RITA DE CÁSSIA MONTEIRO BATISTA

THE ROLE OF MITOCHONDRIAL CARRIER PROTEINS IN PLANTS: MORE THAN JUST THE ENERGETIC ISSUE

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*.

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Co-advisers: Wagner L. Araújo
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Adriano Nunes Nesi
Adviser

I dedicate this work to the Almighty God,
to my beloved husband, Luís, and our dear son, Arthur,
and to our other children who will come.

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“And we know that all things work together for good to them that love God, to them who are the called according to his purpose.”.

(Romans 8:28)

ABSTRACT

Monteiro-Batista, Rita de Cássia, D.Sc., Universidade Federal de Viçosa, april, 2024 **The role of mitochondrial carrier proteins in plants: more than just the energetic issue.** Adviser: Adriano Nunes-Nesi. Co-advisers: Wagner L. Araújo and Paula da Fonseca Pereira.

The transport of metabolites across the inner mitochondrial membrane (IMM) is crucial for cellular metabolism and energy homeostasis. This process is mediated by mitochondrial carrier proteins belonging to the Mitochondrial Carrier Family (MCF). Despite recent advances in understanding the function of these transporter proteins in plants, their full physiological role remains incompletely understood, particularly under varying growing conditions. This study aimed to elucidate the physiological role of mitochondrial transporters under both optimal and adverse conditions, while also characterizing two isoforms of the adenylate transporter ADP/ATP Carrier (AAC1 and AAC2) *in vivo*. Our findings reveal that decreased *AAC1* expression impacts cellular respiration and ATP concentrations, leading to aberrant mitochondrial morphology and reduced root growth. Additionally, *AAC1* downregulation affects both vegetative and reproductive stages, underscoring its involvement in oxidative phosphorylation, energy homeostasis, and plant growth regulation. We further investigated AAC2's role in reproduction, germination, vegetative phase, and root growth. Our results demonstrate AAC2's significance in germination, with lines exhibiting decreased expression showing reduced and delayed germination percentage. Moreover, *AAC2* downregulation affects root growth, silique size, and seed viability, emphasizing its critical role in plant development and reproductive yield regulation. Overall, our study highlights the pivotal role of mitochondrial transport proteins in regulating plant metabolism and development across various environmental conditions. Understanding the functions of these transporters not only provides insights into energy homeostasis but also offers valuable insights into mitochondrial dynamics, a key aspect of cellular metabolism regulation. This comprehensive understanding holds promise for future research on plant adaptation to environmental challenges and the development of strategies to enhance agricultural sustainability and food security.

Keywords: Adenylates; *Arabidopsis thaliana*; metabolism; mitochondria; respiration.

RESUMO

Monteiro-Batista, Rita de Cássia, D.Sc., Universidade Federal de Viçosa, abril de 2024. **O papel das proteínas mitocondriais transportadoras em plantas: muito além da questão da energética.** Orientador: Adriano Nunes Nesi. Coorientadores: Wagner L. Araújo e Paula da Fonseca Pereira.

O transporte de metabólitos através da membrana mitocondrial interna (IMM) é crucial para o metabolismo celular e a homeostase energética. Esse processo é mediado por proteínas transportadoras mitocondriais pertencentes à Família dos Transportadores Mitocondriais (MCF). Apesar dos recentes avanços na compreensão da função dessas proteínas transportadoras em plantas, seu papel fisiológico completo ainda não está totalmente compreendido, principalmente sob diferentes condições de cultivo. Diante disso, este estudo teve como objetivo elucidar o papel fisiológico dos transportadores mitocondriais tanto em condições ótimas quanto adversas, ao mesmo tempo em que caracterizava duas isoformas do transportador de adenilatos ADP/ATP (AAC1 e AAC2) *in vivo*. Os resultados revelam que a diminuição da expressão de *AAC1* afeta a respiração celular e as concentrações de ATP, levando a uma morfologia mitocondrial anormal e crescimento reduzido das raízes. Além disso, a reduzida expressão de *AAC1* afeta estágios vegetativos e reprodutivos, destacando seu envolvimento na fosforilação oxidativa, homeostase energética e regulação do crescimento das plantas. Investigou-se ainda o papel de AAC2 na reprodução, germinação, fase vegetativa e crescimento das raízes. Os resultados demonstram a importância de AAC2 na germinação, com linhagens deficientes na expressão desse transportador apresentando reduzida porcentagem de germinação e maior tempo para a mesma. Além disso, a deficiente expressão de *AAC2* afeta o crescimento das raízes e das síliquas e a viabilidade das sementes, enfatizando seu papel no desenvolvimento das plantas e na regulação do rendimento reprodutivo. No geral, esse estudo destaca o papel das proteínas transportadoras mitocondriais na regulação do metabolismo e desenvolvimento das plantas em diversas condições de cultivo. A compreensão das funções desses transportadores não apenas fornece novos conhecimentos sobre a homeostase energética, mas também oferece informações sobre a dinâmica mitocondrial, um aspecto fundamental da regulação do metabolismo celular. Essa compreensão abrangente pode contribuir para pesquisas futuras sobre a adaptação das plantas a desafios ambientais e o desenvolvimento de estratégias para aumentar a sustentabilidade agrícola e a segurança alimentar.

Palavras-chave: Adenilatos; *Arabidopsis thaliana*; metabolismo; mitocôndria; respiração.

SUMÁRIO

GENERAL INTRODUCTION

Approximately 1.5 billion years ago, an ancestral eukaryotic cell merged with an endosymbiotic α-proteobacteria, leading to the formation of mitochondria (Gray et al., 2001; Roger et al., 2017). This integration endowed the ancestral eukaryotic lineage with a wide spectrum of novel metabolic capabilities, serving as pivotal centers for various additional anabolic and catabolic pathways (Roger et al., 2017). The intracellular transport of mitochondrial metabolites is fundamental to central carbon metabolism, especially in processes like cellular respiration, photosynthesis, and the synthesis of amino acids and fatty acids (Toleco et al., 2020). This transport is facilitated by a diverse array of solute carrier proteins located in the mitochondrial membranes (Nunes-Nesi et al., 2020). These nuclear-encoded Mitochondrial Carrier Proteins transport a wide range of substrates across the inner mitochondrial membrane (IMM), belonging to the Mitochondrial Carrier Family (MCF) (Nunes-Nesi et al., 2020). The MCF family transporters share a common structure with six α-helices, arranged in three tandem repetitive modules, each containing about 100 amino acids with similar molecular weights (30-35 kDa) (Picault et al., 2004). These proteins mediate the specific transport of various substrates, including amino acids, nucleotides, dicarboxylates, cofactors, and H^+ ions (Haferkamp and Schmitz-Esser, 2012; Palmieri et al., 2011).

Among the myriad of metabolites transported by mitochondria, adenylates (Adenosine Monophosphate, AMP; Adenosine Diphosphate, ADP, and Adenosine Triphosphate, ATP) play a crucial role in maintaining energy turnover. Approximately 40% of the mitochondrial proteome is largely dedicated to meeting cellular energy demands (Fuchs et al., 2020). Within the IMM, numerous proteins are specialized in adenylate transport (Nunes-Nesi et al., 2020), highlighting the intricate interplay between mitochondrial dynamics and efficient adenylate transport as potential key components in cellular energy regulation. Three groups of proteins belonging to the MCF are responsible for adenylate transport across the IMM: the ATP/Phosphate Carrier 1 (APCs), which mediate the export of Pi (inorganic phosphate) to the cytosol and the import of adenylates into the mitochondrial matrix (Lorenz et al., 2015); the Adenine Nucleotide Translocator 1 (ADNT1), which promotes the export of ATP to the cytosol in exchange, preferably, for AMP (Palmieri et al., 2008); and the ADP/ATP Carrier (AACs), which facilitates the electrogenic passage of ADP/ATP, transporting a cytosolic ADP molecule in exchange for an ATP molecule from the mitochondrial matrix (Ogunbona & Claypool, 2019). Among previously characterized mitochondrial adenylate transporters (Palmieri et al., 2008; Monné et al., 2015; da Fonseca-Pereira, 2021; Neri-Silva et al., 2023), it has been identified that APCs transporters regulate the adenylate pool in the mitochondrial matrix (Monné et al., 2015). In Arabidopsis, ADNT1's physiological function, as elucidated by Palmieri et al. (2008), da Fonseca-Pereira (2021), and Neri-Silva et al. (2023), encompasses the respiratory process in roots, dark-induced senescence, and early responses of the fermentative pathway under flooding conditions. Adenylate transporters, vital for energy turnover, have also been detected in various organelles such as chloroplasts, peroxisomes, and the plasma membrane (Leroch et al., 2008; Palmieri et al., 2008; Nunes-Nesi et al., 2020), underlining the intricate role of adenylate transport in regulating energy metabolism in plants.

Regarding the identified mitochondrial adenylate transporters in plants so far, proteins from the AACs group are the most abundant (Fuchs et al., 2020). In Arabidopsis, three nuclear genes encode isoforms of AAC proteins (AT3G08580, *AAC1*; AT5G13490, *AAC2*; and AT4G28390, *AAC3*), which are characterized by a N-terminal extension containing approximately 60 residues of 16 amino acids (Haferkamp and Schmitz-Esser, 2012). Among the isoforms, the *AAC1* gene is distinctly the most expressed, regardless of the organ type and developmental stage, while the *AAC2* and *AAC3* genes are positively regulated in the reproductive phase in floral tissues, siliques, and pollen grains, but are expressed at relatively lower levels than *AAC1* in all vegetative tissues (Batista, 2019). Although *in silico* studies have provided insights into the physiological importance of AACs in *A. thaliana* (Fonseca-Pereira et al., 2018), confirmation of their specific roles in plant metabolism is still required. Therefore, it is essential to conduct *in vivo* investigations using genetic approaches to understand better the physiological role of these transporters in plants, especially related to energy homeostasis, redox regulation, and oxidative damage under optimal growth conditions.

In this study, we explored the functional significance of mitochondrial carriers in plant development under optimal conditions and stress. We reviewed the role of different mitochondrial carriers across various metabolic pathways under stress conditions. Additionally, we investigated the physiological functions of the mitochondrial adenylate transporter AAC1 in primary metabolism and development in

A. thaliana. Furthermore, we studied the role of AAC2 in seed germination and developmental stages of *A. thaliana*. Through these investigations, we aimed to provide a more comprehensive understanding of the roles played by mitochondrial carriers in plant growth and adaptation to stress.

Layout and aims of the chapters

This thesis is largely focused on evaluating the functional roles of mitochondrial transporters, especially the role of mitochondrial adenylate ADP/ATP carriers (AAC1 and 2), in the context of plant growth and metabolism. Thus, the main goal of this work was to understand how and to what extent AACs are associated with important biological processes such as photosynthesis, respiration and nitrogen metabolism and how they interact to adjust to metabolic demands. To achieve this goal different and complementary experimental approaches were used. Therefore, the thesis is organized as a compilation of three independent stand-alone chapters discussing the impact of deficient expression of specific adenylate transporters on plant growth and central carbon metabolism. Thus, the first chapter is focused on a literature review addressing the role of mitochondrial carriers under stress conditions. The last two chapters focus on the metabolic characterization of the *aac1* and *aac2* mutants with T-DNA insertion and antisense lines with reduced expression for the *AAC1* and *AAC2* genes, which will be described under optimal growth conditions. Each chapter includes an introduction, discussion, and details of the methods used. At the end of this thesis, a brief general discussion synthesizes the main findings of this work in a broader context, presenting an outlook and future research areas to be pursued.

CHAPTER I- Multifaceted roles of mitochondrial carrier proteins in plant responses to abiotic stress

The transport of metabolites across the IMM plays a critical role in cellular metabolism, impacting various aspects such as intracellular communication, energy balance, and the distribution of metabolic products across different physiological conditions. Particularly under stress—whether biotic or abiotic—mitochondrial function can be significantly affected, triggering complex signaling pathways that activate stress-related gene transcription to modulate metabolism. Numerous studies emphasize the importance of finely regulating the transport of metabolic products through the IMM in response to stress stimuli. In this review, we provide updated insights into the functions of mitochondrial transport proteins in mediating responses to abiotic stress conditions in plants.

CHAPTER II- Functional characterization of the mitochondrial ADP/ATP carrier isoform 1 in *Arabidopsis thaliana*

The ADP/ATP transporter 1 (AAC1) stands out as one of the most abundant proteins found in the mitochondrial inner membrane of *Arabidopsis thaliana*. Its biochemical function involves facilitating the electrogenic exchange of ADP/ATP between the cytosol and the IMM. Despite its prevalence, the physiological role of AAC1 in plants has remained elusive until now. Thus, we investigated the impacts of reduced expression of *AAC1* has profound effects on cellular respiration and disrupts cellular ATP concentrations. Our findings suggest that AAC1 plays a pivotal role in regulating oxidative phosphorylation, maintaining energy homeostasis, and promoting plant growth, while also indirectly influencing the reproductive phase and seed germination. Additionally, we underscore the intricate regulation of mitochondrial adenylate transporters in plants, implying that different isoforms may contribute differentially to essential physiological processes.

CHAPTER III- The protein ADP/ATP carrier 2 (*At*AAC2) influences on seed germination and reproductive yield

The ADP/ATP Carrier 2 (AAC2) is a mitochondrial adenylate protein classified within the MC family. Despite its prevalence, the physiological role of this carrier in plants remains elusive. This study aimed to elucidate AAC2's function during various stages of plant development, including the reproductive phase, germination, vegetative growth, and root development. Through *in silico* analysis, we observed up-regulation of *AAC2* expression in stamens, pollen grains, embedded seeds, and inflorescences. AAC2 demonstrated significance in germination, as evidenced by a reduced germination percentage and delayed germination observed in lines with decreased *AAC2* expression. Photosynthetic efficiency remained unaltered in the mutant lines, however, lines with lower *AAC2* expression exhibited a notable reduction in leaf dark respiration. Although there was an increase in chlorophyll content, primary metabolites were minimally affected. Reduced *AAC2* expression corresponded to diminished initial root growth, silique size, and silique filling. Morphological alterations rendered seeds inviable. In summary, our findings underscore the pivotal role of AAC2 in specific plant stages, particularly in influencing germination and reproductive yield.

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CHAPTER I

Multifaceted roles of mitochondrial carrier proteins in plant responses to abiotic stress

Multifaceted roles of mitochondrial carrier proteins in plant responses to abiotic stress

Abstract- The transport of metabolites across the inner mitochondrial membrane (IMM) is a pivotal aspect of cellular metabolism, influencing intracellular communication, energy homeostasis, and the distribution of metabolic products across diverse conditions. Under (a)biotic stress, the mitochondrial function becomes susceptible to direct or indirect impacts, activating intricate signaling pathways that, in turn, induce the transcription of stress-related genes to regulate metabolism. Several studies underscore the significance of finely tuning the transport of mitochondrial metabolism products through the IMM in response to stress conditions. This review presents updated insights functions of mitochondrial transport proteins in response to abiotic stress conditions in plants.

Keywords: Mitochondria; cell metabolism; mitochondrial carrier family; abiotic stress.

Introduction

As the exposure of plants to adverse conditions is frequent, there is a ready search for novel strategies aiming to increment crop yield as well as to generate more stress-resilient crops (Nowicka *et al.*, 2018; Batista-Silva *et al.*, 2020). In support of this, genetic modification of enzymatic and non-enzymatic antioxidants (Zhu *et al.*, 2013), osmoprotectants (Omari Alzahrani, 2021) and transcription factors (Wang *et al.*, 2016) are among the potential targets currently pursued by the plant scientific community. In general, stress conditions are accompanied by a greater energy demand (De Col *et al.*, 2017). In these circumstances, there is, on the other hand, also a lower energy production of mitochondrial respiration as a consequence of the suppression of the canonical mitochondrial electron transport chain (mETC) (González and Manavella, 2021).

A higher cellular production of reactive oxygen species (ROS) above a defined threshold is a frequent response plants have to various stresses (Sewelam *et al.*, 2016). This increased ROS production is a mark of plant signaling networks capable of indicating the cellular metabolic status. Furthermore, the excessive production of ROS upon stress is followed by an alteration in the energy status of the plant. These alterations include changes in the levels of key metabolites, such as acetyl-CoA, pyridine nucleotides and adenylates (especially ATP, ADP, and AMP). The generation of the different ROS forms takes place in the energy producing and energy-consuming pathways of metabolism, most of which are associated with the electron transport chains in either mitochondria (mETC) or chloroplasts (cETC). That may be sufficient to account for a generic link between the levels of ROS and adenylates in plants. Specifically, there is evidence that a temporal coordination is initiated after seed imbibition during which dynamic changes in the redox status in seeds are associated with modifications in the adenylate energy charge (Nietzel *et al*., 2020).

Because plant metabolism is highly integrated, any fluctuation in the energy status in one compartment may affect the functioning of other parts of the system and has thus the potential to modify fluxes of central metabolism (da Fonseca-Pereira *et al.*, 2022; Sweetlove *et al.*, 2017). Therefore, these metabolic changes inside mitochondrion must be coordinated with changes in other parts of the cell and may simultaneously affect and be affected by processes in these other cell compartments.

Under stress conditions, plants require multiple and complex adjustments in the levels of proteins, metabolites, and transcripts to preserve cell homeostasis (da Fonseca-Pereira *et al.*, 2018). It has been indicated that, among a comprehensive list of genes putatively encoding mitochondrial proteins, genes encoding the Mitochondrial Carrier Family (MCF) proteins were over-represented in stress conditions (Van Aken et al., 2009). This suggests that stress induces changes in the transport demands for metabolites across the mitochondrial inner membrane (Van Aken et al., 2009).

These results demonstrate that under suboptimal conditions, there is a rearrangement in the expression of genes related to metabolite transport (Van Aken *et al.*, 2009; Barreto *et al.*, 2022). Furthermore, compelling evidences suggest a differential regulation of mitochondrial metabolite carriers aiming to reorganize the cell energy metabolism required for a suitable response following stress (Fonseca-Pereira *et al.*, 2018; Welchen and Gonzalez, 2021; Feitosa-Araujo *et al.*, 2020). Here, we review the current information about the *in vivo* function of mitochondrial transport proteins and their proposed metabolic and physiological functions in plants, especially in response to different abiotic stress conditions.

Mitochondrial carriers in plants

An array of nuclear-encoded mitochondrial carrier proteins is responsible for exporting a wide range of substrates across inner mitochondrial membrane (IMM). Most mitochondrial carriers belong to a family of mitochondrial transporters called the mitochondrial carrier family (MCF). MCF members share a standard transmembrane structure of six α-helices, distributed in three tandem repetitive modules, each containing approximately 100 amino acids (Haferkamp and Schmitz-Esser, 2012). Although these proteins are structurally similar, they play different roles in transporting various substrates, such as nucleotides, amino acids, dicarboxylates, cofactors, phosphates, and protons (Nunes-Nesi et al., 2020; Fernie et al., 2020). In the genome of Arabidopsis, 58 members of proteins belonging to the MCF were identified (Palmieri et al., 2011). Until now, most of these transporters have already been biochemically characterized for their specificity in transporting several metabolites (Nunes-Nesi et al., 2020).

In addition to the mitochondrial transporters belonged to the MCF, various groups of mitochondrial and non-mitochondrial transporters have been extensively reviewed regarding their biochemical function and likely physiological roles. For many of these transporters, a potential role in response to abiotic stresses has already been demonstrated. Overall, these studies provide a list of candidate transport proteins that may be applied to investigate stress-responsive response in plants (Sup. Table 1).

Adenylate mitochondrial carriers: essential transporters connecting stress response and energy physiology

In plants affected by exposure to environmental stress conditions, a differential investment in energy and metabolism is required, often at the expense of the energy available for growth and development (Caretto et. al., 2015). In this scenario, cellular metabolism, as a whole, relies on mitochondrial metabolism (Wang et. al., 2018). Among the numerous substrates exported from the IMM, adenylates stand out as one of the most essential components, playing a crucial role in maintaining cellular energy homeostasis.

Three groups of proteins are responsible for adenylate transport through the IMM: Adenine Nucleotide/Phosphate Carriers (APCs), Adenine Nucleotide Transporter 1 (ADNT1), and ADP/ATP Carriers ($AAC_{1\text{-}3}$). Among the proteins belonging to the

MCF, AACs are the most abundant transporters found in the IMM (Haferkamp and Schmitz-Esser, 2012). AACs mediate the electrogenic passage of ADP/ATP, where a cytosolic ADP molecule is exchanged for an ATP molecule from the mitochondrial matrix (Haferkamp et. al., 2002; Ogunbona and Claypool, 2019). The potential roles of AACs in stress conditions were explored by da Fonseca-Pereira et al. (2018) through *in silico* data mining. However, there is still a gap in studies on AACs using reverse genetics approaches in plants conducted *in vivo*.

The group of APC transporters catalyzes the electroneutral exchange of ATP-Mg/Pi, with dependence on Ca^{2+} (Lorenz et al., 2015). Similar to AACs, the physiological function of this transporter in plants under stress conditions has only been discussed through *in silico* experimentation (da Fonseca-Pereira et al., 2018). An additional role of this transporter has been highlighted in responses to Ca^{2+} signaling during stress (Lorenz et al., 2015; Monné et. al., 2015). APCs, as members of the nucleotide transporter category, may play a role in regulating the influx and efflux of Ca^{2+} across cell membranes (Monné et. al., 2015). In this context, we can infer that the regulation of APC transporters may be influenced by changes in intracellular Cytosolic Ca^{2+} concentration ($[Ca^{2+}]$ cyt), a central event in stress responses. ADNT1 promotes the export of ATP to the cytosol in exchange, preferably, for AMP. The physiological role of this transporter in Arabidopsis is related to the respiratory process in roots and leaf senescence (Palmieri et al., 2008; da Fonseca Pereira et. al., 2021). Additionally, ADNT1 has been previously linked to the initial responses of the fermentative pathway under waterlogging stress conditions (further details will be discussed in topic “Hypoxia induces the modulation of the expression of mitochondrial carriers in plants”) (Neri-Silva et. al., 2023).

Crosstalk between mitochondrial and nuclear genomes in stress responses

Mitochondria are dynamic organelles present in eukaryotic cells and play fundamental roles in plant energy metabolism (Sweetlove et al. 2010; Fuchs et. al., 2020; Chen et. al., 2023). In agreement with these roles, 48% of the entire mitochondrial proteome is dedicated to supplying cellular energy demands in plants (Fuchs et. al., 2020). Along with the oxidative phosphorylation system (OXPHOS), the mETC is responsible for the energy supply for plant survival and development (Wurzinger et al., 2018). Therefore, mETC and OXPHOS are involved in the production of ATP for cellular energy supply. In addition, mitochondria are responsible for the production of several other precursors and respiratory intermediates, such as acetyl-CoA, ADP, oxidized (NAD^+) or reduced (NADH) forms of β-nicotinamide adenine dinucleotide, oxidized (FAD) or reduced ($FADH_2$) flavin adenine dinucleotide (Bohovych and Khalimonchuk, 2016).

Due to the connection with central metabolism, plant mitochondria are key organelles for the perception of fluctuating and unfavorable environmental conditions (Crawford et al., 2018). The perception of environmental stresses is directly and/or indirectly connected with mETC and OXPHOS. The perception of the disorder can be transduced into the nuclear genome, where the transcription of stress-related genes is induced as metabolism regulation (Krupinska Karin et. al., 2020). The reduction of ATP synthesis may result in ATP exhaustion, which at one time results in increases in ADP and AMP levels, occurring mainly in conditions of mitochondrial dysfunction or damage (Pedrotti et al., 2018) (Figure 1). This can be observed at the end of the night or in situations of prolonged darkness. Under these conditions, carbohydrates are limited or have been exhausted, leading to energy limitation (Pedrotti et al., 2018). Retrograde signals are sent from the mitochondria to the nucleus as decreases in ATP levels occur, and with this occurs the stimulation of alternative pathways of respiration and catabolism of amino acids and fatty acids (Hildebrandt et. al., 2015; Merendino et. al., 2020). In addition, retrograde signaling can also be indirectly induced by changes in adenylate pools, since they directly influence other metabolites, such as intermediates of the tricarboxylic acid (TCA) cycle can act as a retrograde sign of mitochondria (Wagner et. al., 2018) (Figure 1).

Regulation at the transport level is essential for energy homeostasis, maintenance, distribution of metabolic products, and intracellular communication. A microarray experiment combining a set of 1196 genes of *A. thaliana* plants subjected to stress conditions demonstrated that the members of the MCF were overrepresented among the stress-responsive genes (Van Aken et. al., 2009). The differential regulation of mitochondrial adenylate transporters is differentially regulated under various stress conditions, including drought, heavy metal, oxidative, salt and cold stresses (da Fonseca-Pereira et al., 2018). This result indicates that the transport of metabolites through the IMM is differentially modified upon stress (Van Aken et. al., 2009). The stress responses of MCFs encoding genes are mainly related to the transport of ADP/ATP, dicarboxylic acids, and inorganic phosphate (Pi) (Palmieri et al., 2008;

Hamel et al., 2004), which indicate that there is a greater exchange of respiratory substrates and the TCA cycle in stressful conditions (Van Aken et. al., 2009).

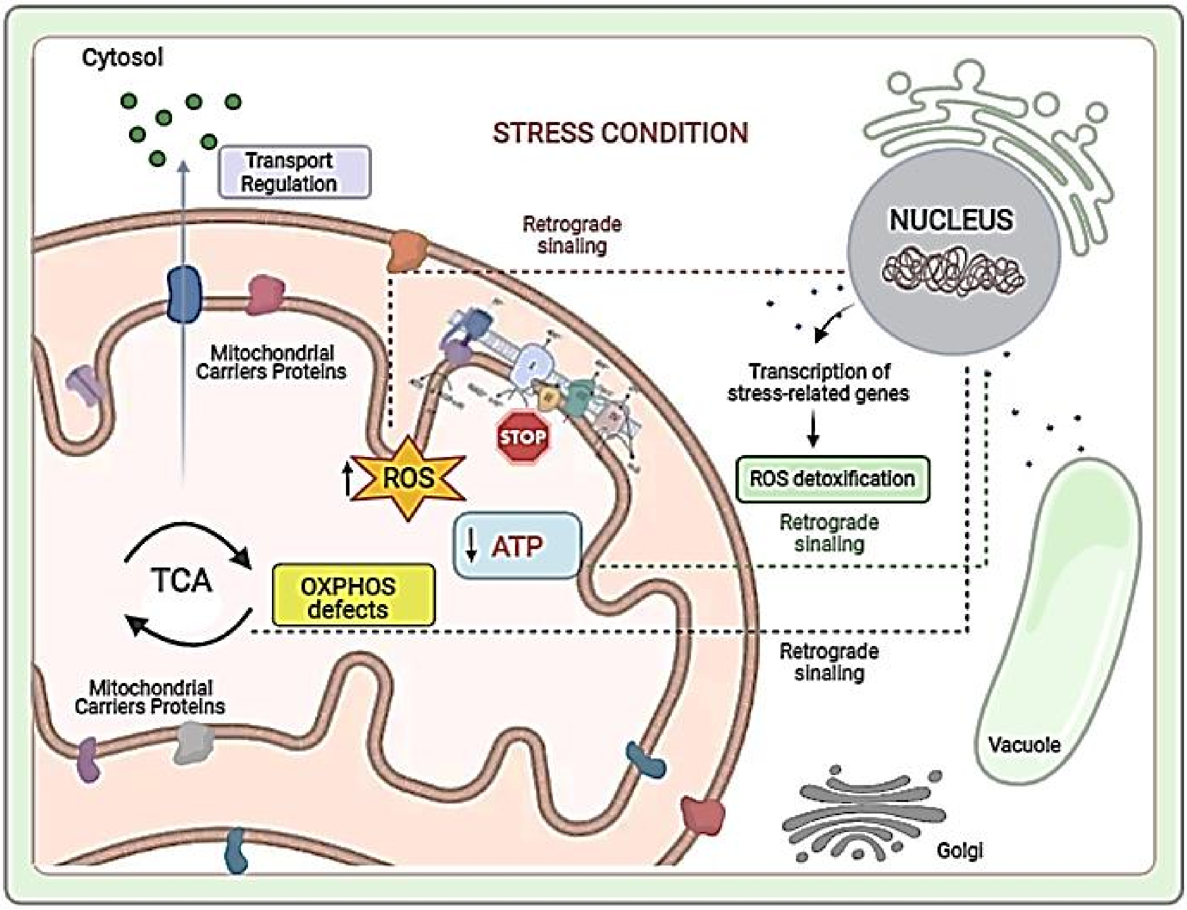

Figure 1. Stress signal arised from mitochondria. Mitochondria, as organelles, play a crucial role in responding to fluctuating and unfavorable environmental conditions, acting as key sensors due to their connection with central metabolism. Dysfunction in the mitochondrial electron transport chains (mETC) and oxidative phosphorylation (OXPHOS) during stress disrupts adenylate levels and elevates the production of reactive oxygen species (ROS). This imbalance triggers retrograde signals to the nuclear genome, inducing the transcription of genes related to ROS detoxification and regulating metabolite transport across the IMM. Furthermore, retrograde signaling can also be indirectly initiated by alterations in adenylate pools, as they directly impact other metabolites. For instance, intermediates of the tricarboxylic acid (TCA) cycle can function as retrograde signals from mitochondria. The regulation of mitochondrial protein transporters, such as the MCF family, plays a crucial role in retrograde signaling in response to stress. These transporters, responsible for importing and exporting metabolites and ions across mitochondrial membranes, dynamically modulate metabolic flux and signaling pathways. By adjusting the transport of key molecules, they contribute to coordinating cellular responses to environmental signals, enhancing the integration of mitochondrial function with cellular metabolism and stress adaptation. This intricate interplay between mitochondria and central metabolism positions these organelles as finely-tuned regulators of metabolic processes and stress-responsive gene expression.

The involvement of mitochondrial transporters in responses to drought stress

In plants under water limitation, the expression of genes related to energy transport seems to be regulated so that energy turnover is modulated to allow plant survival. Bioinformatics analysis indicated that mitochondrial APC1 and AAC3 are highly induced in *A. thaliana* under drought and osmotic stress conditions (da Fonseca et. al., 2018). These carriers may be acting to indirectly ensure the high rates of synthesis of osmoprotective compounds, mainly from the catabolism of amino acids in plants submitted to situations that promote dehydration (da Fonseca et. al., 2018; Araújo et. al., 2011). In tobacco, it was observed that the overexpression of the *Uncoupling Protein 1*(*UCP1*) gene resulted in increases in respiration and a reduction of hydrogen peroxide levels in leaves during water deficit (Barreto et. al., 2017). It became evident that UCP1 has a potential mitigating role in responses to drought stress (Barreto et. al., 2017).

Proteomic analyses demonstrate that the transport of various metabolites from mitochondria to the cytosol can be altered by the regulation of protein levels in plants submitted to water deficit (Ford, Cassin, and Bacic, 2011; Wang et. al., 2016). Mitochondrial carrier proteins of dicarboxylate/tricarboxylate carrier (DTC) responsible for transporting dicarboxylic acids (such as malate, malate, oxaloacetate, and 2-oxoglutarate) and tricarboxylic acids (such as citrate, isocitrate, cis-aconitate, and trans-aconitate) (Picault et. al., 2002) were demonstrated as being downregulated under drought stress conditions (Ford, Cassin and Bassic, 2011). In contrast, it was observed that voltage-dependent anionic channel proteins (VDACs), which are located in the outer membrane of mitochondria can regulate the flows of Ca^{2+}, ATP/ADP exchange, and metabolites are upregulated by water deficit (Ford, Cassin, and Bassic, 2011). Changes in the abundance of mitochondrial carriers are indicative of stress tolerance (Ford, Cassin, and Bassic, 2011). Among the various mitochondrial carrier proteins, VDAC proteins seem to be directly related to drought tolerance (Wen et. al., 2014). A study conducted by Wen et. al., 2014, showed that overexpression of the *AtVDAC2* gene may confer drought resistance in *A. thaliana*.

Investigating the mechanisms involved in stomatal movements modulated by abscisic acid (ABA) in plants under water deficiency, the mitochondrial protein NRGA1 (Negative Regulator of ABA 1 signaling of the Guard cell) was identified as a probable mitochondrial pyruvate carrier (PMC) involved in the function of the guard cell (Li et al., 2014). Studies with *A. thaliana* mutants for *NRGA1* demonstrated not only the involvement of NRGA1 in the regulation of somatic movements mediated by ABA but also that the content of cellular pyruvate is important to modulate responses to drought stress (Li et. al., 2014; Shen et. al., 2017). The physiological characterization of NRGA1 demonstrated that this protein is capable of mediating the production of ABA stimulated by drought, thereby controlling the stomatic opening through the activities of the guard cell ion channel (Li et. al., 2014). In the same study, it was observed the existence of a relation between the transport of mitochondrial pyruvate and the signaling of the guard cell. Since *NRGA1* encodes a putative PMC, the reduction of its activity led to an accumulation of pyruvate in the cytosol, possibly resulting in a reduction of the products of the mitochondrial TCA cycle, which conferred a greater tolerance to drought stress due to a higher ABA sensitivity (Li et. al., 2014).

The role of pyruvate transport modulating response to drought and ABA was observed in mutant plants of *mcp1* (Shen et. al., 2017) (Figure 2). The physiological characterization indicated that A*t*MPC1 has a function similar to that of NRGA1 in the regulation of stomatal closure. MPC1 function loss mutants (*mpc1*) showed hypersensitivity to ABA-induced stomatic closure, in addition to a lower rate of water loss (Shen et. al., 2017). Furthermore, *mpc*1 mutant plants exhibited higher pyruvate content. This increase in pyruvate levels would have induced stomatal closure and made the anionic channels of the slow type of guard cells active (Figure 2). Increased pyruvate production can be an indicator of specific metabolic needs as well as differential energy demand (Le et. al., 2022) and can be a factor in the mitochondrial response to stress. Plants have two distinct pyruvate pools that are operated depending on conditions and energy demand. One pool is formed from the import of pyruvate and operates to sustain the TCA cycle, while the second pool of pyruvate is derived from the NAD-malic enzyme and acts as an emergency valve, under conditions that the pyruvate imported into the matrix is too little to meet the energy requirement (Le et. al., 2022). Indeed, the mutations in the NAD-malic enzyme and mitochondrial pyruvate carrier (MPC1) resulted in impaired plant growth and an elevation in cellular pyruvate levels. This elevation indicated a downregulation of respiratory metabolism, concurrently raising the levels of branched-chain amino acids at night, signifying an alternative substrate supply for respiration (Le et. al., 2021). The absence of both enzymes led to a metabolic blockade, highlighting the collaborative role of pyruvate-supplying enzymes in supporting the TCA cycle for sustained plant growth (Le et. al., 2021).

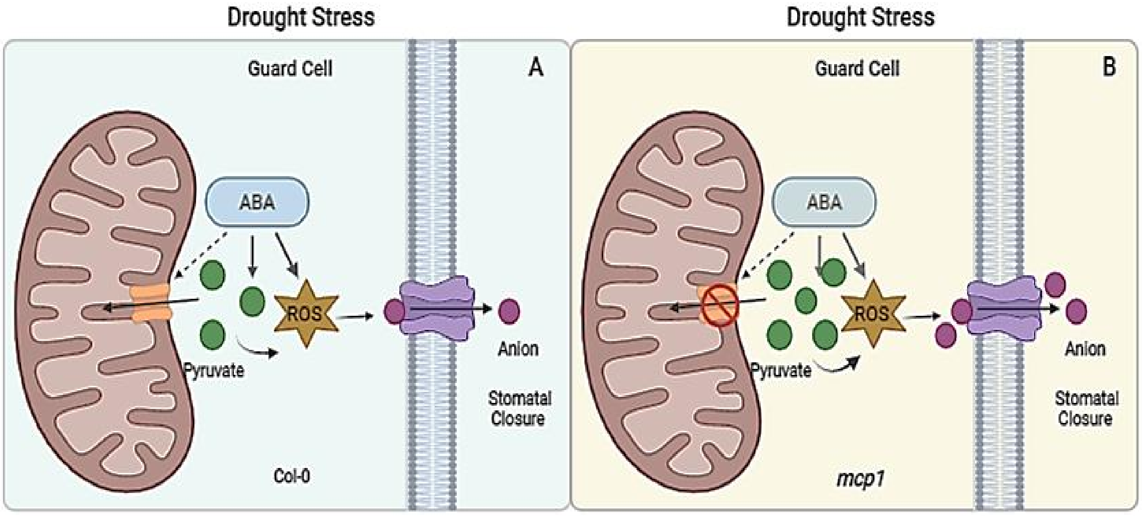

Figure 2. The role of the MCP1 transporter in stomatal opening in response to water deficit and ABA. A) Responses in Col-0 plants. B) Responses in mutant plants *mcp1*. AtMPC1 plays an important role in regulating stomatal movement in response to water deficit. Plants subjected to drought conditions have a higher level of ABA, causing an increase in cellular pyruvate. Pyruvate accumulation activates slow-type anion channels that increase ROS production which promotes stomatal closure. *Mpc1* mutant plants showed a greater accumulation of cytosolic pyruvate, favoring the increase of ROS levels, which promotes a rapid stomatal closure. Figure adapted from the model proposed by Shen et. al., 2017.

The responsive role of mitochondrial carriers by heavy metal stress

Heavy metals are non-essential elements capable of generating phytotoxicity problems, even when at low concentrations (Keunen et. al., 2011). These elements promote a negative impact on physiological and biochemical processes, human health, and the environment. In plants, exposure to heavy metals can trigger oxidative stress mainly by increasing levels of ROS promoting an unbalanced pro-oxidant and antioxidant homeostasis in plant cells (Keunen et. al., 2011; Sharma et. al.,2009). This cellular redox imbalance caused by heavy metals can negatively affect mitochondrial respiration and impair ATP levels, further altering the activity of the TCA cycle activity. Under heavy metal stress, plants modulate various metabolic pathways, including photosynthesis and mitochondrial respiration. This modulation helps them produce more reducing power, such as NADPH, NADH, and $FADH_2$, as well as assimilatory ATP, to meet the high energy demand caused by heavy metal exposure (Hossain and Komatsu, 2013). Consequently, plants tolerate heavy metals by adjusting their metabolism to ensure the continuity of ATP supply and the TCA cycle.

Among the mechanisms influencing plant tolerance to cadmium (Cd), it was observed that the formation of MPC protein complexes, such as *AtMPC1* and *AtMPC2,* can prevent Cd stress by ensuring ATP production and allowing the continuity of the TCA cycle, as well as relieving glutamate (He et. al., 2019). In this study, it was observed that *AtMPC1* silencing resulted in a reduction in ATP content, negatively affecting the TCA cycle, which promoted Cd accumulation in Arabidopsis (He et al., 2019).

Several physiological processes are susceptible to arsenic (As) toxicity. One of the physiological effects of As is to cause damage to cell membranes which results in electrolyte leakage (Singh et al., 2006). Furthermore, after exposure to As the entire energy state of the plant is disturbed resulting in a drastic reduction in cellular efficiency for ATP biosynthesis (Shukla et. al., 2015; da Fonseca et. al., 2018). *In silico* analyses demonstrated that two adenylate carrier proteins are positively regulated (AAC_{2-3}) upon arsenate exposure, this possibly happens so that there is a differential regulation of adenylate transporters, thus ensuring the supply of energy for processes related to detoxification (da Fonseca-Pereira et. al., 2018). In the face of these complex mitochondrial responses, understanding the underlying molecular mechanisms becomes crucial for developing effective strategies to manage heavy metal stress in plants, aiming not only at plant health but also at food and environmental safety.

Mitochondrial carriers in aluminum stress responses

Acidified soils make up around 30% of the Earth's surface due to their high aluminum (Al) content (FAO, ITPS, 2015). This phenomenon represents a global agricultural challenge, impairing plant growth and limiting productivity in acidic environments (Kochian et al., 2004). Mitochondrial metabolism plays a pivotal role in determining plant resistance to Al and, consequently, their tolerance or resistance to acidic soils (Nunes-Nesi et al., 2014). The presence of Al^{3+} alters the cation exchange capacity of roots, influencing water and nutrient uptake by plants (Nunes-Nesi et al., 2014). This inadequate absorption becomes a limiting factor for plant productivity, resulting in yield losses ranging from 25 to 80%, depending on the species (Sade et al., 2016).

In the presence of Al^{3+}, mitochondrial respiration is inhibited due to the formation of reactive oxygen species (ROS), leading to oxidative damage in plants (Nunes-Nesi et al., 2014). In response, the production and exudation of carboxylic acids, such as citrate and malate, become crucial for combating this stress. These organic acids, when exuded by the roots, play a key role in chelating Al in the root's immediate environment (Ma et. al., 2001; Yan et al., 2021). The types of these acids can vary among species, with malate playing a central role in detoxification in *A. thaliana* (Liu et al., 2009). However, the production and exudation of these acids through the TCA cycle depend on NAD$^+$ production, relying on the proper functioning of the electron transport chain (Sweetlove et al., 2010; Zhang and Fernie, 2018).

Mitochondrial carriers, responsible for exporting ATP and importing other phosphorylated molecules, sustain the electron transport chain, providing the necessary NAD$^+$ for the TCA cycle. It is anticipated, therefore, that mitochondrial carriers emerge as strong candidates for maintaining respiratory metabolism homeostasis under aluminum deficiency. *In silico* co-expression analyses indicate a close relation between Al resistance genes (11 genes) and members of the mitochondrial carrier family (22 genes) (Nunes-Nesi et al., 2014). This correlation is possibly associated with the transport of carboxylic acids, playing a crucial role in this context, though this association still requires validation (Nunes-Nesi et al., 2014). Understanding the intricate interplay between Al stress, mitochondrial metabolism, and the exudation of these acids is essential for developing strategies to enhance plant resilience in acidic soils. Further validation and exploration of the identified gene associations are imperative for elucidating the molecular mechanisms underlying plant responses to Al stress, paving the way for targeted interventions to mitigate its adverse effects on agricultural productivity

Hypoxia induces the modulation of the expression of mitochondrial carriers in plants

Results from *in silico* expression analyses support evidence that the expression of most adenylate carriers is altered during the early stage of hypoxia (da Fonseca et al., 2018). However, large changes in the expression pattern of adenylate transporters are also observed in later stages of hypoxia stress period. Under hypoxic stress conditions, the expression pattern indicates that most genes encoding adenylate transporter proteins are downregulated, including AAC$_{1\text{-}3}$, APC2, and PNC$_{1\text{-}2}$ transporters, which were positively regulated (da Fonseca et al., 2018). Additional evidence points out that AAC3 activity is responsible for supplying ATP to mitochondria from glycolysis (Visser et al., 1994; Klingenberg, 2008). Under hypoxic conditions, membrane potential maintenance is believed to be performed by AAC3 and APC2, which would act to favor membrane potential formation by pumping protons coupled to ATP hydrolysis (Stael et al., 2011; Traba et al., 2009). Moreover, it has been shown that under conditions of low oxygen supply, UCP1 activates the expression of nuclear genes associated with hypoxia responses (Barreto et al., 2022). In *A. thaliana*, these responses occur due to the inhibition of an important mechanism that senses the presence of nitric oxide (NO), the Plant Cysteine Oxidase (PCO) branch of the Proteolysis (PRT)6 N-degron pathway (Barreto et al., 2022). Thus, by inhibiting the PCO N-degron pathway, UCP1-modulated retrograde mitochondrial signaling acts as a facilitator of hypoxic stress responses.

Another mitochondrial carrier that has its expression altered as a result of hypoxic stress is ADNT1. In Arabidopsis, *ADNT1* is expressed in seedling roots, cotyledon vascular tissues, and leaf primordia (Palmieri et al., 2008). Mutant plants and transgenic lines with lower expression of *ADNT1* in *A. thaliana* under waterlogging conditions showed a significant reduction in the maximum quantum yield of PSII electron transport after flooding, indicating a more pronounced impact of hypoxic stress on these lines (Neri-Silva et al., 2023). Additionally, the authors observed that ADNT1-deficient lines exhibited higher levels of AMP in the roots under non-stress conditions. ADNT1-deficient plants also displayed a differential gene expression pattern related to hypoxia, with increased expression of non-fermentation-related kinase 1 (*SnRK1*) and positive regulation of adenylate kinase (*ADK*) under both stress and non-stress conditions (Neri-Silva et al., 2023). These findings suggest that the negative regulation of *ADNT1* influences adenylate levels under hypoxic stress. These findings indicate that reduced expression of *ADNT1* is associated with an early "hypoxic state" due to disruption of the adenylate pool caused by the reduced import of AMP by mitochondria. This disturbance, detected by SnRK1, results in a metabolic reprogramming associated with the early induction of the fermentative pathway in ADNT1-deficient plants (Neri-Silva et al., 2023).

Besides the regulation of adenylate carriers in plants, there is strong evidence that DICs play an important role in hypoxia stress responses in *Oryza sativa* (Narsai and Whelan, 2013), *A. thaliana*, and *Eucalyptus grandis* (Barreto et al., 2022). In the study conducted by Barreto et al. (2022), it was shown that *AtDIC*s and *EgDIC*s are induced by submergence stress in *A. thaliana* and *E. grandis*, while overexpression of *AtDIC2* improved the survival of seedlings under submergence. Under conditions of low O_2 supply, DICs appear to be involved in the metabolic flux of organic acids, being important players in the export of malate to the cytosol, where it can be oxidized to oxaloacetate and NADH. Therefore, the involvement of DICs in hypoxia responses provides reducing equivalents for ethanolic fermentation (Borecký et al., 2006) and thus supports the respiration process in conditions where O_2 is limited. *In vitro* and organelle analyses demonstrate that DIC2 predominantly imports malate over citrate export, aligning with the altered utilization of malate and citrate in response to prolonged darkness in *dic2-1* plants (Lee et al., 2021). Additionally, isotopic glucose tracing reveals a diminished flow toward citrate in *dic2-1*, leading to a metabolic redirection for amino acid synthesis. These observations unveil the physiological role of DIC2 in mediating the flux of malate and citrate between the mitochondrial matrix and other cellular compartments (Lee et al., 2021). The intricate regulatory network involving DICs, exemplified by the role of DIC2 in Arabidopsis and its broader implications in hypoxia stress responses across different plant species, underscores the importance of mitochondrial carriers in plant adaptation mechanisms. Understanding these mechanisms not only sheds light on the complex interplay within plant cellular processes but also offers potential applications for enhancing plant resilience to environmental stresses.

Overall, the modulation of mitochondrial carriers, including adenylate carriers and DICs, is important for plant adaptation to hypoxia. The dynamic expression observed during the early and prolonged phases of hypoxia influences energy metabolism, cellular homeostasis, and stress response pathways. Understanding these intricate mechanisms contributes to our knowledge of plant cellular processes and offers promising applications for enhancing plant resilience to environmental stresses induced by hypoxic conditions.

Mitochondrial transporter BAC2 and its relation with osmotic stress in *Arabidopsis*

Basic Amino Acid Carrier 2 (BAC2), belonging to MCF family, transports basic amino acids (Picault et al., 2004; Planchais et al., 2014). In Arabidopsis, BAC2 regulates arginine metabolism, responding to stress signals during hyperosmotic stress and dark-induced senescence (Toka et al., 2010). Studies on *bac2* mutant plants reveal that *BAC2* overexpression activates arginine catabolism, depleting arginine and causing urea accumulation in leaves. Mutants also exhibit compromised recovery under hyperosmotic stress, particularly during leaf expansion initiation (Planchais et al., 2014). Transcriptome analysis under hyperosmotic stress shows disturbances in genes related to stress transcription factors and arginine metabolism enzymes in *bac2* mutants compared to control conditions (Planchais et al., 2014).

Notably, mutants with reduced *BAC2* expression show elevated expression of the proline biosynthetic gene P5CS1, resulting in increased proline accumulation (Toka et al., 2010). These responses underscore BAC2's role in regulating arginine levels and related metabolites, emphasizing its physiological significance in orchestrating plant responses and stress recovery (Toka et al., 2010; Planchais et al., 2014).

The mitochondrial transporter BAC2 regulates arginine metabolism in Arabidopsis, influencing arginine levels in both the cytosol and mitochondria. Elevated BAC2 expression (BAC2-OE) decreases arginine content, attributed to enhanced transport into the mitochondria, where arginase-mediated catabolism occurs. This process produces ornithine and urea, leading to notable accumulations of urea, ornithine, and citrulline in BAC2-OE lines, indicating heightened arginine catabolism (Planchais et al., 2014). Urea predominantly accumulates in aerial parts, consistent with ARGAH2 expression in leaves. The urea degradation process involves DUR3 and TIP transporters, followed by specific gene expression and urease-mediated degradation (Planchais et al., 2014). The absence of nickel (Ni) in the growth medium may limit urease activity, resulting in excessive urea accumulation in BAC2-OE plants. Beyond metabolism, *BAC2* influences gene expression, affecting stress-related transcription factors (TFs) such as SZF1, WRK33, CZF1, and ERF5. Altered expression of genes related to arginine metabolism, polyamines, and transporters in *bac2* mutants emphasizes BAC2's comprehensive role in metabolic homeostasis and stress responses, positioning it as an essential component in Arabidopsis' adaptation to challenging environments (Planchais et al., 2014).

The function of the uncoupling protein in response to stress in plants

Uncoupling protein 1 (UCP1) is encoded in the nucleus and localized in the IMM (Nedergaard et al., 2005). Through its action in uncoupling the electrochemical proton gradient in the mETC, it dissipates energy in the form of heat (Kajimura et al., 2010). The biological function of plant uncoupling proteins (pUCPs) and their role under stress conditions has been a subject of considerable debate. Some studies suggest that *UCP1* and *UCP2* transcripts aren’t induced by stress conditions (Van Aken et al., 2009; Sweetlove et al., 2006). UCP1 plays an important role in the plant's response to oxidative stress. The absence of *AtUCP1* resulted in localized oxidative stress, underscoring the significance of this protein in maintaining the redox balance of the mitochondrial electron transport chain. Surprisingly, the plant retains the ability to resist a broad range of abiotic stresses, suggesting that UCP1 isn't essential for these specific stress responses. However, in the same study, the authors reported a substantial impact on the photosynthetic phenotype in the knockout lines of *AtUCP1* (Sweetlove et al., 2006).

Furthermore, several studies have demonstrated the role of UCPs in stress tolerance responses in tobacco and tomato (Barreto et al., 2016; Barreto et al., 2017; Chen et al., 2013). In an experiment using *Nicotiana tabacum* plants overexpressing UCP1, a faster recovery from water stress was observed upon rehydration (Barreto et al., 2017). In the same study, *UCP1* mutant lines exhibited higher respiration rates, a reduction of H_2O_2 in leaves during water stress exposure, and an increase in seed size and number under both control and water stress conditions (Barreto et al., 2017). Additionally, in tomato (*Solanum lycopersicum*), UCP overexpression altered the homeostasis of cellular redox and antioxidant capacity under heat conditions and in response to the necrotrophic plant pathogen Botrytis cinerea (Chen et al., 2013). While the exact mechanisms of action and the full scope of functions of plant uncoupling proteins are still under investigation, the existing evidence underscores their significance in stress adaptation and opens avenues for further exploration of their potential applications in enhancing plant resilience to environmental challenges.

Mitochondrial Phosphate Transporter participates in salt stress responses in Arabidopsis

The response to salt stress exposure in plants demands a series of signaling and metabolic adjustment processes, involving a high energy cost. Thus, mitochondrial function is affected to provide an adequate supply of ATP and reduction power for the essential reactions that combat salt toxicity to be continuing (Chen-Othman et. al., 2017). This mitochondrial supply role includes modulations in ATP transport, ions, and ROS detoxification that allow the cellular function to remain stable at high salt concentrations (Chen-Othman et. al., 2017).

In this salt exposure survival process, the mitochondrial Phosphate Transporter (MPT), which belongs to the MCF, plays a crucial role in ATP production in plant cells (Zhu et. al.,2012). The mitochondrial phosphate transporter (MPT/PiC) catalyzes phosphate (H_2PO^{4-})/proton symport, phosphate/hydroxyl ion antiport, and mitochondrial matrix exchange with cytosolic phosphate (Mayr et. al., 2007). This ability of MPT to capture orthophosphate (Pi) in the mitochondrial matrix is an essential process for the oxidative phosphorylation of ADP into ATP (Haferkamp, 2007). It has already been shown that in plants overexpression of *AtMPT* exhibits higher sensitivity to salt stress compared to wild-type plants during the stages of seed germination and seedling establishment (Zhu et. al.,2012). With that, the varying levels of ATP may be connected to the plant’s response to salt stress. Furthermore, in the same study, a change in gene expression of genes related to gibberellin synthesis was promoted by a change in energy state in *AtMPT* overexpressing lines. With this, it became evident that there is a link between gibberellin metabolism and energy homeostasis mediated by MPTs in responses to high salinity stress in *A. thaliana* (Zhu et. al.,2012).

The plant's response to salt stress involves intricate signaling and metabolic adjustments, imposing a substantial energy burden. Mitochondrial function, crucial for ATP and reduction power supply, undergoes modulations in ATP transport, ion regulation, and ROS detoxification to maintain cellular stability under high salt concentrations. This also highlights a potential connection between ATP levels, gibberellin synthesis, and energy homeostasis mediated by MPTs in response to high salinity stress in plants.

Conclusion

Under stress conditions, not only intermediates of the respiratory process can be regulated, but also transport is regulated to balance, as appropriately as possible, the consumption, requirement, and distribution of these metabolites. Thus, it is tempting to believe that mitochondrial carrier proteins are regulated as exposure to stressful conditions occurs. Proteomic analysis, *in silico* expression analysis, and some *in vivo* assays already show real evidence that not only the levels of metabolites from the respiratory process but also the transport mediated by mitochondrial proteins are affected. However, there is a growing and notorious need for more *in vivo* studies to be conducted to understand more deeply how the regulation at the transporter level happens, as well as the impacts on the whole metabolism in plants subjected to abiotic stress conditions.

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Material Supplemental

Sup. Table 1. Mitochondrial Carrier proteins are identified as having potential roles in response to abiotic stress.

Plant Species	Carrier	Description	Substrate	Related Abiotic Stress	Reference
Arabidopsis thaliana	APC1	Adenine nucleotide/Phosphate Carriers 1	ATP-Mg/Pi	Drought Osmotic stress	da Fonseca et. al., 2018
Arabidopsis thaliana	APC2	Adenine nucleotide/Phosphate Carriers 2	ATP-Mg/Pi	Hypoxia	da Fonseca et. al., 2018
Arabidopsis thaliana	ADNT1	Adenine Nucleotide Transporter 1	ATP/AMP > ADP	Hypoxia	Neri-Silva et. al., 2023
Arabidopsis thaliana	AAC1	ADP/ATP Carrier 1	ATP/ADP	Hypoxia	da Fonseca et. al., 2018
Arabidopsis thaliana	AAC2	ADP/ATP Carrier 2	ATP/ADP	Arsenic toxicity Hypoxia	da Fonseca et. al., 2018
Arabidopsis thaliana	AAC3	ADP/ATP Carrier 3	ATP/ADP	Drought Osmotic stress, Arsenic toxicity Hypoxia	da Fonseca et. al., 2018
Nicotina tabacum	UCP1	Uncoupling Protein 1	H^+/ Fatty Acids	Drought	Barreto et. al., 2017
Arabidopsis thaliana	UCP1	Uncoupling Protein 1	H^+/ Fatty Acids	Hypoxia	Barreto et. al., 2022
Solanum lycopersicum	UCP1	Uncoupling Protein 1	H^+/ Fatty Acids	Heat Stress	Chen et. al., 2013
Arabidopsis thaliana	DTC		Dicarboxylic/Tricarboxylic acids	Drought	Ford, Cassin and Bassic, 2011

Arabidopsis thaliana	VDACs	Voltage-dependent anionic channel	Ca^{2+}-ATP/ADP	Drought	Ford, Cassin, and Bassic, 2011; Wen et. al., 2014
Arabidopsis thaliana	NRGA$_1$	Negative Regulator of ABA 1	Pyruvate	Drought	Li et. al., 2014; Shen et. al., 2017
Arabidopsis thaliana	MPC1	Mitochondrial Pyruvate Carrier 1	Pyruvate	Drought	Shen et. al., 2017
Arabidopsis thaliana	MPC1-2	Mitochondrial Pyruvate Carrier 1 and 2	Pyruvate	Cadmium toxicity	He et. al., 2019
Oryza sativa	DIC2	Mitochondrial Dicarboxylate Carrier 2	Sulfate/malate, oxaloacetate, phosphate and succinate > 2-oxoglutarate	Hypoxia	Narsai and Whelan, 2013
Arabidopsis thaliana	DIC2	Mitochondrial Dicarboxylate Carrier 2	Sulfate/malate, oxaloacetate, phosphate and succinate > 2-oxoglutarate	Hypoxia	Barreto et. al.,2022
Eucalyptus grandis	DIC2	Mitochondrial Dicarboxylate Carrier 2	Sulfate/malate, oxaloacetate, phosphate and succinate > 2-oxoglutarate	Hypoxia	Barreto et. al.,2022
Arabidopsis thaliana	BAC2	BAC2: Basic Amino Acid Carrier 2	Basic amino acids	Hyperosmotic stress Dark-induced senescence	Toka et al., 2010 Planchais et. al., 2014
Arabidopsis thaliana	MPT	Mitochondrial Phosphate Transporter	Phosphate inorganic	Salinity Stress	Zhu et. al.,2012

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CHAPTER II

Functional characterization of the mitochondrial ADP/ATP carrier isoform 1 in *Arabidopsis thaliana*

Functional characterization of the mitochondrial ADP/ATP carrier isoform 1 in *Arabidopsis thaliana*

Abstract: The ADP/ATP transporter 1 (AAC1) is one of the most abundant proteins in the mitochondrial inner membrane of *Arabidopsis thaliana*. Biochemically, it is characterized by mediating the electrogenic exchange of ADP/ATP between the cytosol and the mitochondrial inner membrane. To date, the physiological function of AAC1 in plants remains unknown. Here, we report that reduced expression of *AAC1* affects cellular respiration and alters cellular ATP concentrations. Electron microscopy analysis showed that reduced activity of the AAC1 transporter led to a dramatic increase in the number of abnormal mitochondria, with an advanced degree of vacuolar degeneration in root cells. Our results demonstrated, there were significant decreases in root oxygen consumption, reduced root growth, and increased levels of soluble carbohydrates. Impacts on the vegetative and reproductive stages were reported, such as a reduction in the number of leaves and an increase in silique size. Our results suggest that AAC1 plays an impact of mitochondrial respiration, maintaining energy homeostasis and plant growth, as well as influencing the reproductive phase and seed germination.

Keywords: Mitochondria, adenylates, cell respiration, bioenergetics.

Introduction

Mitochondria are dynamic organelles with distinct mechanisms to adapt their function and composition actively. Changes in mitochondrial metabolism primarily result from its capacity to exchange molecules with the cytosol, regulating the import and export of substrates, products, and signaling molecules (Baena-González et al., 2010; da Fonseca-Pereira et al., 2018; Dourmap et al., 2020). A constant flow of ATP out of the mitochondrial matrix, in exchange for ADP, is needed to supply energy to the cytosol and thus meet the demands of numerous biosynthetic processes outside the mitochondria. Three groups of proteins belonging to the Mitochondria Carrier Family (MCF) are responsible for transporting adenylates across the inner mitochondrial membrane: the ATP/Phosphate Carrier 1 (APCs), responsible for mediating the export of Pi (inorganic phosphate) to the cytosol and the import of adenylates into the mitochondrial matrix (Lorenz et al., 2015); the Adenine Nucleotide Translocator 1 (ADNT1), which promotes the export of ATP to the cytosol in exchange, preferentially, for AMP; and the ADP/ATP Carrier (AACs), which acts in the electrogenic passage of ADP/ATP, transporting a molecule of cytosolic ADP in exchange for a molecule of ATP from the mitochondrial matrix (Ogunbona and Claypool, 2019). To date, some mitochondrial adenylate transporters have been previously characterized for their physiological role in plants (Palmieri et. al., 2008; Monné et. al., 2015; da Fonseca-Pereira, 2021; Neri-Silva et., 2023). Among these, it has been identified that APC transporters are capable of regulating the pool of adenylates in the mitochondrial matrix (Monné et. al., 2015). In Arabidopsis, the physiological function of ADNT1 is related to the respiratory process in roots, dark-induced senescence, and the early responses of the fermentative pathway in waterlogged conditions (Palmieri et al., 2008; da Fonseca-Pereira, 2021; Neri-Silva et. al., 2023). Due to the importance of adenylate transport for energy turnover, adenylate transporters have been identified in other organelles, such as chloroplasts, peroxisomes, and the plasma membrane (Leroch et al., 2008; Palmieri et al., 2008; Nunes-Nesi, Cavalcanti and Fernie, 2020). The identification of these adenylate transporters in different organelles suggests the complexity and importance of adenylate transport in regulating energy metabolism in plants.

The proteins of the AAC group are the most abundant among the mitochondrial transporters located in the inner membrane of mitochondria (Fuchs et al., 2020). In addition to acting as adenylate exchangers, studies on mammalian mitochondria suggest that these transporters also can transport protons (H^+) (Bertholet et al., 2019). AAC transporters are highly related to their orthologs in animals and yeast, both in structure and function (Haferkamp and Schmitz-Esser, 2012). In humans, the most expressed isoform of the ADP/ATP transporter is adenine nucleotide translocase type 1 (ANT1), present in tissues such as skeletal muscle, heart, and brain (Dolce et al., 2005). Mutations associated with the *ANT1* gene can cause mitochondrial disorders, including mitochondrial myopathy, cardiomyopathy, and Senger syndrome, due to multiple deletions of mitochondrial DNA (Sharer, 2005; Echaniz-Laguna et al., 2012). In *Saccharomyces cerevisiae*, the absence of the main mitochondrial ADP/ATP transporter results in a defect in the activity of the cytochrome *c* oxidase enzyme (Ogunbona and Claypool, 2019). In *Trypanosoma brucei*, reduced expression of the *TbAAC* transporter resulted in altered growth, reduced ATP synthesis, and an increase in membrane potential and the amount of reactive oxygen species (Gnipová et al., 2015).

In Arabidopsis, three nuclear genes encode isoforms of AAC proteins (AT3G08580, *AAC1*; AT5G13490, *AAC2*; and AT4G28390, *AAC3*), which are characterized by an N-terminal extension containing around 60 residues of 16 amino acids (Haferkamp and Schmitz-Esser, 2012). Among the isoforms, the *AAC1* gene is distinctly the most expressed, regardless of the type of organ and stage of development. *AAC2* and *AAC3* genes are positively regulated in the reproductive phase in flower, silique, and pollen grain tissues, but are expressed at relatively lower levels than AAC1 in all vegetative tissues (Picault et. al., 2004; da Fonseca-Pereira et. al., 2018). The Michaelis-Menten constant (*Km)* and the maximal velocity (*Vmax)* values indicate that the AAC_{1-3} transporters in Arabidopsis have a high affinity for ATP and ADP transport, with *Km* of 10 µm (AAC1 and AAC3) and 14 µm (AAC2) for ADP and a *Km* value of 15 µm (AAC1) and 22 µm (AAC2) and 12 µm (AAC3) for ATP (Haferkamp et. al., 2002). Differential kinetic patterns may contribute to the functional diversity of mitochondria, enabling the regulation of adenylate flow by the cell's energy requirements. (Haferkamp et. al., 2002).

Based on its transport properties, we hypothesized that the AAC1 could play a critical role in oxidative phosphorylation, impacting different physiological processes. Here, we investigated the effects of reduced *AAC1* expression on growth parameters, reproductive phase, and metabolic responses, focusing on tissues where mitochondrial oxidative phosphorylation exclusively meets energy demands. We show that reduced *AAC1* expression affects cellular respiration in leaves and roots, alters cellular ATP levels, changes mitochondrial morphology, and impacts the reproductive phase, germination, and root growth. The results are discussed in the context of mechanisms related to the exchange of adenylates between the cytosol and mitochondria of plant cells.

Material and methods

Plant material

The $aac1^{-1}:aac1^{-1}$ T-DNA insertion line was obtained from the Institute Genome Analysis Laboratory - SIGnAL Salk collection (SALK_134240C) and has a T-DNA insertion, via *Agrobacterium tumefaciens*, in the promoter region of the transporter gene (Figure 2Ai). The flanking sequence contains 473 bp. The zygosity was analyzed by PCR screening, using the following primers: forward 5' CGTAACACATGGGAGAACAATAGAGAA-3', reverse 5'-TAAACTAAAGTCAACAGATCCAAACGATTTGA- 3' and LBb1.3: 5'-ATTTGCCGATTTCGGAAC-3'. The homozygous $aac1^{-1}:aac1^{-1}$ was line was crossed with homozygous transgenic lines equipped with the genetically encoded biosensor ATeam1.03-nD/nA. The zygosity of *aac1* loci was tested using the abovementioned primers while the ATeam1.03-nD/nA loci was selected by fluorescence. The resulting F3 population which were homozygous at both loci were used for experiments.

The antisense lines were created by expressing the complete AAC1 coding sequence in the opposite orientation in the Gateway vector with 35S as promoter and *t* terminator (Figure 2A). The transgenic lines selected were derived from heterozygous progeny (T2) by analyzing the kanamycin resistance segregation pattern. Approximately 50 seeds from the T3 generation of each antisense line were sterilized and sown in Petri dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 50 mg L^{-1} kanamycin and screened for expression. After 24 hours in the dark at 4°C, the plates were transferred to a short-day regime and selection was carried out according to the criterion of survival up to 7 days after germination. The plants selected at T4 showed 100% resistance to kanamycin (T4 generation). The selected lines were named *p35s::AAC1:L05*; *p35s::AAC1:L07*, and *p35s::AAC1:L04* and *p35s::AAC1:L02*, which were used side by side with the mutant line and the wild type (WT) in the experiments carried out.

Growth conditions

The seeds were surface sterilized and germinated on half-concentration MS medium, supplemented with 1% (w/v) sucrose and the selective agent corresponding to the genotype used. The seeds were stratified for four days at 4 °C in the dark and then kept in a growth chamber at 22 ± 2 °C, relative humidity of 60%, irradiance of 150 μmol photons m^{-2} s^{-1}, and photoperiod of 8 hours of light and 16 hours of darkness for ten days. After 10 days, the seedlings were transplanted into 0.1 L plastic pots in commercial Carolina Soil Standard EC 0.7 - 8 Kg substrate. After transplanting, the seedlings were kept in a growth chamber under the same conditions as above for five weeks.

Biometric evaluations

Plants grown under the conditions described above were collected at six weeks of age to assess the following growth parameters: rosette fresh mass (RFM), rosette dry mass (RDM), rosette leaf area (RLA), rosette specific leaf area 20 (RSLA), number of leaves (NL) and specific leaf area (SLA). RLA was determined using the digital image method, where the leaves were scanned using a scanner (HP Scanjet G2410 1200X1200), and the images obtained were processed using ImageJ software (Baker et al., 1996). SLA was estimated using the following formula: SLA = RLA /RDM.

Gas exchange parameters

Gas exchange was assessed 1 h after the start of the light period using an open-system infrared gas analyzer (IRGA - Infrared Gas Analyzer) model LI 6400XT (LI-COR, Lincoln, NE, USA) with an attached fluorometer (6400-40 LI-COR Inc.) on 5-week-old plants. The reference CO_2 concentration was set at 400 mmol CO_2 mol^{-1} of air. All measurements were carried out using the 2 cm^2 leaf chamber, keeping the block temperature at 25°C and 150 μmol photons m^{-2} s^{-1}. Respiration rates were determined during the dark period, after 30 minutes for the plant to acclimatize to this condition.

Evaluation of respiration rates in roots and leaves

Respiration rates were assessed in six-week-old plants using a Clark-type O_2 electrode, as described by Jiang et al. (2019). Before measuring respiration rates, the fresh weight of roots and leaves was obtained and then incubated in a buffer containing 50 mM HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid), 10 mM MES (2-N-morpholinoethanesulfonic acid) (pH 6.6) and 0.2 mM $CaCl_2$. Measurements were made in the dark to avoid interference from light for 20 minutes. The O_2 consumption rate was calculated assuming that the O_2 concentration in the air-saturated buffer was 260 μM at 23ºC.

ATP export assay in isolated mitochondria

Arabidopsis seedlings were grown in hydroponic pots for 14 days as described by Sweetlove et al. (2007) under long-day conditions (16 hr 50–75 µmol photons m^{-2} s^{-1} at 22°C; 8 hr dark at 18°C). Approximately 20 g of whole seedlings from WT and *aac1:aac1* lines were used for mitochondrial isolation as described by Fuchs et al. (2022). The genetically encoded $MgATP^{2+}$ biosensor was purified as described by De Col et al. (2017). Purified ATeam and isolated mitochondria were quantified using a Pierca BCA protein quantification kit as recommended by the manufacturer. The dynamics of ATP export in isolated mitochondria was carried out as previously described (De Col et al., 2017) A total of 20 µg of isolated mitochondria was added to basic incubation medium (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH, pH 7.5, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% (w/v) BSA) supplemented with either 0.5mM or 4mM ADP in a total volume of 200 µL in 96-well plates. Before starting the assay 1 µM purified ATeam1.03-nD/nA was added to each well containing the isolated mitochondria. Fluorescent background of the basic incubation medium was recorded and subtracted from all data before analysis. ATeam1.03-nD/nA was excited with monochromatic light at a wavelength of 435 ± 10 nm in a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). Emission was recorded at 483 ± 9 nm (mseCFP) and 539 ± 6.5 nm (cp173-mVenus). The internal temperature was kept at 25°C, and the plate was orbitally shaken at 400 rpm for 10 s after each cycle. Fluorescence insentity was measured in 2 minutes intervals.

Determination of adenylate levels by bioluminescence assay

Adenylate levels in leaf and root tissues were determined according to Neri-Silva et al., (2023), using the ATP/ADP/AMP Assay Kit (Biomedical Research Service Center, University at Buffalo, Buffalo, NY). Measurements were taken on a luminometer (VictorTMX5 2030 Multilabel Reader) in parallel with the ATP curve.

Root growth

The seeds were sown on plates on half strength MS medium supplemented with 1% sucrose. The plates containing the seeds were stratified in the dark at 4ºC for three days. After stratification, the germination rate was assessed for three days, at the beginning and end of the light period. Subsequently, root growth was carried out as previously described (Palmieri et al. (2008) with some modifications. Five-day-old seedlings were transferred to Petri dishes (90x15mm) on half strength MS medium supplemented with 1% sucrose and placed vertically in a growth room at 22 ± 2 ºC, relative humidity of 60%, irradiance of 150 µmol photons m^{-2} s^{-1} and photoperiod of 8 hours of light and 16 hours of dark. Root length was assessed daily over a total period of ten days. Root growth was then measured using ImageJ software, quantifying the distance between successive points marked along the axial growth.

Transmission electron microscopy

Eighteen-day-old seedlings of WT, *aac1⁻¹:aac1⁻¹*, *p35s::AAC1:L05,* and *p35s::AAC1:L07* were submerged and vacuum infiltrated for 2 h in fixative solution (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). The samples were washed in 0.1 M cacodylate buffer and post-fixed in a solution containing 1% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide ($KFeCN_6$) for 1 hour. The samples were then washed three times in water and incubated in 1% uranyl acetate in maleate buffer for 1 hour. The samples were then washed 3 times in maleate buffer and subsequently dehydrated in a graded series of ethanol (50%, 70%, and 90%, 10 min each; 100%, 2 x 10 min). The samples were placed in propylene oxide for 1 h and infiltrated in a 1:1 mixture of propylene oxide and TAAB 812 resin mixture (Marivac Canada, St. Laurent, Canada). The samples were soaked in a TAAB 812 resin mixture and polymerized at 60°C for 48 hours. Ultra-thin sections of approximately 90 nm were cut using a Reichert Ultracut-S microtome, placed on copper grids, stained with lead citrate, and examined using a Tecnai G2 Spirit BioTWIN transmission electron microscope. The images were recorded with an AMT 2k CCD camera. Mitochondrial number and area were quantified in 25 field images representing each genotype. Image analysis was performed using ImageJ. Normal and abnormal mitochondria were classified according to the absence or occurrence of mitochondrial vacuolar degeneration, as described by Chaanine (2019).

Real-time monitoring of cytosolic $MgATP^{+2}$ dynamics in isolated root cells

Wild-type and T-DNA insertion lines for the AAC1 locus (*$aac1^{-1}$:$aac1^{-1}$)* equipped with the MgATP2 genetically encoded biosensor were grown as abovementioned in vertical petri dishes. Each well of transparent 384-well plates were filled with 100 µL plate reader assay medium (10 mM MES, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM KCl, pH 5.8 with KOH). Single roots were excised from 7-day old seedlings and transferred each well of the microplate using a pair of soft tweezer. The 384-well plates containing the excised roots were dark-adapted for one hour inside the plate reader chamber pre-equilibrated at 25 ºC. Fluorescence intensity was measured in plate mode with top optics using a standard protocol with 54 excitation flashes per well and cycle, an orbital averaging with 2 mm diameter and 0.1 s settling time. Excitation and emission wavelengths for ATeam1.03-nD/nA was: Ex = 435 ± 10 nm / Em = 485 ± 10 (gain = 1300) and 540 ± 7.5 nm (gain = 1100). Fluorescence intensity was measured for about two hours before performing any treatment as indicated in the figures. Chemical treatments were carried out by pipetting 1 µL into each well to reach a maximum concentration of the solvent of 1% into each well. Chemicals were dissolved in absolute ethanol. Each time point is an average of 16 biological replicates. The experiment was repeated twice.

Sample processing and metabolite extraction

Roots of each strain were collected in the middle of the light period and immediately frozen in liquid nitrogen and stored at -80 ºC until analysis. Subsequently, the samples were homogenized and aliquots of approximately 20 mg of fresh matter were subjected to methanolic extraction as described by Salem et al. (2016). After extraction, the insoluble fraction was quantified for starch (Fernie et al., 2001) and protein (Bradford MM, 1976) and the soluble fraction for chlorophylls, nitrate (Sulpice et al., 2009), glucose, fructose and sucrose (Fernie et al., 2001) and the organic acids malate and fumarate (Nunes-Nesi et al., 2007).

Phenotypic characterization during the reproductive stage and germination

To characterize the role of AACs during the reproductive phase, seedlings were transferred to commercial substrate Carolina Soil Standard EC 0.7 - 8 Kg and kept in a growth room at 22 ± 2°C, 60% relative humidity, with a photoperiod of 12h light and 12h dark and irradiance of 150 µmol photons m^{-2} s^{-1} for twelve weeks to produce seeds. The siliques of wild and mutant plants were collected and clarified in a solution of 0.2N NaOH and 1% sodium dodecyl sulfate (SDS) (Yoo et al., 2012). Ten clarified siliques were collected and the length, width, and number of seeds per silique were analyzed under a dissecting microscope (Stemi 2000-C, Zeizz) with an adapter (SOLIGOR tube for Canon A650 IS B52 Wide) for a CANON 12.1-megapixel camera (Power Shot A650 IS). In addition, the number of branches and siliques per plant was quantified. Subsequently, ten seeds from different siliques on each plant were evaluated, using six replicates per genotype, for length and diameter using the aforementioned devices. In addition, the weight of a thousand seeds and the weight of all the seeds produced by the same plant will be quantified. The biometric data obtained from photographic records was evaluated using the ImageJ program.

Expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from 5-week-old leaves using TRIzol reagent (Ambion, Life Technology, Carlsbad, CA, USA) according to the manufacturer's recommendations. Total RNA was treated with DNase I (InvitrogenTM). The integrity of the RNA was checked on a 1% agarose gel (w/v) and the concentration were measured using a Nanodrop spectrophotometer. Finally, 500 ng of total RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Thermo Fischer, Waltham, MA, USA) according to the manufacturer's recommendations. Real-time PCR was performed in a 96-well MicroAmp™ optical reaction plate (Applied Biosystems, Waltham, MA, USA) using Power SYBR® Green PCR Master Mix (Life Technologies/Applied Biosystem) according to the manufacturer's recommendations. The primers used here were designed using the primer design tool of the open-source program QuantPrime-qPCR and are described in Table S1. Relative transcript levels were calculated by relative quantification and normalized using two constitutively expressed genes ACTIN2 (AT2G37620). Three biological replicates were processed for each experimental condition.

Statistical analysis

The experimental design was entirely randomized with six replicates. The results obtained were subjected to analysis of variance and compared by Student's t-test using the algorithm contained in Microsoft Excel software (Microsoft Corporation, Seattle, WA, USA), with values with $P < 0.05$ being considered significant.

Results

Lines with reduced *AAC1* expression show a reduction in growth parameters without changes in photosynthetic efficiency

Previously, we selected homozygous plants from the *aac1*$^{-1}$*:aac1*$^{-1}$ line with T-DNA insertion and four homozygous antisense lines under the control of the 35S promoter (*p35s::AAC1:L05; p35s::AAC1:L07, and p35s::AAC1:L04* and *p35s::AAC1:L02*) (Figure 1A). To do this, we assessed the level of *AAC1* expression by qRT-PCR. The relative expression of *AAC1* in the *aac1*$^{-1}$*:aac1*$^{-1}$ line was 8.75%. The antisense lines showed 6.6% (*p35s::AAC1:L05*), and 10.6% (*p35s::AAC1:L07*). The other antisense lines showed a reduced and non-significant expression of *AAC1*, with 20.7% (*p35s::AAC1:L04*) and 21,4% (*p35s::AAC1:L02*) of the expression observed in WT, normalized with the ACTIN reference gene (Figure 1B). These independent mutant lines were used for further analysis to investigate the function of AAC1 in plants.

Given the notorious importance of adenylate transport for plant growth and development (Fonseca-Pereira et al., 2018), we further investigated whether reduced *AAC1* expression affects shoot growth. Lower *AAC1* expression affected growth parameters in *A. thaliana*. We observed a significant reduction in fresh mass in leaves (Figure 1C) and roots (Figure 1D), and dry mass of leaves (Figure 1E) and roots (Figure 1F) compared to WT. In addition, the transgenic *aac1* lines had a lower number of leaves (Figure 1G) and a smaller leaf area (Figure 2H). However, no visual/aberrant changes in phenotype and leaves were observed in the mutant and transgenics during vegetative growth (Figure 1I).

We evaluated the parameters related to gas exchange in leaves from six-week-old plants. The deficiency in the expression of *AAC1* didn't affects the photosynthetic parameters. There isn't observed significant alteration in internal CO_2 concentration (C*i*) (Sup. Figure 1A), the ratio of internal concentration about the air's CO_2 concentration (C*i*/C*a*) (Sup. Figure 1B), and stomatal conductance (*gs*) (Sup. Figure 1C), and photosynthesis (*A*) (Sup. Figure 1D). However, was observed a reduction of dark respiration, with a significant reduction of 21.4% and 11.4% in the *p35s::AAC1:L07* and *p35s::AAC1:L02* lines, respectively (Sup. Figure 2E).

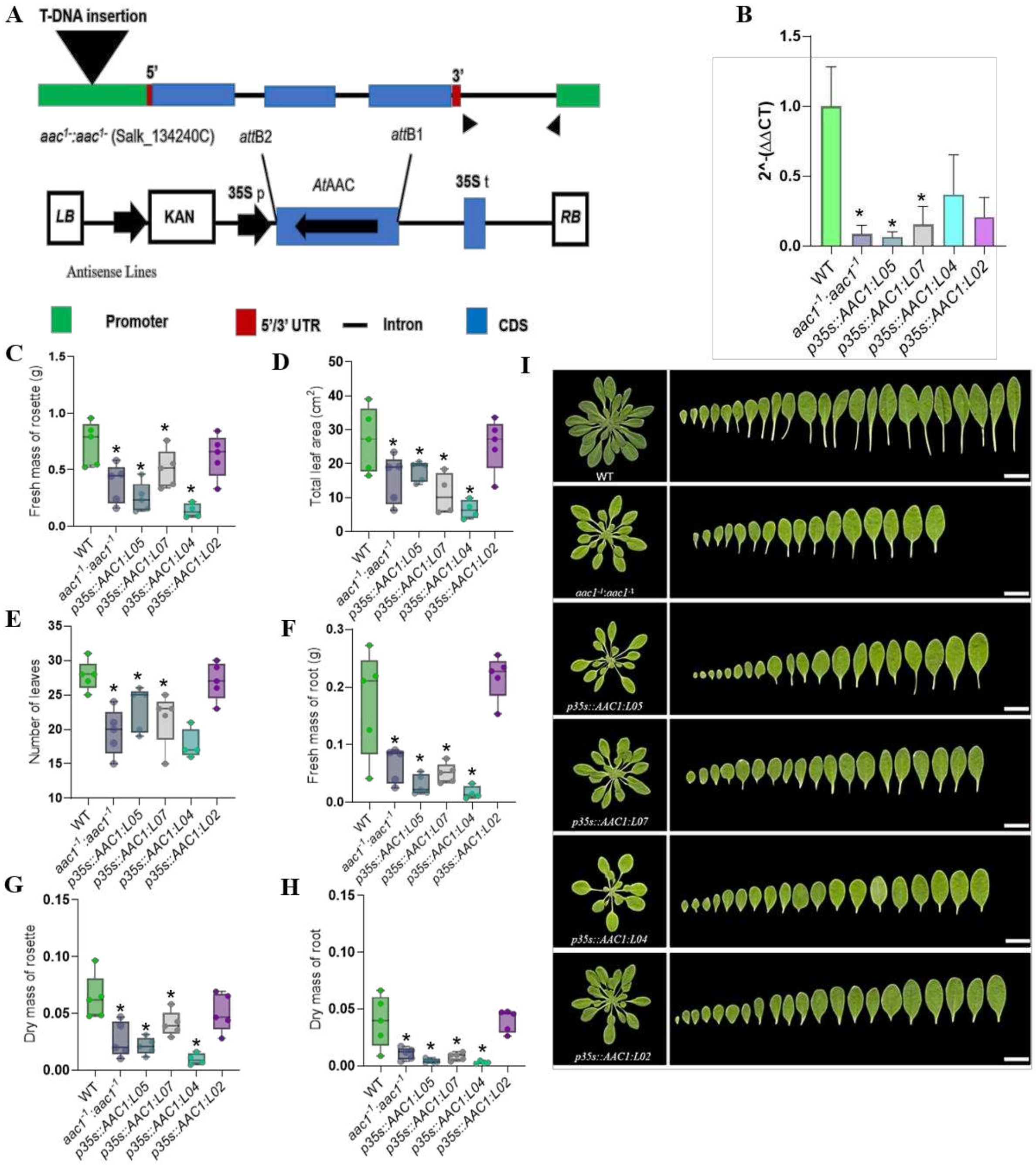

Figure 1. Isolation and phenotypic characterization of a mutant and antisense lines for the mitochondrial ATP transporter (AAC1) in *A. thaliana*. Schematic representation of the AtAAC1(AT3G08580) gene T-DNA insertion. The boxes represent the exons and the arrows next to the T-DNA are the position of the primers used to select the mutant plants (A). The antisense lines have the resistance gene (CAN), the 35S promoter, and the AAC1 gene in the antisense orientation and a35S terminator. Expression of *AAC1* in wild-type (WT) and the respective transgenic lines (B). Fresh mass of rosette (C). Fresh mass of root (D). Number of leaves (E). Total area leaf (F). Dry mass of rosette (G). Dry mass of root (H). The visual phenotype of rosette and line leaves with reduced expression of *AAC1* and WT (I). The plants evaluated were five weeks old at the time of collection and were grown under optimal conditions. Three biological replicates were processed for RT qPCR.Values represent the mean ± standard error of six individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the WT.

Low expression of *AAC1* does not impact ATP export capacity *ex situ* but decreases respiratory capacity and cellular ATP content in leaves and roots

The observed decrease in dark respiration suggest AAC1 was limiting mitochondrial oxygen consumption most likely by limiting ATP export from the mitochondria to the cytosol. We sought to investigate this hypothesis by monitoring ATP export in isolated mitochondria in the presence of purified $MgATP^{-2}$ biosensor in the incubation media as previously described (De Col et al., 2017). Isolated mitochondria in the presence of 4 mM ADP resulted in an increase in $MgATP^{-2}$ content in the media, even in the absence of respiratory substrate. This was reported to be a result of the outer membrane located Adenylate Kinase (AK) activity (de Col et al., 2017). Also, AK activity did not rely on AAC-mediated ADP or ATP transport, since it is unaltered in the presence of the AAC inhibitor Carboxyactractyloside (cAT, Figure 2A). However, when the respiratory substrate succinate was added to the media an additional increase in the $MgATP^{-2}$ accumulation in the media was observed which is partially inhibited by cAT. No differences were observed between WT and *aac1*$^{-1}$*:aac1*$^{-1}$ lines in the presence of 4mM ADP (Figure 2A). However, when ADP concentration was lower in the incubation media no $MgATP^{-2}$ accumulation was observed in the absence of succinate (Figure 2B). When succinate was added $MgATP^{-2}$ rapidly accumulates in the media meaning that ATP accumulation in the media is strictly dependent on respiratory activity. Also, addition of cAT completely abolishes $MgATP^{-2}$ accumulation confirming this accumulation is dependent of AAC export of ATP from the mitochondrial matrix to the incubation media. However, no differences could be observed between WT and *aac1*$^{-1}$*:aac1*$^{-1}$ mutants, suggesting that at least *ex situ* lower content of AAC1 does not limit mitochondrial ATP export.

Reduced expression of *AAC1* in *A. thaliana* plants led to impacts on cellular respiration compared to WT. The results showed significant decreases in the rate of oxygen consumption in leaves (Figure 2C) and roots (Figure 2D). A significant reduction of 52 % was observed for line *aac1*$^{-1}$*:aac1*$^{-1}$ and 38 % for line *p35s::AAC1:L02*. In the roots, there was a significant reduction in all the lines with reduced expression for *AAC1* compared to WT. This reduction was 56 % for line *aac1*$^{-1}$*:aac1*$^{-1}$ and 70 % for *p35s::AAC1:L05*, 42 % for *p35s::AAC1:L07*, 67 % for *p35s::AAC1:L04* and 70 % for *p35s::AAC1:L02*. Changes in adenylate levels were also observed in the lines with reduced expression. Interestingly, in the leaves the *aac1*$^{-}$

^{1}:*aac1^{-1}* line had a significantly higher ATP level compared to the WT, however, the antisense lines (*p35s::AAC1:L05* and *p35s::AAC1:L07*) showed the opposite behavior, with significant decreases in the ATP level (Figure 2E). In the roots, significant increases in ATP content were observed in all the lines with reduced expression for *AAC1* compared to WT (Figure 2F).

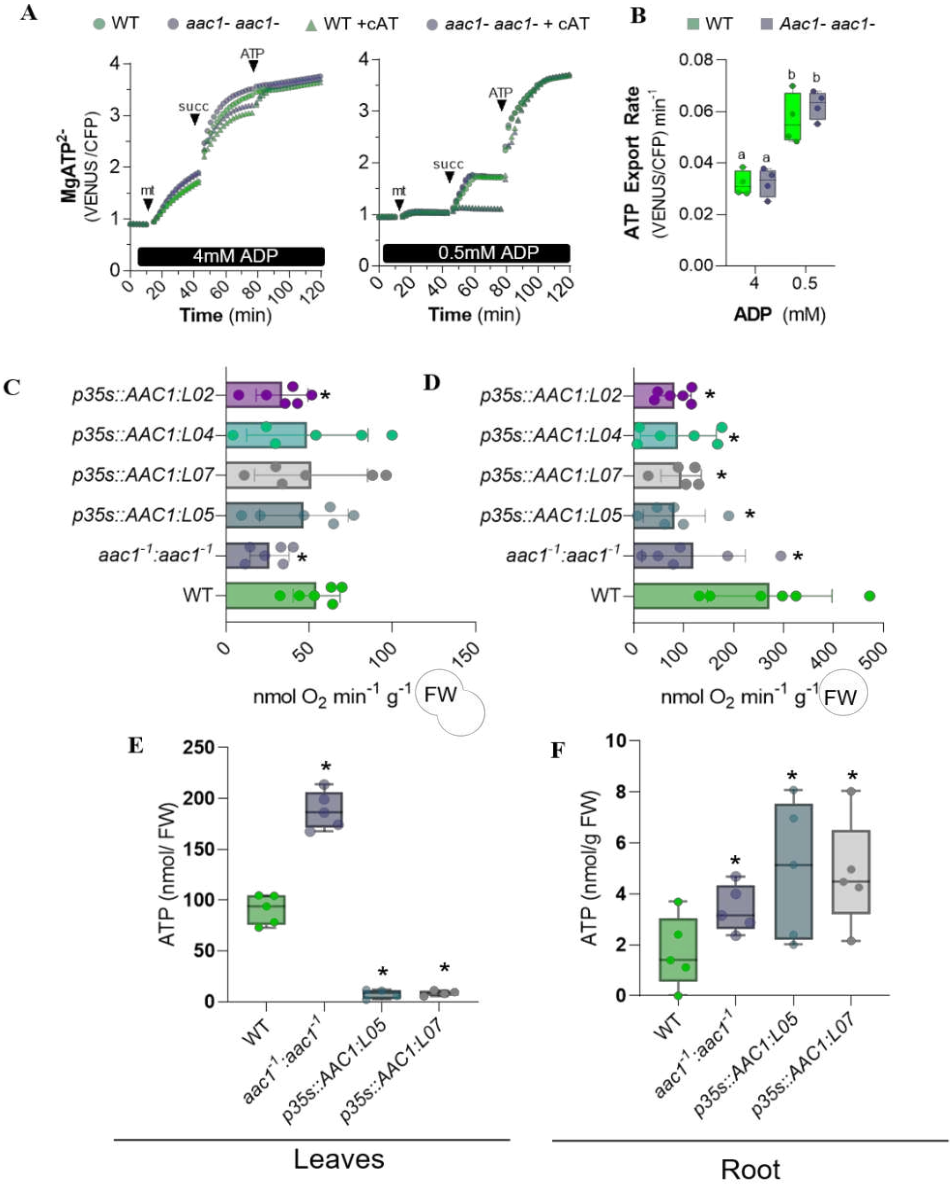

Figure 2. ATP export, oxygen consumption rate and ATP level in leaves and roots of plants with low expression of *AAC1* and WT in *A. thaliana*. Mitochondria isolated from WT and *aac^{1-}:aac^{1-}* seedlings were incubated in the presence of 1µM purified MgATP^{-2} sensor in the presence of 4mM or 0.5mM ADP with or without the presence of cAT. Purified mitochondria (10µg), Succinate (5mM) and ATP (5mM) were added at the indicated time points (black triangles) (A). The ATP export rate was calculated for WT and *aac^{1-}:aac^{-1}* mitochondria in the presence of 4mM ADP (without succinate) or 0.5mM ADP (with succinate) (B). Oxygen consumption rate in leaves (C) and roots (D). Level of intracellular ATP in leaves (E) and roots (F). The plants evaluated were five weeks old at the time of collection and were grown under optimal conditions. Values represent the mean ± standard error of six (for oxygen uptake analysis) and four individual plants (ATP level assay). Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the wild type (WT**).**

AAC1 deficiency promotes stunted root growth and impacts mitochondrial morphology in root cortex cells

Considering that root cells depend exclusively on mitochondrial oxidative phosphorylation to meet their energy demands, we evaluated the role of AAC1 in roots. Analysis of root growth on vertical agar plates with sucrose (Figure 3A) and without sucrose supplementation (Figure 3B) indicated that the root lengths of plants with reduced expression for *AAC1* were shorter than WT at all times evaluated (Figure 3C).

As cellular respiration and root growth were more strongly affected in root tissues, we decided to investigate whether the reduction of *AAC1* could be promoting structural/morphological damage to the mitochondria. Transmission electron microscopy (TEM) revealed that the lines with reduced *AAC1* expression showed advanced mitochondrial vacuolar degeneration (Figure 4A). Mitochondrial cristae were less apparent and even absent in several mitochondria of the low *AAC1* expression lines compared to WT (Figure 4B). There were no significant changes in the number of mitochondria per area (Figure 4C), in mitochondrial length (Figure 4D), and in mitochondrial area (Figure 4E). The percentage of abnormal mitochondria was significantly higher in the transgenic lines (around ~70%), while only 20% of defective mitochondria were observed in the WT (Figure 4F).

The presence of defective mitochondria in the *AAC1* deficient mutants suggest these lines might be more sensitive to inhibition of mitochondrial function. We monitored cytosolic MgATP^{-2} dynamics in isolated roots after treatment with known chemical inhibitors of mitochondrial respiratory activity such as Oligomycin (Omy, ATP synthase), Antimycin A (AA, Complex III) and n-propyl gallate (nPG, Alternative Oxidase) (Figure 5). We found no steady-state distinction in MgATP^{-2} content in the solvent treated plants and, interestingly, MgATP^{-2} content in the cytosol is highly stable within 10 hours of treatment (Figure 5A). The addition of Omy resulted in a constant decrease in cytosolic MgATP^{-2} over the course of 10 hours but no differences were observed between WT and the mutant (Figure 5B). However, it was evident after AA treatment that *aac1*$^{-}$ *aac1*$^{-}$ mutants were more sensitive, displaying lower MgATP^{-2} cytosolic content immediately after addition of AA when compared to the WT, which was maintained throughout the experiment. While AA inhibits Complex III, therefore limiting Cytochome Oxidase (COX) dependent oxygen reduction to H_2O, AA + nPG simultaneously inhibits both terminal oxidases in plant mitochondria, COX and

Alternative Oxidase (AOx). The addition of AA + nPG resulted in a faster and sharper decrease in $MgATP^{-2}$ content in the cytosol when compared to the other treatments but there were no differences between WT and *aac1* mutants. The $MgATP^{-2}$ content inferred from the VENUS/CFP ratio at the indicated time-points (black arrows, Figure 6A) reveals a clear distinction in $MgATP^{-2}$ content between the WT and *aac1*$^{-1}$*:aac1*$^{-1}$ when roots were treated with AA, but not other inhibitors.

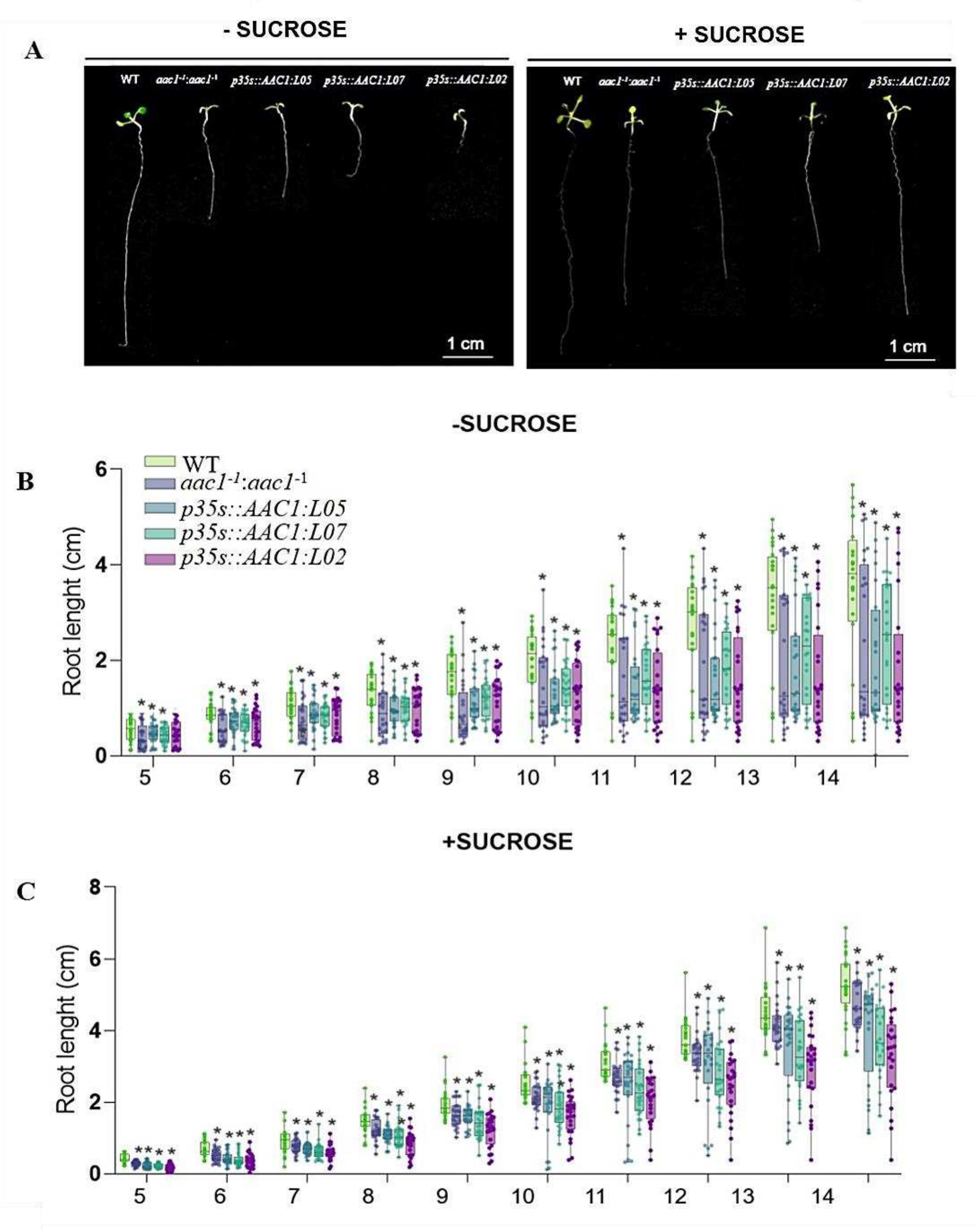

Figure 3. Root growth in lines with reduced expression for AAC1 and WT in *A. thaliana.* Root length was assessed over 10 days with seeds grown in medium without sucrose (A) and with 1% sucrose (B). The values represent the mean ± standard error of 6 plates, each with four seeds per genotype.

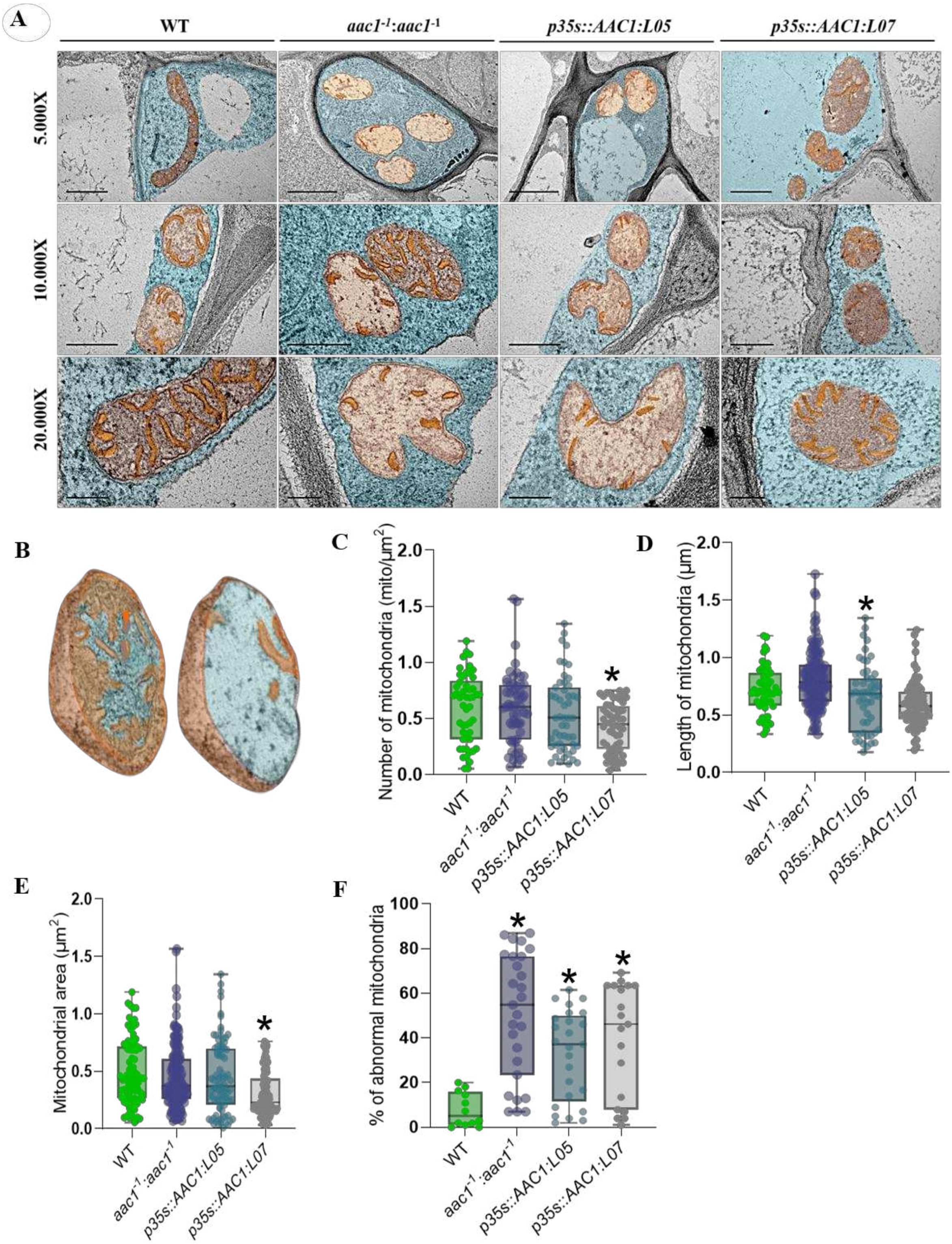

Figure 4. Lower *AAC1* leads to mitochondrial vacuolar degeneration. Representative MET micrograph of the root cortex of WT and AAC1 mutant and transgenic strains of 18-day-old seedlings (A). Illustrative three-dimensional model comparing a normal mitochondrion and a mitochondrion with damaged/absent ridges, commonly observed in the MET images of this assay (B). Quantification of mitochondrial number per μm^2 (C). Length of mitochondria (D) and mitochondrial area (E). Percentage of abnormal and abnormal mitochondria (F). MET image analysis was performed on 20 field images (n = 25) for each genotype. *P < 0.05 compared to WT. Error bars, mean ± SEM Scale bars, 1 μm.

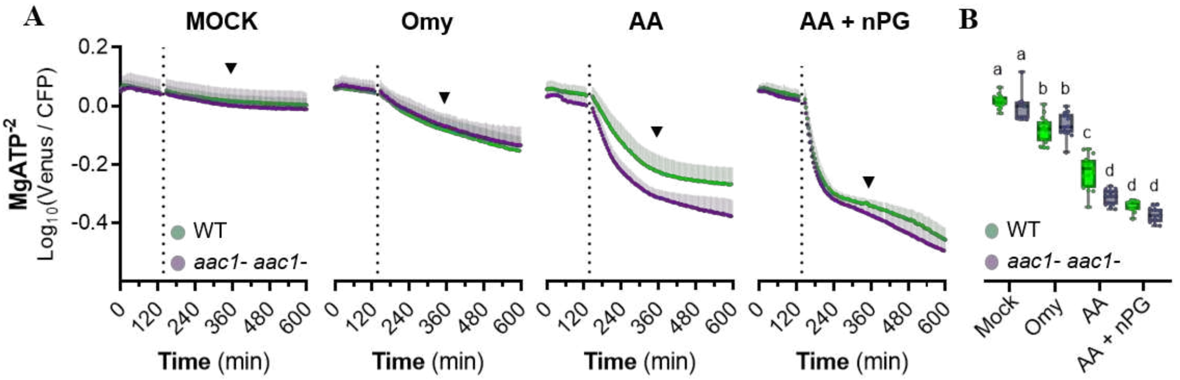

Figure 5. MgATP^{-2} dynamics in isolated roots treated with mitochondrial oxidative phosphorylation inhibitors. Roots from WT and *aac^{1-}:aac^{1-}* lines were excised from 7-day old seedlings and placed in 384-well transparent microplates. Fluorescence intensity from individual channels were recorded for 600 minutes and MgATP^{-2} content (VENUS / CFP ratio) was calculated (A). Mitochondrial electron transport chain inhibitors were added at the indicated time-points (dashed lines). Ethanol 0.1% final concentration was used as a mock control. Oligomycin (Omy), Antimycin (AA) and n-propyl gallate (nPG) were used at 100µM, 50µM and 500µM concentrations respectively. Error bars = ±SD (B). The ratios at the indicated time points (black triangles) were plotted separately. Statistical significance was tested by one-way ANOVA and different letters represent values statistically significant by Tukey`s multiple comparisons test ($P < 0.05$). Boxes show means and whiskers show min to max with all points. Each time point for A and B is a mean of 16 replicates.

Low expression of *AAC1* alters levels of primary metabolites in root tissues under optimal growth conditions

For a more detailed characterization of the function of the AAC1 transporter, biochemical analyses were carried out on the roots of -week-old plants (Figure 6A, B, and C). About soluble carbohydrate levels (sucrose, glucose, and fructose), a significant increase in glucose and fructose was observed in the lines with low *AAC1* expression in roots collected in the middle of the day. An upward trend was also observed in sucrose levels, but it was only significant in the *p35s::AAC1:L07* line. Concerning organic acid levels, there was a general trend towards a reduction in malate (Sup. Figure 2A) and fumarate (Sup. Figure 2B) levels in the roots of the low *AAC1* expression lines, but significant values were only found in the *aac1^{-1}:aac1^{-1}* line. No changes in amino acid levels were observed in the *aac1* mutant lines compared to the WT (Sup. Figure 2C).

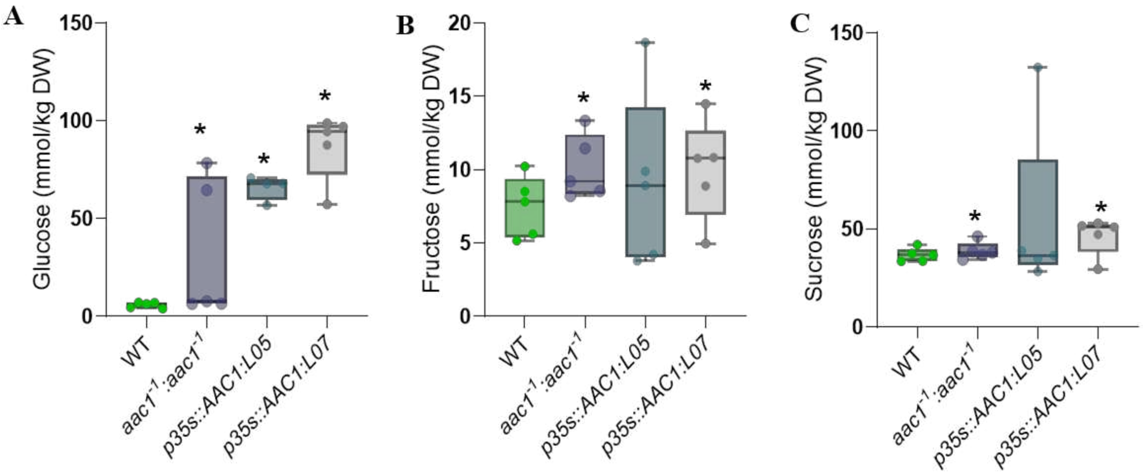

Figure 6. Impact of *AAC1* reduction on soluble carbohydrate levels in roots. Glucose (A), Fructose (B) and Sucrose (C). The plants evaluated were five weeks old at the time of collection and were grown under optimal conditions. The values represent the mean ± standard error of six individual plants. The asterisks indicate a significant difference (P < 0.05) according to Student's t-test, compared to the WT.

AAC1 deficiency and its role during the reproductive phase and seed germination

Considering the relation between mitochondrial respiration and seed development, we decided to investigate further the impact of reduced expression of the *AAC1* transporter on the reproductive stage (Sup. Figure 3A). Interestingly, *aac1* mutant and transgenic plants exhibited higher numbers of branches (Sup. Figure 3B), and open flowers than WT (Sup. Figure 3C), while no changes were observed in the number of flower buds (Sup. Figure 3D). The siliques were slightly larger in the lines with reduced *AAC1* expression (Figure 7A-B), and the length of the siliques was significantly longer in all *AAC1* mutant genotypes than in WT (Figure 7B). In addition, an increase in the number of seeds per silique per plant was observed (Figure 7C). However, the number of siliques per plant was not different between the WT and the mutant lines (Figure 7D). The weight of a thousand seeds was significantly higher in the *aac1* mutants compared to the WT (Figure 7E). The phenotypes of WT and lines with lower expression of AAC1 in the siliques and seeds can be seen in Figure 7F.

The impact of reduced *AAC1* expression on seed germination has been verified. To this end, seeds were germinated in the presence and absence of 1% (w/v) sucrose. Germination was significantly lower in the *aac1* lines compared to WT. In medium without sucrose supplementation, the germination percentage in the lines with reduced *AAC1* expression after 3 days of the evaluation was around ~70% while in WT germination was close to 100% (Figure 7G). Sucrose supplementation increased the germination percentage in the transgenic lines to around ~78%, although these values are lower than in the WT. The germination rate was not significantly different between the genotypes and in both treatments (Sup. Figure 4 A-B).

Reduced expression of *AAC1* do not affect the expression of other adenylate carriers under optimal growth conditions

We investigated whether the expression of different isoforms of the ADP/ATP carrier and other mitochondrial adenylate transporters is differentially regulated when *AAC1* expression is reduced. Leaves from WT, mutant, and transgenic *aac1* lines were collected from five-week-old plants. Interestingly, there were no significant differences in the relative expression of *AAC2* and *AAC3* isoforms, nor for A*DNT1*, in mutant *aac1* plants compared to WT. Interestingly, there was a minor increase in the expression of *APC1*, suggesting that this mitochondrial adenylate transporter may play a role in conditions where AAC1 activity is impaired (Figure 8).

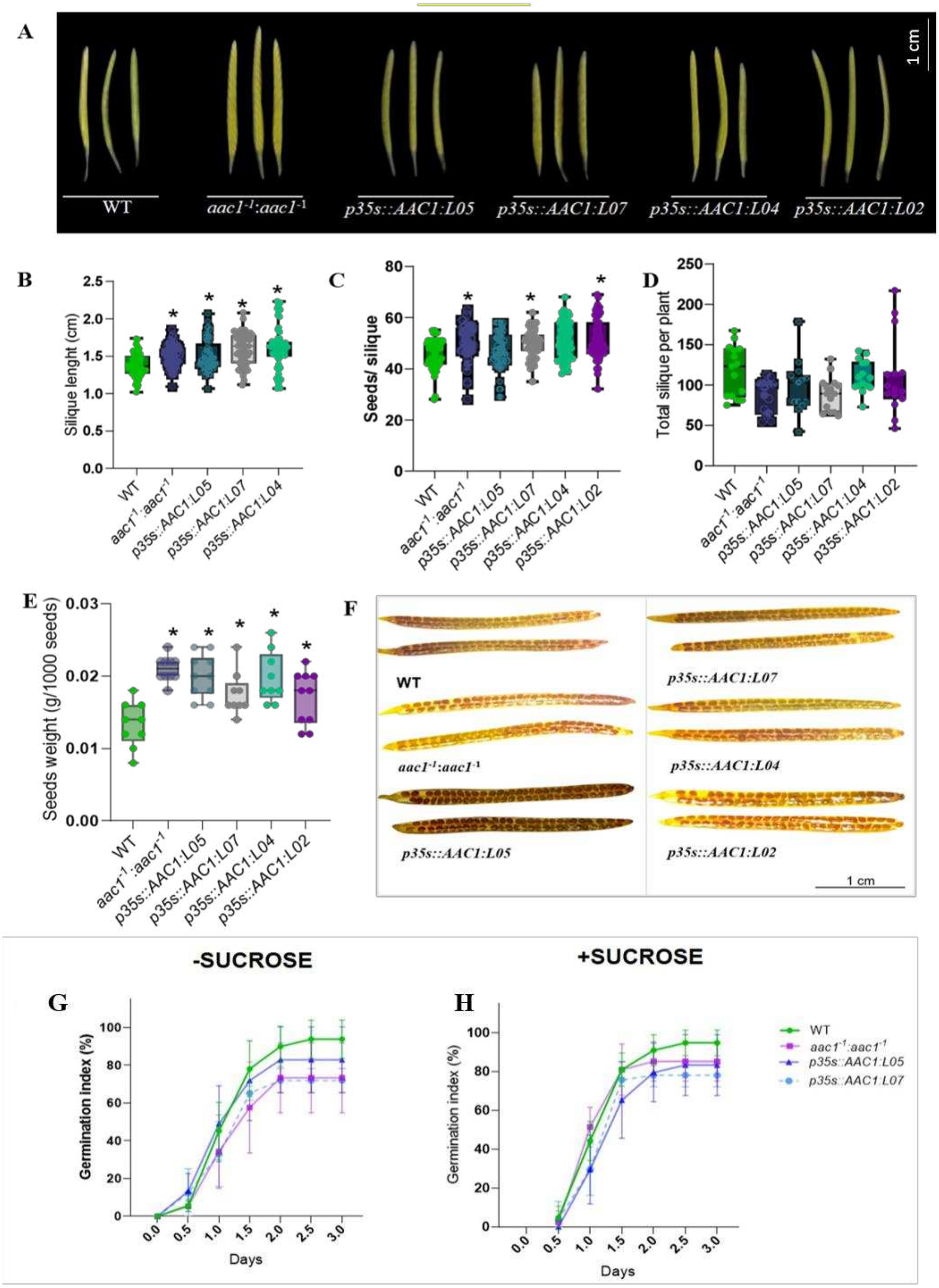

Figure 7. Impact of the decrease in *AAC1* on the reproductive stage. Visualization of the silique (A). Silique length (B). Number of seeds per silique (C). Total silique per plant (D). Weight of a thousand seeds (E). Silique was clarified in 0.2N NaOH solution and 1% SDS (F). Germination percentage in medium without sucrose (G) and with 1% sucrose (H). The values represent the mean ± standard error of twelve individual plants (for assessing the reproductive stage) and of 6 plates, each with 50 seeds per genotype The asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the *wild type* (WT).

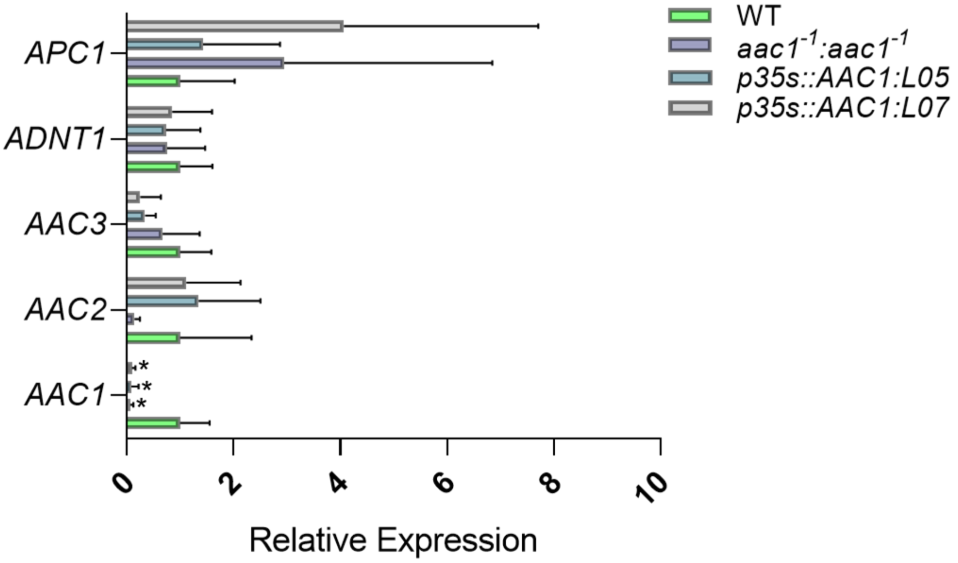

Figure 8. Relative expression of mitochondrial adenylate transporters in lines low for *AAC1* and WT. Abbreviations: AAC1, ADP/ ATP transporter 1; AAC2, ADP/ ATP transporter 2; AAC3, ADP/ ATP transporter 3; ADNT1, Adenine Nucleotide Translocator 1; APC1, ATP/Phosphate transporter. Values represent the mean ± standard error of three individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test compared to *wild type* (WT).

Discussion

AAC1 is an important mitochondrial protein for oxidative phosphorylation in *A. thaliana*

Oxidative phosphorylation is a crucial process for the production of ATP, which in turn drives cellular functions. The flow of ATP generated during mitochondrial oxidative phosphorylation involves a complex interaction of various mitochondrial adenylate carrier proteins, which may have redundant or complementary functions (Nunes-Nesi et al., 2020). Our findings demonstrate that the reduction in AAC1 expression, and consequently in protein levels, resulted in decreased respiration rates, particularly pronounced in roots, as evidenced by measurements of oxygen consumption rates (Figure 2C and D).

This decrease in cellular respiration is consistent with observations in knockout mutants of the ADNT1 adenylate transporter in *A. thaliana* (Palmieri et al., 2008) and mutants of genes crucial in the regulation of respiratory fluxes, such as mutant plants of *AtSFC1* (Brito et al., 2020), *NDUFS4* (Meyer et al., 2009) and *LRK1* in rice plants (Qu et al., 2020). The reduction in cellular respiration observed in the *aac1* lines provides initial support for our model that the mitochondrial adenylate transporter AAC1 plays a relevant role in the oxidative phosphorylation process. In addition, the biochemical characterization of this transporter indicated that AAC1 plays a critical role in regulating the availability of ADP in the IMM, directly influencing these cellular respiration maintenance processes (Haferkamp et. al., 2002). Levels adequate of ADP in the intermembrane space play a fundamental role in maintaining respiratory homeostasis. An adequate supply of this substrate is essential to prevent unwanted side effects resulting from the accumulation of NADH and other reduced by-products of metabolism (Halliwell, 2006). These adverse effects include the potential formation of superoxide and other ROS, known to cause substantial damage to cell structures and, in extreme cases, lead to cell apoptosis (Halliwell, 2006).

The role of AAC1 in cellular ATP levels, energy metabolism, and mitochondrial morphology in *A. thaliana* roots

Investigating ATP export in isolated mitochondria revealed that AAC1 deficiency didn't compromises ATP accumulation in the medium (Figure 3A). The lack of significant differences between WT and aac1 mutant lines suggests that a reduced ex situ content of AAC1 does not necessarily limit mitochondrial ATP export. However, our results indicated that the impaired export of ATP promoted the accumulation of this metabolite in the root tissues of aac1 mutant roots (Figure 3D). The discrepancy observed between the lack of alteration in ATP export in isolated mitochondria and the increased ATP levels in root tissues of aac1 mutant roots could potentially be attributed to mitochondrial structural damage (Figure 5), which may subsequently impact proper ATP export. While the deficiency in AAC1 did not compromise ATP accumulation in the medium of isolated mitochondria, suggesting compensatory mechanisms or redundancies in adenylate transport, the observed impairment in ATP export could be attributed to structural abnormalities within mutant root mitochondria. Such structural damage might disrupt ATP export machinery, leading to ATP accumulation within root tissues. The integrity of mitochondrial structure likely plays a crucial role in ATP export processes. Disruptions in mitochondrial morphology could impair ATP export efficiency, possibly explaining the observed ATP accumulation within root tissues. Thus, the lack of significant changes in ATP export in isolated mitochondria may not fully reflect the in vivo conditions within mutant roots, where structural abnormalities could be affecting ATP export dynamics.

The buildup of ATP in root tissues of aac1 deficient lines may trigger changes in ATP levels within both the IMM and the cytosol, potentially leading to an accumulation of ATP within the IMM. Subsequently, this accumulation could induce a decrease in ATP levels in the cytosol. Decreases in the level of cytosolic ATP suppress ATPases and activate calcium ion channels (VDAC), releasing $Ca^{2}+$ into the cytosol (Demidchik et. al., 2007; Demidchik et. al., 2015). Calcium overload in the mitochondrial matrix activates TCA cycle enzymes and increases the production of ROS by mCTE complexes I and III (Feissner et. al., 2009). As a consequence, oxidative phosphorylation is interrupted and the mitochondrial permeability transition pore (mPTP) is activated, leading to its opening and increased permeability to ions and water (Brustovetsky et. al., 2002). This process causes mitochondrial swelling and eventually results in mitochondrial vacuolar degeneration, with permeabilization of the inner membrane and rupture of the mitochondrial outer membrane (Garrido et. al., 2006; Chaanine, 2019). We showed that reduced expression of AAC1 caused damage to the mitochondrial structure of A. thaliana root cells, resulting in advanced degrees of mitochondrial vacuolar degeneration (Figure 5A). As mitochondrial swelling occurs and progresses to more advanced stages of vacuolar degeneration, most of the mitochondrial cristae are lost, impairing mitochondrial oxidative capacity. Consequently, the reduction of AAC1 negatively affected oxidative phosphorylation in the roots (as discussed in more detail in the previous topic) which may be closely related to the mitochondrial dysfunction observed in micrographs of the mitochondria of *aac1* lines plants (Figure 4 and 9).

The reduction in the activity of the mitochondrial transporter AAC1 led to an increase in soluble carbohydrates in the roots of *A. thaliana*. The accumulation of these metabolites may be associated with the reduction in respiration observed in the aac1 lines. Lower ATP export and ADP import can inhibit the mitochondrial electron transport chain and generate an accumulation of reductants in the mitochondrial matrix (O'Leary et al., 2019). In addition, it has already been reported that increased glucose induces increased expression of the calcium-dependent mitochondrial ATP-Mg/Pi transporter (Sal1p) in Saccharomyces cerevisiae (Cavero et. al., 2005). In this study, the authors reported that Sal1p is not essential in wild-type cells, but becomes essential for ATP transport to the mitochondria in yeasts lacking ADP/ATP translocases (Cavero et. al., 2005). In Arabidopsis, Ca^{2+} dependent ATP-Mg/Pi transport is mediated by mitochondrial APC transporters (Monné et. al., 2015). The regulation of APC1 in plants with reduced expression for AAC1 may favor ATP export to the cytosol, thus alleviating ATP overload in the IMM. In addition, this transporter is highly expressed in root tissues (Monné et al., 2015). The regulation of APC1 in plants with reduced expression for AAC1 may favor ATP export to the cytosol, thus alleviating ATP overload in the IMM. In addition, this transporter is highly expressed in root tissues (Monné et al., 2015).

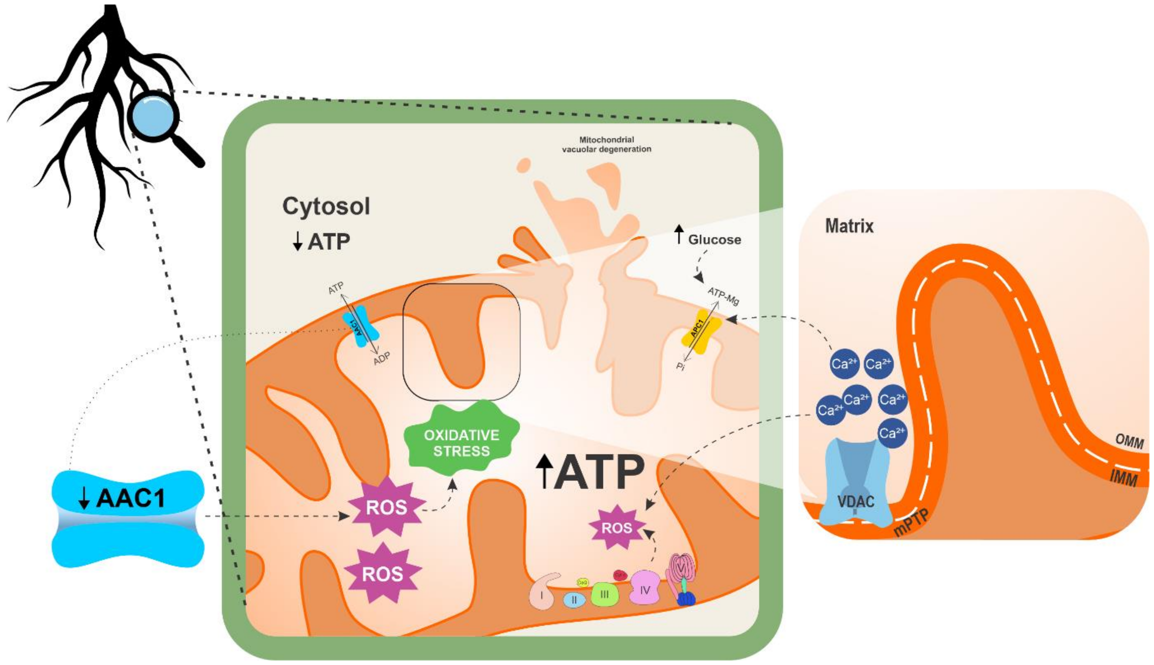

Figure 9. Hypothetical model of mitochondrial energy metabolism regulation in roots with reduced AAC1 expression. Reduced AAC1 activity results in the accumulation of ATP in the inner mitochondrial membrane (IMM), which in turn can lead to a decrease in ATP levels in the cytosol. The decrease in cytosolic ATP levels suppresses ATPases and activates calcium ion channels (VDAC), releasing Ca^{2+} into the cytosol. Calcium overload in the mitochondrial matrix activates Krebs cycle enzymes and increases the production of reactive oxygen species (ROS) in the mCTE. As a consequence, oxidative phosphorylation is disturbed and the mitochondrial permeability transition pore is activated, leading to its opening and an increase in permeability to ions and water. This process causes mitochondrial swelling and eventually results in mitochondrial vacuolar degeneration, with permeabilization of the inner mitochondrial membrane and rupture of the outer mitochondrial membrane (OMM). At the same time, the reduction in oxidative phosphorylation leads to an increase in glucose levels, which, together with changes in Ca^{2+} levels, increases the expression of the *APC1* favoring the export of ATP to the cytosol in this situation.

The reduction in the activity of the mitochondrial transporter *AAC1* led to an increase in soluble carbohydrates in the roots of *A. thaliana*. The accumulation of these metabolites may be associated with the reduction in respiration observed in the *aac1* lines. Lower ATP export and ADP import can inhibit the mitochondrial electron transport chain and generate an accumulation of reductants in the mitochondrial matrix (O'Leary et al., 2019). In addition, it has already been reported that increased glucose induces increased expression of the calcium-dependent mitochondrial ATP-Mg/Pi transporter (Sal1p) in *Saccharomyces cerevisiae* (Cavero et. al., 2005). In this study, the authors reported that *Sal1p* is not essential in wild-type cells, but becomes essential for ATP transport to the mitochondria in yeasts lacking ADP/ATP translocases (Cavero et. al., 2005). In Arabidopsis, Ca^{2+} dependent ATP-Mg/Pi transport is mediated by mitochondrial APC transporters (Monné et. al., 2015). The regulation of *APC1* in plants with reduced expression for *AAC1* may favor ATP export to the cytosol, thus alleviating ATP overload in the IMM. In addition, this transporter is highly expressed in root tissues (Monné et al., 2015). The regulation of APC1 in plants with reduced expression for *AAC1* may favor ATP export to the cytosol, thus alleviating ATP overload in the IMM. In addition, this transporter is highly expressed in root tissues (Monné et al., 2015). Therefore, it may contribute to continued root growth in plants with lower *AAC1* expression, even if this growth is significantly reduced by the impact of AAC1 on oxidative phosphorylation and damage to mitochondria (Figure 9).

AAC1 contributes to modulating growth and reproductive stage in *A. thaliana*

Mitochondrial energy metabolism is essential for plant germination, growth, reproduction, and survival (da Fonseca-Pereira et. al., 2018). We showed that, as observed in the roots, *aac1* lines showed significant decreases in leaf respiratory activity, both in terms of O_2 consumption (Figure 2C and D) and CO_2 evolution (Sup. Figure 1E). However, plants with reduced *AAC1* expression didn’t show changes in photosynthetic parameters under optimal growth conditions, but they did have impaired growth, with lower fresh mass, leaf area, and number of leaves (Figure 1 C-H). Reduced respiration associated with unbalanced ADP/ATP ratios between the cytosol and the IMM can lead to changes in the redox potential of the mitochondrial matrix, increasing ROS levels and, as a consequence, altering redox homeostasis and cell cycle progression. It has already been proven that cell cycle alterations in the apical meristem result in changes in vegetative growth (Dolzblasz et al., 2016). *In silico* co-expression analysis showed that *AAC1* is highly expressed in the vegetative apex in *A. thaliana* (Sup. Figure 5). Cell cycle progression is dependent on a substantial amount of mitochondrial energy. Phosphorylation of mitochondrial substrates mediated by cyclin B1/Cdk1 allows cells to perceive and respond to increased energy demand and positively regulate mitochondrial respiration so that cell cycle progression occurs effectively. However, when mitochondrial function is impaired, the necessary supplement of ATP is compromised, which can result in cell cycle arrest (Wang et al., 2014) and as a consequence, growth is negatively affected.

Despite the damage to growth, our results present an intriguing finding: the reduction in AAC1 expression resulted in a significant increase in silique size, number of seeds per silique, and 1000-seed weight (Figure 7 A-F). The reduction in AAC1 expression likely reduces maintenance costs due to its negative impact on respiration. This means that less energy is required for cellular upkeep. Furthermore, when combined with the unchanged photosynthesis observed in our study, it leads to shifts in carbon allocation towards seed production. In essence, with reduced energy expenditure on cellular maintenance and a stable photosynthetic rate, more resources become available for directing towards seed development. Notably, while AAC1 is expressed at significant levels during seed formation, in silico co-expression analysis suggests that AAC2 expression is relatively higher in reproductive tissues, playing an important role in seed and silique formation (da Fonseca-Pereira et al., 2018). This implies that during the reproductive stage, AAC1 may not play a crucial role but instead may influence reproductive. The reduction in *AAC1* expression may have led to a compensatory increase in the expression of *AAC2*, which is already strongly expressed at this stage, resulting in significant benefits in terms of silique and seed formation.

Are the ADP/ATP carrier protein isoforms redundant?

We demonstrated that, in leaves during the vegetative period, the reduction of *AAC1* didn’t result in increases in the relative expression of the other AAC isoforms under optimal growth conditions (Figure 8). However, there are indications that a certain degree of functional redundancy may exist in specific situations and stages (da Fonseca-Pereira et. al., 2018). The reduction in *AAC1* expression triggered changes in phosphorylate activity, and metabolite levels and affected growth. Due to the importance of cellular energy homeostasis, it is expected that any modifications in adenylate transport will trigger a complex regulatory network of adjustment mechanisms to ensure plant survival. This becomes particularly critical in situations where there is an energy overload in the IMM, but there is little availability of ATP for biosynthetic reactions and therefore for growth. In this sense, the differential regulation of the different AACs isoforms may guarantee survival at different stages of plant life, while the regulation of another adenylate transporter occurs more specifically in response to disturbances in the levels of specific compounds. We observed an increase in the expression of *APC1*, which indicates that this transporter seems to have a functional role that is more intertwined with the physiological function of AAC1 in plants, as demonstrated in *S. cervisae* (Cavero et. al., 2005). Therefore, the differential regulation of APC1 suggests that the maintenance of plant adenylate pools in aac1 lines plants seems to be indirectly modulated by the level of cytosolic Ca^{2+}. Taken together, our results highlight the complexity of plant adaptation mechanisms in response to variations in energy homeostasis. Furthermore, they provide evidence that different isoforms and transporters of adenylate may differentially contribute to crucial physiological processes.

Conclusion

In this study, we have provided convincing evidence for the role of AAC1 in oxidative phosphorylation in *A. thaliana*. Lower expression of *AAC1* resulted in reduced rates of respiration, especially in the roots, which negatively affected growth. In addition, we demonstrated that AAC1 plays an important role in cellular energy homeostasis, affecting the concentration of ATP. The reduction of *AAC1* also impacted the morphology of mitochondria, leading to advanced mitochondrial vacuolar degeneration, which resulted in reduced respiration and the accumulation of soluble carbohydrates in the roots. Intriguingly, despite the negative effects during the vegetative phase. Lower *AAC1* expression resulted in a surprising increase in seed size and weight, suggesting that its role may be more prominent at specific stages. Our results indicate that the different isoforms of ADP/ATP transporters may not be redundant, performing distinct functions in specific situations and stages of plant development.

Future perspectives

Our results suggest that AAC1 plays a vital role in oxidative phosphorylation, affecting ATP production and cellular energy homeostasis in *A. thaliana.* Although this study has provided valuable information on the function of AAC1, there are still many questions to be explored. Future research could focus on gaining a deeper understanding of the underlying mechanisms that regulate adenylate transport by this transporter and how it interacts with other proteins involved in oxidative phosphorylation. Another interesting prospect would be to investigate how the different isoforms of ADP/ATP transporters contribute to the plant's energy metabolism and development at different stages of its life cycle. Furthermore, exploring the impact of *AAC1* reduction in plants under abiotic or biotic stress may provide additional insights into its functional importance in challenging situations.

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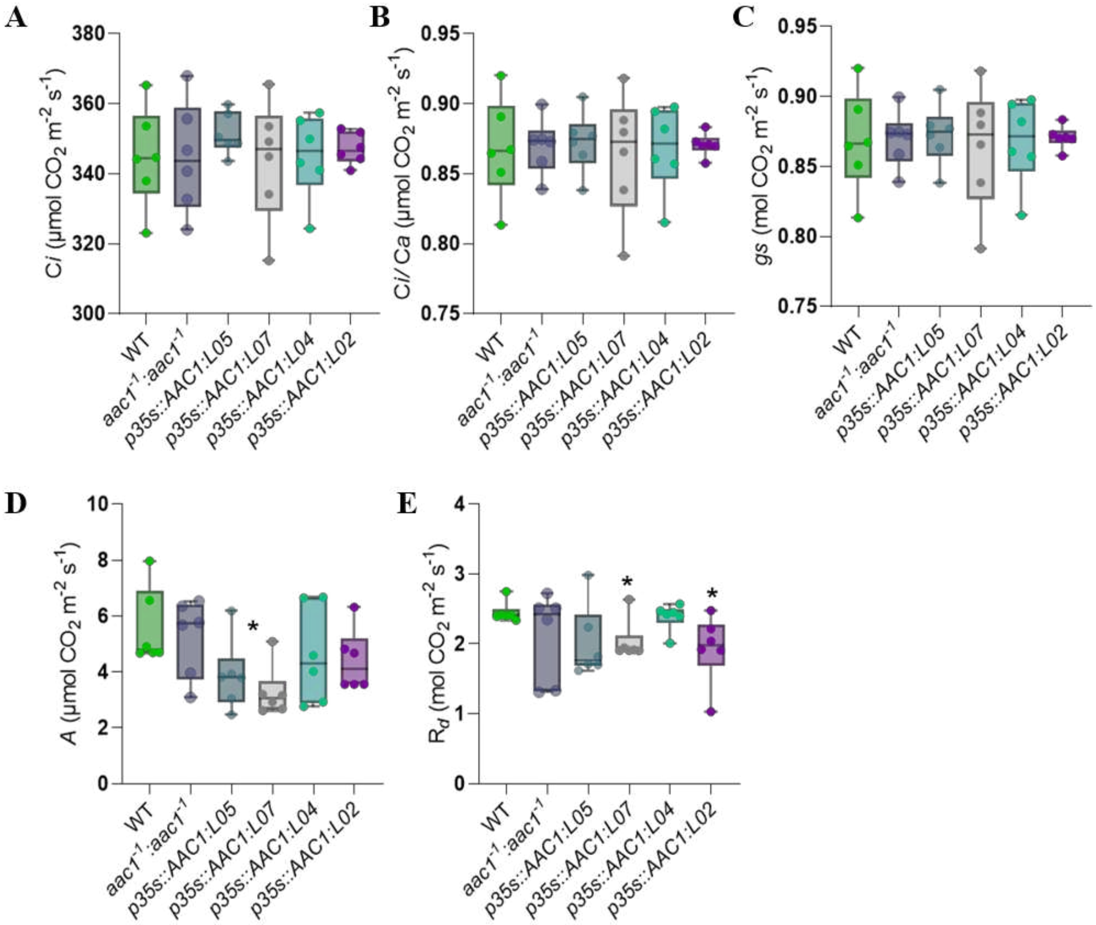
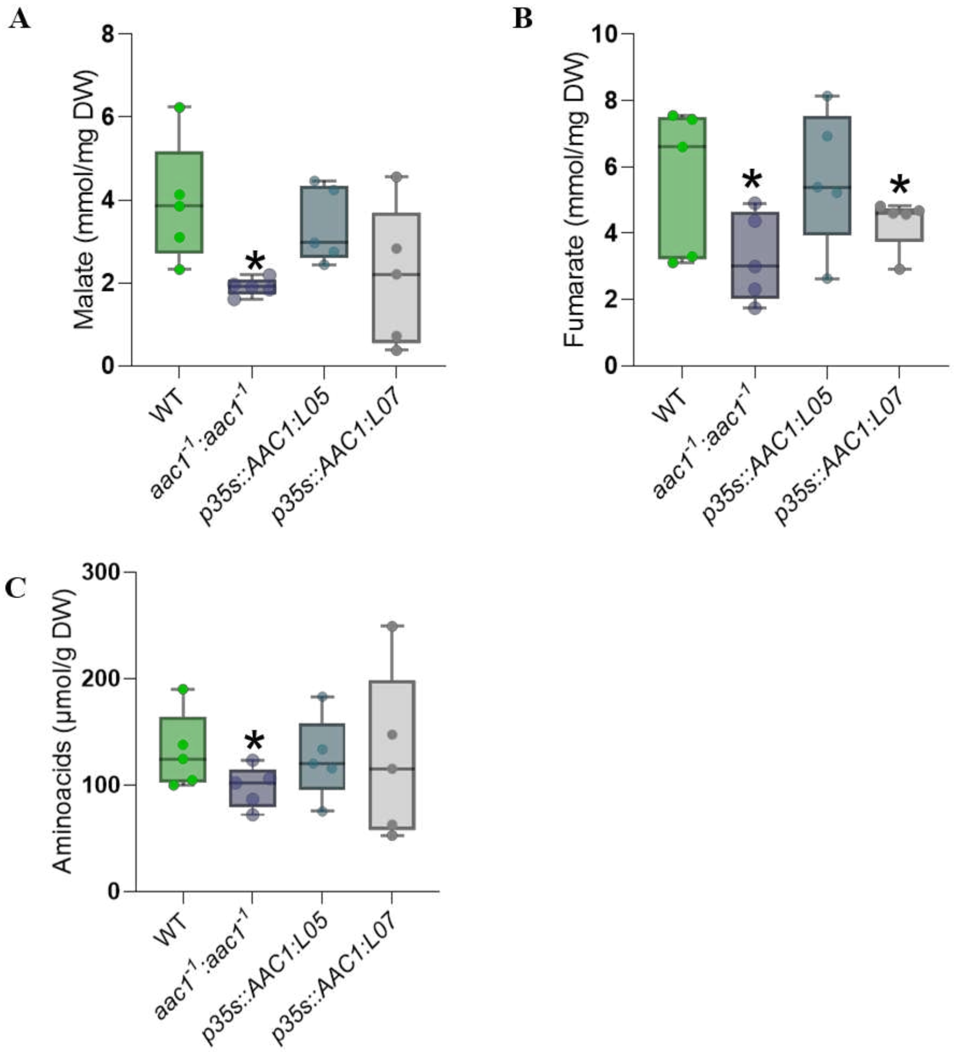
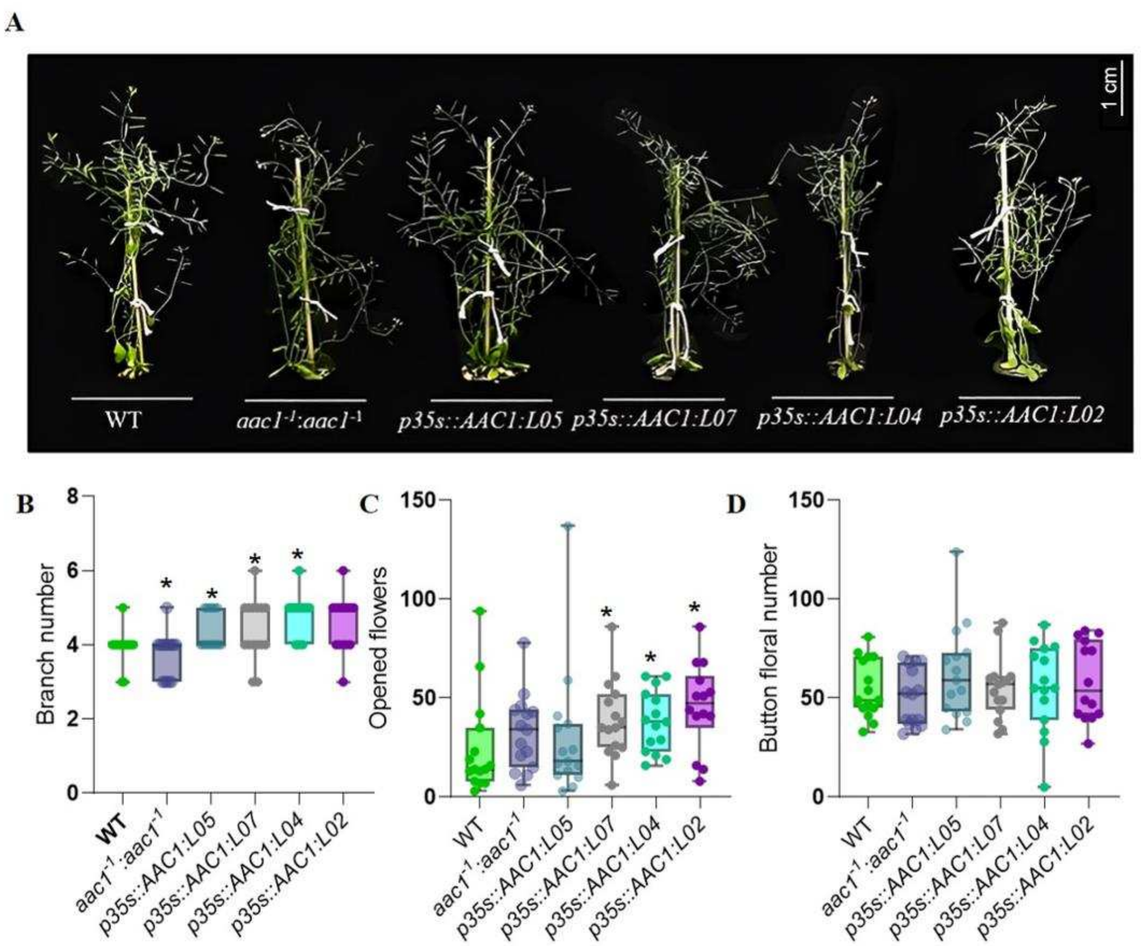
Material Supplemental



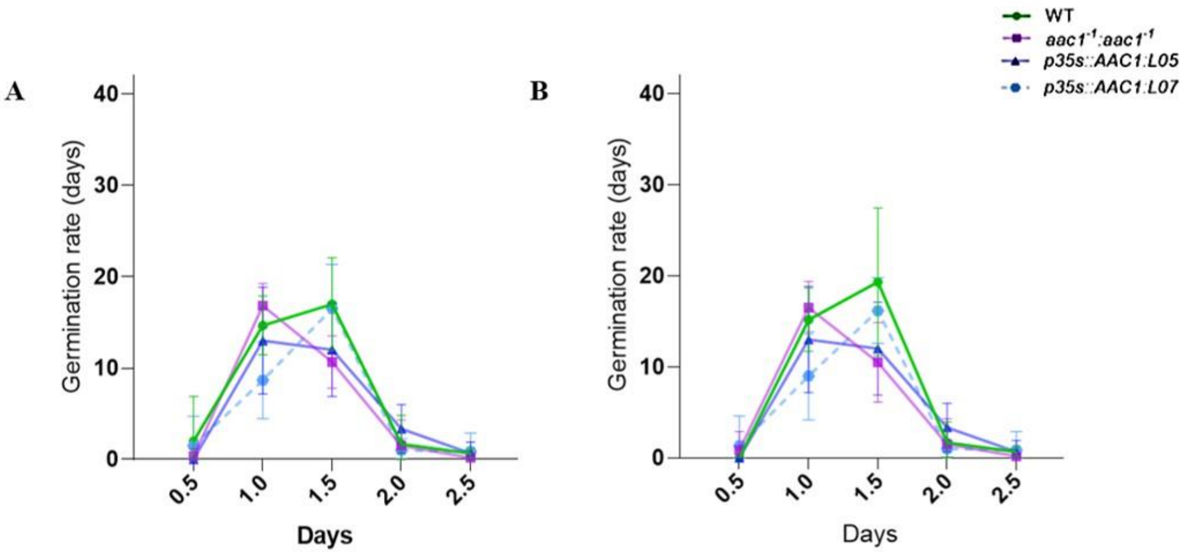
Sup. Figure 1. Gas exchange parameters in lines with reduced expression of AAC1 and WT. Internal CO_2 concentration (C*i*) (A). The ratio between internal concentration and air CO_2 concentration (Ci/Ca) (B). Stomatal conductance (*gs*) (D). Photosynthesis (*A*) (E). Respiration in the dark (*F*). The plants evaluated were five weeks old at the time of collection and were grown under optimal conditions. The values represent the mean ± standard error of six individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's *t-test*, compared to the *wild type* (WT)



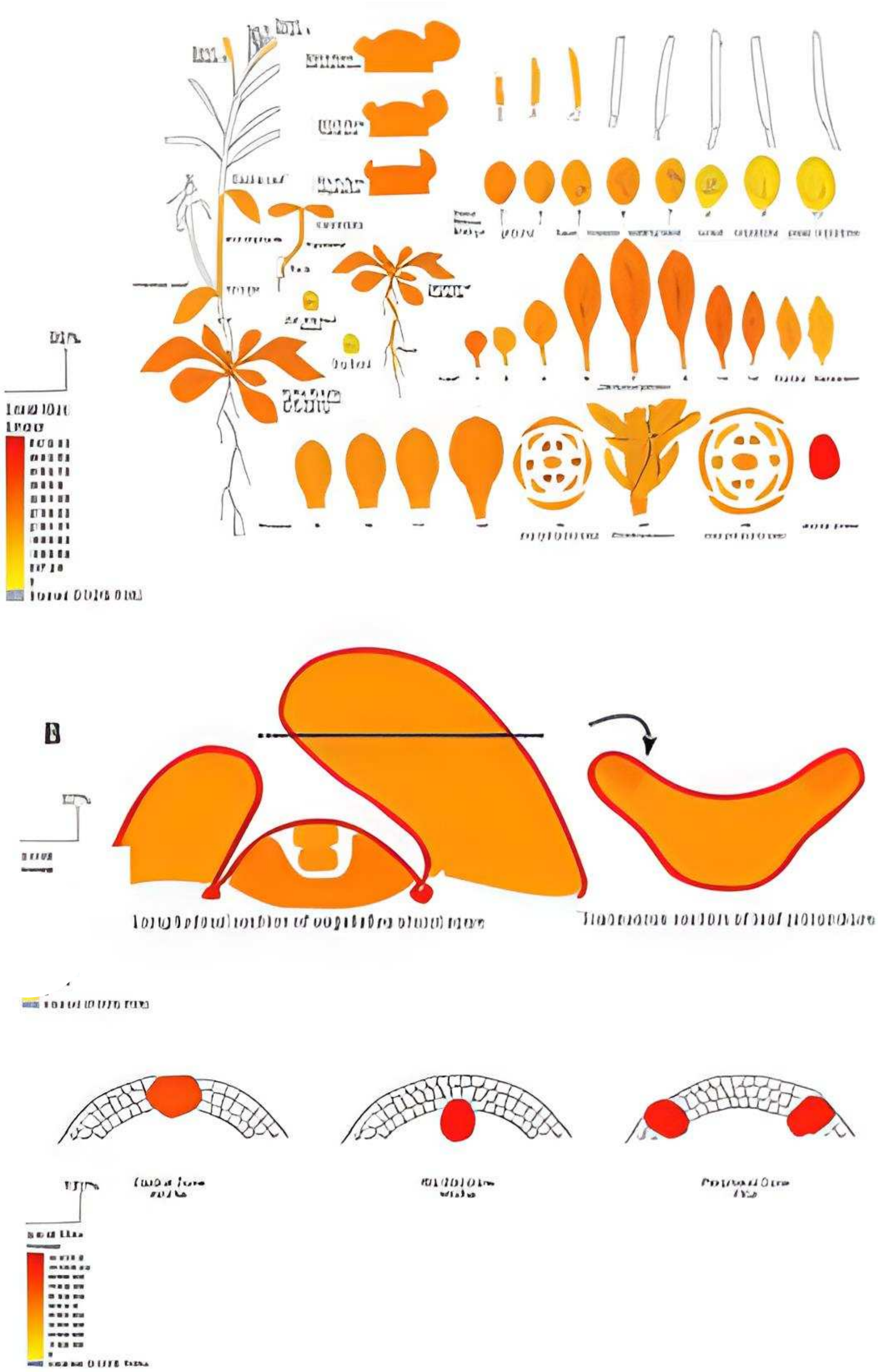
Sup. Figure 2. Levels of organic acids and amino acids in roots of lines with reduced expression of *AAC1* and WT. Malate (A), fumarate (B) and amino acids (C). The plants evaluated were five weeks old at the time of collection and were grown under ideal conditions. The values represent the mean ± standard error of six individual plants. The asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the *wild type* (WT).



Sup. Figure 3. The impact of *AAC1* on the reproductive stage in *A. thaliana*. Flowering phenotype (A). Number of branches (B), open flowers (C), and flower buds (D). The plants evaluated were five weeks old at the time of collection and were grown under ideal conditions. The values represent the mean ± standard error of twelve individual plants. The asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the *wild type* (WT).

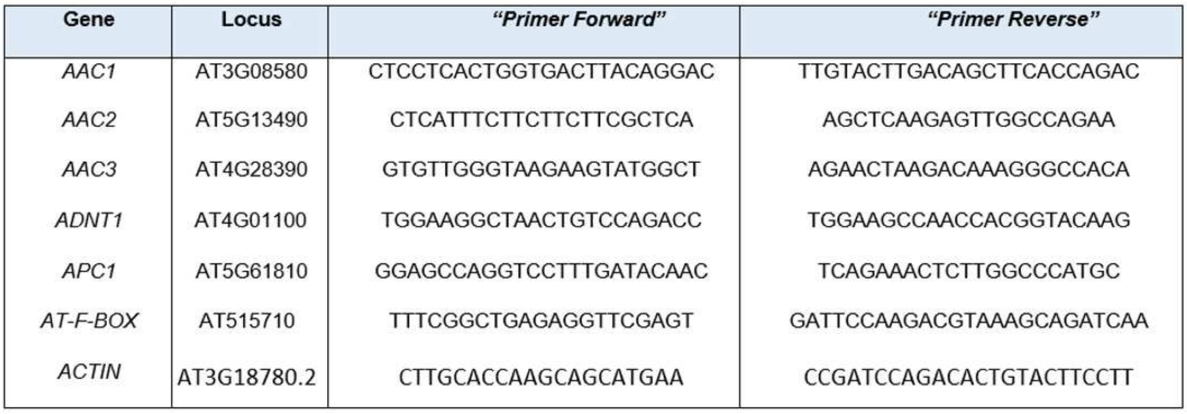


Sup. Figure 4. Germination rate in reduced expression *AAC1* lines and WT. Seed germination index in MS medium with 0% sucrose (A) and with 1% sucrose (B). Values in figures are presented as mean ± standard error of six individual plates with 50 seeds each per line; an asterisk indicates values that were determined by the student's t test to be significantly different ($P < 0.05$) from the WT.



Sup. Figure 5. *In silico* analysis of the expression of the *AAC1* in different tissues (A) and the apical meristem (B). Source: Arabidopsis eFP Browser

Sup. Table 1. List of primers used for QuantPrime-qPCR analyses



Gene	Locus	*"Primer Forward"*	*"Primer Reverse"*
AAC1	AT3G08580	CTCCTCACTGGTGACTTACAGGAC	TTGTACTTGACAGCTTCACCAGAC
AAC2	AT5G13490	CTCATTTCTTCTTCTTCGCTCA	AGCTCAAGAGTTGGCCAGAA
AAC3	AT4G28390	GTGTTGGGTAAGAAGTATGGCT	AGAACTAAGACAAAGGGCCACA
ADNT1	AT4G01100	TGGAAGGCTAACTGTCCAGACC	TGGAAGCCAACCACGGTACAAG
APC1	AT5G61810	GGAGCCAGGTCCTTTGATACAAC	TCAGAAACTCTTGGCCCATGC
AT-F-BOX	AT515710	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
ACTIN	AT3G18780.2	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT

CHAPTER III

The protein ADP/ATP carrier 2 (AtAAC2) influences on seed germination and reproductive yield

The protein ADP/ATP carrier 2 (*At*AAC2) influences on seed germination and reproductive yield

Abstract: ADP/ATP Carrier 2 (AAC2) is a mitochondrial adenylate protein belonging to the Mitochondrial Family Carrier. To date, the physiological role of this carrier in plants remains unknown. This study aimed to elucidate AAC2's function during the reproductive stage, germination, vegetative phase, and root growth. Through *in silico* expression analysis, AAC2 is up-regulated in stamens, pollen grains, embedded seeds, and inflorescences. This carrier demonstrated significance in germination, as evidenced by reduced germination percentage and delayed germination in lines with decreased *AAC2* expression. Photosynthetic efficiency remained unaltered, however, lines with lower AAC2 expression exhibited a notable reduction in respiration. Although there was an increase in chlorophyll content, primary metabolites were minimally affected. Reduced *AAC2* expression corresponded to diminished initial root growth, silique size, and silique filling. Morphological alterations were observed in unviable seeds. These findings underscore AAC2's role in specific plant stages, particularly in influencing germination and reproductive yield.

Key-words: Mitochondria, Arabidopsis, bioenergetics

Introduction

Oxidative phosphorylation in mitochondria is the most efficient metabolic pathway for regenerating ATP in eukaryotes. ATP, as the main class of molecules related to energy transfer, plays a crucial role in this process. In addition to ATP, other adenine nucleotides play essential roles in both the metabolism and physiology of plants (da Fonseca-Pereira et. al., 2018). In this context, the ATP regenerated in the mitochondrial matrix must be released, while the substrates for ATP synthesis, ADP and phosphate, must imported by the organelle. A constant flow of ATP out of the mitochondrial matrix, in exchange for ADP, is vital for supplying energy to the cytosol, where numerous biosynthetic processes are outside. The mitochondrial proteome is largely dedicated to meeting cellular energy demands, with the inner mitochondrial membrane (IMM) housing several transporter proteins specialized in the transport of adenylates (Nunes-Nesi et. al.,2020).

Three groups of proteins belonging to the Mitochondrial Carrier Family are responsible for transporting adenylates through the IMM: ATP/Phosphate Carrier 1 (APCs), responsible for mediating the export of Pi (inorganic phosphate) to the cytosol and the import of adenylates into the mitochondrial matrix (Lorenz et al., 2015); Adenine Nucleotide Translocator 1 (ADNT1), which promotes the export of ATP to the cytosol in exchange, preferentially, for AMP; and the ADP/ATP Carrier (AACs), which acts in the electrogenic passage of ADP/ATP, transporting a molecule of cytosolic ADP in exchange for a molecule of ATP from the mitochondrial matrix (Ogunbona and Claypool, 2019). Among these, the group of AACs is the most abundant (Fuchs et. al., 2020). Apart from their role as adenylate exchangers, research on mammalian mitochondria indicates that these transporters may also facilitate the movement of protons (H^+). (Bertholet et al., 2019).

The nuclear genome encodes three isoforms of AAC (Adenine Nucleotide Translocase) proteins: AAC1 (AT3G08580), AAC2 (AT5G13490), and AAC3 (AT4G28390) in Arabidopsis. These proteins feature an N-terminal extension consisting of approximately 60 residues of 16 amino acids. The kinetic parameters, such as *Km* and *Vmax* values, reveal that $AAC_{1\text{-}3}$ transporters in Arabidopsis exhibit significant affinity for ATP and ADP transport. Specifically, the *Km* values are 10 µm (AAC1 and AAC3) and 14 µm (AAC2) for ADP, while for ATP, they are 15 µm (AAC1), 22 µm (AAC2), and 12 µm (AAC3) (Haferkamp et al., 2002). These diverse kinetic profiles likely contribute to the functional versatility of mitochondria, facilitating the adjustment of adenylate flux in response to cellular energy demands (Haferkamp et al., 2002).

During the reproductive phase, a large part of the cellular energy demands is aimed at guaranteeing the survival and perpetuity of the species (Selinski and Scheibe, 2014). Inefficient transport of ATP from the mitochondria can lead to over-reduction of the electron transport chain, leading to increased levels of Reactive Oxygen Species (ROS), redox imbalance and mitochondrial dysfunction. Busi et. al. (2011) showed that mitochondrial dysfunction leads to an inhibition of glycolysis that is accompanied by a decrease in respiration, low levels of ATP and ADP, and induction of the transcription of genes involved in mitochondrial respiration responses, which can directly impact flower formation and metabolic regulation in plants. Therefore, the adenylate turnover can contribute to maintaining adequate levels of ROS and proper mitochondrial function. Because of this, we have directed our efforts towards understanding the role of AAC2 during the reproductive phase. However, due to the lack of information and *in vivo* experimentation on the role of AAC2 in plants, we carried out a broader basic characterization, also investigating the effects of reduced *AAC2* expression on seed germination, vegetative phase, and root growth.

Given the crucial role of mitochondrial adenylate transporters, particularly AAC2, in regulating energy metabolism and cellular respiration during the reproductive phase in plants, we hypothesize that reduced expression of *AAC2* will lead to impaired energy homeostasis, resulting in altered seed germination, vegetative growth, and root development in *A. thaliana*. Our findings demonstrate that reducing *AAC2* expression affects cellular respiration and germination, alters seed morphology and silique size, and influences initial root growth. *In silico* expression data indicates that *AAC2* expression is positively regulated during the flowering and mature pollen grain stages. These results are discussed in the context of energy homeostasis facilitated by the mitochondrial adenylate transporter and its influence on the reproductive phase in *A. thaliana*.

Materials and methods

Plant material

The *aac2^{-1}:aac2^{-1}* strain, obtained from the Institute Genome Analysis Laboratory - SIGnAL Salk (AT5G13490) collection, has a T-DNA insertion via *Agrobacterium tumefaciens* in the intron region of the transporter gene (Figure 1A). The flanking sequence contains 473 bp. The zygosity was analyzed by PCR screening, using the following primers: Foward 5- AACTACTACTTCTCCTGTGTTTGTCCAA-3', reverse 5'- GAAACTGCAAAAAAAATGGAGAAAAAAACAAAAGC- 3', and LBb1.3: 5'-ATTTTGCCGATTTCGGAAC-3'.

The antisense line was created by expressing the complete *AAC2* coding sequence in the opposite orientation in the Gateway vector with 35S as promoter and *t* terminator (Figure 2A). The transgenic line was selected from the heterozygous progeny (T2) by analyzing the kanamycin resistance segregation pattern. Approximately 50 seeds from the T3 generation of each antisense line were sterilized and sown in Petri dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 50 mg/L^{-1} kanamycin and screened for expression. After 24 hours in the dark at 4°C, the plates were transferred to a short-day regime and selection was carried out according to the survival criterion up to 7 days after germination.

Growth conditions

The seeds were sterilized on the surface and then placed on a medium containing half-strength MS, supplemented with 1% (w/v) sucrose and the appropriate selective agent for each genotype. After stratification in darkness at 4°C for four days, they were transferred to a growth chamber set at 22 ± 2°C, with a relative humidity of 60%, an irradiance of 150 μmol photons m^{-2} s^{-1}, and a photoperiod of 8 hours light and 16 hours darkness for a period of ten days. Following this, the seedlings were transplanted into 0.1-liter plastic pots filled with commercial Carolina Soil Standard EC 0.7 - 8 Kg substrate. Post-transplantation, the seedlings were maintained in the growth chamber under the same aforementioned conditions for five weeks.

Expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from 6-week-old leaves following the TRIzol reagent protocol (Ambion, Life Technology, Carlsbad, CA, USA) as per the manufacturer's guidelines. The RNA samples underwent DNase I treatment (InvitrogenTM) to remove any genomic DNA contamination. RNA integrity was assessed on a 1% agarose gel (w/v), and the concentration was determined using a Nanodrop spectrophotometer. Subsequently, 600 ng of total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Thermo Fischer, Waltham, MA, USA) according to the manufacturer's instructions. Real-time PCR was conducted in a 96-well MicroAmp™ optical reaction plate (Applied Biosystems, Waltham, MA, USA) using Power SYBR® Green PCR Master Mix (Life Technologies/Applied Biosystem) following the manufacturer's protocol. Specific forward primers 5‟-CTCATTTCTTCTTCTTCGCTCA-3” and reverse primers 5’-AGCTCAAGAGTTGGCCAGAA-3” were used. Relative transcript levels were determined by relative quantification and normalized using constitutively expressed gene, ACTIN2 (AT2G37620), forward primer 5‟- CTTGCACCAAGCAGCATGAA-3” and reverse 5‟- CCGATCCAGACACTGTACTTCCTT -3”. Three biological replicates were analyzed for each experimental condition. Three biological replicates were analyzed for each experimental condition.

Root growth

Seeds were planted on plates containing half-strength MS medium. These plates were then subjected to stratification in darkness at 4°C for three days. Following stratification, the germination rate was evaluated for three days, both at the beginning and end of the light cycle. Subsequently, root growth was conducted following the method outlined by Palmieri et al. (2008) with some modifications. Seedlings aged five days were transferred to Petri dishes (90x15mm) containing half-strength MS medium and positioned vertically in a growth chamber set at 22 ± 2 ºC, with a relative humidity of 60%, irradiance of 150 µmol photons m^{-2} s^{-1}, and a photoperiod of 8 hours light and 16 hours darkness. Root length was measured daily over a span of ten days using ImageJ software, which quantified the distance between successive points marked along the axial growth.

Gas exchange parameters

Gas exchange was evaluated one hour after the onset of the light period using an open-system infrared gas analyzer (IRGA - Infrared Gas Analyzer) model LI 6400XT (LI-COR, Lincoln, NE, USA) equipped with a fluorometer (6400-40 LI-COR Inc.) on 6-week-old plants. The reference CO_2 concentration was maintained at 400 mmol CO_2 mol^{-1} of air. All measurements were conducted using the 2 cm^2 leaf chamber, with the block temperature set at 25°C and irradiance at 150 μmol photons m^{-2} s^{-1}. Respiration rates were determined during the dark period, following a 30-minute acclimatization of the plants to this condition.

Sample processing and metabolite extraction

Roots from each line were harvested during the mid-light period and immediately flash-frozen in liquid nitrogen, preserving them at -80 ºC until further analysis. Following this, the samples underwent homogenization, and approximately 20 mg of fresh tissue aliquots were extracted using a methanol-based method, following the procedure outlined by Salem et al. (2016). Subsequent analysis focused on the soluble fraction, evaluating chlorophylls (Sulpice et al., 2009), glucose, fructose, and sucrose (Fernie et al., 2001), along with organic acids (Nunes-Nesi et al., 2007).

Phenotypic characterization during the reproductive stage and germination

To characterize the role of AAC2 during the reproductive phase, seedlings were transferred to commercial substrate Carolina Soil Standard EC 0.7 - 8 Kg and kept in a growth room at 22 ± 2°C, relative humidity of 60%, with a photoperiod of 12h light and 12h dark and irradiance of 150 μmol photons m^{-2} s^{-1} for twelve weeks for seed production. The siliques of wild and mutant plants were collected and clarified in a solution of 0.2N NaOH and 1% sodium dodecyl sulfate (SDS) (Yoo et al., 2012). Ten clarified siliques were collected and the length, width, and number of seeds per silique were analyzed under a dissecting microscope (Stemi 2000-C, Zeizz) with an adapter (SOLIGOR tube for Canon A650 IS B52 Wide) for a CANON 12.1-megapixel camera (Power Shot A650 IS). In addition, the number of branches and siliques per plant was quantified. Subsequently, ten seeds from different siliques on each plant were evaluated, using six replicates per genotype, for length and diameter. In addition, the weight of a thousand seeds and all the seeds produced by the same plant will be quantified. The biometric data obtained from photographic records was evaluated using the ImageJ program.

Seeds from Col-0 plants and mutants were properly sterilized and placed to germinate in a half strength MS medium supplemented with 1% sucrose for 72 hours. Following this, the seeds underwent a stratification process for five days at 4°C in the dark. Subsequently, the plates were transferred to a growth chamber set at 22 ± 2°C, 60% relative humidity, irradiance of 150 μmol photons m^{-2} s^{-1}, and a photoperiod of 8 hours light and 16 hours dark. Germination percentage and rate, seed germination index, and germination speed index were evaluated using both half strength MS medium with 0% sucrose and the same medium with 1% sucrose. Six replications with 50 seeds for each genotype were included in the analysis, and the variables were calculated according to Bruginski et al. (2009).

Bioinformatic analysis

Gene expression data were obtained from the expression browser tool at the Bioarray Resource (BAR; www.bar.utoronto.ca; (Waese and Provart, 2017)). Throughout the ePlant (Waese et al., 2017) an analytic tool for exploring multiple levels of *A. thaliana* data.

Statistical analysis

The experimental design was entirely randomized with six replicates. The results obtained were subjected to analysis of variance and compared by Student's t-test using the algorithm contained in Microsoft Excel software (Microsoft Corporation, Seattle, WA, USA), with values with $P < 0.05$ being considered significant.

Results

AAC2 gene expression profile

To analyze the expression pattern of the *AAC2* gene in different stages and tissues, we performed an *in-silico* expression analysis using mRNA level data obtained through the Expression Browser tool on Bioarray Resource (BAR), a publicly accessible source. Our finding reveals that the relative expression of *AAC2* is positively regulated in tissues associated with the reproductive phase (Figure 1). The highest levels of expression are noted in floral stamens, mature pollen, inflorescences, and seeds immersed after 24 hours. On the other hand, *AAC2* expression levels are relatively lower in roots, vegetative leaves, hypocotyls, cotyledons, and dry seeds. These results highlight a differential pattern of *AAC2* gene expression in different tissues and stages of development (Figure 1).

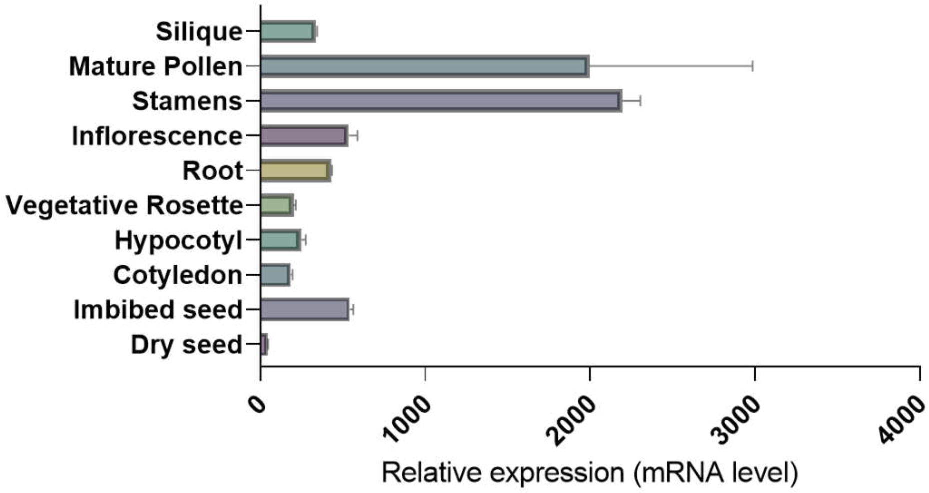


Figure 1. Expression profile of gene encoding mitochondrial adenylate carrier AAC2 in different organs and developmental stages of *Arabidopsis thaliana*. The expression data corresponding to mRNA levels was retrieved from the expression browser tool at the Bio array Resource (BAR) (www.bar.utoronto.ca).

Generation and phenotypic characterization of lower expression *AAC2* lines

To investigate the physiological role of the AAC2 transporter, homozygous plants of the *aac2*$^{-1}$*:aac2*$^{-1}$ line with T-DNA insertion and a homozygous antisense transgenic line under control of the 35S promoter (*p35s::AAC2*) were selected (Figure 2A). The homozygous lines were selected by PCR using specific primers (Figure 1B). We assessed the level of *AAC2* expression by qRT-PCR. The relative expression of *AAC2* in the *aac2*$^{-1}$*:aac2*$^{-1}$ line was 24.6% and for the *p35s::AAC2* line 4.8% of the expression observed in WT, was normalized with the ACTIN reference gene (Figure 2C). These independent mutant lines were used for further analysis to investigate the function of AAC2 in plants. Visually, the lines with reduced expression did not show an aberrant phenotype and exhibited no visible differences compared to WT (Figure 2D).

Given the importance of adenylates for seed germination (Raveneau et. al, 2017), we decided to investigate the impact of reducing *AAC2* expression on seed germination. To this end, seeds were germinated in the presence and absence of 1% (w/v) sucrose. Germination was significantly lower in the *aac2* mutant lines compared to WT. The evaluation 12 hours after the test was set up showed that the *aac2* lines had a significantly lower rate than the WT in the MS medium without sucrose supplementation. The reduction was 37 % for the *aac2*$^{-1}$*:aac2*$^{-1}$ line and 56 % for the *p35s::AAC2* line (Figure 3A). Despite sucrose supplementation promoting an increase in germination, the germination percentage of the *aac2* lines remained lower than the WT (Figure 3B). At the end of the test, the germination percentage on plates without sucrose remained lower in the *aac2* lines, with ~93% germination for the WT, ~84% for *aac2*$^{-1}$*:aac2*^{1}*,* and ~79% for *p35s::AAC2*. With sucrose supplementation, the germination percentage was ~98% for WT, 80% for *aac2*$^{-1}$*:aac2*$^{-1}$*,* and ~87% for *p35s::AAC2*. The germination rate was significantly lower at 12h of evaluation in the seeds with reduced expression for *AAC2* compared to the WT in the plate without sucrose supplementation (Figure 3C). In the treatment with sucrose supplementation, the the germination rate did not differ so much between the mutants and the WT, although it was slightly lower in the low *AAC2* expression lines throughout the evaluations (Figure 3D). Interestingly, in the last evaluation, the speed germination index of the *aac2* lines was relatively higher than the WT. The average time for 50% of the seeds to germinate, calculated by the germination rate, was higher for lines with reduced expression for *AAC2*. We demonstrated that lines with reduced expression of *AAC2* exhibited a significantly higher germination speed index compared to the WT in both treatments (Figures 3 E-F).

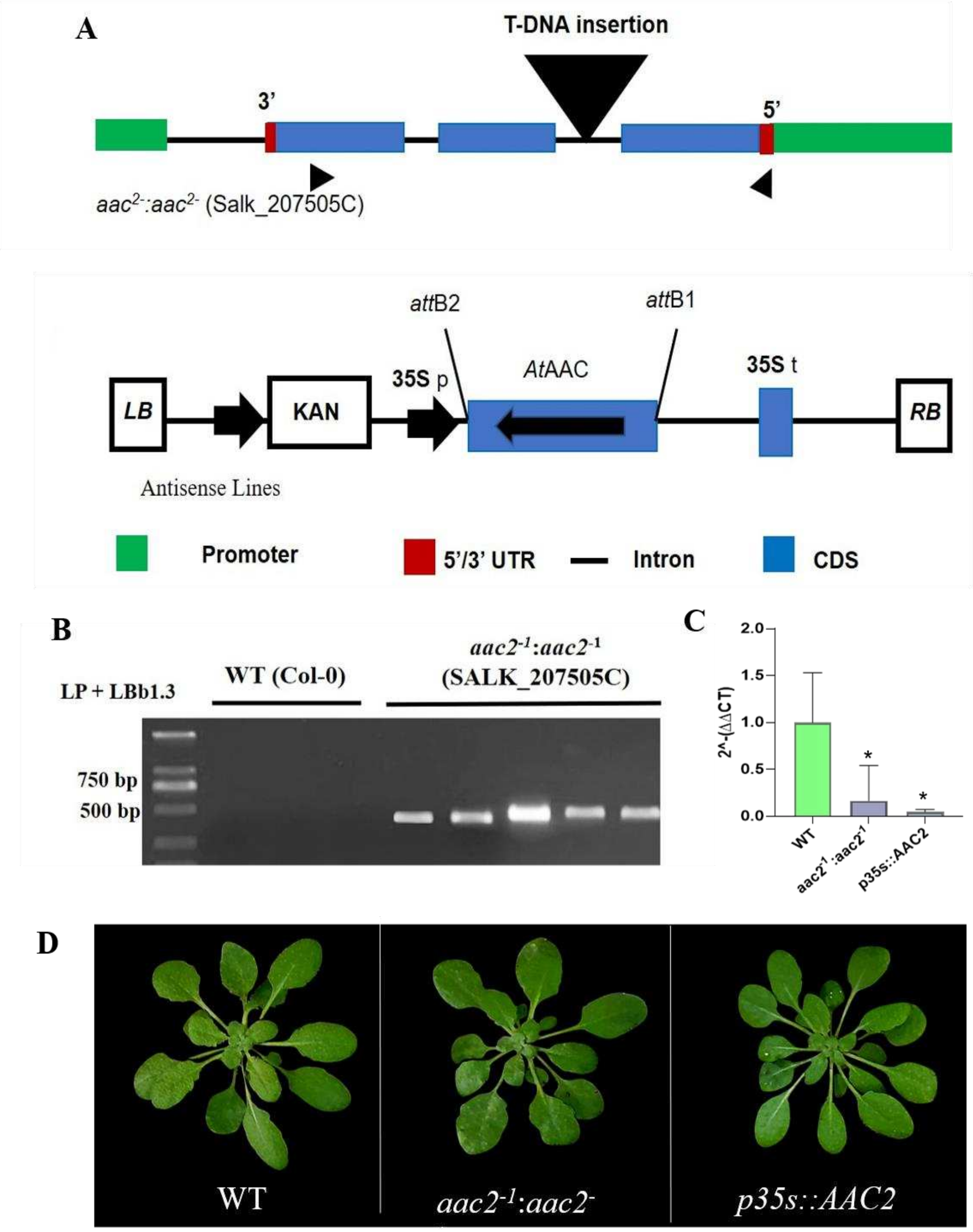


Figure 2. Isolation and phenotypic characterization of a mutant and antisense line for the mitochondrial ATP/ADP transporter (AAC2) in *A. thaliana*. Schematic representation of the At*AAC2* gene T-DNA insertion. The boxes represent the exons and the arrows next to the T-DNA are the position of the primers used to select the mutant plants (A). Amplication of the PCR product in a 1.5% agarose gel, the absence of bands in the wild type (WT), and the presence of a band in the mutant lines indicate homozygous plants for *AAC2* (B). Relative expression of mutant and transgenic (C). The visual phenotype of the rosette with reduced expression of *AAC2* and WT (D). Values represent the mean ± standard error of six individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the WT.

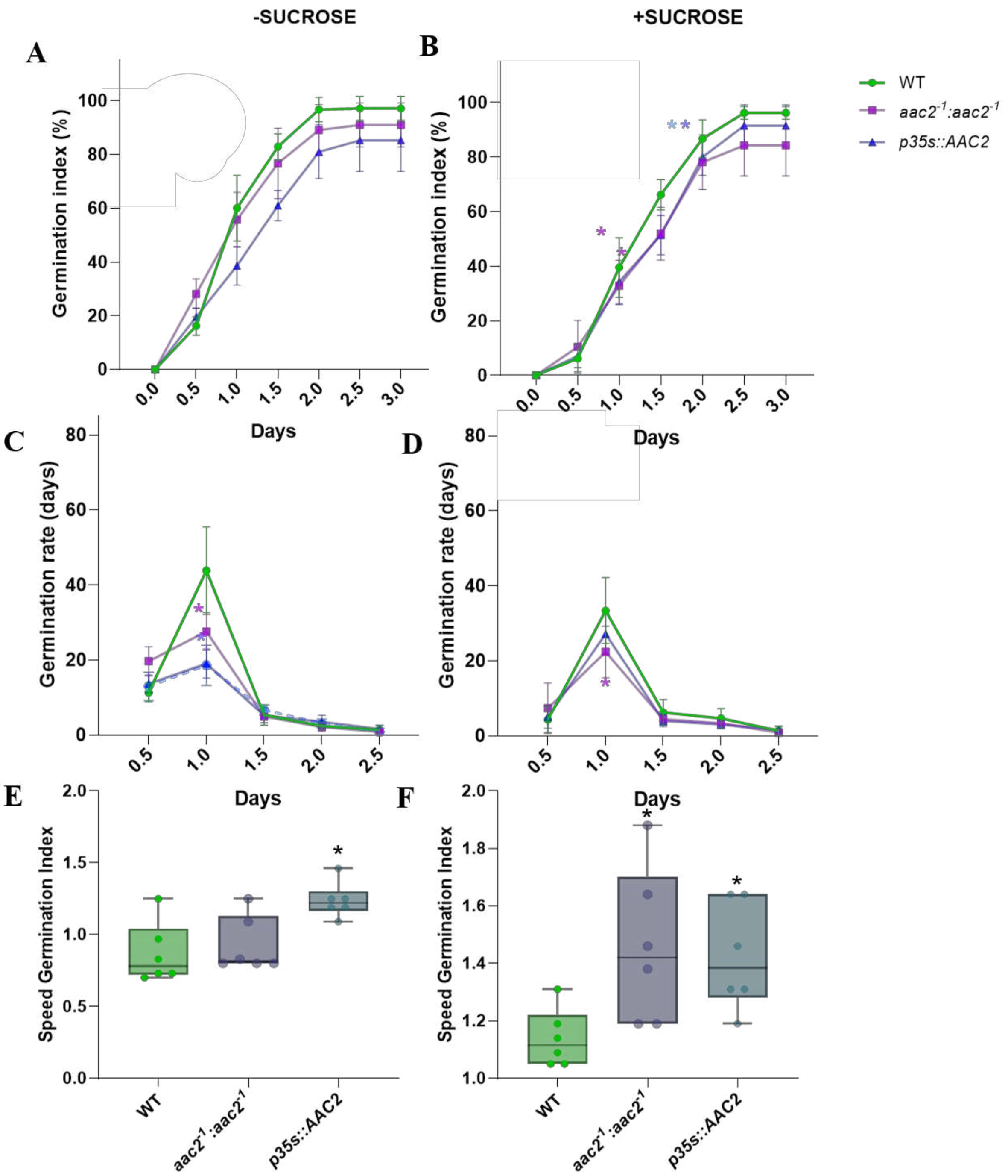


Figure 3. Germination of Arabidopsis thaliana mutant and transgenic line deficient in *AAC2* expression. Seed germination on half strength MS medium without sucrose (A) and with 1% sucrose (B). Germination rate in half strength MS medium without sucrose (C). Germination rate in MS medium with 1% sucrose (D). Seed germination index in half strength MS medium without sucrose (E). Seed germination rate in half strength MS medium without sucrose (F). Values in figures are presented as mean ± standard error of six individual plates with 35 seeds each per line. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the WT.

Effects of *AAC2* deficiency on seedling root growth and development

Considering that root cells rely strictly on mitochondrial oxidative phosphorylation to meet their needs, we chose to evaluate the function of the adenylate transporter AAC2 in roots of young plants. Analysis of root growth on vertical agar plates indicated that the root lengths of plants with reduced expression for *AAC2* were initially shorter compared to WT rootlets (Figure 4A). However, root growth was restored over the days, and at the end of the experiment, the length of the roots of the *aac2* lines didn't differ from the WT. The root growth rate was also lower in the lines with low *AAC2* expression in the first few days, with no further significant differences at the end of the experiment (Figure 4B).

Photosynthetic capacity of plants with reduced expression of *AtAAC2* under optimal growth conditions

To gain more insight into the impact of the reduction in *AAC2* expression under optimal conditions, we grew the plants and evaluated parameters related to gas exchange in leaves from five-week-old plants. We assessed the rate of photosynthesis in the aerial parts of the plants and nocturnal respiration. Lines with low *AAC2* expression exhibited unchanged CO_2 assimilation rates. No significant differences were observed in internal carbon conductance (Ci) (Sup. Figure 1A), ratio between the intercellular CO_2 concentration of the leaf and that of the air (*Ci/Ca*) (Sup. Figure 1B), and stomatal conductance (*gs*) (Sup. Figure 1C), transpiration (Sup. Figure. 1D) and net CO_2 assimilation rates (Sup. Figure 1E). Interestingly, we observed a significant reduction in the root respiration rates of the mutant compared to the WT, this reduction being significantly 8.31% for the $aac2^{-1}{:}aac2^{-1}$ line (Sup. Figure 1F).

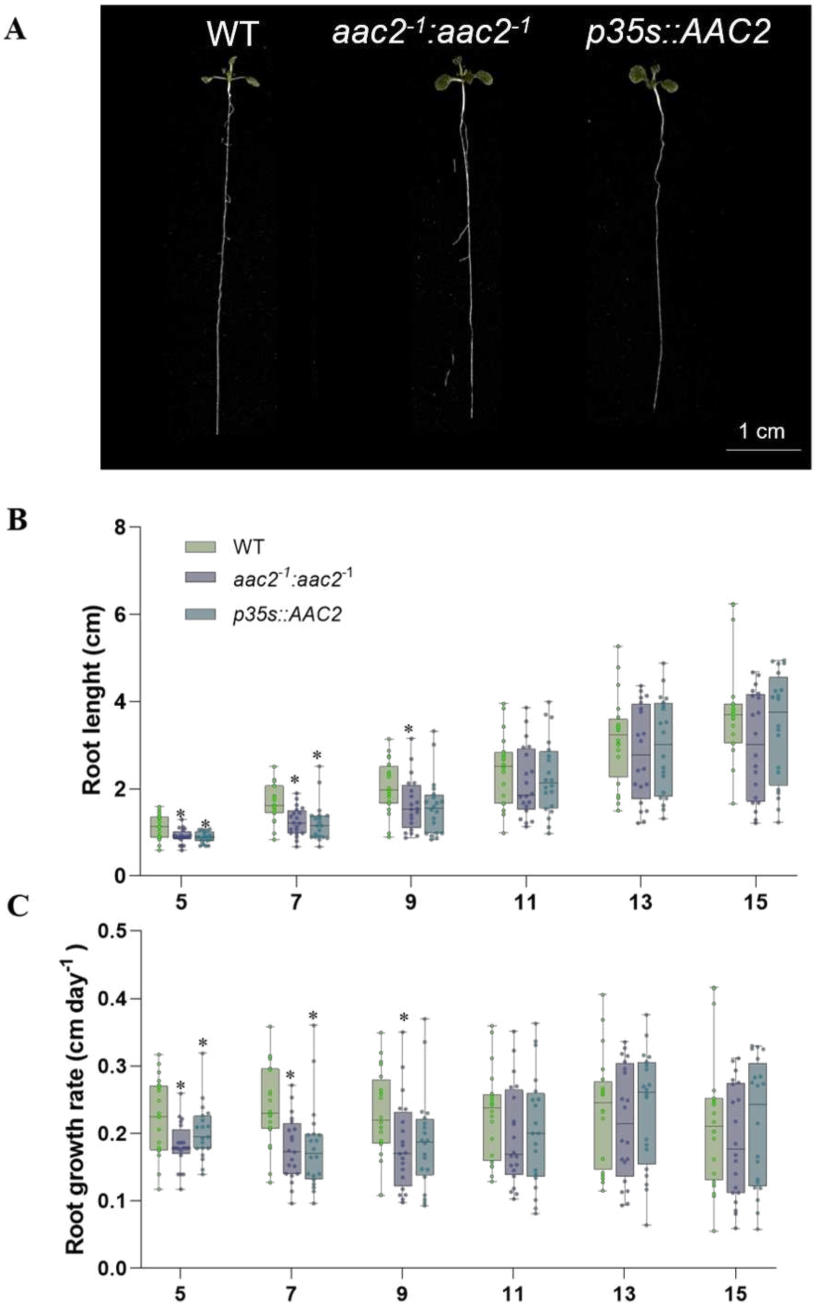


Figure 4. Root growth in lines with reduced expression for *AAC2* in *A. thaliana*. Root length was assessed over 10 days with seedlings grown on half strength MS medium (A), root growth rate (B). The values represent the mean ± standard error of 6 plates, each with four seeds per genotype. Wild type (WT).

Primary metabolite levels in leaf and root tissues of *AtAAC2* lower expression lines under optimal growth conditions

To explore the influence of AAC2 on the metabolism of *A. thaliana*, we analyzed the levels of primary metabolites in leaves and roots throughout the day in 5-week-old plants grown under optimal growth conditions. Reducing the expression of *AAC2* resulted in an increase in the levels of photosynthetic pigments in fully expanded young leaves. A significant increase was observed in the levels of chlorophyll *a* (Figure 5A) and chlorophyll *b* (Figure 5B), total chlorophyll content (Figure 5C), particularly noticeable in the *p35s::AAC2* line, and in the carotenoid level (Figure 5D). In our investigation of soluble carbohydrate levels in leaves over the course of the day, we found no significant differences in the levels of glucose (Sup. Figure 2A), fructose (Sup. Figure 2B), and sucrose (Sup. Figure 2C). Similarly, in roots, we observed no significant differences in the levels of glucose (Sup. Figure 2D), fructose (Sup. Figure 2E), and sucrose (Sup. Figure 2F) evaluated mid-light period.

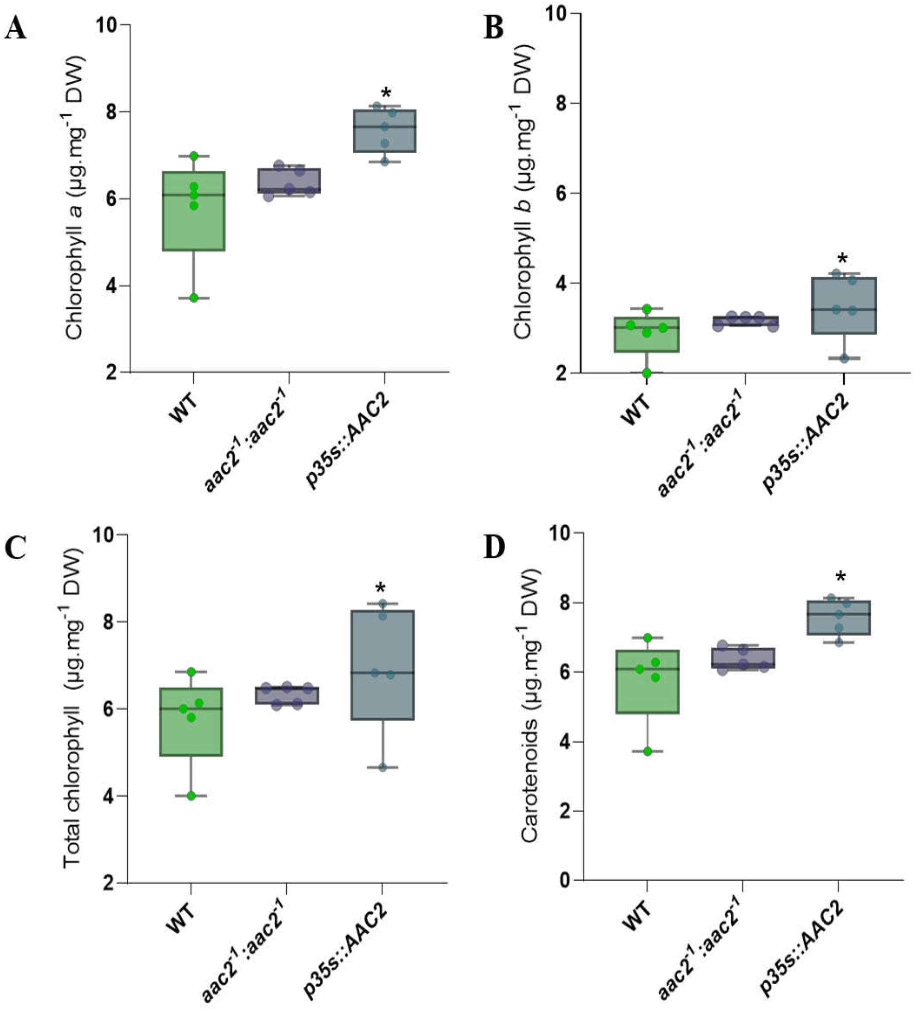


Figure 5. Photosynthetic pigments evaluated in plants with low expression of *AAC2* plants. Contents of chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C), and total carotenoids (D). Values represent the mean ± standard error of six individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the wild type (WT).

The lines with low expression for *AAC2* exhibited an increase in amino acid levels in leaves collected at midday, being significant for the *aac2* line (Sup. Figure 3A). However, no significant changes in amino acid levels were observed in roots between the mutant and transgenic lines compared to WT (Sup. Figure 3B). We also investigated whether the expression of *AAC2* would have an impact on the levels of two abundant organic acids in roots. However, no significant differences were observed in the levels of malate (Sup. Figure 4A) and fumarate (Sup. Figure 4B) between the lines with low *aac2* expression compared to the WT.

Effects of reduced *AtAAC2* expression on seed development and silique filling

Given the close relation between mitochondrial respiration and seed development, we verified the impact of reduced expression of the *AAC2* transporter during the flowering and seed formation phases. The analyzed parameters were slightly smaller in the lines with reduced *AAC2* expression (Figure 6A). The length (Figure 6B), width (Figure 6C), and total number of siliques (Figure 6D) per plant were significantly lower in the *aac2*$^{-1}$*:aac2*$^{-1}$line. In addition, there were reductions in the number of seeds per silique in both lines with reduced *AAC2* expression, showing incomplete filling of the siliques compared to the WT control group (Figure 6E).

For a more detailed understanding, we investigated the phenotype of the seeds in the lines used in the study. The reduction in *AAC2* resulted in morphological changes in the seeds, characterized by apparent shrinkage and unviability (Figure 7A). There was no reduction in the width of *aac2* seeds compared to WT (Figure 7B). Interestingly, there was a significant increase in length (Figure 7C) and thousand-seed weight in the lines with low *aac2* expression compared to the WT group (Figure 7D). Despite a downward trend, no decreases were observed in the total weight of the mutant and transgenic seeds compared to the WT group.

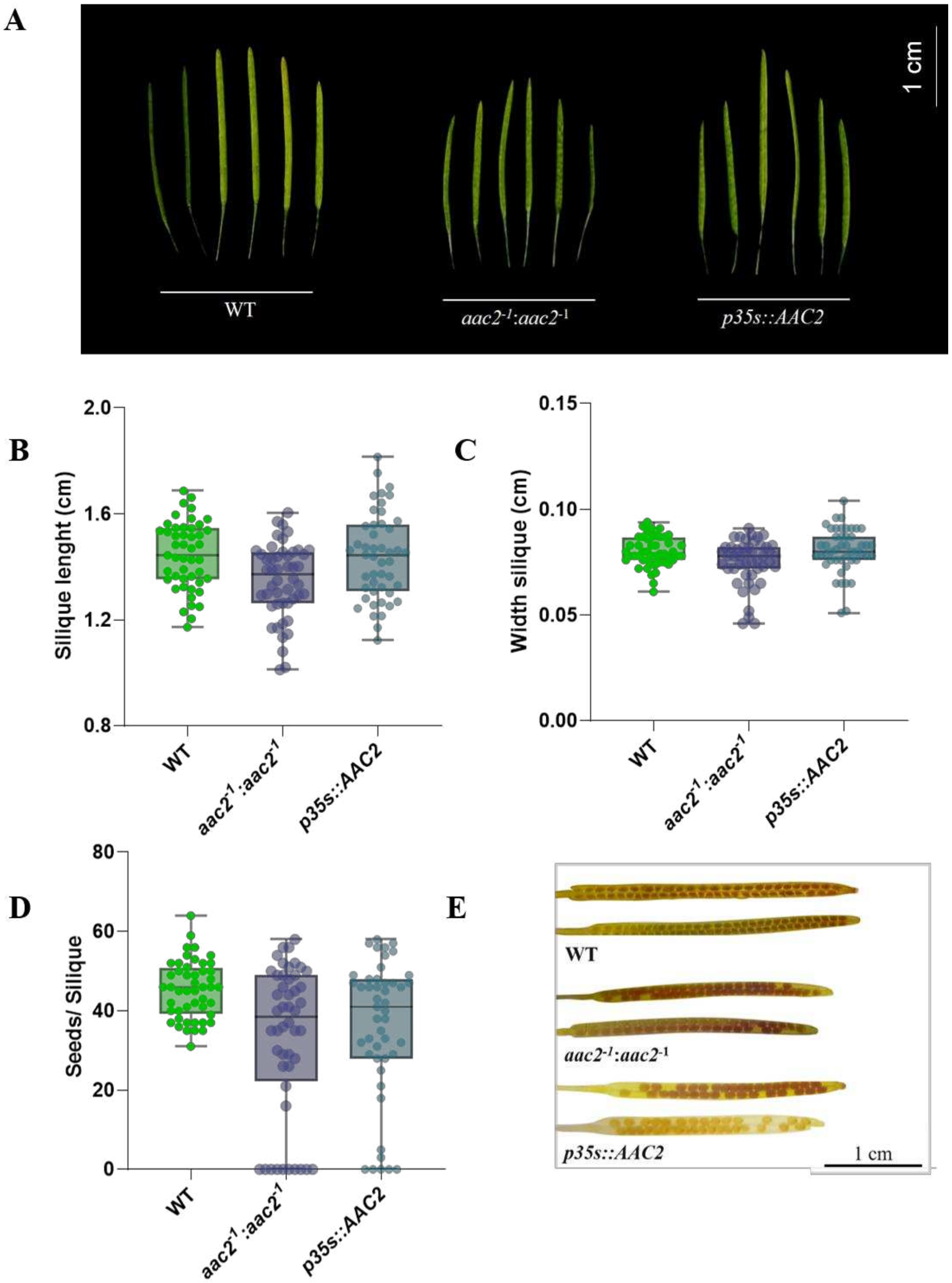


Figure 6. Impact of the decrease in *AAC2* expression on the reproductive stage of Arabidopsis. Visualization of the silique (A), silique length (B), silique width (C), and number of seed per silique (D). Silique was clarified in 0.2N NaOH solution and 1% SDS (E). Values represent the mean ± standard error of ten individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the wild type (WT).

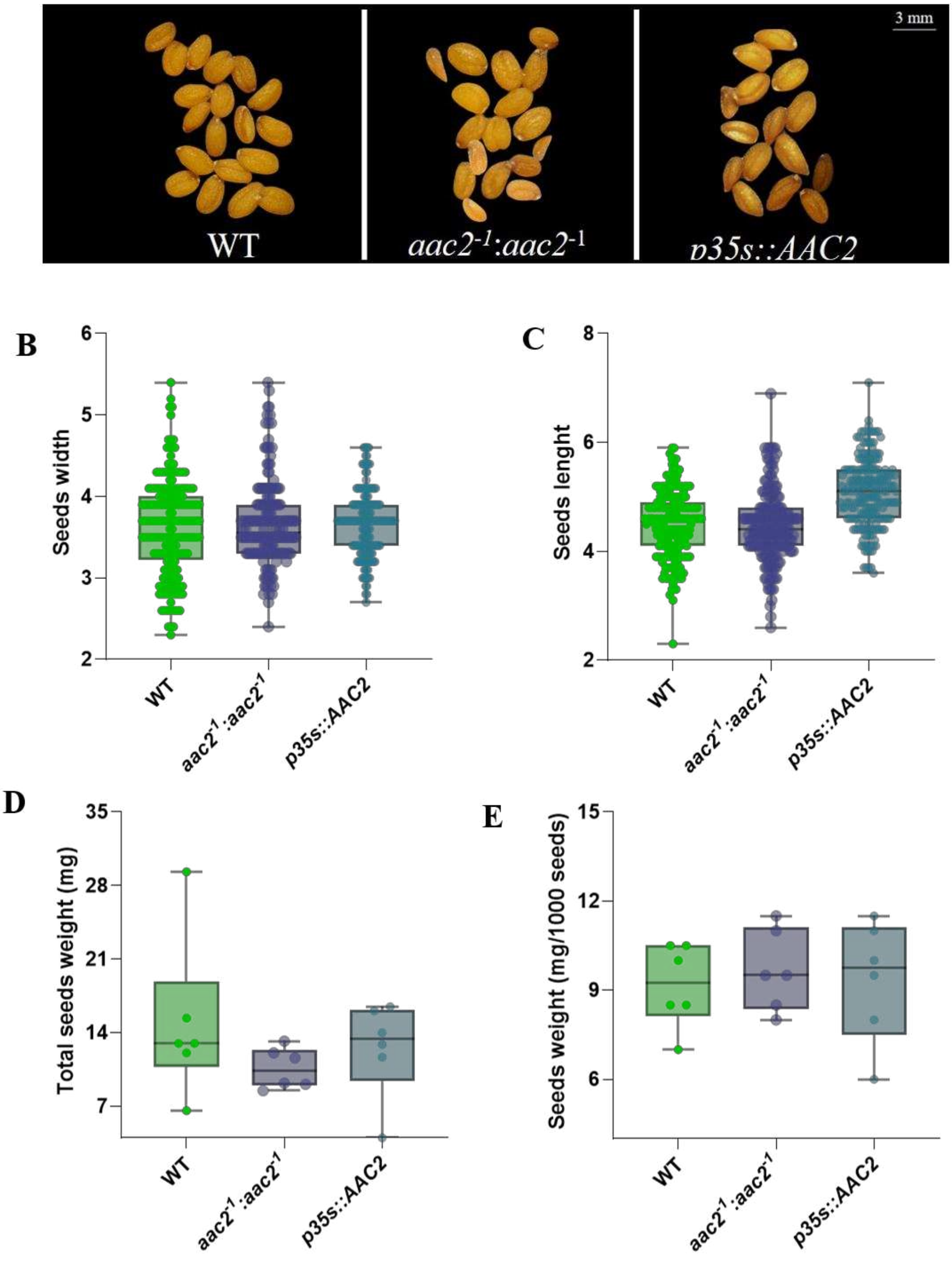


Figure 7. Impact of the decrease in *AAC2* expression on the seeds of Arabidopsis. Visualization of the seeds with lower expression *AAC2* lines and wild type (WT) (A). Seed width (B), silique lenght (C), total seeds weight (D), and seeds weigh of 1000 seeds (E). Values represent the mean ± standard error of ten individual plants. Asterisks indicate a significant difference ($P < 0.05$) according to Student's t-test, compared to the WT.

The *aac2* mutant and transgenic plants exhibited no significant differences in plant height (Sup. Figure 5A and B), although there was a trend toward an increase in the number of branches (Sup. Figure 5C), especially noticeable in the *p35s::AAC2* line. No changes were observed in the number of open flowers in the lines with reduced *aac2* expression (Sup. Figure 5C), although there was a trend towards an increase in the number of flower buds, which was only significant for the *aac2*$^{-1}$*:aac2*$^{-1}$ line (Sup. Figure 5C).

Discussion

AtAAC2 is important for energy turnover during seed soaking

The results presented here offer an enlightening perspective on the regulation of energy metabolism during seed germination, focusing on the important role of the mitochondrial adenylate transporter, AAC2. As seeds begin the soaking process, fluctuations in adenylate levels occur, highlighting the crucial importance of the efficient transport of these compounds for successful germination (Raveneau et. al., 2017). In lines deficient in *AAC2*, we observed a significant reduction in germination percentage and germination rate 24 hours after the test (Figure 2 A-D), indicating a direct association between the expression of *AAC2* and the efficiency of the germination process. This reduction can be attributed, in part, to the negative impact of low *AAC2* expression on energy homeostasis during the germination process (Figure 8).

During germination, the rapid resumption of mitochondrial biogenesis is vital for providing the energy needed immediately after seeds emerge from metabolic stasis (Raveneau et. al., 2017). The efficiency of this process depends largely on the efficient transport of adenylates from the mitochondria to the cytosol (da Fonseca-Pereira et. al., 2018). Adenylate transport is a key component in ensuring an adequate supply of ATP, which is essential for meeting the increased energy demands during germination. The efficient transfer of these energy compounds allows for a smooth transition of seeds from the inactive to the active germination state, sustaining initial growth and cell division. The fine regulation of genes related to adenylate transport emerges as a crucial strategy to ensure successful germination by promoting energy efficiency (da Fonseca-Pereira et. al., 2018).

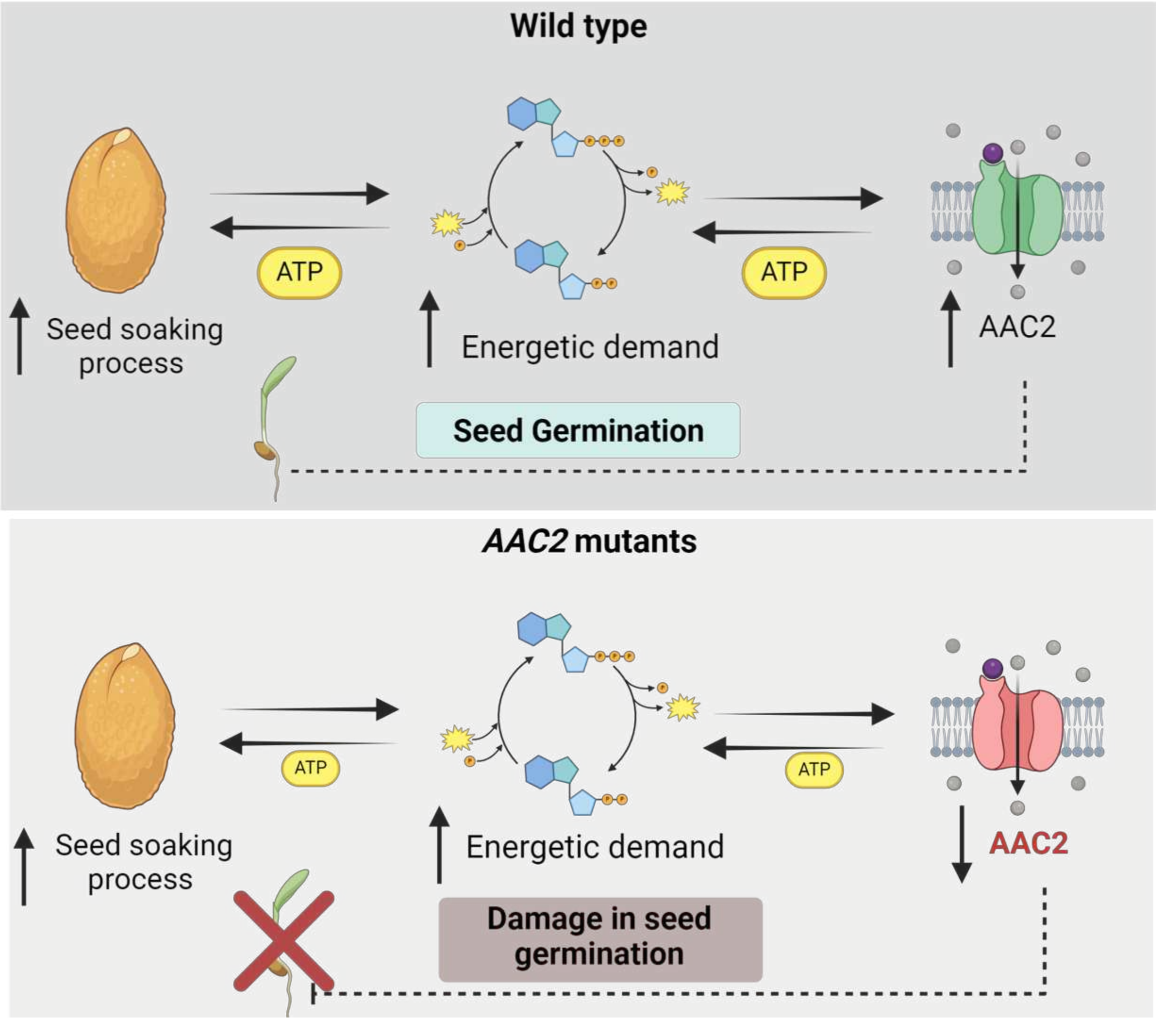


Figure 8. Illustrative comparison between wild-type (WT) and AAC2-deficient lines' seeds during germination. As seeds absorb water, fluctuations in adenylate levels underscore the importance of their efficient transport for successful germination. AAC2-deficient lines displayed a significant decrease in both germination percentage and rate, indicating a direct link between AAC2 expression and germination efficiency. This reduction may stem from *AAC2*'s impact on energy balance during germination.

Recent discussions have underscored the intricate link between hormones and energy metabolism during seed germination, with brassinosteroids emerging as key regulators. Mahati and Padmasree et al. (2023) elucidated the significant role of brassinolide (BL), a phytohormone belonging to the brassinosteroids class (BRs), in integrating the mitochondrial electron transport chain (mETC) and chloroplasts, thereby boosting the efficiency of the Calvin-Benson cycle (CBC) in *A. thaliana* cells. BL stimulates enzymatic activity or gene expression associated with the CBC and glucose-6-phosphate. Under restricted mETC conditions, BL induces the expression of genes linked to the malate valve and antioxidant systems, indicating its crucial role in coordinating chloroplasts and mitochondria to regulate cellular redox homeostasis.

In silico gene expression profiling revealed that *AAC2* expression is dramatically up-regulated 24h after soaking (Figure 1). A previous *in silico* heterologous expression study points to the up-regulation of mitochondrial adenylate transporters $AAC_{1\text{-}2}$ as important during seed germination in *A. thaliana* (da Fonseca-Pereira et. al., 2018). The reduction in germination percentage in lines with low expression of *AAC2* at establishment, after 24h of test establishment, coincides exactly with the increase in *AAC2* expression during soaking. Our results from the *in vivo* assay confirm a possible role for AAC2 during germination, corroborating the *in-silico* assays (Figure 1), and demonstrating that the mitochondrial adenylate transporter appears to play an important role during germination in *A. thaliana*.

AtAAC2 impact on respiration, photosynthetic pigment levels, and initial root growth

Mitochondrial oxidative phosphorylation is a crucial process for synthesizing energy molecules. AAC2, as a mitochondrial transporter protein, plays a vital role in facilitating energy turnover by exporting mitochondrial ATP to the cytosol. Our study reveals that deficiencies in *AAC2* expression can significantly impact fundamental physiological processes. Reduced *AAC2* expression resulted in a notable decrease in cellular respiration (Figure 1F), while having no discernible effect on photosynthetic parameters (Figure 1A - E). Similar outcomes were observed in mutant plants with low *AAC1* expression, suggesting a conserved role for AAC proteins in cellular respiration (See Sup. Figure 1, in Chapter 2 for further details). These findings are consistent with those reported by Palmieri et al. (2008), who reported that plants mutant for the *ADNT1* showed unchanged assimilation rates, as well as unchanged chloroplast electron transport and transpiration rates, but reduced root respiration (Palmieri et. al., 2008).

Most of the primary metabolites evaluated in leaves and roots did not show significant changes when *AAC2* expression was reduced. However, we observed an increase in the levels of photosynthetic pigments in lines with low *AAC2* expression (Figure 5A - D). The complexity of the impact of reducing the *AAC2* transporter on photosynthetic pigments, even though it is little expressed in leaves during the vegetative phase, suggests a variable response, influenced by various factors. The plant may activate compensatory mechanisms to maintain homeostasis and adequate photosynthetic function in the face of this reduction. It is important to note that although *AAC2* is less expressed in leaves during the vegetative phase (Figure 1), it is crucial to consider the multifaceted nature of proteins and transporters, which are often expressed in different organs or at different times in the plant's life cycle (da Fonseca-Pereira et. al., 2018; Nunes-Nesi et. al., 2020).

Initial root growth was impacted in plants with low *AAC2* expression (Figure 3A). As the roots develop, other adenylate transporters may compensate for the lack of AAC2, highlighting the possible importance of *AAC1* and *ADNT1*, which have high expression in root tissues (da Fonseca-Pereira et. al., 2018; Palmieri et. al., 2008). ADNT1 also plays a significant role in root growth, as evidenced by the significant reduction in root growth in ADNT1 mutant plants, associated with decreased respiration (Palmieri et al., 2008). These complex interactions between adenylate transporters highlight the plasticity and redundancy in the mechanisms regulating energy metabolism in plants, influencing their development at different stages of the life cycle.

The versatile impact of *AtAAC2* on energy dynamics and reproductive phase

Gene profile analysis revealed that *AAC2* exhibits higher expression levels in leaf stamens, mature pollen grains, and inflorescences, indicating its importance during the reproductive phase of plants when there is an increase in energy demands (Figure 1). Pollen formation, an energetically demanding process, relies exclusively on the mitochondria in developing pollen grains, as these lack differentiated plastids and amyloplasts (Selinski and Scheibe 2014).

The decrease in *AAC2* expression directly impacted cellular respiration, resulting in decreased respiration rates (Sup. Figure 1F). This reduction likely led to decreased oxidative phosphorylation, subsequently reducing the activity of the electron transport chain and potentially increasing the formation of ROS. Previously, we demonstrated that the reduction in *AAC1* expression in *A. thaliana* affected oxidative phosphorylation, adenylate levels, and mitochondrial vacuolar degeneration in root cells with high energy demand, leading to mitochondrial dysfunction (See Figures 2 C-F, Figure 4A, in Chapter 2 for further details). In pollen grains, mitochondrial dysfunction significantly influences their development, as discussed by Hanson and Bentolila (2004). The significance of AAC2 in mature pollen grains is further supported by the reduced expression of this transporter protein in mutant and transgenic lines. This reduction may have contributed to the significant decrease in silique filling (Figure

6D) and silique dimensions (Figure 6 A-B) observed in lines with reduced *AAC2* expression compared to the WT.

The production of shorter siliques can be attributed to dysfunctional gametophytes associated with low expression of *AAC2*. Previous results suggest that mutations in the energy-related gene succinate dehydrogenase 1 (SDH1) interrupt male gametophyte development (León et al., 2007). The mutation in *SDH1* drastically decreases ATP synthesis, leading to the death of pollen grains in the early stages of development. Additionally, ATP deficiency and high levels of ROS can cause mitochondrial dysfunction and subsequently programmed cell death (PCD). In studies on female gametophyte patterns, AAC2 is crucial for the coordinated development of gametic cells (Kägi et al., 2010). In the context of female gametophyte regulation, a mutation in the *AAC2* gene plays a vital role, highlighting its importance in mitochondrial integrity and the complex interaction between gametic and non-gametic cells associated with PDC (Kägi et al., 2010).

Our previous findings demonstrated that mutant plants with low expression of AAC1 exhibited a significant increase in yield, displaying larger silique sizes and enhanced seed formation compared to WT (see Figure 7 A-F, in Chapter 2 for further details). These results suggest that the decrease in *AAC1* expression might have triggered a compensatory upregulation of *AAC2* expression, which is already highly expressed during this stage, resulting in significant improvements in silique and seed development. In this study, we further elucidate these findings by demonstrating that a reduction in *AAC2* expression led to more pronounced yield losses, particularly affecting silique size, silique filling, and seed formation. These findings reinforce the notion of compensation between AAC1 and AAC2 in regulating plant yield.

In summary, our findings indicate that AAC2 is vital for regulating the reproductive phase in *A. thaliana*, affecting different stages of development, from germination to the growth of female and male gametophytes. This highlights the possible role of AAC2 for plant reproduction and vitality.

Conclusion

The results presented here reinforce the understanding of the important role of the mitochondrial transporter AAC2 in the regulation of different physiological processes in *A. thaliana*. From the modulation of seed germination to the marked influence on the reproductive phase, AAC2 emerges as a central element in the plant's energy balance and reproductive success. The results reveal that the dynamic expression of *AAC2* during soaking is strategically important, highlighting its direct participation in the transition between metabolic states and its direct influence on germination efficiency. Furthermore, the significant presence of AAC2 in reproductive organs highlights its relevance in the critical phases of reproduction, providing valuable information for a broader understanding of plant physiology.

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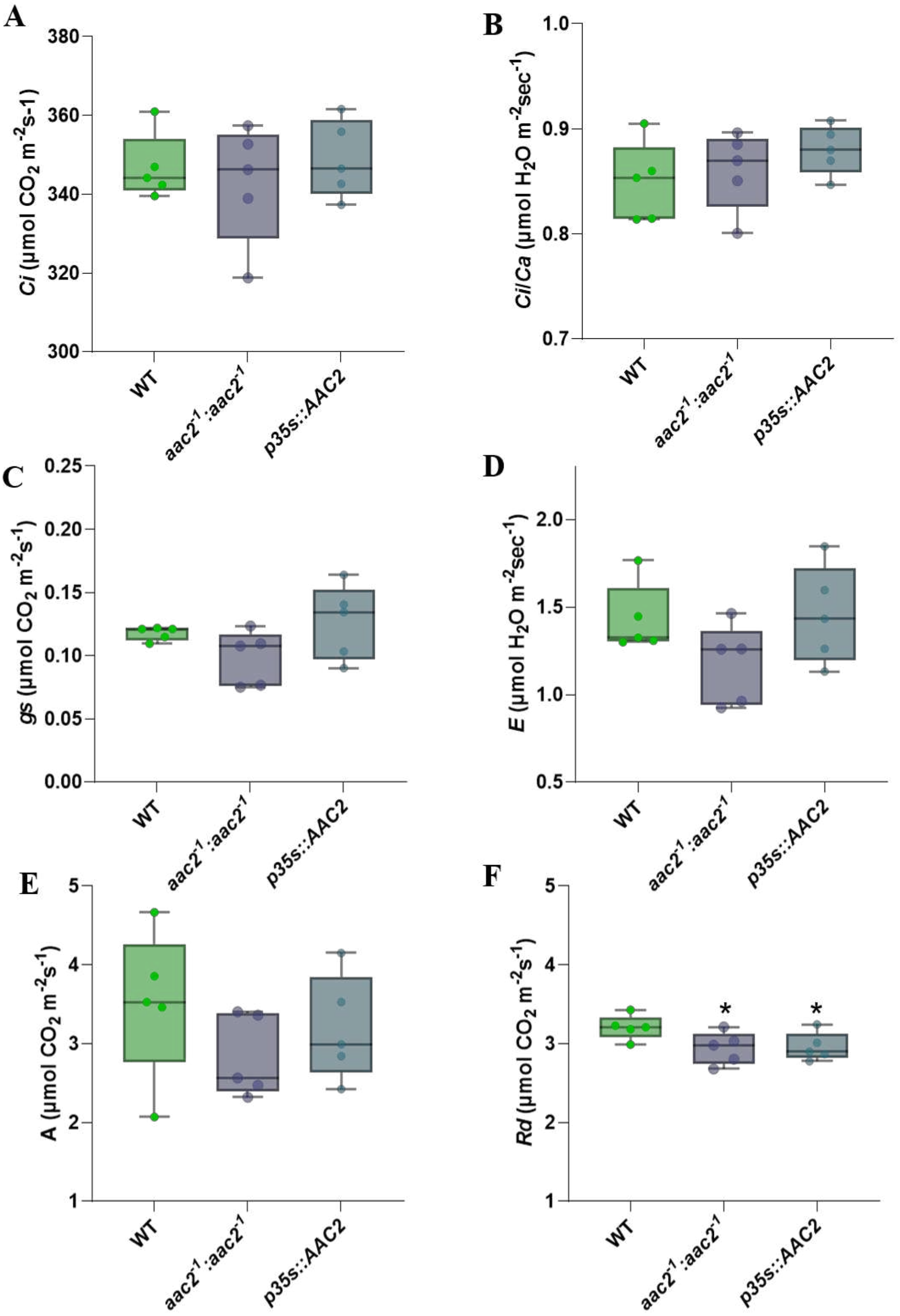
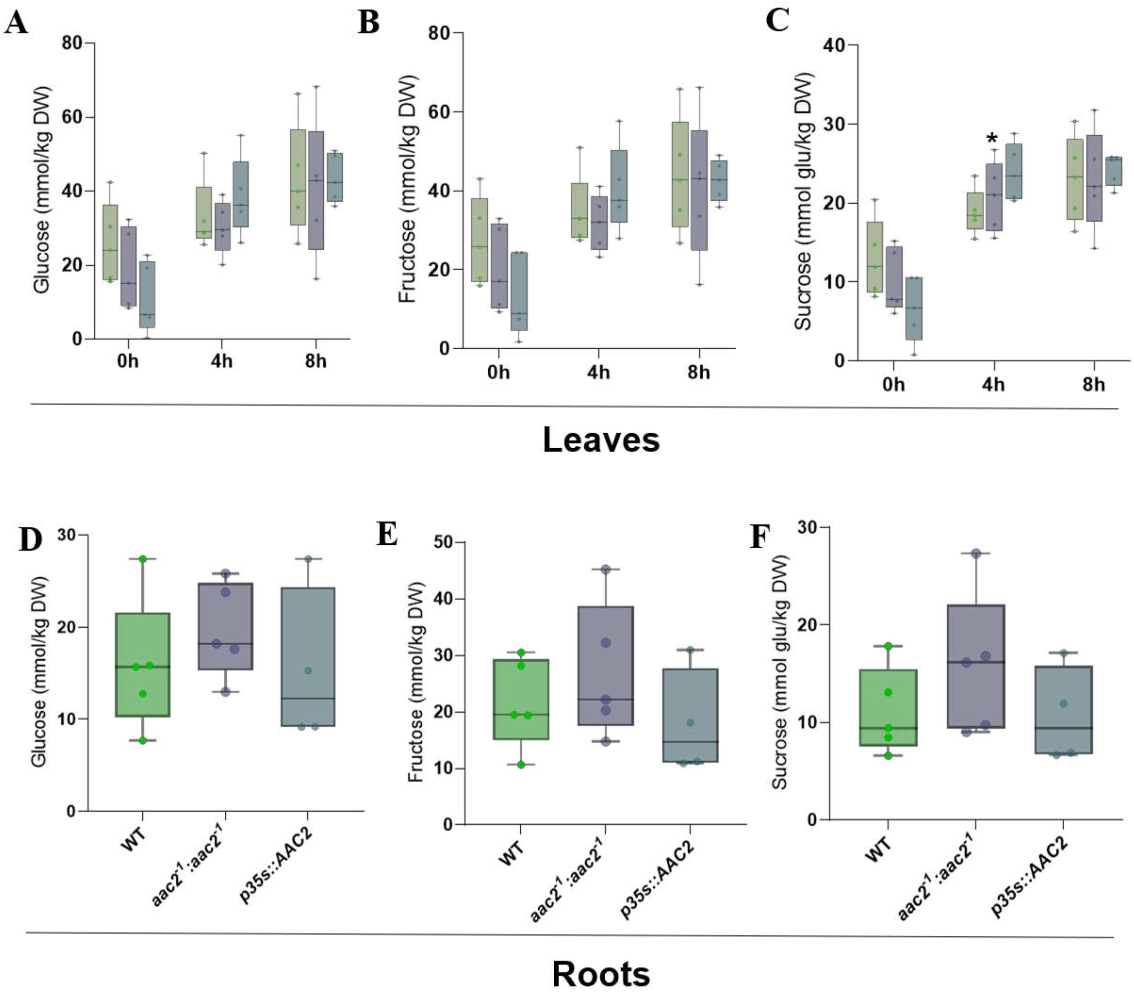
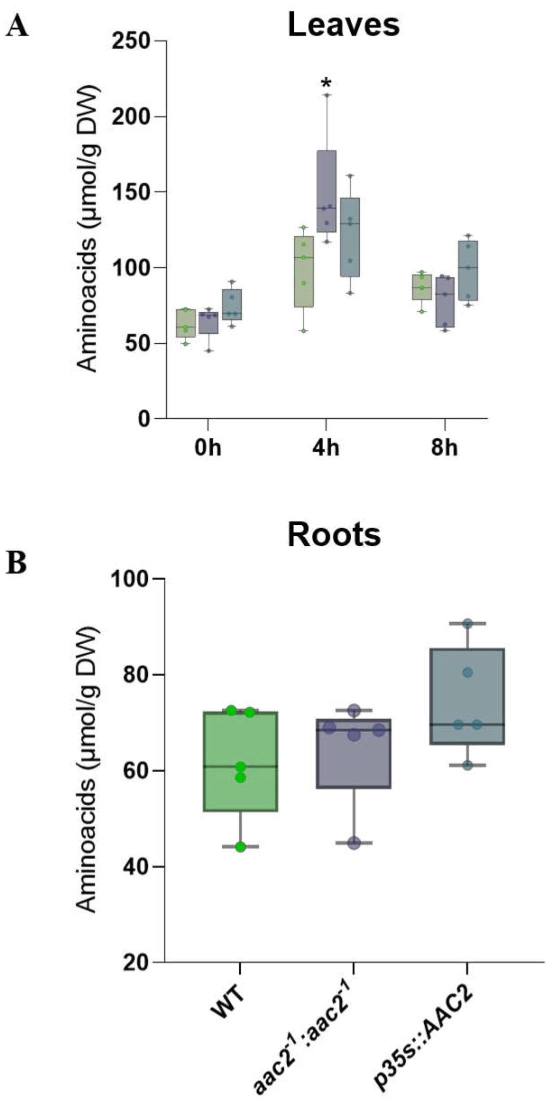
Material Supplemental



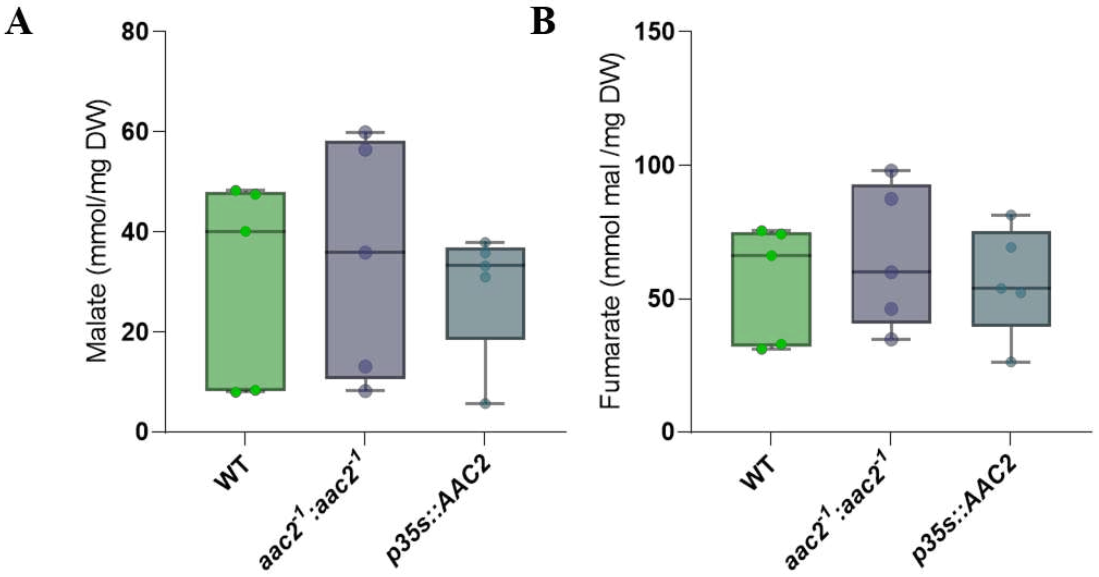
Sup. Figure 1. Gas exchange parameters in 6-week-old *Arabidopsis thaliana* genotypes deficient in *AAC2* and WT. Internal CO_2 concentration (C*i*) (A). The ratio between the intercellular CO_2 concentration of the leaf and that of the air (C*i*/C*a*) (B). Stomatal conductance (g_s) (C). Transpiration (*E*) (D). Assimilation rate (*A*) per area unit at 150 µmol photons m^2 s^{-1} (E). Dark respiration (R_d) (F). Values are presented as mean ± SE of five individual plants per line; an asterisk indicates values that were determined by *t*-test to be significantly different ($P < 0.05$) from the WT



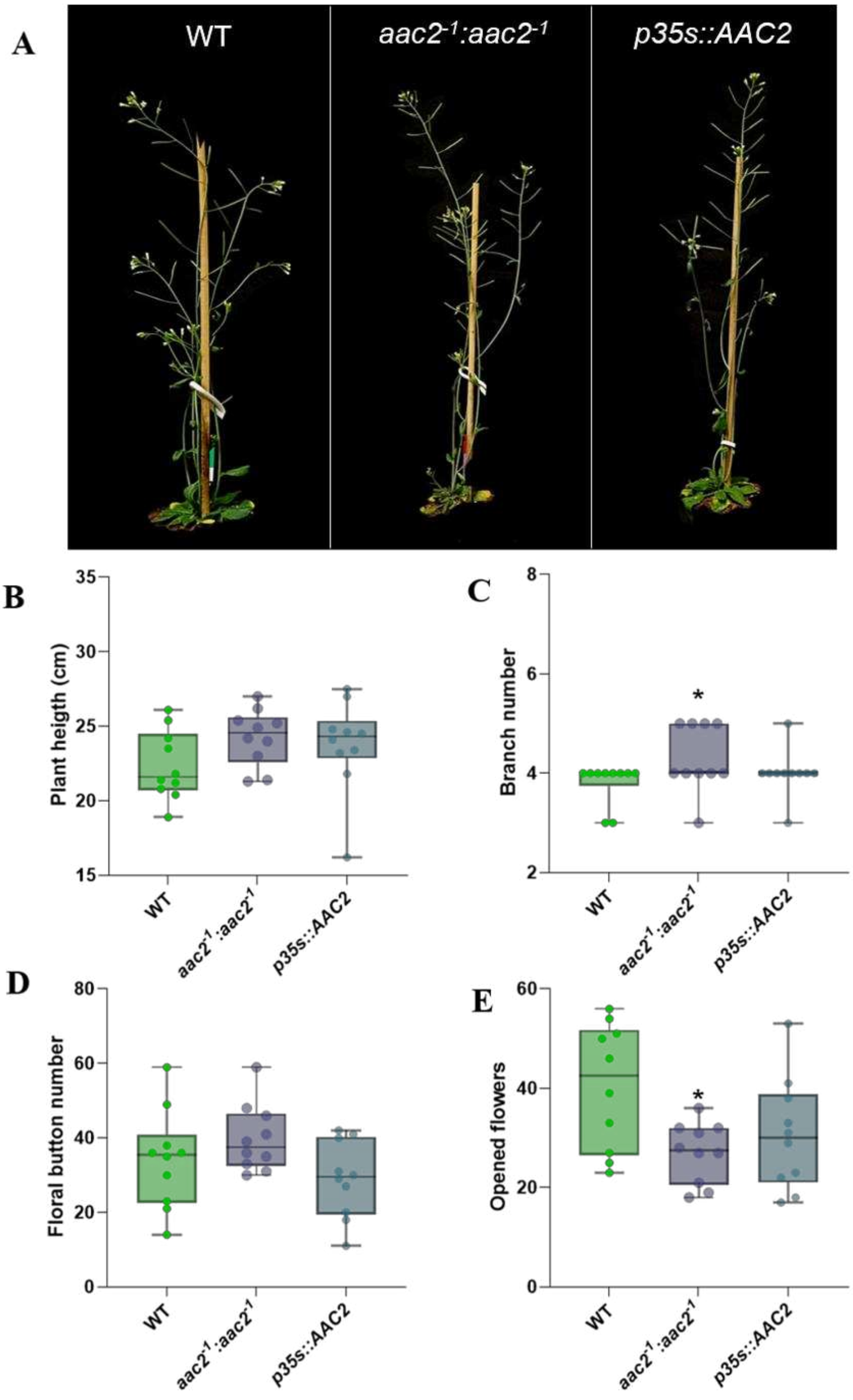
Sup. Figure 2. Soluble carbohydrate levels in leaves and roots at five weeks of age in plants with low expression of *AAC2* and WT. Glucose in leaves (A). Fructose in leaves (B). Sucrose in leaves (C). Glucose in roots (D). Fructose in roots (E). Sucrose in roots (F). Values are presented as mean ± SE of five individual plants per line; an asterisk indicates values that were determined by *t*-test to be significantly different ($P < 0.05$) from the WT.



Sup. Figure 3. Amino acids levels in leaves and roots at five weeks of age in plants with low expression of *AAC2* and WT. Amino acid levels in leaves (A). Amino acid levels in roots (B). Values are presented as mean ± SE of five individual plants per line; an asterisk indicates values that were determined by *t*-test to be significantly different ($P < 0.05$) from the WT.



Sup. Figure 4. Organics acid contents in roots at five weeks of age in plants with low expression of *AAC2* and WT. Malate (A). Fumarate (B). Values are presented as mean ± SE of five individual plants per line; an asterisk indicates values that were determined by *t*-test to be significantly different ($P < 0.05$) from the WT.



Sup. Figure 5. Impact on the phenotype at the reproductive stage of lines with low *AAC2* expression and WT plants. A. Visualization of the plants (A). Plant height (B). Number of branches (C). Number of open flowers (D). Number of flower buds (E). Values are presented as mean ± SE of ten individual plants per line; an asterisk indicates values that were determined by *t*-test to be significantly different ($P < 0.05$) from the WT.

GENERAL CONCLUSION

The experiments and data discussed here underscore the role of adenylate carriers in plant metabolism and physiology. These carriers not only facilitate energy transport within the cell but also contribute to a complex network displayed during stress responses. The study of mitochondrial carrier proteins under stress conditions reveals their regulation in response to environmental challenges. Proteomic analysis, *in silico* expression studies, and some *in vivo* assays provide evidence that not only the levels of respiratory metabolites but also the transport mediated by mitochondrial proteins are affected by stress. However, further *in vivo* studies are still needed to fully understand how transporter regulation occurs and its impacts on plant metabolism under abiotic stress conditions.

In our investigation, we demonstrated important role of AAC1 in oxidative phosphorylation in *A. thaliana*. Reduced expression of *AAC1* resulted in decreased respiration rates, particularly in roots, negatively impacting growth. Additionally, AAC1 influenced cellular energy homeostasis and ATP concentration, affecting mitochondrial morphology and root carbohydrate accumulation. Surprisingly, lower *AAC1* expression led to increased seed size and weight despite negative effects during the vegetative phase, suggesting its stage-specific functions.

Our findings emphasize the importance of AAC1 in oxidative phosphorylation, ATP production, and cellular energy homeostasis. However, there are still many unanswered questions regarding the regulation and function of this transporter, as well as the roles of other ADP/ATP transport isoforms in plant metabolism and development. Furthermore, our results shed light on the critical role of AAC2 in regulating various physiological processes in *A. thaliana*, from seed germination to the reproductive phase. Its dynamic expression during waterlogging suggests its strategic importance in metabolic state transitions and germination efficiency. Moreover, AAC2's significant presence in reproductive organs underscores its relevance in crucial phases of reproduction, offering valuable insights into plant physiology. Determining the biochemical parameters of AAC proteins in plants, such as Michaelis-Menten (Km) and maximum reaction velocity (Vmax) and substrate specificity, is crucial for understanding their metabolic and physiological roles. These parameters provide insights into substrate affinity, maximum transport capacity, and specificity of different isoforms of AACs and others adenylates carriers. Understanding these parameters can clarify how AACs contribute to plant energy metabolism and adenylate cycling, enhancing our understanding of energetic molecule transport from the mitochondria and plant metabolism.

In conclusion, our study enhances understanding of the pivotal role of mitochondrial adenylate carriers in plant metabolism, stress responses, and development. These carriers represent promising targets for future research aiming to improve plant performance under both optimal and challenging environmental conditions.