ABA and increased whole plant wilting in response to drought, and are therefore required for ABA-induced stomatal closure (Eisenach et al., 2017). Similarly, mutants of the anion efflux channel SLAC1 delayed light-induced stomatal opening, low CO_2, and high air humidity in intact plants, which were associated with repression of the organic acid influx transporter AtABCB14, but primarily, due to a compensatory feedback control in slac1 plants involving the elevation of elevated cytosolic Ca^{2+} concentrations, as a mechanistic basis for the negative regulation of K^+ influx channel activity, thus providing an adaptive response of guard cells to counteract the tendency of stomata of *slac1* mutants to over-open (Laanemets et al. , 2013).

Stomatal opening was reduced in epidermal fragments enriched with guard cells in all mutant malate channels and transporters after incubation with ABA (Fig. 8), unlike what was observed in *almt4* plants (Eisenach et al., 2017). It is worth noting that activation of other S- and R-type anion efflux channels can transmit malate efflux from guard cells in response to ABA (Van Kirk and Raschke, 1978; Keller et al., 1989; Pandey et al., 2007). The R-type channel, ALMT12/QUAC1, has been described to mediate the release of malate from guard cells into the apoplastic space during stomatal closure (Meyer et al., 2010). In this same study, *almt12* plants were shown to respond to exogenous ABA and stomata remained wide open, also exhibiting a slower decline in stomatal conductance in response to light-dark and high CO_2 transitions. Furthermore, in the light-dark transitions, *slac1* showed slow and modest response in stomatal closure, while at high CO_2 (800 ppm) it invariably kept its stomata open (Negi et al., 2008; Vahisalu et al., 2008). It is worth noting that *slac1* guard cells exhibit greatly reduced S-type anion channel function, whereas R-type anion channels and ABA-activated Ca^+ permeable channels remain functional (Vahisalu et al., 2008). Plants *quac1 slac1* are nearly insensitive to darkness, elevated CO_2, reduced humidity, and ABA, indicating that both types of anion channels are required to launch the sequence of events leading to stomatal closure (Jalakas et al., 2021). Whereas *slac1 slah3* double mutants exhibit reduced stomatal closure in response to abiotic and ABA treatments, showing that S-type anion channels alone are not sufficient to elicit response to these stimuli and that the QUAC1 channel is sufficient to abolish stomatal responses (Jalakas et al., 2021). Therefore, that guard cells exhibit greatly reduced S-type anion channel function, whereas R-type anion channels and ABA-activated Ca^+ permeable channels remain functional (Vahisalu et al., 2008). This could be the reason why the removal of ALMT6 and ALMT4 in single and double mutants were not sufficiently affected the stomatal responses to darkness, elevated CO_2 and ABA, explained by the possible activity of the QUAC1 channel. That said, we do not rule out the possibility that efflux channels for malate and other ions operate together to provide efficient stomatal regulation, but that different members of the ALMT family may be specifically required under particular conditions. Gene expression aborages related to ion transport in guard cells would add in a clearer understanding about the compensatory and regulatory mechanisms within these cells regarding these stimuli.

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Chapter 4

Concluding g remarks

5. CONCLUDING REMARKS

This thesis was directed largely toward explaining how organic acid metabolism and impaired vacuolar transport, especially malate, in guard cells disrupt stomatal movements under proper and adverse growth conditions. In an effort to achieve the objectives, we used complementary experimental approaches and the results were obtained in an independent chapter.

In chapter 3, we used knockout lines for a vacuolar organic acid transporter tDT, and two vacuolar malate channels ALMT6 (influx and efflux) and ALMT4 (efflux). Our efforts were directed at understanding the functional redundancy suggested previously, given that impaired malate and fumarate accumulation by tDT repression does not impact stomatal function (Medeiros et al., 2017). Guard cell-specific ALMT6 (Meyer et al., 2011) was identified as a potential candidate vacuolar anion channel to compensate for this transport in *tdt-1* lines. Furthermore, repression of the ALMT4 efflux channel closes stomata in a delayed and incomplete manner in response to abscisic acid (ABA) (Eisenach et al., 2017), in contrast, malate efflux currents by ALMT6 activity do not bypass this impaired phenotype in *almt4* plants. That said, we used double mutant lines with combined action for tDT and ALMT6 (*almt6 tdt-1* lines), suggested to directly impact stomatal opening; and double mutants for ALMT6 and ALMT4 (*almt6 almt4* lines) targeting stomatal closure. Our intent was to verify whether the alteration in malate levels causes impairment to the activity of malate channels in the vacuolar membrane of guard cells and stomatal function. As well as the possible mechanism of compensation by this transporter and members of the ALMT family under specific environmental conditions.

It was shown that inefficient regulation of stomatal opening through repression of the ALMT6 channel culminates in lower photosynthetic rates through decreased gs and AN, with impaired growth phenotypes in the *almt6 tdt-1* double mutants. Differential growth was followed by metabolic reprogramming in the accumulation of organic acids and sugars in leaves. The absence of ALMT6 leads to an imbalance in malate uptake and release in guard cells, which in turn keeps stomata more closed, consequently the decreases in gs and gm influenced lower photosynthesis. The combined action of ALMT6 and tDT, and non-functional ALMT6 and ALMT4, caused impacts of the same order, regarding photosynthetic parameters in conditions for proper growth. However, primary metabolism is distinctly altered as a strategy to avoid carbon loss and ensure respiration rates and growth. Contents of organic acids, mainly malate, decrease in influx mutant lines related to stomatal opening, whereas they accumulate in leaves of the *almt6 almt4* double mutants also involved in impaired malate efflux during stomatal closure. Increased sugar and starch levels are addressed to coordinate nocturnal respiration in *tdt-1*, *almt6*, *almt4*, *almt6 almt4*; and *almt6 tdt-1*, respectively. We proposed that ALMT6 does indeed present a crucial role in regulating stomatal opening and compensates for malate transport in non-functional *tdt* in stomatal opening, yet this transporter is essential for proper storage of dicarboxylates in the vacuole of mesophyll cells for maintenance of respiration rates and growth, as is the ALMT4 channel. We extended our approach to understanding how impaired organic acid accumulation impacts gs behavior across diverse stimuli. Notably, lack of functional ALMT6 affected stomatal opening, with slower opening kinetics and lower gs in response to dark-light transition, consistent with biochemical (lower V_{cmax} on basa a C_{c} basis) and anatomical (lower density) limitations found in these plants. Interestingly, repression of the ALMT4 efflux channel also exhibits phenotype of a slower stomatal opening response to light, and normal stomatal closure in guard cell-enriched epidermal fragments incubated with ABA, as well as all other mutants that were responsive to this stimulus. Unexpectedly, we observed no impact on stomatal responses under normal and high CO_2 variations for individual and combined loss of function of tDT, ALMT6 and ALMT4.

We assume that, due to the high complexity of stomatal regulation in response to the environment, the activity of influx and efflux channels of organic acids and solutes, especially those located in the plasma membrane of guard cells, are operating by mechanisms that detect perturbations in the water balance of guard cells to these environmental and exogenous variations. Therefore, one should keep in mind that guard cells are able to make use of different anions and solutes to alter their water potential and perform their movements. Thus, functional approaches at the guard cell level and the expression of genes related to malate and ion transport specific to guard cells may provide additional information on how the functioning of stomata is effectively affected when malate accumulation is altered. Important to add also that, given the metabolic particularities found for the tDT transporter and ALM6 and ALMT4 channels, they may present relative importance under adverse abiotic stress conditions in plants.

5.1. LITERATURE CITED

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