ROBERTO NERI DA SILVA

PHYSIOLOGICAL AND METABOLIC CHARACTERIZATION OF ADENYLATES CARRIERS IN *Arabidopsis thaliana*

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

Adviser: Adriano Nunes Nesi

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ABSTRACT

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, March, 2021. **Physiological and metabolic characterization of adenylates carriers in *Arabidopsis thaliana***. Adviser: Adriano Nunes Nesi.

Adenylate transporters play a vital role in animal and plant cells, connecting ATP production sites to consumption regions. So far, 16 of these transporters have been identified in plants. Although progress has been made in recent years regarding the understanding of the functions performed by these proteins, there are still gaps to be filled. In this context, this work initially presents a study of the role of these proteins in plant responses to a series of different types of stress. Here, using bioinformatics data, we demonstrate that the expression pattern of adenylate transporters is variable in different environmental conditions, suggesting that these genes have distinct and non-redundant functions in plants. In the second chapter of the thesis, we investigated the function of mitochondrial adenylate transporter ADNT1 in *A. thaliana* plants under flooding conditions. ADNT1 deficiency caused greater leaf loss and a lower maximum quantum yield of PSII electron transport. Stress responses occurred prematurely in ADNT1 deficient plants, due to increased levels of alanine and other stress marker metabolites, such as aspartate, trehalose, and inositol. We also observed increased expression of *SnRK1*, *ADK*, *APY*, *PDC1*, and *PPDK* genes, indicating that the disturbance in the adenylate pool caused by ADNT1 deficiency caused alterations in gene expression and metabolism related to stress responses. In the third chapter, The CRISPR / Cas9 system was used through the vector pKAMA-ITACHI (pKI1.1R) to obtain mutant plants to characterize the APC1 transporter in *A. thaliana*. We observed that APC1 mutant plants have a shorter silique length and a smaller number of seeds. Furthermore, APC1 deficiency led to the production of short roots and a reduction in seedling fresh weight in a medium without sucrose. Together, our results suggest that the APC1 transporter plays an important role in reproductive organs, and seedling development.

Keywords: ATP. Stress responses. Bioinformatic. CRISPR/Cas9.

RESUMO

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, março de 2021. **Caracterização fisiológica e metabólica de transportadores de adenilatos em *Arabidopsis thaliana*.** Orientador: Adriano Nunes Nesi.

Os transportadores de adenilatos desempenham um papel vital em células animais e vegetais, conectando os sítios de produção de ATP as regiões de consumo. Até o momento foram identificados 16 desses transportadores em plantas. Embora se tenha avançado nos últimos anos com relação ao entendimento das funções desempenhadas por essas proteínas, ainda existem lacunas a serem preenchidas. Neste contexto, este trabalho apresenta inicialmente um estudo do papel dessas proteínas nas respostas das plantas a uma série de diferentes tipos de estresse. Aqui, utilizando dados de bioinformática, demonstramos que o padrão de expressão dos transportadores de adenilatos é variável em diferentes condições ambientais, sugerindo que esses genes têm funções distintas e não redundantes nas plantas. No segundo capítulo da tese, investigou-se a função do transportador de adenilatos mitocondrial ADNT1 em plantas de *A.thaliana* sob condições de alagamento. As respostas ao estresse ocorreram prematuramente em plantas deficientes em ADNT1, devido ao aumento dos níveis de alanina e de outros metabólitos marcadores de estresse, como aspartato, trealose e inositol. Também observamos maior expressão dos genes *SnRK1, ADK, APY, PDC1 e PPDK,* indicando que a perturbação no pool de adenilatos causado pela deficiência de ADNT1 causou alterações na expressão gênica e no metabolismo relacionado as respostas ao estresse. No terceiro capítulo O sistema CRISPR / Cas9 foi utilizado por meio do vetor pKAMA-ITACHI (pKI1.1R) para obter plantas mutantes para caracterizar o transportador APC1 em *A. thaliana*. Nós observamos que as plantas mutantes APC1 apresentam um menor comprimento de síliquas e um menor número de sementes. Além disso, a deficiência de APC1 levou à produção de raízes curtas e redução do peso fresco em plântulas em meio sem sacarose. Juntos, nossos resultados sugerem que o transportador APC1 desempenha um papel importante nos órgãos reprodutivos, e desenvolvimento de plântulas.

Palavras-chave: ATP. Respostas a estresse. Bioinformática. CRISPR/Cas9.

SUMMARY

GENERAL INTRODUCTION

Plants are constantly submitted to unfavorable environmental conditions that impair growth and development. The stress caused by these situations can be divided into biotic stress, like pathogen infection and herbivory, and abiotic stress, such as drought, flooding, heat, cold, salinity, or toxic metals like aluminum (Zhu, 2016). Climate change increases the occurrence and time of these stresses, and it is expected that these effects grow in the next decades (Fedoroff et al., 2010). Since the world population continues to grow, increasingly difficult to produce food to sustain that growth, which is even more complicated in a climate change scenario, food security becomes one of the major issues to countries around the world (Mora et al., 2020). To guarantee food production in the near future is fundamental continuous research to reveal and understand the mechanisms of stress responses in plants.

Mitochondrial metabolism has been one of the main targets to understand and improve stress responses. A common consequence when plants are submitted to stress conditions is the production of reactive oxygen species (ROS) (Foyer and Noctor, 2013), which can cause oxidative damage to lipids, proteins, and nucleic acids (Mittler et al., 2011). The mitochondrion is a central site of ROS production but also acts neutralizing the oxidative damage by antioxidant mechanisms through mitochondrial ascorbate peroxidase (Chew et al., 2003) and glutathione peroxidase (Navrot et al., 2020). Also, in the ETC (Electron Transport Chain) the mitochondria play a significant role in suppressing ROS production by the activity of alternative oxidase (AOX), likely in conjunction with alternative NAD(P)H dehydrogenases (NDs) (Van Aken et al., 2009a). However, mitochondria also play roles in a variety of important cellular processes associated with carbon, nitrogen, phosphorus, and sulfur metabolism in plants (Jacoby et al., 2012). Connecting the metabolism by transmembrane proteins located in the inner membrane of the mitochondria (Palmieri et al., 2011b). Most of these transporters belong to a mitochondrial carrier family (MCF) (Picault et al., 2004a; Monné and Palmieri, 2014). Currently, 58 MCF members were identified in the *Arabidopsis thaliana* genome (Toleco et al.; Nunes-Nesi et al., 2020). Although MCF members have a large part of their structure highly conserved in different organisms (Palmieri et al., 2011b), they also exhibit high variability in the mode of transport, substrate, and specificity (Palmieri et al., 2008a). Among the MCF proteins, there are a group of transmembrane proteins called adenylate transporters that are present also in the plastid, peroxisome, endoplasmic reticulum, and plasma membrane (da Fonseca-Pereira et al., 2018). The adenylates (ATP, ADP, and AMP) are adenine-based nucleosides linked to phosphate groups playing a vital role in all living organisms, providing energy to various metabolic processes necessary for the maintenance of cellular homeostasis, both in optimal growth conditions and under stress conditions (da Fonseca-Pereira et al., 2018b).

At the dawn of the genomic era around the year 2000, the development and application of Bioinformatics techniques began, which from data generated by sequencing, transcriptional analysis, proteomic and metabolomic, allowed us to integrate these data in a way never seen before. In the last 20 years, the programs have become more robust and more operator-friendly, allowing a greater number of researchers to develop hypotheses, identify new genes, propose and investigate new functions of these genes.

In this work, we investigate the importance of adenylate transporters in plant development and stress responses. Through *in silico* expression analysis, we discussed the changes in the plant's energy metabolism and the relevance of adenylate transporters in different metabolic pathways. Moreover, we investigate the physiological role of the mitochondrial adenylate transporter ADNT1 in primary metabolism under waterlogging conditions. Additionally, we studied the role of adenylate transporter APC1 in the developmental stages of *A. thaliana.*

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CHAPTER I

Data-Mining Bioinformatics: Connecting Adenylate Transport and Metabolic Responses to Stress

Data-Mining Bioinformatics: Connecting Adenylate Transport and Metabolic Responses to Stress[1]

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HIGHLIGHTS

ATP is mainly produced inside the chloroplast and mitochondrion, and efficient adenylate transport systems are required for intracellular energy partitioning. Several adenine nucleotides carriers have been identified and their biochemical functions determined. However, their physiological roles under optimal and suboptimal conditions remain poorly investigated and, thus, poorly understood. The large amount of publicly available transcriptomic data coupled with improvements in high-quality microarray data in databases has emerged together with myriad tools to identify candidate genes not previously associated with a given biological question. The *in silico* survey of the expression pattern of adenine nucleotide carriers demonstrated that their expression is variable under different conditions, suggesting that specific adenylate carriers have distinct and nonredundant functions.

RESUME

Adenine nucleotides are essential in countless processes within the cellular metabolism. In plants, ATP is mainly produced in chloroplasts and mitochondria through photophosphorylation and oxidative phosphorylation, respectively. Thus, efficient adenylate transport systems are required for intracellular energy partitioning between the cell organelles. Adenylate carriers present in different subcellular compartments have been previously identified and biochemically characterized in plants. Here, by using data-mining bioinformatics tools, we propose how, and to what extent, these carriers integrate energy metabolism within a plant cell under different environmental conditions. We demonstrate that the expression pattern of the corresponding genes is variable under different environmental conditions, suggesting that specific adenylate carriers have distinct and nonredundant functions in plants.

Why Study Adenylate Carriers in Plants?

ATP is the major molecule related to energy transfer. In addition to ATP, other adenine (see Glossary) nucleotides have pivotal roles in both the metabolism and physiology of plants (Haferkamp et al., 2011). Remarkably, the distribution of cellular adenylate pools is adjusted according to adenylate metabolism, which includes de novo synthesis in plastids and mitochondria, catabolism in the cytosol and peroxisomes, and the salvage pathway in cytosol (Zrenner et al., 2006; Ashihara et al., 2018). Moreover, the equilibrium between ATP, ADP, and AMP optimizes ATP synthesis in plant mitochondria and chloroplasts (Roberts et al., 1997; Igamberdiev and Kleczkowski, 2006; Gout et al., 2014; Raveneau et al., 2017), and their ratios have been proposed to be key factors for metabolic regulation (Igamberdiev and Kleczkowski, 2009). Many adenine nucleotide carriers present in several subcellular compartments have been systematically identified and characterized at the biochemical level in land plants (Figure 1 and Table 1) and other organisms. With the exception of the plastidic nucleotide transporters 1 and 2 (NTT1- 2), which are ATP/ADP transporters that belong to a phylogenetically unrelated family of plastidic nucleotide transporters (NTT-type) (Schmitz et al., 2010a; Schippers et al., 2015), all adenylate carriers identified so far in plants are members of the mitochondrial carrier family (MCF) (Picault et al., 2004a). Many MCF proteins are located in other cell compartments, such as peroxisomes and chloroplasts, where they also have pivotal roles. For instance, an adenine nucleotide transporter in the endoplasmic reticulum (ER) membranes of *Arabidopsis thaliana* (ER-ANT1) was demonstrated to be essential for growth, as well as for the accumulation of ER-related storage lipids and proteins (Leroch et al., 2008; Hoffmann et al., 2013) and photorespiratory metabolism (Hoffmann et al., 2013). In most cases, reverse genetics approaches have been used to elucidate the physiological significance of genes encoding adenylate carriers in Arabidopsis (Reiser et al., 2004; Kirchberger et al., 2008; Leroch et al., 2008; Palmieri et al., 2008b; Schmitz et al., 2010b; Yin et al., 2010; Rieder and Neuhaus, 2011; Hoffmann et al., 2013). However, there is still the major challenge of determining their expression patterns and physiological roles in plant metabolism. The simultaneous presence of different adenylate carriers within a cell compartment, their possible overlapping functions, and fluctuating metabolite concentrations under different physiological conditions are the main reasons behind this limitation (Picault et al., 2004a; Palmieri et al., 2011a).

Given the importance of adenylate carriers, coupled with the lack of information concerning the role of their encoding genes, here we used transcriptional responses of adenylate carrier genes in plants growing under optimal and/or adverse conditions to propose their roles in metabolic and physiological functions in Arabidopsis. To this end, we first performed a systematic *in silico* survey of the expression of adenylate carriers encoding genes in response to different abiotic stress conditions by collecting information derived from public available microarray data sets.

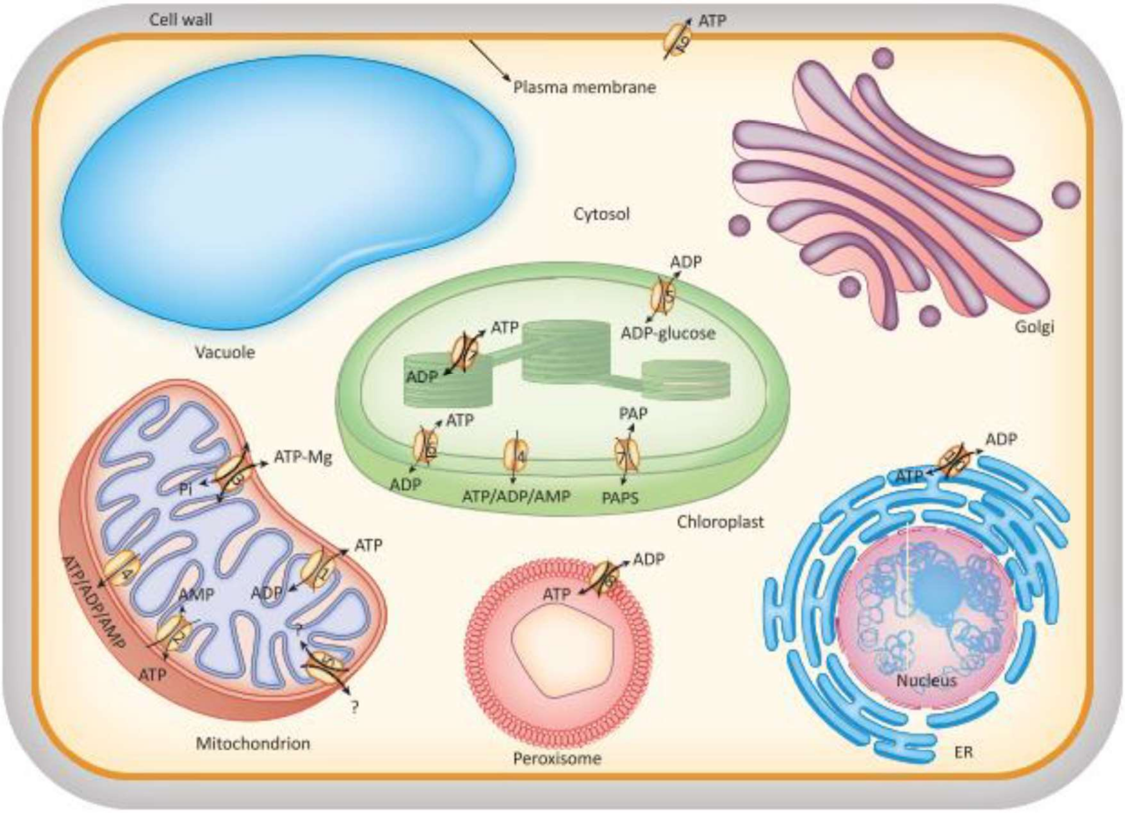

Figure 1. Model Illustrating the Adenine Nucleotide Carriers Identified in Membranes from Plant Cells. Carriers localized in the inner membrane of mitochondrion: (1) AAC1-3, ADP/ATP carriers; (2) ADNT1, AMP/ATP carrier; (3) APC1-3, ATP-Mg/Pi carriers; (4) AtBT1, *Arabidopsis thaliana* Brittle1 transporter suggested to export ATP/ADP/AMP from mitochondria to the cytosol; and (5) ZmBT1, Zea mays Brittle1 transporter, the substrate and transport mode of which are unclear at mitochondria, as indicated by the question mark (?). Carriers localized in plastids: (4) AtBT1, ATP/ADP/AMP carrier; (5) ZmBT1, ADP-Glucose/ADP carrier of maize (*Z. mays*); (6) NTT1-2, ATP/ADP carriers; (7) TAAC/PAPST1, a PAPS/PAP carrier in plastids and an ATP/ADP carrier in the thylakoid membrane. The other carriers are localized in peroxisome (PNC1-2), plasma membrane (PM-ANT1), and endoplasmic reticulum (ER-ANT1), numbered from 8 to 10, respectively. The transporters PNC1-2, PM-ANT1, and ER-ANT1 perform the counter exchange of ATP with AMP or ADP, the export of ATP, and the exchange of ATP/ADP, respectively. Both the form and transport direction of adenylates shown here are in accordance with the production and consumption sites of these molecules under optimum conditions of metabolism. Abbreviations: ER, endoplasmic reticulum, PAP, 50-phosphoadenosine 30-phosphate; PAPS, phosphoadenosine 50-phosphosulfate. For additional definitions, please see the main text.

Table 1. Adenylate carriers identified in plants [a,b]

Carrier	AGI/Accession	Location	Substrates	Mode of transport	Reverse genetic approach	Refs
AAC1	At3g08580	Mitochondria	ATP⇔ADP	Antiporter	No	[23]
AAC2	At5g13490	Mitochondria	ATP⇔ADP	Antiporter	No	[23]
AAC3	At4g28390	Mitochondria	ATP⇔ADP	Antiporter	No	[23]
APC1	At5g61810	Mitochondria	ATP-Mg⇔Pi	Antiporter	No	[24–26]
APC2	At5g51050	Mitochondria	ATP-Mg⇔Pi	Antiporter	No	[25,26]
APC3	At5g07320	Mitochondria	ATP-Mg⇔Pi	Antiporter	No	[24–26]
ADNT1	At4g01100	Mitochondria	ATP⇔AMP > ADP	Antiporter	Yes	[15]
*At*BT1	At4g32400	Plastid and mitochondria	ATP/ADP/AMP⇒	Uniporter	Yes	[16,88,89]
*Zm*BT1	BT016796	Plastid and mitochondria[c]	ADP-glucose⇔ADP	Antiporter	No	[88,100]
TAAC/PAPST1	At5g01500	Plastid	PAPS⇔PAP	Antiporter	Yes	[74,75]
		Thylakoid	ATP⇔ADP			
NTT1	At1g80300	Plastid	ATP⇔ADP	Antiporter	Yes	[9,10]
NTT2	At1g15500	Plastid	ATP⇔ADP	Antiporter	Yes	[9,10]
PNC1	At3g05290	Peroxisome	ATP⇔ADP	Antiporter	Yes	[27,28]
PNC2	At5g27520	Peroxisome	ATP⇔ADP	Antiporter	Yes	[27,28]
ER-ANT1	At5g17400	Endoplasmic reticulum	ATP⇔ADP	Antiporter	Yes	[12,13]
PM-ANT1	At5g56450	Plasma membrane	ATP⇒	Uniporter	Yes	[17]

[a] Only transporters the primary function of which is the transport of adenylates have been included.

[b] Other transporters, such as plastidic NAD^+ transporter (AtNDT1) (Palmieri et al., 2009), mitochondrial NAD^+ transporter (AtNDT2) (Palmieri et al., 2009), and peroxisomal NAD^+ carrier (AtPXN) (Agrimi et al., 2012; van Roermund et al., 2016), also have the ability to transport adenylates as secondary substrates.

[c] Given that mitochondria do not synthesize starch, ZmBT1 is likely involved in processes other than transporting ADP-glucose for starch biosynthesis (Bahaji et al., 2011b).

Considering that plant responses to abiotic stresses are often controlled by complex regulatory networks, we also analyzed how, and to what extent, the expression patterns of adenylate carriers are associated with other metabolic pathways. Furthermore, we discuss novel findings regarding the regulation of adenylate carriers and present compelling evidence suggesting that the proper expression of adenylate carriers is pivotal for transcriptional responses of genes associated with metabolic responses to abiotic stresses. In parallel, we list adenylate carrier candidate genes that may be applied to investigate stress-responsive genes in plants. We conclude by highlighting the questions that need addressing to formally clarify the dynamics of the complex network connecting adenylate transporters and how they are integrated at the cellular level to the different metabolic processes under constraining environmental conditions.

Expression Patterns of Adenylate Carriers Encoding Genes in Response to Different Stress Conditions

Plants face constantly fluctuating environments that are often unfavorable or stressful for growth and development (Simontacchi et al., 2015; Zhu, 2016). Plants also differ from other organisms in having a more extensive subcellular compartmentation, which requires additional intracellular transport activities for efficient adenylate exchange (Geigenberger et al., 2010). Abiotic stresses induce multiple responses resulting in changes in proteins, metabolites, and transcripts (Urano et al., 2010). For instance, several MCF proteins, including ADP/ ATP carrier 3 (AAC3) (Haferkamp et al., 2002), ATP-Mg/phosphate carrier 1 (APC1) (Lorenz et al., 2015; Monné et al., 2015; Monné et al., 2017), and peroxisomal adenine nucleotide carrier 2 (PNC2) (Arai et al., 2008; Linka et al., 2008), are among the most stress-responsive transcripts encoded by the Arabidopsis proteome (Van Aken et al., 2009b; Palmieri et al., 2011a). Thus, a rearrangement of adenylate carrier expression levels is expected under suboptimal conditions. Therefore, a complete understanding of the mechanisms associated with the responses of adenylate carriers to stress conditions appears crucial for enhancing plant tolerance to abiotic stress and, thus, the identification of genes associated with stress adaptation.

Transcriptional profiling using microarrays and/or RNA-seq data has recently developed into one of the most prominent tools for functional genomics and, as such, is a powerful approach for gene function prediction and hypothesis generation (Dopazo and Aloy, 2006; Usadel et al., 2009; Araújo et al., 2012b). Although transcriptome analysis has not been applied in studies using adenylate carrier mutants to date, the large amount of high-quality microarray data in public databases has emerged together with myriad computational data-mining tools (e.g., Genevestigator, Bio-Analytic Resource for Plant Biology, and ArrayExpress) (Brazma et al., 2003; Zimmermann et al., 2004; Toufighi et al., 2005). The incorporation of these various bioinformatics tools has been used to extract relevant information from those large-scale data sets (Toufighi et al., 2005; Hruz et al., 2008; Less and Galili, 2008; Cavalcanti et al., 2014;

Kolesnikov et al., 2015; Tohge et al., 2015), allowing a survey by bait genes (in this case adenylate carries) in a range of environmental conditions. Thus, here we used available transcriptomic data to generate hypotheses concerning the impact of abiotic stresses on the expression level of adenylate carriers in Arabidopsis.

The Significance of Adenylate Carriers Under Hypoxia

Under environmental conditions in which O_2 levels decrease to values lower than those required to sustain oxidative phosphorylation, most ATP production is mediated by glycolysis in the cytosol (Loreti et al., 2016). Therefore, adenylate transport should be coordinated to provide the energy distribution required for a suitable response following hypoxia. Interestingly, it appears that the expression of most adenylate carriers significantly changes during the early phase of hypoxia (see Figure S1A in the supplemental information online). By contrast, the long-term maintenance at low oxygen concentrations and the posterior recovery resulted in major changes in the expression pattern of adenylate carriers. Under these conditions, *AAC3*, *APC2* (Lorenz et al., 2015; Monné et al., 2015; Monné et al., 2017), *PNC1–2* (Arai et al., 2008; Linka et al., 2008), and *ER-ANT1* were upregulated (see Figure S1A in the supplemental information online), while most of the genes, including *AAC1–2* (Haferkamp et al., 2002), were downregulated under hypoxic conditions.

Clear contrasting expression profiles of the three AAC genes after seed imbibition (Box 1) and under cellular oxygen deficiency (see Figure S1A in the supplemental information online) were noticeable. Transcripts of *AAC1* and *AAC2* were induced, while *AAC3* decreased its expression after imbibition (~20-fold) compared with the expression observed in dry seeds. At the same time, *AAC3* was upregulated, while *AAC1–2* was downregulated after 1 h of hypoxic treatments and following recovery. These results indicate that, under the conditions described above, *AAC3* is expressed oppositely in relation to *AAC1* and *AAC2*. Studies using yeast (*Saccharomyces cerevisiae*) as a model demonstrated that *yAAC1* and *yAAC2* are expressed under aerobic conditions, whereas *yAAC3* is specifically expressed under anaerobic conditions (Kolarov et al., 1990; Visser et al., 1994; Klingenberg, 2008). Therefore, *yAAC3* is suggested to act in reverse order, supplying ATP from glycolysis to the mitochondria (Visser et al., 1994; Klingenberg, 2008). Thus, it appears plausible to assume that, in plants, similar to the observations in yeast, *AAC3* has an alternative role with respect to the other two AAC isoforms. Noteworthy, *AAC3* was upregulated after 9 h of hypoxia and even more after reoxygenation (see Figure S1A in the supplemental information online), suggesting that the induction of this mRNA is required for both hypoxia and following recovery from oxygen deficiency. Interestingly, promoter sequence analysis of ADP/ATP carriers in yeast demonstrated that the oxygen repression of *yAAC3* is mediated through the expression of the transcription factor *ROX1* (ŠABOVÁ et al., 1993), a heme-dependent repressor of hypoxic genes in yeast (Zitomer and Lowry, 1992; Zhang and Hach, 1999; Zhang et al., 2017). Moreover, *yAAC2*, which encodes the major mitochondrial ADP/ATP carrier in yeast, is controlled at the transcriptional level by oxygen and heme and also by the HAP2 transcription factor (Betina et al., 1995). Given the differential expression pattern of *AAC1–3* under hypoxia, it seems reasonable to suggest that, as observed in yeast, the expression of AACs in plants is probably specifically controlled by transcription factors related to hypoxia conditions.

In addition to *AAC3*, *APC2* showed the highest expression under hypoxic stress and following recovery (see Figure S1A in the supplemental information online). Hypoxic conditions are associated with the loss of oxidative phosphorylation and altered mitochondrial respiration (Rocha et al., 2010). Deficient oxidative phosphorylation in the mitochondria might be counter-balanced by ADP/ATP and ATP-Mg/Pi carriers, which could provide the mitochondria matrix with ATP (Traba et al., 2009a; Laco et al., 2010; Stael et al., 2011). Thus, the electrogenic exchange of ATP^{4-} for ADP^{3-} carried out by AAC3 would preserve the mitochondrial membrane potential by a reversal of the ATP synthase (Traba et al., 2009a; Stael et al., 2011) and, along with the electroneutral exchange of ATP-Mg for Pi by APC2, would also contribute to the generation of membrane potential through the proton pumping coupled to the hydrolysis of ATP (Traba et al., 2009a; Stael et al., 2011) under oxygen deficiency. Moreover, the fact that the APCs contain N-terminal EF-hand calcium (Ca^{2+})-binding domains (Monné et al., 2015), which are found in a collection of 'calcium sensor' proteins, some of which with a demonstrated role in oxygen deprivation (Lokdarshi et al., 2016), also suggest that APC is part of the core of hypoxia-response proteins in plants. Altogether, these results indicate the existence of a functional interplay (Traba et al., 2009a) between ADP/ATP and ATP- Mg/Pi carriers in plants, as well as in yeast, which is most likely involved in ATP import into mitochondria during oxygen deprivation.

The induction of ER-ANT1 and PNC1-2 might be associated with signaling events caused by oxygen depletion. It is well known that eukaryotic cells suffering oxygen depletion display an immediate increase in free cytosolic Ca^{2+} released by channels from the plasma membrane or storage compartments (tonoplast, ER, or mitochondria) (Subbaiah et al., 1994; Hetherington and Brownlee, 2004; Dodd et al., 2010; Wang et al., 2016). Accordingly, in vitro experiments showed that intracellular Ca^{2+} release is coupled to an increase of ATP within the ER (Vishnu et al., 2014). Such findings point to the likely involvement of ER-ANT1 in mediating ATP transport into the ER lumen under stress and specifically under oxygen deficiency. In this vein, recent studies demonstrated that increased cytosolic $[Ca^{2+}]$ can be detected by peroxisome-located Ca^{2+} sensor proteins, such as calcium-dependent protein kinases (CDPKs) (Costa et al., 2010; Corpas, 2018; Corpas and Barroso, 2018), suggesting that Ca^{2+} signaling is also involved in a transduction cascade that affects ER-ANT1, PNC1, and PNC2. In summary, the close co-expression profile of these genes encoding peroxisome (*PNC1–2*), ER (*ER-ANT1*) and mitochondrial (*AAC3* and *APC2*) proteins suggest that these transporters are regulated by common mechanisms under hypoxia.

Box 1. Role of Adenylate Carriers during Seed Germination Process

A range of processes occurs during seed germination, including changes associated with the hydration of tissues, which are normally characterized by the production of large amounts of ROS (Wojtyla et al., 2016). Therefore, seed germination success may be closely associated with internal ROS levels (Bailly et al., 2008), being similar to other environmental stresses in this aspect.

Once water uptake by the seed is stable, metabolism is reactivated and energy demands increase to support reserve mobilization, organelle repair (Narsai et al., 2011), and alterations in transcripts, proteins, and metabolites (Weitbrecht et al., 2011). Furthermore, fluctuations in the content of different adenylates during the stages of seed imbibition are observed [6]. Interestingly, there is a large transcript abundance for genes involved with transport functions during early germination (Howell et al., 2009; Narsai et al., 2011). Moreover, given the importance of AMP, ADP, and ATP for seed germination capacity (Raveneau et al., 2017), the participation of adenylate carriers in different organelles might be fundamental for the supply of ATP-utilizing systems during germination and seedling development.

Given the association between adenylates and seed germination, we performed a temporal expression analysis of adenylate carrier genes following seed imbibition. Four genes (*AAC1*, *AAC2*, *NTT1*, and *NTT2*) were dramatically upregulated, while three other genes (*ADNT1*, *AtBT1*, and *APC2*) showed transient increases in their transcript level after imbibition (Figure I). Four of the most expressed adenylate carrier genes of the mitochondrion (*AAC1–2*) and plastids (*NTT1–2*) increased their expression level after imbibition. The fact that plastidic ATP/ADP transport activity exerts significant control over lipid synthesis for Arabidopsis seed development (Reiser et al., 2004) explains, at least partially, the upregulation of NTT expression after imbibition. Moreover, plastidial protein biosynthesis also requires a significant amount of ATP (Allen, 2015), which is probably imported by NTT from the cytoplasm. Notably, the higher similarity between the expression profile of the two plastidic ATP/ADP carriers after imbibition supports previous observations that these transporters exhibit similar biochemical and molecular features (Reiser et al., 2004). By contrast, a divergent expression profile between AAC genes was observed; whereas *AAC3* showed strong downregulation during germination, especially at 48 h after imbibition, *AAC1* and *AAC2* showed strong induction. This expression pattern suggests that, due to duplication gene events, *AAC1* and *AAC2* maintain similar functions (sub functionalization) displaying overlapping functions, while *AAC3* has an alternative role (neofunctionalization) during the early seed imbibition phase.

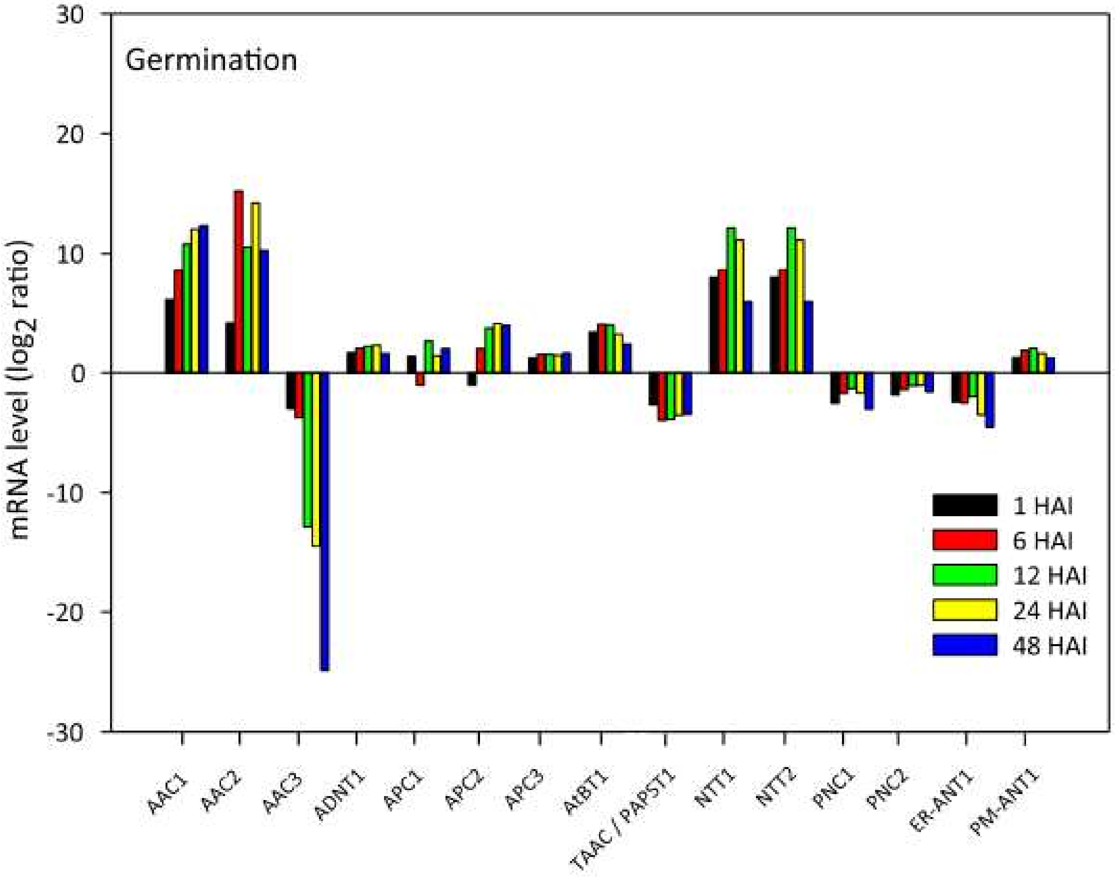

Figure I. Expression Analysis of *Arabidopsis thaliana* Genes Encoding Adenylate Carriers during Seed Germination. The time points selected were from 1 to 48 h after imbibition (HAI). Gene expression data were obtained from microarray data stored in the GENEVESTIGATOR database (Zimmermann et al., 2004). The data set was obtained from the Gene Expression Omnibus (GEO) within the National Centre for Biotechnology Information (NCBI) database or from the MIAME ArrayExpress database (www.ebi.ac.uk/arrayexpress/). The conditions tested are according to Narsai et al. (Narsai et al., 2011). All original microarray data files have been deposited to the GEO at the NCBI under accession number GSE30223. Gene expression values were normalized to dry seed data, converted to log2, and are shown on the y axis. For a complete description of the gene names, see Table 1 in the main text. Data Set III in the supplemental information online shows the differences in gene expression data obtained in stress experiments as revealed by Student’s t-test at *P* <0.05. For definitions of gene symbols, please see the main text.

Arsenic Toxicity Impacts Transcript Levels of Adenylate Carriers

Arsenic toxicity is a consequence of the competition between arsenate (V) and/or arsenite (III) with phosphate during phosphorylation reactions, resulting in negative impacts on energy metabolism (Finnegan and Chen, 2012). Upon arsenic exposure, there is a disturbance in the plant energetic status, with a dramatic reduction in the cellular efficiency to produce ATP [64] despite the concomitant increased demand for ATP (Srivastava et al., 2011; Srivastava et al., 2013). Thus, it appears reasonable to anticipate a differential regulation of adenylate carriers to provide energy for the reactions related to As detoxification. In agreement with this assumption, two mitochondrial ADP/ATP carriers (AAC2-3) were upregulated, with a more pronounced expression for *AAC3* at both arsenate concentrations analyzed (see Figure S1B in the supplemental information online). It was previously demonstrated that ATP synthesis is negatively affected in mitochondria under As treatment (Finnegan and Chen, 2012). By contrast, it has been postulated that the remaining ATP in mitochondria is enough to be exported and to be used, for example, in the biosynthesis of the antioxidant glutathione under As stress (Noctor et al., 2012). In accordance, *AAC3* was previously identified as a putative candidate gene for As tolerance associated with the mitochondrial electron transport chain (mETC) in Arabidopsis (Fu et al., 2014).

Collectively, these results highlight the involvement of plant mitochondrial metabolism in the As responses and reinforce the importance of *AAC3* as a stress-responsive gene in Arabidopsis. Lastly, the higher expression of *ER-ANT1* suggests compensation for the lower cytosolic ATP levels observed under As exposure. Thus, ER-ANT1 would supply the ATP-dependent chaperones that are crucial for the proper folding and assembly of ER or secretory proteins inside the organelle throughout the transport process (Pfeffer and Rothman, 1987; Walter and Ron, 2011) to alleviate the protein damage and preserve cellular homeostasis under As stress.

The Behavior of Adenylate Carriers under a Range of Abiotic Stress Conditions

To obtain further insights into the importance of adenylate carriers under a range of stressful conditions (e.g., drought, salt, and osmotic stress), an overview of the changes in transcript levels of adenylate carrier genes in shoots and roots with respect to nonstressed conditions was obtained (Figure 2). At first glance, shoot and root systems appear to display significant molecular plasticity, as observed by the different expression patterns between the systems. Accordingly, the transcriptional response in shoots is more affected by environmental changes than in roots. This might reflect the higher complexity of autotrophic organs in terms of the metabolic requirements to associate energy metabolism with several other metabolic pathways (e.g., photosynthesis, respiration, and photorespiration) in contrast to the situation observed in heterotrophic organs, in which energy production is mainly driven by mitochondrial metabolism (Araújo et al., 2012a; Cavalcanti et al., 2014).

The *AAC2*, *AAC3*, *PNC2*, and *APC1* genes were generally the most inducible under most of the situations evaluated (Figure 2). *APC1* was strongly induced, whereas, in agreement with (Monné et al., 2015), *APC2–3* were only slightly induced by cold both in roots and shoots. Interestingly, adenylate carrier genes encoding the mitochondrial APC1 and AAC3 and the peroxisomal PNC2 were generally highly induced compared with other transporters by stresses causing dehydration, namely drought, salt, and osmotic stresses. These stresses have been characterized to involve ATP-dependent protein degradation resulting in the accumulation of branch chain amino acids (BCAAs) (Araújo et al., 2011). Given that BCAAs are suggested to function as alternative electron donors for them ETC (Araújo et al., 2011), it is tempting to speculate that AAC3 and APC1 are involved in mediating the export of ATP generated from mitochondrial amino acid catabolism to support the high rates of osmoprotectant synthesis (Obata and Fernie, 2012).

The higher expression of *AAC3* was detected in roots under osmotic stress, salinity conditions, oxidative stress, heat stress (Figure 2D, F, H, J, respectively), as well as UV-B radiate on and plants with mechanical injuries (see Figure S2 in the supplemental information online). The significant impact of AAC3 in roots is consistent with the complete reliance on mitochondrial oxidative phosphorylation to meet the energy demands in heterotrophic organs. AAC3 was also induced in shoots by osmotic (Figure 2C) and heat (Figure 2I) stresses. By contrast, *AAC1* carrier expression remained stable under most of the stress conditions assessed. In turn, *AAC2* exhibited higher expression in shoots after UV-B radiation (see Figure S2 in the supplemental information online), and osmotic and oxidative stresses (Figure 2C, G). The carriers adenine nucleotide transporter 1 (ADNT1) (Palmieri et al., 2008b), NTT1 (Reiser et al., 2004; Schmitz et al., 2010b), plasma membrane-located adenylate transporter 1 (PM-ANT1)

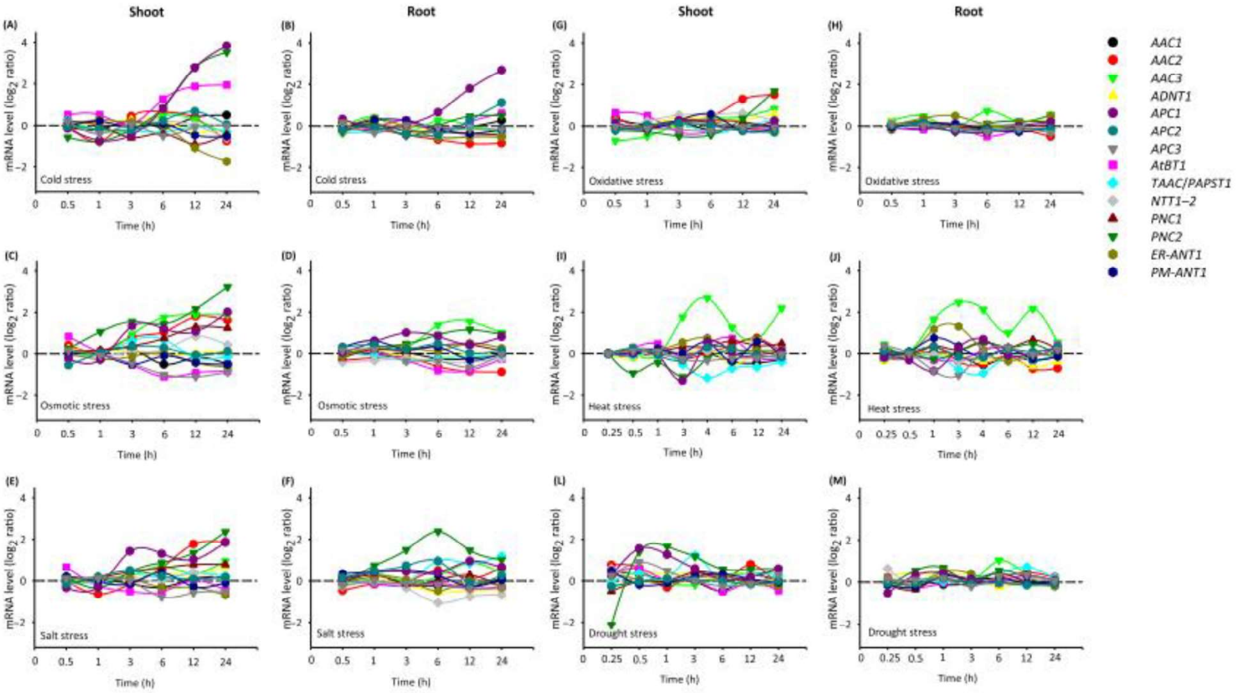

Figure 2. Expression Analysis of *Arabidopsis thaliana* Genes Encoding Adenylate Carriers under Stress Conditions. Gene expression data were obtained from the expression browser tool at the Bioarray Resource (BAR; www.bar.utoronto.ca; (Toufighi et al., 2005)). The conditions tested are according to AtGenExpress (AtGenExpress, abiotic stress treatments in roots and shoots of 18-day-old plants with time points from 0.25 h and/or 0.5 h to 24 h after the onset of stress treatment) (Kilian et al., 2007).

cont/Figure 2. Abiotic stress data sets were from AtGen Express [accession numbers: ME00325 (cold), ME00327 (osmotic), ME 00328 (salt), ME00340 (oxidative), ME00339 (heat), andME00338(drought)](www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). Gene expression values were normalized to control data, converted to log2 and are shown on the y axis. For a complete description of the gene symbols, see Table 1 in the main text. Data Set III in the supplemental information online shows the differences in gene expression data obtained in stress experiments as revealed by Student's t test at $P < 0.05$.

(Rieder and Neuhaus, 2011) and *A. thaliana* Brittle1 transporter (AtBT1) (Kirchberger et al., 2008) displayed similar profiles, showing mild modifications under all conditions evaluated (Figure 2). ER-ANT1 and thylakoid/plastid envelope membrane ATP/ADP carrier (TAAC/PAPST1) (Thuswaldner et al., 2007; Gigolashvili et al., 2012) also showed few changes, with exception of the upregulation of *ER-ANT1* in roots (by wounding and heat) and *TAAC/PAPST1* in shoots (by salt and drought). Interestingly, the transcript pattern of *TAAC/PAPST1* obtained by Northern blotting revealed the highest expression in leaves undergoing senescence or abiotic stress, probably because of the role of TAAC/PAPST1 in supplying ATP to the thylakoid lumen for mobilization of nitrogen resources (under senescence) and their degradation (under stress) (Thuswaldner et al., 2007). The additional role of TAAC/PAPST1 in supplying plastidic phosphoadenosine 50-phosphosulfate (PAPS) to the cytosol (Gigolashvili et al., 2012) supports its association with stress and senescence, given that sulfated compounds are involved in responses to various stresses (Anjum et al., 2015; Bielecka et al., 2015). The *in silico* analysis of adenylate carriers at specific stages of development (see Figure S3 in the supplemental information online) also supports a strong induction of TAAC/PAPST1 in senescent leaves. Interestingly, a conspicuous induction of the transcripts of TAAC/PAPST1, AAC3, ADNT1, ER-ANT1, and PNC1–2 is observed in senescent leaves, concomitantly with a reduction of AAC1 expression. Thus, it appears that not only environmental stresses but also natural senescence cause notable changes in the expression of different adenylate carriers in plants.

PNC2, AAC3, and APC1 showed similar expression patterns in both systems, being the only carriers that exhibited increased expression in either shoots or roots under stress conditions. It appears reasonable to assume that this can be explained by the subcellular localization of these carriers in mitochondria or peroxisome, organelles that have important roles in the scavenging of reactive oxygen species

(ROS), a common consequence of different stresses (Mittler, 2017). Mitochondria and peroxisomes produce ROS and many metabolites, some of which may serve as retrograde signals that are important for stress responses (Zhu, 2016). Moreover, AAC3, APC1, and PNC2 are the most upregulated MCF adenylate carriers in Arabidopsis under stress conditions (Van Aken et al., 2009b; Palmieri et al., 2011a). Collectively, these results suggest that plant metabolism is dependent on energy transport under stresses. Perhaps more important, given the central role of mitochondria and peroxisome in stress responses, it appears that adenylate carriers in these compartments, especially PNC2, AAC3, and APC1, are particularly induced under adverse situations. Noteworthy, ATP is also essential for ribosomal protein biosynthesis and the activity of chaperones and/or heat shock-related processes (Sangster and Queitsch, 2005). Thus, upregulation of the expression of specific adenylate transporters might not be directly related to metabolism, but rather to provide energy for protein biosynthesis, folding/unfolding, and/or protein transport. Further experimentation focusing on the identification of the precise mechanisms regulating this expression pattern is clearly required.

Co-expression Analyses of Adenylates Carriers

Co-expression analyses have been successfully used to verify changes in expression within a previously known pathway or a group of genes as well as for the identification of genes not previously associated with a given biological question (for reviews, see (Usadel et al., 2009; Bordych et al., 2013) and references therein). In this context, this approach is suitable for indicating candidate genes that might be associated with adenylate carriers. Thus, we initially identified which genes are co-expressed with genes encoding adenylate carriers in Arabidopsis (Figure 3). Briefly, to perform this analysis, we used two data sets: all available experiments (Figure 3A) and stress-related datasets (Figure 3B). Furthermore, we grouped them according to their biological function to understand the correlations between the expression patterns of genes encoding adenylate carriers and genes involved in other functions. The analysis of ‘all experiments’ revealed that the number of genes co-expressed with adenylate carriers was relatively small and mainly associated with energy metabolism [tricarboxylic acid (TCA) cycle, glycolysis, mETC, amino acid, and protein metabolism, and redox regulation] (Figure 3A).

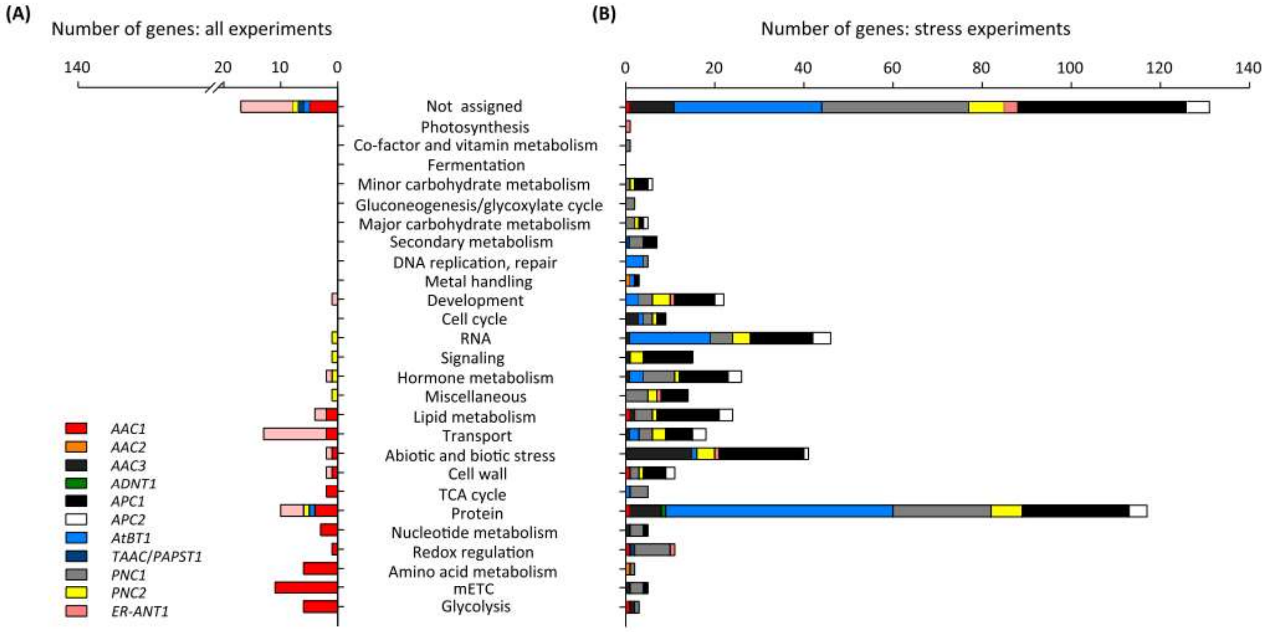

Figure 3. Co-expression Analysis of *Arabidopsis thaliana* Genes Encoding Adenylate Carriers. Transcriptional data mining was performed using co-expression PRIMe database (Kenji Akiyamaa, Eisuke Chikayamaa, Hiroaki Yuasab, Yukihisa Shimadaa, Takayuki Tohgea, Kazuo Shinozakia, Masami Yokota Hiraia, Tetsuya Sakuraia, Jun Kikuchia and Saitoa, 2008); http://prime. psc.riken.jp/?action=coexpression_index)

Cont/ Figure 3. using as a trap the *A. thaliana* genes encoding adenylate carriers listed in Table 1 in the main text. Corresponse connection was performed using coefficient values calculated by a microarray data set of all experiments covering 1388. Gene Chip data (A) and 'stress experiments' covering 298 Gene Chip data (B) using the 'union of sets' method through the PRIMe platform co-expression website. Positive correlations (r > 0.65) were used to identify candidate genes. Later, these Genes were grouped into functional categories based on the Mapman platform using the Mercator tool (www.plabipd.de/portal/web/guest/mercator-sequence-annotation). The bars represent the number of genes co-expressed with each adenylate carrier. Abbreviations: mETC, mitochondrial electron transport chain, TCA, tricarboxylic acid. For a complete description of the trap and candidate gene symbols, see Table 1 in the main text and Data Sets I and II in the supplemental information online, respectively.

Notably, the only adenylate carriers that exhibited co-expressed genes in 'all experiments' were *AAC1*, *ER-ANT1*, *PNC1*, *AtBT1*, and *TAAC/PAPST1*. One possible explanation for the low number of co-expression patterns could be the existence of genes with constitutively low basal transcript abundance because these are genes with multiple isozymes that are coregulated with components outside central metabolic pathways, or by gene regulation through post-transcriptional processes (Lee and Millar, 2016). Interestingly, the number of genes co-expressed with adenylate carriers was significantly higher under stress conditions (Figure 3B), demonstrating that these transporters display more complex expression patterns across different tissue types under adverse conditions. Despite this clear trend, particularly in the case of *AAC1* and *ER-ANT1*, our co-expression analysis also revealed that the number of genes co-expressed with them in 'all experiments' was considerably higher (44 and 32 genes, respectively) than the number of genes co-expressed under stress conditions (six and eight, respectively).

The 44 candidate genes co-expressed with *AAC1* were grouped mainly in the following categories: mETC (11 genes), glycolysis (six genes), and TCA cycle (six genes). Notably, five genes encoding proteins of mitochondrial ATP synthase complex were related with AAC1 in 'all experiments' (see Data Set I in the supplemental information online), which indicates a strong association of *AAC1* with genes involved in mitochondrial respiration, especially with those related to the mETC. This is in good agreement with previous studies demonstrating that the ATP generated via oxidative phosphorylation is transported by AAC1 in exchange for cytosolic ADP (Laloi, 1999;

Haferkamp et al., 2002). Most of the genes co-expressed with *ER-ANT1* in ‘all experiments’ were classified in signaling pathways (11 genes), including three calcium-dependent protein kinases and four others that, in agreement with the high expression of the *ER-ANT1*-promoter GUS in pollen (Leroch et al., 2008), are related to metabolic changes occurring in pollen.

AAC3 showed a considerable number of co-expressed candidate genes (43 genes) under stress conditions. Fifteen of them were categorized as stress-responsive genes, eight of which encode heat shock proteins. By contrast, only two candidate genes were co-expressed with *AAC2* under the same conditions, the dihydrolipoamide succinyl transferase, related to the TCA cycle, and cytochrome c oxidase (see Data Set II in the supplemental information online). It was further observed that neither *AAC2* nor *AAC3* is co-expressed with any genes in ‘all experiments’; neither do they have any co-expressed candidate genes in common under stress conditions. Taken together, these results suggest that the expression of the AAC family members is nonredundant and that they have different functions during plant growth and under stress conditions. Moreover, the expression of *AAC3* under stress conditions appears to be strongly correlated with genes involved in processes that rely on ATP-dependent enzymes belonging, for example, to protein degradation pathways, such as ubiquitin-associated proteins. During stress situations, the mobilization of alternative substrates, such as proteins and amino acids, may be necessary for the maintenance of ATP synthesis (Araújo et al., 2011). Notably, the oxidation of certain amino acids can directly feed electrons into the mETC and the physiological significance of amino acids has been demonstrated under conditions faced by most plants at some stage of their life cycle, such as drought and senescence (Araújo et al., 2011; Krüßel et al., 2014; Barros et al., 2017). Therefore, it can be anticipated that, to maintain normal metabolic function, the homeostasis of individual amino acids must be tightly regulated due to their specific functions in signaling, stress responses, and the production of secondary metabolites (Hildebrandt et al., 2015). As such, further investigation of the connections between protein degradation, amino acid metabolism, and the expression of adenylate carriers should provide significant insights into an integrated overview of the metabolic connections of these pathways in higher plants.

Both *AtBT1* and *PNC1* were co-expressed with 118 and 117 genes, respectively, under stress, whereas *APC1* and *APC2* were co-expressed with 169 and 29 genes, respectively (see Data Set II in the supplemental information online). APCs not only catalyze the electroneutral exchange of ATP-Mg for Pi (Monné et al., 2017), but also transport adenosine 50-phosphosulfate (APS), a key intermediate of the sulfur assimilation pathway, which leads to the biosynthesis of cysteine, methionine, and glutathione (Monné et al., 2015). APS is synthesized in the cytosol and plastids, but not in mitochondria, whereas the enzymes necessary for converting APS to cysteine exist in the cytosol, plastids, and mitochondria (Lunn et al., 1990). Therefore, APS imported into mitochondria by the APCs may be used for mitochondrial cysteine biosynthesis and, hence, its requirement in the mitochondrial matrix could increase to enhance the synthesis of glutathione under stress conditions (Kopriva et al., 2012).

Under non-optimal conditions, *AtBT1* was co-expressed mainly with genes involved in protein synthesis (44 genes) and transcriptional regulation, and RNA processing and degradation (18 genes). *AtBT1* has a dual-targeting to plastids and mitochondria (Bahaji et al., 2011b) and is the only identified transport protein that might function in the export of ATP from chloroplasts (Kirchberger et al., 2008; Bahaji et al., 2011b). Moreover, specific delivery of AtBT1 to mitochondria was shown to complement the phenotype of homozygous Atbt1 mutants, indicating that further investigations are required to elucidate the role(s) of AtBT1 proteins in mitochondria (Bahaji et al., 2011a). Thus, its higher induction under stresses might be a consequence of the use of ATP for energy-consuming processes under stress conditions in the cytosol and other organelles and also of yet unidentified roles of AtBT1. In the same vein, *PNC1* was also co-expressed with genes involved in different metabolic processes, with an emphasis on protein degradation (17 genes) and amino acid metabolism (eight genes). It is not surprising that *PNC1* is co-expressed with genes associated with protein degradation, since this is a common activity in peroxisomes, especially following stress situations (Corpas et al., 2009). In general, not only *PNC1*, but also *AtBT1*, *AAC3*, and *APC1* were co-expressed mainly with genes involved in protein degradation. These four transporters were also mainly associated with a considerable number of genes the functions of which are not yet known. This reveals the importance of characterizing the function of these genes to determine their relationship with energy metabolism associated with plant responses following stress conditions. Further functional characterization of these candidate genes will likely help us to identify genes that control and regulate plant energy metabolism as well as facilitating the discovery of novel gene functions with potential biotechnological applications.

Outstanding Questions

How exactly do adenylate carriers integrate energy metabolism between organelles within a plant cell, particularly under different adverse conditions?

What is the role of adenylate carriers in global climate change? Is there a particular stress where specific transporters would be more or less important?

How can we distinguish between functional redundancy and specialization of distinct adenylate carriers? Can we expect a higher degree of specialization of these transporters under different environmental conditions?

Can the long-term effects of key abiotic stress factors promote adaptive changes in cellular metabolism and adenylate transport also simultaneously affecting primary metabolism?

Are there any key leaf physiological traits associated with temporal variability in the expression of adenylate transporters among different species? How can bioinformatics and co-expression data be validated?

How can energetic status modulation associated with adenylate transport be linked to metabolism in general under different environmental conditions? Do environmental conditions modify the cellular response and adenylate transport in a cell type-specific manner?

Can the manipulation of adenylate transporters unlock further opportunities to improve stress tolerance and also enhance important agronomical traits, such as seed germination and plant yield?

How could such information guide crop breeding programs to develop crops adapted to suboptimal environmental conditions?

Concluding Remarks

Although there are several available mutants for adenylate carriers in the model plant Arabidopsis, insights into the regulatory mechanisms underpinning tolerance to stresses, in general, remain limited. Furthermore, it appears that there is currently an incomplete understanding of the in vivo function of adenylate carriers and, as such, our understanding of the impact of these carriers in plant metabolism remains fragmentary. Nevertheless, the revolution afforded by next-generation sequencing coupled with available bioinformatics tools means that resources are ready to fully unveil the function of such carriers in plants. However, it is tempting to speculate that the pathways of energy metabolism and adenylate transporters are tightly regulated at the plant level in a suitable manner to allow the plant to prioritize survival under suboptimal conditions.

Here, we present evidence that thein *in silico* screen of public gene expression databases, as carried out in plants, can aid the identification of connections between genes and processes, thus providing information about how each gene is connected to others and their possible involvement in different metabolic processes. Also, we show that an *in silico* co-expression survey using public transcriptome data sets provides new perspectives concerning how these carriers might integrate into metabolism under stress conditions to maintain cellular homeostasis. We are certain that the co-expression approach perse cannot provide a sufficiently short list of candidate genes to be investigated *in vivo* (Cavalcanti et al., 2014). Thus, further experimental validation is required (including, but not limited to those in the Outstanding Questions) to ensure that the observed co-expression is biologically relevant and prove the predictive hypothesis (Aoki et al., 2007). However, the co-expression approach is undeniably a powerful tool to select and rank the best putative candidate genes for further characterization and to determine their functional roles in plant energy metabolism. We posit that this will further open new routes for enhancing yield and stress tolerance attributes by tailoring approaches to tap into more specific attributes in crop species under future global climate change scenarios. Moreover, our analysis presents itself as a powerful approach allowing hypothesis construction and gene prioritization that highlights the role of the *AAC* family, *APC* family, and *PNC2* as important responsive genes under environmental stress, and provides novel insights into the system-level understanding of adenylate transport in higher plants.

Future analyses are necessary to identify the degree of redundancy and specialization of the adenylate carriers, especially for those found in the same organelle. Since T-DNA insertional lines are not currently available for all the transporters discussed here, it appears reasonable to anticipate that the generation of single and multiple adenylate carrier mutants by novel tools, including CRISPR-Cas9, will provide information concerning the significance of these different adenylate carriers and aid their validation as appropriate targets for plant development and stress tolerance.

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CHAPTER II

FUNCTIONAL CHARACTERIZATION OF ADNT1 CARRIER IN *Arabidopsis thaliana* UNDER WATERLOGGING CONDITIONS

ABSTRACT

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, March, 2021. **Functional characterization of adnt1 carrier in *Arabidopsis thaliana* under waterlogging conditions.** Adviser: Adriano Nunes Nesi.

Adenine nucleotides are present in numerous vital processes, acting as energy carriers throughout the cell, constituents of nucleic acids, and also acting as cofactors for numerous enzymes. Here we investigate the impact of reduced expression of *ADNT1* in *A. thaliana* plants submitted to waterlogging conditions, assessing physiological, molecular, and metabolic changes along the stress period. *Arabidopsis thaliana* (ecotype Columbia [Col-0]) seeds from wild-type (WT), *adnt1* T-DNA mutant (GABI-Kat 451B06), and two *ADNT1* antisense lines (Line 10 and 22) were used in all experiments. Among seven adenylate carriers analyzed, our results indicate that the only adenylate carrier that displayed an increase in expression levels under waterlogging stress was *ADNT1*. The absence of ADNT1 generates greater leaf loss and a minor maximum quantum yield of PSII electron transport indicates that ADNT1 deficient plants suffered great damages in comparison with WT. The stress responses occur prematurely in ADNT1 deficient plants, which can be seen by the increase of alanine levels, and other stress metabolites markers, such as aspartate, trehalose, and inositol. The expression of *SnRK1* also was higher in ADNT1 deficient plants already in non-stress conditions, and over time stress period. We also observed that Adenylate Kinase *(ADK)* and Apyrase (*APY)* have higher expression already under non-stress conditions in the ADNT1 deficient lines. Additionally, the greater expression of *PDC1* and *PPDK* during the waterlogging stress period was also observed in the ADNT1 deficient plants, which suggests an early and more expressive induction of the fermentative pathway. Our findings indicate that the ADNT1 carrier performs an important role in Arabidopsis during waterlogging stress. We hypothesize that the absence of this carrier generates an early "hypoxic status" due to perturbation of the adenylate pool caused by reduced AMP transport to mitochondria.

Keywords: Hypoxia conditions, ATP/AMP, stress responses, SnRK1.

RESUMO

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, março de 2021. **Caracterizaçâo funcional do transportador adnt1 sob condições de alagamento em *Arabidopsis thaliana*.** Orientador: Adriano Nunes Nesi.

Os adenilatos estão presentes em vários processos vitais, atuando como transportadores de energia em toda a célula, constituintes dos ácidos nucléicos, e também atuando como cofatores para várias enzimas. Aqui nós investigamos o impacto da expressão reduzida de *ADNT1* em plantas de *Arabidopsis thaliana* submetidas a condições de alagamento, avaliando mudanças fisiológicas, moleculares e metabólicas ao longo do período de estresse. Sementes de *A. thaliana* (ecótipo Columbia [Col-0]) do tipo selvagem (WT), mutante T-DNA (*adnt1)* (GABI-Kat 451B06) e duas linhas *ADNT1* antisenso (Linha 10 e 22) foram usadas em todos os experimentos. Entre sete transportadores de adenilatos analisados, nossos resultados indicam que o único transportador de adenilatos que apresentou um aumento nos níveis de expressão sob alagamento foi o *ADNT1*. A deficiência de *ADNT1* gerou maior perda de folhas e um menor rendimento quântico máximo de transporte de elétrons PSII indicando que plantas deficientes em ADNT1 sofreram maiores danos em comparação com o WT. As respostas ao estresse ocorreram prematuramente em plantas deficientes em ADNT1, o que pode ser observado pelo aumento dos níveis de alanina e de outros metabólitos marcadores de estresse, como aspartato, trealose e inositol. A expressão de *SnRK1* também foi maior em plantas deficientes em ADNT1 tanto em condições controle quanto ao longo do período de estresse. Observamos também que a Adenilato Quinase (ADK) e a Apirase (APY) apresentam maior expressão já em condições controle nas linhagens deficientes em ADNT1. Além disso, a maior expressão de *PDC1* e *PPDK* durante o período de estresse por alagamento também foi observada nas plantas deficientes em ADNT1, o que sugere uma indução precoce e mais expressiva da via fermentativa. Nossos resultados indicam que o transportador ADNT1 desempenha um papel importante em Arabidopsis durante o estresse por alagamento. Nossa hipótese é que a ausência desse transportador gera um “estado de hipóxia” precoce devido à perturbação do pool de adenilato causada pela redução do transporte de AMP para as mitocôndrias.

Palavras chave: Condições de hipoxia, ATP/AMP, respostas ao estresse, SnRK1.

INTRODUCTION

In the context of cellular metabolism, nucleotides are present in numerous vital processes, acting as energy carriers throughout the cell, constituents of nucleic acids, and also acting as cofactors for numerous enzymes (Haferkamp et al., 2011). In the last few years, some nucleotide adenylate transporters of plant cells have been identified, described, and reconstituted into proteoliposomes for transport assays (Haferkamp et al., 2011; Palmieri et al., 2011b). These transporters mediate a flow of these metabolites through the cell's organelles connecting their metabolism. Among all the compounds transported between the organelles, adenosine triphosphate (ATP) is perhaps the most important, as it is involved in most of the biochemical pathways present in a plant cell (Haferkamp et al., 2011). In this context, ATP transporters play an essential role in coupling endergonic and exergonic reactions during non-stressful and stressful conditions (da Fonseca-Pereira et al., 2018b). Bearing in mind that ATP is synthesized mainly in the mitochondrial and chloroplast electron transport chain (ETC), ATP transporters are necessary to guarantee the supply of energy for the metabolic reactions that occur in the cytosol and other organelles (Gardeström and Igamberdiev, 2016).

In Arabidopsis, most ATP transporters biochemically characterized act as an antiport, preferably by adenosine diphosphate (ADP), but also by adenosine monophosphate (AMP) (Kirchberger et al., 2008; Klingenberg, 2008; Leroch et al., 2008; Palmieri et al., 2008b). Unlike other mitochondrial adenylate transporters, the ADNT1 (At4g01100) transporter performs the transport of ATP in exchange for AMP preferentially to ADP (Palmieri et al., 2008b). This difference between ADNT1 and other adenine nucleotide transporters indicates that it has a particular function, acting in organs and situations that accumulate AMP such as carbon starvation, and ETC inhibition like root system under hypoxia conditions (Chen et al., 2017; Cho et al., 2021). Also, it has already been demonstrated that the expression of *ADNT1* occurs mainly in heterotrophic organs, where the ATP supply from mitochondria becomes more crucial such as seedlings, flowers, and roots of adult plants (Palmieri et al., 2008b). Likewise, considerable expression of *ADNT1* was observed in senescent leaves, evidencing a possible physiological function of this transporter in these processes (Palmieri et al., 2008b).

It has been shown that in heterotrophic plant organs, such as roots, AMP is an abundant nucleotide in the cytosol (Roberts et al., 1997). It has also been reported that cytosolic AMP levels increase markedly during abiotic stresses, such as hypoxia (Saglio et al., 1980; Zhang et al., 2013). The ATP exported from the mitochondrial matrix in exchange for cytosolic AMP by ADNT1 can be converted in the intermembrane space by the mitochondrial adenylate kinase (ADK) into two ADP molecules. This ADP re-enters in the mitochondrial matrix via the ADP / ATP transporter to support the synthesis of ATP through oxidative phosphorylation (Roberts et al., 1997). Thus, given the transport characteristics of ADNT1, which transport preferably AMP in exchange for ATP. It is likely that plants with reduced expression of this transporter are more sensitive to stress conditions due to hypoxia. Since, in such circumstances, the absence of the import of cytosolic AMP by the mitochondria results in an elevated AMP/ATP ratio in the cytosol, which active signalization by SnRK1 (SNF-related-kinase 1) that triggers the waterlogging responses (Limami et al., 2014) prematurely. Here we investigate the impact of reduced expression of *ADNT1* in *A.thaliana* plants submitted to waterlogging conditions, assessing physiological, molecular, and metabolic changes along the stress period.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia [Col-0]) seeds from wild-type (WT), *adnt1* T-DNA mutant (GABI-Kat 451B06), and two *adnt1* antisense lines were used in all experiments. All genotypes were kindly provided by Dr. Alisdair R. Fernie, Max Planck Institute for Molecular Plant Physiology, Golm-Potsdam, Germany. The seeds were germinated on half-strength Murashige and Skoog (MS) medium plates, pH 5,7 (Murashige and Skoog, 1962), containing 1% (w/v) sucrose in a growth chamber (150 μmol photons m^{-2} s^{-1} white light, 21°C) under short-day conditions (8 h of light/16 h of dark). After 10 days, the seedlings were transferred to a commercial substrate (Carolina Soil) and kept in a growth chamber under the same conditions previously mentioned.

Waterlogging stress conditions

The root system of 4-week-old plants was submitted to hypoxia by waterlogging, submerging the pots in trays with deionized water treated with N_2 gas to eliminate O_2 dissolved, forming approximately 0.2 cm of water blade above the substrate. During the experiment samples from leaves and roots were collected in the middle of the light period, snap-frozen in liquid nitrogen, and stored at -80ºC until analyses. The samplings were performed before (time 0) and 3, 7, and 12 days after the beginning of the waterlogging condition.

Chlorophyll fluorescence measurements

The chlorophyll fluorescence was measured on fully expanded leaves of 4-weeks-old plants submitted to waterlogging conditions with the miniaturized pulse-amplitude-modulated photosynthesis yield analyzer (Mini-PAM) of H. Walz (Effeltrich, Germany) with the leaf clip holder for small size plants. After the dark adaptation of plants, the minimum fluorescence at open PSII centers in the dark-adapted state (Fo) was determined. A saturating pulse of white light (800 msec, 3000 µmol photons m^{-2} sec^{-1}) was applied to determine the maximum fluorescence at closed PSII centers in the dark-adapted state (F_m), and during actinic light illumination ($F_{m'}$) (Oh et al., 1996). The ratio of variable fluorescence to maximal fluorescence (F_v/F_m) - representing the potential quantum yield of PSII photochemistry - was measured in dark-adapted leaf tissue.

Metabolite measurements

Frozen samples (50 mg), harvested in the middle of the light period, were homogenized with a mixture of methanol-chloroform-water (1:1:2.5), without Ribitol, according to Medeiros et al., (2017) for metabolites extraction. The methanol soluble phase was transferred to a 1.5 mL tube for the quantification of sugars, organic acids, and amino acids. The resulting pellet was subjected to three washes with the same extracting solution. Starch and total protein concentrations were quantified in the pellet obtained (Fernie et al., 2001b; Cross et al., 2006). The supernatants and pellets were stored at −20 °C until further analyses. The starch and soluble sugars (glucose, fructose, and sucrose) were analyzed as described by Daloso et al., (2015) and Stitt et al., (1989), with minor modification. The concentrations of total proteins and amino acids were quantified as described by Cross et al., (2006). The concentrations of malate and fumarate were determined as described by Nunes-Nesi et al., (2007). The chlorophyll *a* and *b* were determined before adding the mixture of chloroform-water. The absorbance readings were taken from each sample at 645 and 665 nm as previously described by Porra et al., (1989). All measurements were performed in a VersaMaxTM Microplate Reader (Molecular Devices®).

Metabolite Profile

Aliquots of 50–60 mg frozen leaf and root material were extracted with a mixture of chloroform–methanol-water for analyses of sugars, organic acids, and amino acids by gas chromatography-mass spectrometry (GC–MS) (Lisec et al., 2006). Peak integration was evaluated using TAGFINDER 4.0 software (Luedemann et al., 2008). The mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005). The amount of each metabolite was determined as the relative metabolite abundance, calculated by normalization of signal to that of Ribitol (internal standard) as described by Lisec et al. (2006). The data were calculated based on the fresh weight for leaves and roots.

Expression Analysis by qRT-PCR

Total RNA was isolated from root material using TRIzol reagent (Ambion, Life Technology) according to the manufacturer’s recommendations. Total RNA was treated with DNase I (DNase I Rnase Free; Celco). The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured using a Nanodrop spectrophotometer. Finally, 500 ng of total RNA were reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer) according to the manufacturer’s recommendations. Real-time PCR was performed on a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems) using Ludwig SYBR Green qPCR Mix (Biotec) according to the manufacturer’s recommendations. The primers used here were designed using the open-source program QuantPrime-qPCR primer design tool (Arvidsson et al., 2008) and are described in Supplemental Table 1. Relative transcript levels were calculated by relative quantification (standard curve method) and normalized using two constitutively expressed genes *ACTIN2* (AT2G37620) and *EF1a* (AT5G60390). Three biological replicates were processed for each experimental condition.

Experimental design and statistical analysis

The experiments consisted of four genotypes (WT, *adnt1*, Line 10, and Line 22) submitted to waterlogging in an extended experiment with four-time points (0, 3, 7, and 12 days) and in the experiment of short time with five-time points (0, 12, 24, 48, and 72h), six replicates of each genotype were used for biochemical analyses, ten replicates for fluorescence analyses, and three replicates were used to RT-PCR analyses. *t*-test was performed using the algorithm incorporated in Microsoft Excel (Microsoft Corporation, Seattle, WA, USA). Uneven variances were assumed and taken into account in the calculations. Values with $P<0.05$ with the *t*-test were considered significant.

RESULTS

Expression of *ADNT1* in all genotypes characterized

To evaluate the level of *ADNT1* expression in the different lines used in all experiments, we perform an RT-PCR analysis in leaves of four-week-old plants (Fig. 1). The three lines used exhibit a very different expression pattern between then, when the T-DNA line *aac1* show a greater reduction in *ADNT1* expression, around 95%, follow by Line 10 which exhibits a reduction of 48%, at last Line 22 shows 30% of *ADNT1* expression reduction.

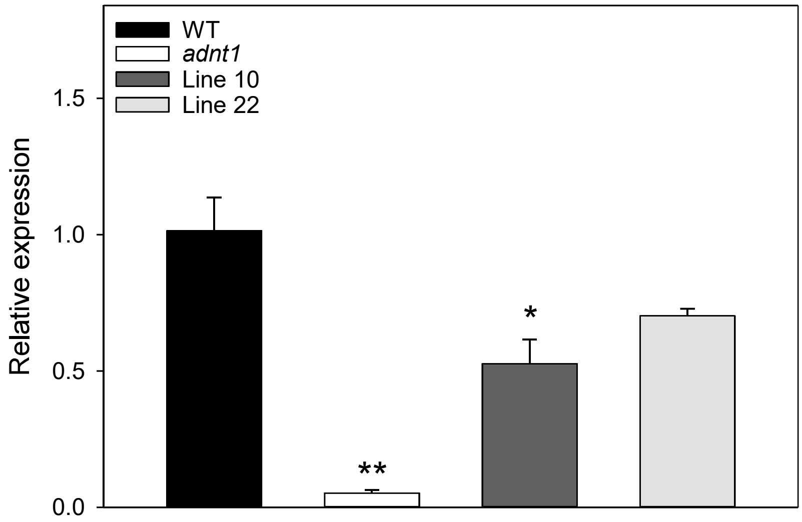

Figure 1. Expression analysis of ADNT1 by quantitative real-time reverse transcription (RT)-PCR in leaves of the *Arabidopsis thaliana* wild-type (Col-0), homozygous mutants (*adnt1*), and antisense lines (line 10 and 22). Values are means ± standard error of three independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$) from the wild-type (WT).

The expression of mitochondrial adenylates carriers during waterlogging stress

To evaluate the importance and response of adenylates carriers during the root waterlogging stress, we analyzed the expression of the seven mitochondrial carriers in roots of wild-type plants (Fig. 2). The only gene encoding adenylate carrier that displayed altered expression levels under this condition was *ADNT1*. The expression of *ADNT1* was higher at all time points with a peak 24 hours after the start of waterlogging stress in comparison with the other carriers. This result supports the hypothesis that under hypoxia conditions the ADNT1 carrier plays an important role during the stress establishment.

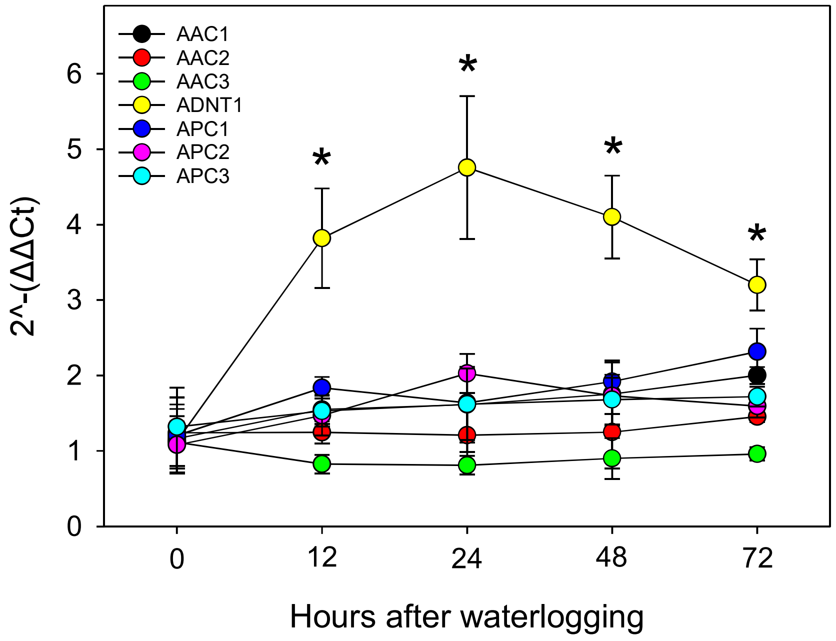

Figure 2. Changes in transcript levels of mitochondrial adenylates carriers in roots of four-week-old Arabidopsis plants during root waterlogging treatment for 0, 12, 24, 48, and 72 hours. Values are means ± standard error of three independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$) from the control time point (0 hours).

Phenotypic characterization of ADNT1 deficient plants during waterlogging

Under optimal conditions for Arabidopsis growth, the phenotype of plants with reduced expression of the ADNT1 carrier and wild-type was similar to the phenotypes observed in the previous study, with no abnormal phenotypes visible in the mutants during vegetative growth (Palmieri et al., 2008b). After four weeks of cultivation, the plants were transferred to waterlogging conditions. Under this condition plants from all genotypes exhibited mild signs of chlorosis and leaf abscission after seven days of oxygen deficiency (Fig. 3A). Interestingly, the *adnt1* mutant and both antisense lines showed slightly more leaf abscission signs as compared to the wild-type at the end of the stress period (Fig. 3A).

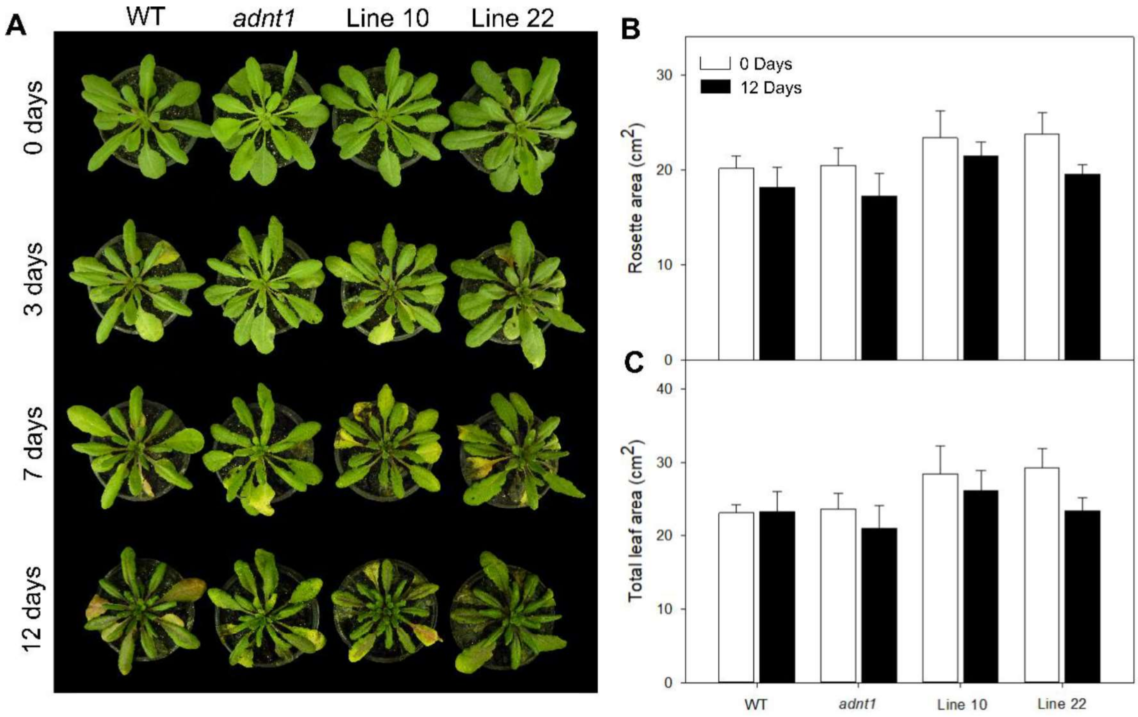

Figure 3. Phenotypic characterization of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days (**A**). Area of the rosette from four weeks plants in time 0 and 12 days of stress (**B**) and total leaf area in time 0 and 12 days of stress (**C**). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

After 12 days of waterlogging, visible stress signs were observed in the plants caused by the condition of low oxygen availability. In the genotypes deficient in the ADNT1 carrier expression, the presence of purplish leaves to a slightly greater extent was observed in comparison with those observed in the wild-type. Because of the leaf abscission exhibited by plants during the waterlogging stress, we evaluated the rosette area and total leaf area of the plants before and at the end of the stress period. In terms of rosette area occurred a reduction in all genotypes, being that the *adnt1* and Line 22 exhibited greater reduction, while wild-type and Line 10 a minor reduction was observed (Fig. 3B). Although the total leaf area in the lines with lower *ADNT1* expression had a reduction, the wild-type remained stable with no loss of leaf area (Fig. 3C), but the difference between the time 0 and 12 days of stress was not statistically different.

Chlorophyll fluorescence analysis in ADNT1 carrier deficient plants under root waterlogging stress

To assess the possible damage caused to the photosynthetic apparatus due to root waterlogging, the measurement of the maximum quantum electron transport yield of PSII was measured in leaves. Plants deficient in the expression ADNT1 carrier, in particular *adnt1* and Line 10, displayed lower Fv/Fm values at 7 and 12 days when compared to the wild-type (Fig. 4E).

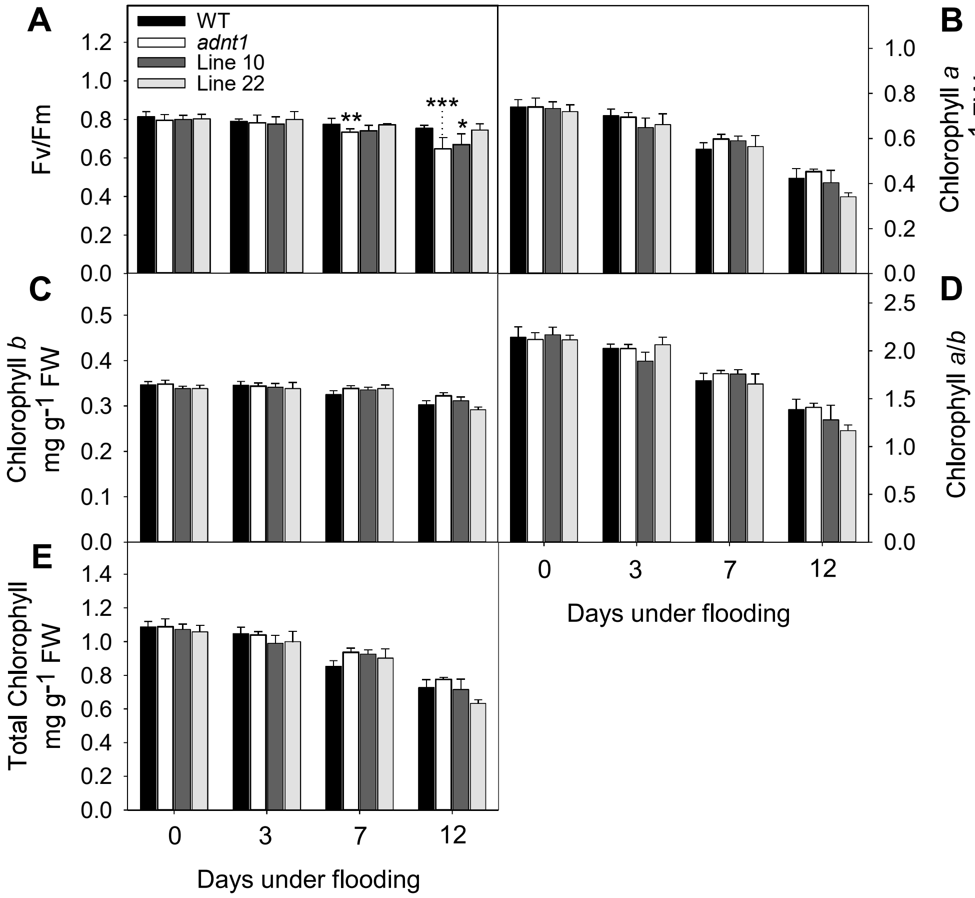

Figure 4. Photosynthetic pigments content and maximum quantum yield of PSII electron transport of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. Fv/Fm (**A**), Chlorophyll *a* (**B**), Chlorophyll *b* (**C**), Chlorophyll *a*/*b* (**D**), and Total Chlorophyll (**E**). Values are means ± standard error of six independent samples to pigment analyses and ten independent samples to fluorescence

analysis. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

We further investigated the effects of reduced ADNT1 carrier expression on the contents of photosynthetic pigments. The content of chlorophyll *a* (Fig. 4B), *b* (Fig. 4C), chlorophyll *a*/*b* ratio (Fig. 4D), and total chlorophyll (Fig. 4E) were measured. During the period of stress, the levels of chlorophyll *a*, chlorophyll *a*/*b* ratio, and total chlorophyll decreased over time. The levels of chlorophyll *b*, however, remained stable during the period of root waterlogging. However, there were no statistical differences between genotypes as compared to wild-type in any of the parameters mentioned above.

Changes in primary metabolites in leaves of plants deficient in the expression of ADNT1 carrier under root waterlogging stress

For a more detailed characterization of the function of the ADNT1 carrier, biochemical analyzes were performed on leaves. Regarding the levels of soluble carbohydrates (glucose, fructose, and sucrose; Fig. 5B, C, and D, respectively), an increase was observed throughout the stress period in all genotypes. However, three days after the beginning of stress, the ADNT1 antisense lines exhibited lower glucose content when compared to the wild-type. The same was observed for sucrose levels in the antisense lines, which in addition to presenting low levels on the third day also showed low levels on the twelfth day of stress. In fructose, there was a significant difference in two points along with the stress in plants with reduced expression of *ADNT1* compared to the wild-type. On the first, plants from Line 10 had a lower fructose content on the third day of stress, and the *adnt1* mutant had a higher level of fructose on the seventh day of stress compared to the wild-type (Figure 5C). The starch levels before the stress period were greater in *adnt1* mutant and Line 10, while wild-type and Line 22 exhibit minor levels. During the times three and seven days of stress, we observed variation in starch levels without a clear correlation with the *ADNT1* expression pattern of the lines. On the twelfth day of stress, all ADNT1 deficient plants showed a lower starch level compared to the wild-type (Fig. 5A).

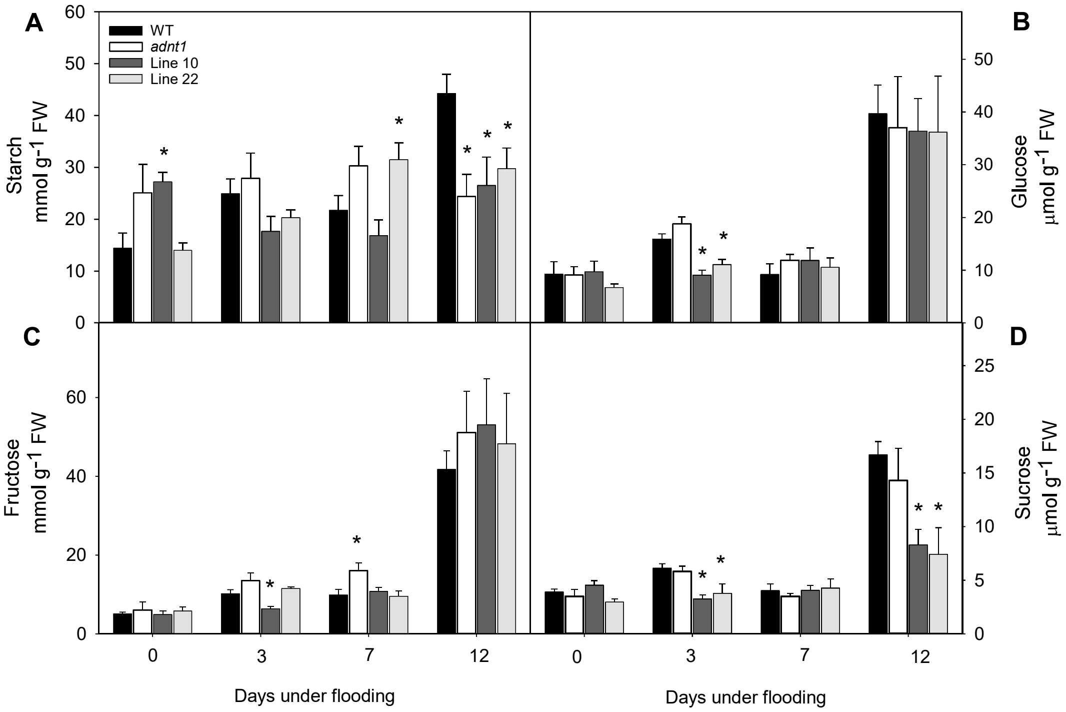

Figure 5. Variation of the main carbon-related compounds in leaves of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. Levels of starch (**A**), glucose (**B**), fructose (**C**), and sucrose (**D**). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$).

Additionally, to verify the influence of ADNT1 deficiency on nitrogen metabolism in plants under waterlogging conditions, the protein levels (Fig. 6A) and amino acids (Fig. 6B) were evaluated. Both total free amino acids and soluble protein contents remained stable throughout the stress period, except for the twelfth day of stress, where the *adnt1* mutant exhibited a slight increase in the total amino acid content.

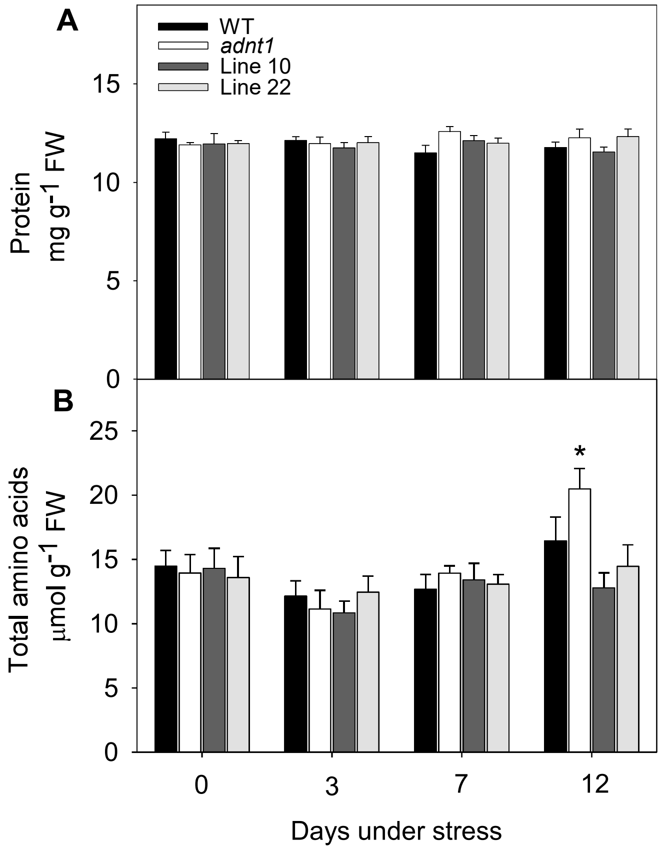

Figure 6. Changes in the main nitrogenous compounds in leaves of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. Levels of protein (**A**), and total amino acids (**B**). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

Deficiency of ADNT1 leads to a differential metabolic response following waterlogging conditions

To obtain a deeper view of metabolic changes during the waterlogging stress, we performed metabolite profiling analysis in samples from leaves and roots. We were able to identify 35 metabolites in leaves and 30 metabolites in roots. In the levels of leave metabolites (Fig. 7 and 8) we observed few differences in time 0 between the genotypes, such as higher levels of aspartate, and reduced levels of glyceric acid on ADNT1 deficient lines compared with wild-type.

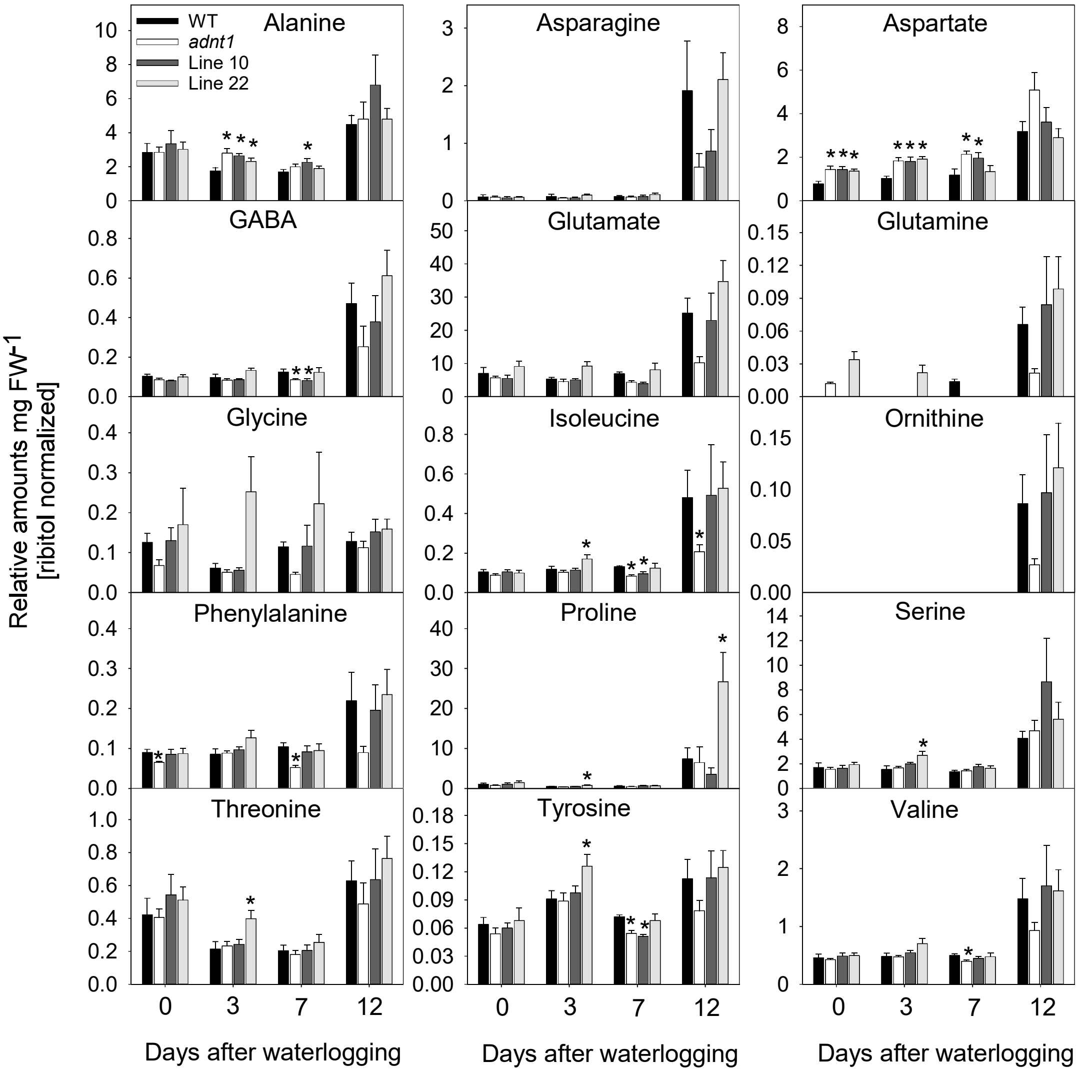

Figure 7. Amino acids extracted from leaves of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$).

Although in the time of 3 days we observed a great number of changes in metabolites of plants with *ADNT1* low expression in comparison to wild-type. In general, we observed a decrease in levels of organic acids, intermediates of the tricarboxylic acid (TCA) cycle, such as fumarate, and malate. At the same time, we verified an increase of key metabolites related to stress responses, such as alanine, aspartate, nicotinic acid, trehalose, and inositol in plants with reduced expression

ADNT1. Additionally, the levels of some sugars also were increased at 3 days of stress, such as fucose, and maltose.

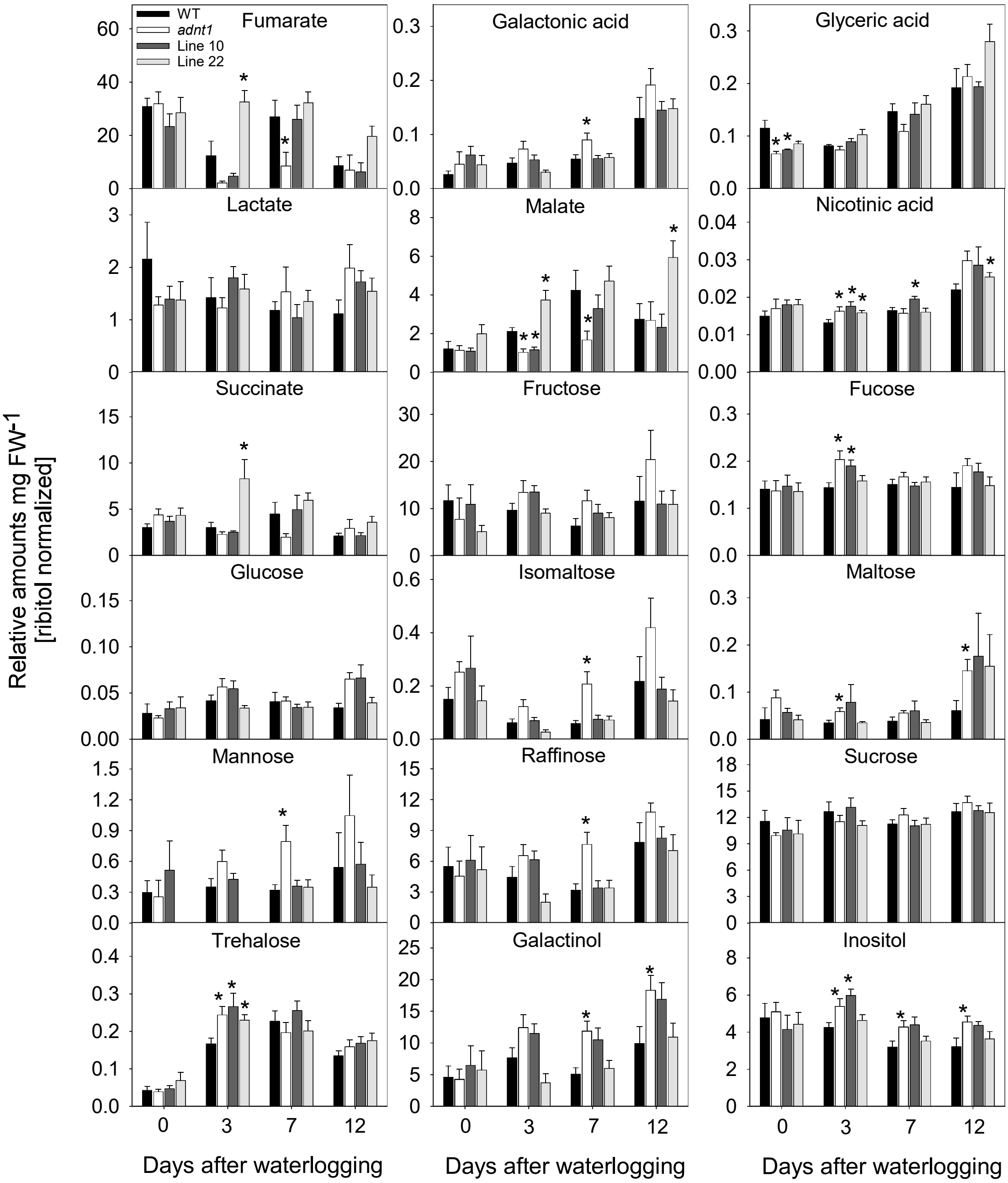

Figure 8. Organic acids, sugars, and alcohol sugars extracted from leaves of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student’s t-test to be significantly different (*P* < 0.05).

After 7 days under waterlogging, we observed that the levels of isoleucine, phenylalanine, tyrosine, valine, and GABA were reduced. Notwithstanding, the levels of aspartate, galactonic acid, isomaltose, mannose, and raffinose were increased mainly in the *adnt1* mutant in comparison with the wild-type. Later on, at 12 days of root waterlogging, we observed few differences between genotypes, with higher levels of maltose, galactinol, and inositol in plants with reduced *ADNT1* expression.

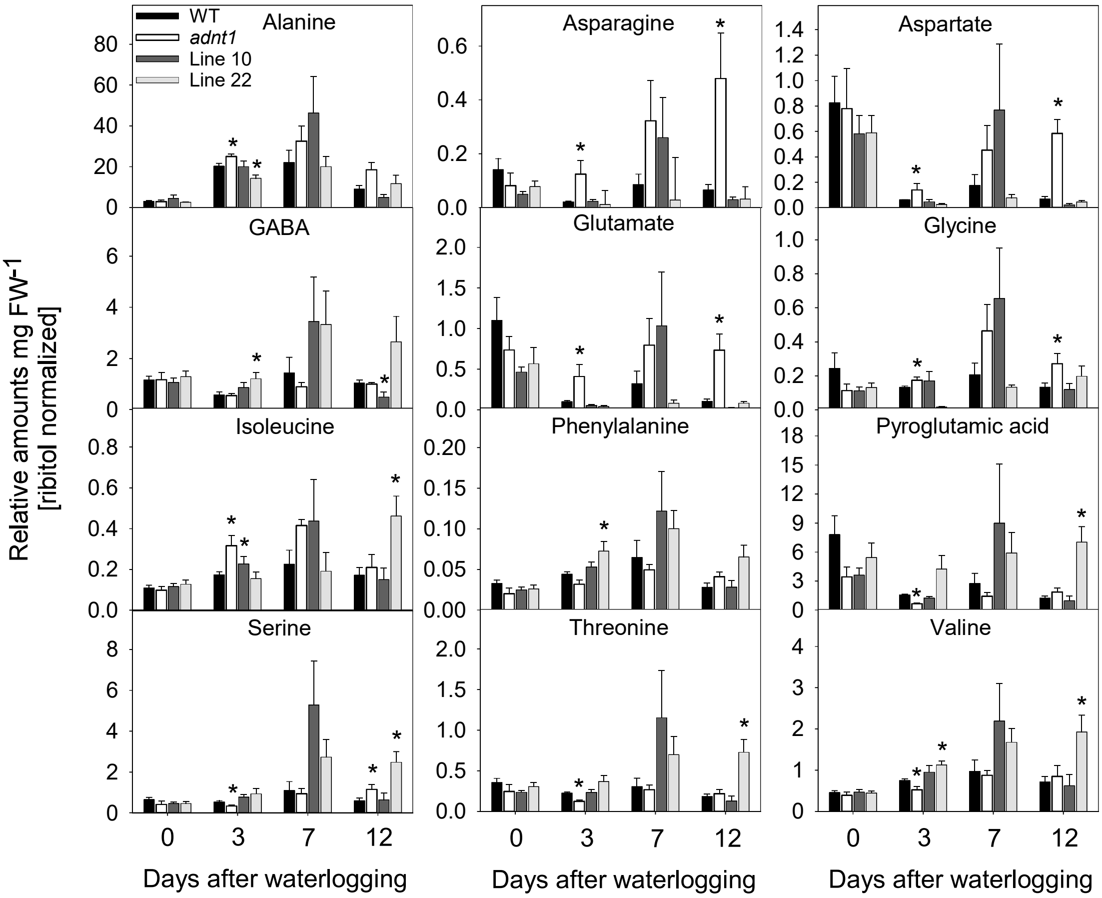

Figure 9. Amino acids extracted from roots of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

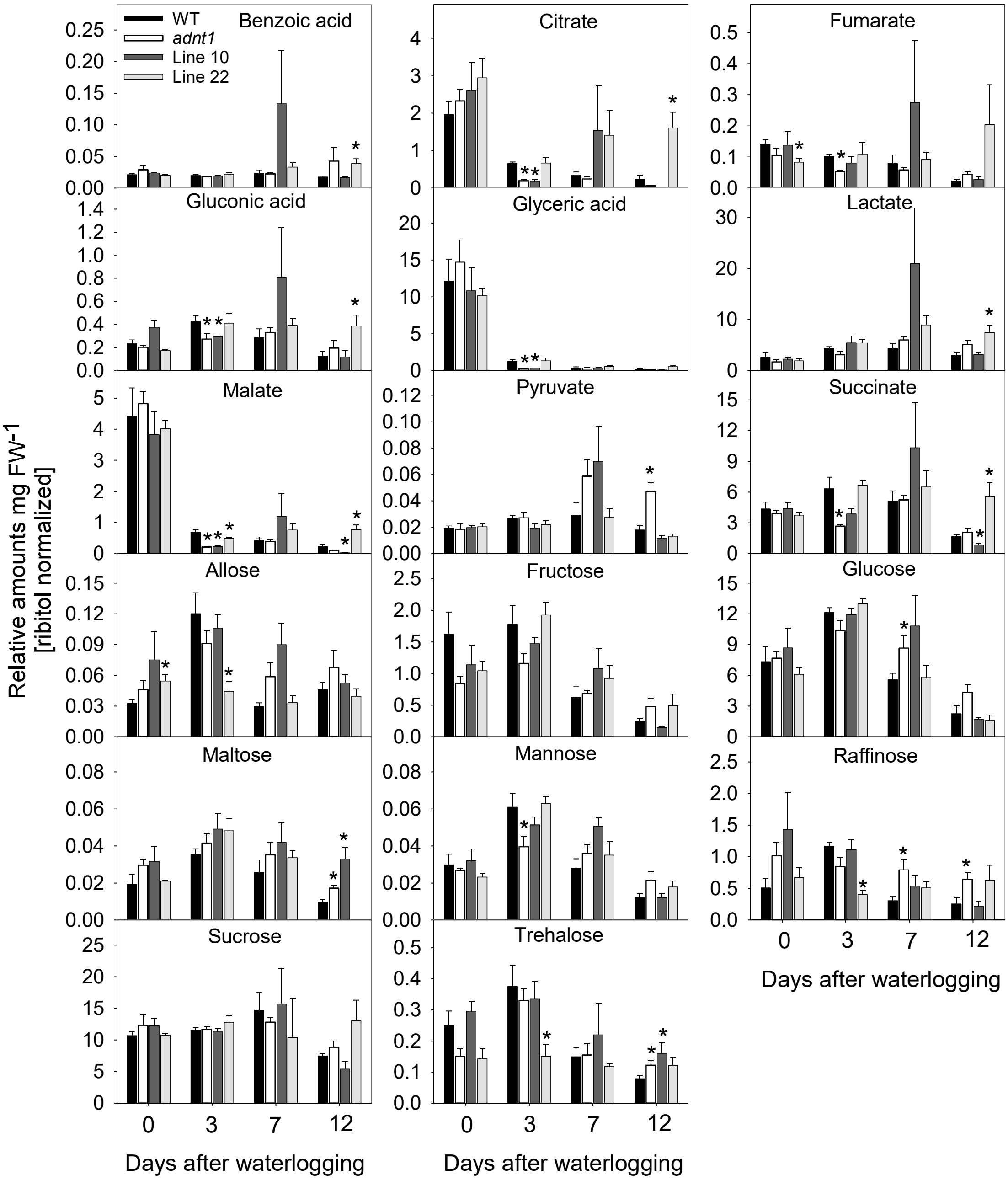

Figure 10. Organic acids, sugars, and alcohol sugars extracted from roots of four-week-old Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples. Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$).

Before the start of the stress period, we did not observe alterations in root metabolite levels in the ADNT1 deficient lines. While in the time 3 days of stress, we observed in roots an increase in the levels of alanine, isoleucine on *adnt1* mutant and Line 10. Additionally, occur an increase statically different in comparison of wild-type on the levels of asparagine, aspartate, glutamate, and glycine only in the *adnt1* mutant. On the other hand, citrate, fumarate, gluconic acid, and glyceric acid exhibit a decrease on *adnt1* mutant and Line 10 in comparison with wild-type. At 7 days of waterlogging only two metabolites show a difference between the genotypes, they are glucose, and raffinose with an increase in the *adnt1* mutant in the last two metabolites cited when compared with wild-type. At last, in time 12 days, the level of maltose and trehalose are bigger in *adnt1* mutant and Line 10, and asparagine, aspartate, glutamate, glycine, pyruvate, and raffinose are bigger just in *adnt1* mutant.

Expression of genes encoding proteins related to energy status in roots of ADNT1 carrier deficient plants under waterlogging stress

To evaluate the impact of reduced expression of *ADNT1* in plants submitted to waterlogging conditions, we analyzed the expression of genes related to the energy status of the cell (Fig. 11). The gene selection for this analysis was based on signaling of energy deficiency associated with stress, *SnRK1* (SNF1-related kinase 1), modulation of adenylates pool, *ADK* (Adenylate Kinase), diphosphate kinase activity which produces AMP, *APY* (Apyrase), and *PPDK* (Pyruvate phosphate dikinase), and a gene marker of stress response to hypoxia conditions, *PDC1* (Pyruvate decarboxylase 1). The gene *SnRK1* was highly expressed in ADNT1 deficient plants throughout the waterlogging period in times 0, 24, and 48 hours of stress with a significant difference in time 24 hours to *adnt1* mutant, and in all-time points for Line 10. The expression of *ADK* was significantly higher only at times 0, and 12 hours in the *adnt1* mutant line. The *APY* showed a pattern with a reduction in expression during the analyzed stress period, with the *adnt1* mutant and Line 10 exhibiting higher expression at time 0 and lower at time 48 and 72 hours in comparison with wild-type. The ADNT1 deficient lines showed higher expression of *PDC1* at 48, and 72 h of root waterlogging in comparison with wild-type, with a statistical difference in *adnt1* mutant at time of 72 hours, and in Line 10 in times 48 and 72 hours. Also, the expression of *PPDK* was analyzed and the lines with low expression of *ADNT1* display a bigger level in times 48, and 72 h, although without a statistical difference in comparison with wild-type.

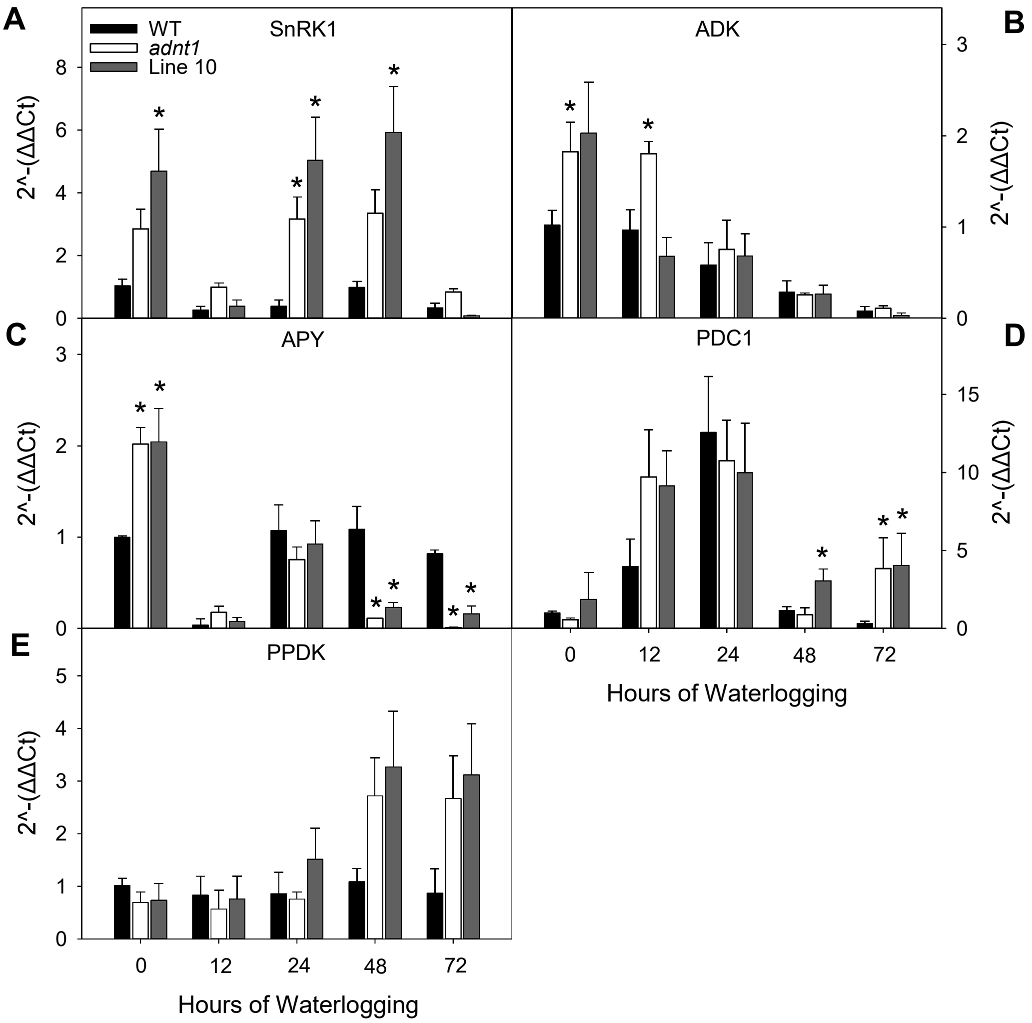

Figure 11. Changes in transcript levels in 4-week-old, short-day-grown Arabidopsis plants with reduced expression of ADNT1 carrier under root waterlogging treatment for 0, 3, 7, and 12 days. Transcript abundance is shown for genes associated with the change in energy status of the cell *SnRK1* (A), *ADK* (B), *APY* (C), *PDC1* (D), and *PPDK* (E). Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$) from the wild-type at each time point analyzed.

DISCUSSION

Does the reduced expression of ADNT1 carrier cause leaf senescence during waterlogging stress?

When plants are submitted to hypoxic conditions such as soil waterlogging, this affects not just the root system but also the aerial part (Mustroph et al., 2014). When O_2 becomes limited, this affects primarily aerobic respiration, resulting in an energy deficit and ultimately in cell and tissue death (Bailey-Serres et al., 2012). The adenylate pool is perturbed by inhibition of ETC, the ratios ATP/ADP and ATP/AMP decrease, and this triggers metabolic changes, that ultimately result in an early senescence process. In *ADNT1* expression deficient plants this process occurs early than wild-type plants, because without the ADNT1 carrier the AMP accumulates more rapidly in the cytosol, leading to senescence of aerial part in these plants. This can be explained by the action of ethylene in the response to prolonged waterlogging stress (Bailey-Serres et al., 2010; Loreti et al., 2016). Although the deficiency of ADNT1 doesn’t generate visible alterations in phenotypic (Fig. 3A) in chlorophyll content (Fig. 4E), but the greater leaf loss (Fig. 3C) and a minor maximum quantum yield of PSII electron transport (Fig. 4A) indicate that ADNT1 deficient plants suffered great damages in comparison with wild-type.

Deficiency in ADNT1 expression leads to early “hypoxic status” in *A. thaliana*

During the hypoxic conditions, the ratios of adenylates are perturbed due to a decrease in ETC activity in the function of oxygen limitation (Geigenberger, 2003). Because of that, the ratios of ATP/ADP and ATP/AMP decrease (Phukan et al., 2015). We observed an increase in the expression of *SnRK1*, *ADK*, and *PDC1* in ADNT1 deficient plants. The increase of expression of these genes was followed by metabolic changes, such as, alanine accumulation, which is one of the first responses of plants in conditions of low O_2 concentration (Rocha et al., 2010). The fact that ADNT1 carrier deficient plants exhibited increased alanine levels in both leaves and roots than wild-type (Fig. 7 and 9) indicates that these plants achieve hypoxic status prematurely. All this added to the fact that also other metabolites like, aspartate, trehalose, and inositol are common metabolites that accumulate during hypoxic stress (Mustroph et al., 2014), support the hypothesis that ADNT1 deficient plants are more sensitive to root waterlogging.

When the plants are submitted to hypoxia, the ETC decreases the activity considerably, increasing the NADH/NAD$^+$ ratio in the mitochondrial matrix, consequently, the TCA cycle also slows down the activity leading to a higher level of your intermediates at first, but follow to a decrease in function of pyruvate metabolization in the cytosol, reducing substrate do TCA Cycle (Abadie et al., 2017). In agreement, the ADNT1 deficient plants exhibited a large reduction in organic acids (citrate, fumarate, malate, and succinate) than wild-type plants in particular on roots that are directly exposed to hypoxic conditions. Because ETC is inhibited under hypoxic conditions, ATP production is considerably affected making glycolysis the main pathway for supply energy to metabolism. Because of the lower capacity of glycolysis to produce ATP in comparison with ETC, larger sugar consumption may occur. However, low O_2 may also cause an increase in sugar content in the aerial part due to the impairment of sugar translocation to roots (Bailey-Serres and Voesenek 2010). The last case is probably what's happened with all ADNT1 deficient genotypes, because leaves accumulated sugars in general over time (Fig. 8), while in roots sugars drop at the end of the stress period after a primary increase (Fig. 10). That said, these results suggest an early response of ADNT1 deficient plants to hypoxic conditions, due to a probably higher AMP level in the cytosol of these plants. This promotes stress responses prematurely in ADNT1 deficient plants, which can be seen by the increase of alanine levels, and other stress metabolites markers, such as aspartate, trehalose, and inositol.

Reduced expression of ADNT1 carrier affects the expression of genes related to waterlogging stress responses

SnRK1 is well known as a key sensor of the energy status of the cell and can be regulated by the level of sugars and adenylates (Baena-González et al., 2007; Baena-González and Sheen, 2008; Geigenberger et al., 2010). The expression of this gene is greater in ADNT1 deficient plants already in non-stress conditions, and over time stress period. This result suggests that the expression of *SnRK1* might be altered by the perturbation in the adenylate pool caused by ADNT1 disruption and not directly by the waterlogging stress as already suggested (Bailey-Serres et al., 2010; IM et al., 2014). We also observed that *ADK* and *APY* have higher expression already under non-stress conditions in the ADNT1 deficient lines. In the case of *ADK*, the absence of ADNT1 probably causes a higher level of AMP in the cytosol which requires the action of ADK to equilibrate the levels of adenylates and the ratios (Dzeja and Terzic, 2009; De Col et al., 2017). Although is unexpected that greater expression of *APY* in non-stress conditions in ADNT1 deficient plants. Apyrase produces AMP through ATP hydrolysis by diphosphate activity, which helps in adjusts the adenylate pool (Igamberdiev and Kleczkowski, 2006; Clark and Roux, 2018). Since we expect that ADNT1 deficient plants under waterlogging conditions possess a greater AMP level in the cytosol, it is intriguing the high expression of *APY* under non-stress conditions. After 48 and 72h of waterlogging stress, the expression of *APY* drops in ADNT1 deficient plants (Fig. 11C), which cooperates with the hypothesis that higher AMP level in these plants results in the dispensability of APY to increase the AMP levels to equilibrate the adenylate pool. Additionally, the greater expression of *PDC1* and *PPDK* during the waterlogging stress period was observed in the ADNT1 deficient plants (Fig. 11D, and E). Suggests an early and more expressive induction of fermentative pathway in ADNT1 deficient plants, in the response of drop of O_2 levels, which follow pyruvate accumulate in the time course of stress.

CONCLUSIONS

Our findings indicate that the ADNT1 carrier performs an important role in plants during waterlogging stress, being the only mitochondrial adenylate carrier up-regulated in roots of plants submitted to waterlogging stress. We hypothesize that the absence of this carrier generates an early “hypoxic status” due to perturbation of the adenylate pool caused by reduced AMP transport to mitochondria. This perturbation in the adenylate pool causes a decrease in ATP/AMP ratio that is sensed by SnRK1, resulting in metabolic changes, induces the fermentative pathway prematurely in ADNT1 deficient plants. These premature responses can result in rapidly reserves consumption, in prolonged stress, which can be disadvantageous to plant leading to a great susceptibility to waterlogging conditions.

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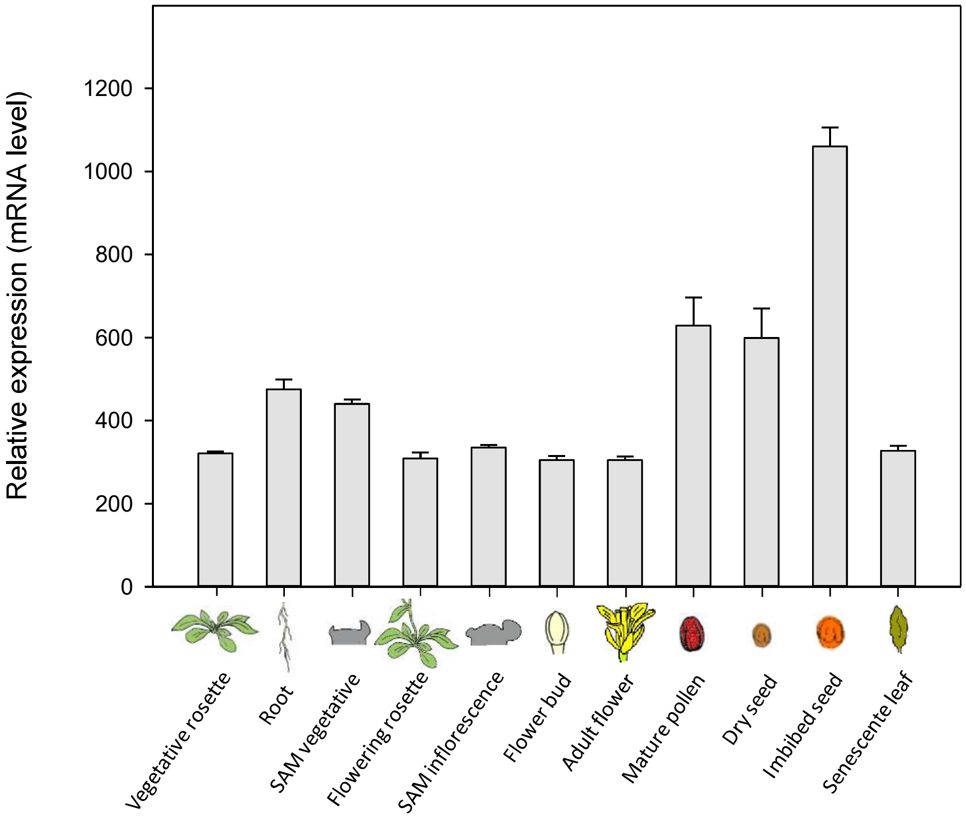
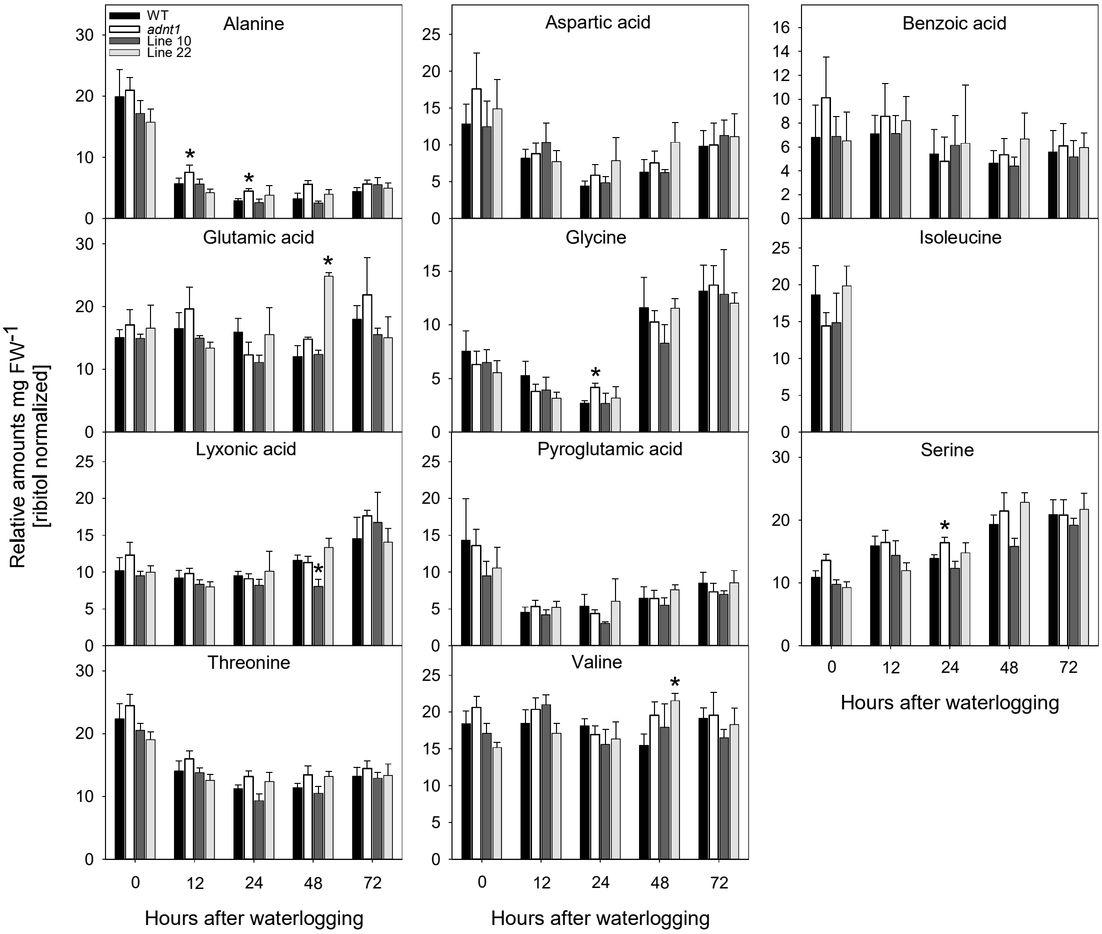
SUPPLEMENTAL DATA

Supplemental Table 1. List of primer used in RT-PCR analysis

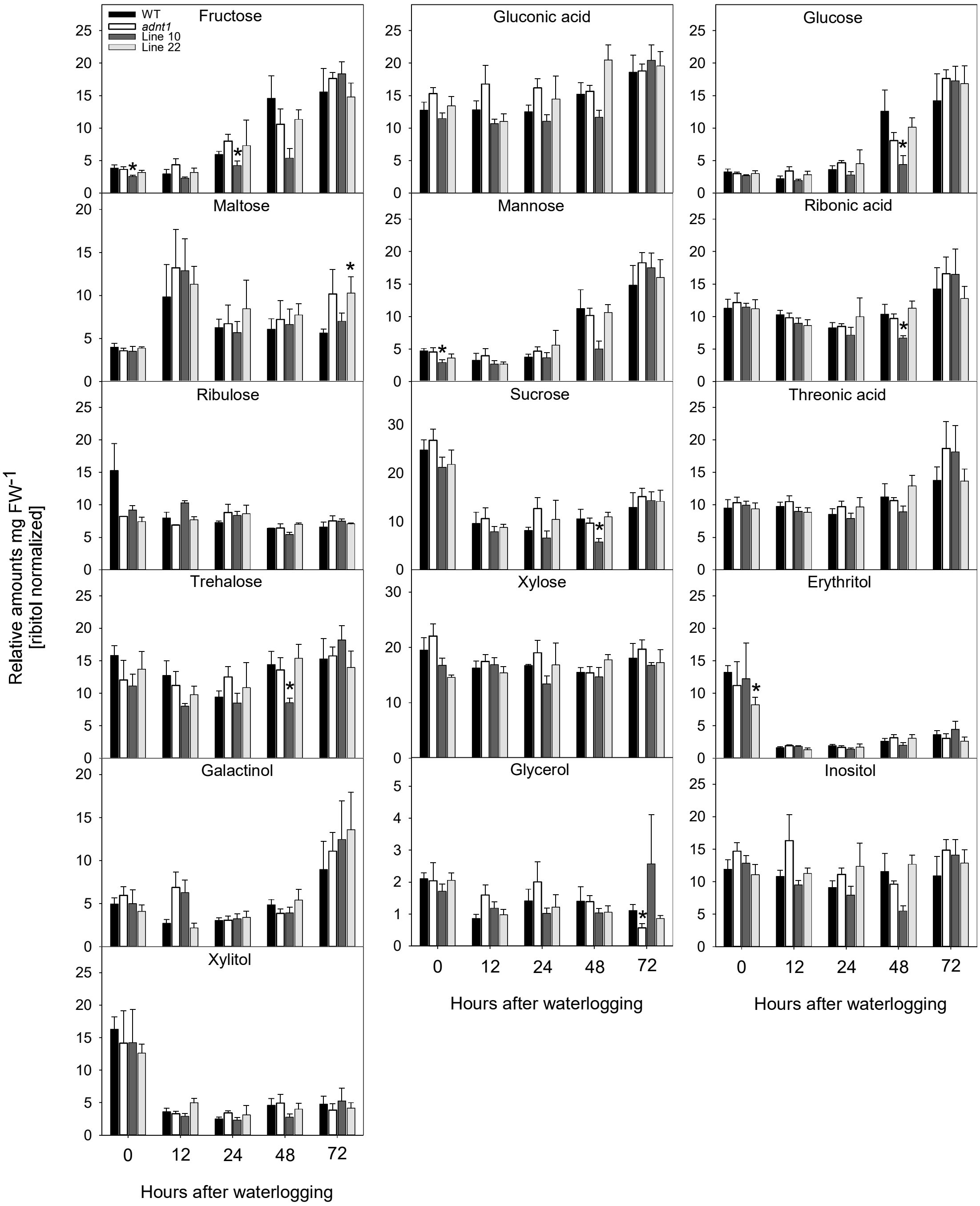
Gene	ID	Forward primer	Revese primer
Actin	AT3G18780.2	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
EF1a	AT5G60390.1	TTCGCTGTTAGGGACATGAGG	GTCGATCATAACGAAAGTCTCA
AAC1	AT3G08580.1	CTCCTCACTGGTGACTTACAGGAC	TTGTACTTGACAGCTTCACCAGAC
AAC2	AT5G13490.1	GGTTCGTTGTGGAGAGGAAACACC	ATGCAAAGTTCAAGGCCTGAGTGG
AAC3	AT4G28390.1	CGAAGGAATGCTTGCTCTTTGGAG	CGCAAAGTTTAAAGCCTGAGTGG
ADNT1	AT4G01100.1	GGGGCTTAGCCAAACTCTTT	TGAGAAAAGAGGAGCCCAAA
APC1	AT5G61810.1	GGAGCCAGGTCCTTTGATACAAC	TCAGAAACTCTTGGCCCATGC
APC2	AT5G51050.1	GAAGACAAAGCGGATATAGGCAC	TCGTGTAAGTCTGCAACCGAGTT
APC3	AT5G07320.1	CGAGATGGGCGTGTTGATTACC	AATGCCGGCCTTAACAAGAGC
SnRK1.2	AT3G01090.1	CAACCGAACCCAGAATGATGGC	AGGCACGGAAACGATTGTCCAG
ADK	AT2G37250.1	ATGGAGTTTGACTTACCTGGAGG	GTGAACTTATGCTGCGACAGAC
APY	AT3G04080.1	GGATTCGGATTGAAGCCATCAC	GCAAATACCCAAAATTGCCTTCCT
Pdci	AT4G33070.1	CGACCGTTGGGTTTAATCATGT	CCATGATAAAGCGTACATGGAAA
PPDK	AT4G15530.5	TGAGAAGGGTCATACCGTGAGC	ACTCCGCCTCTTTCGCAATCTC



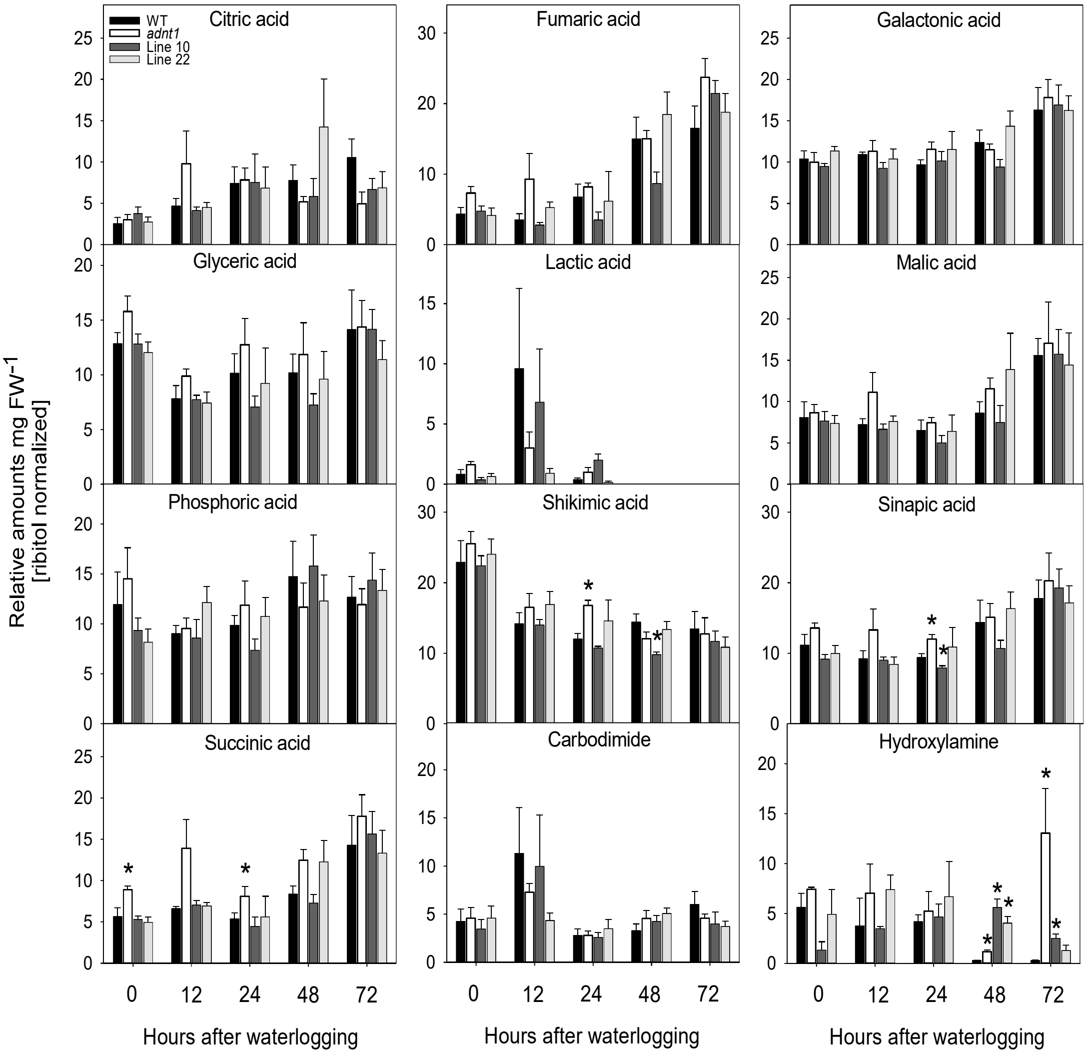
Supplemental Figure 1. Expression profile of gene encoding mitochondrial adenylate carrier ADNT1 in different organs and developmental stages of *Arabidopsis thaliana*. The expression data corresponding to mRNA levels retrieved from the expression browser tool at the Bioarray Resource (BAR) (www.bar.utoronto.ca).



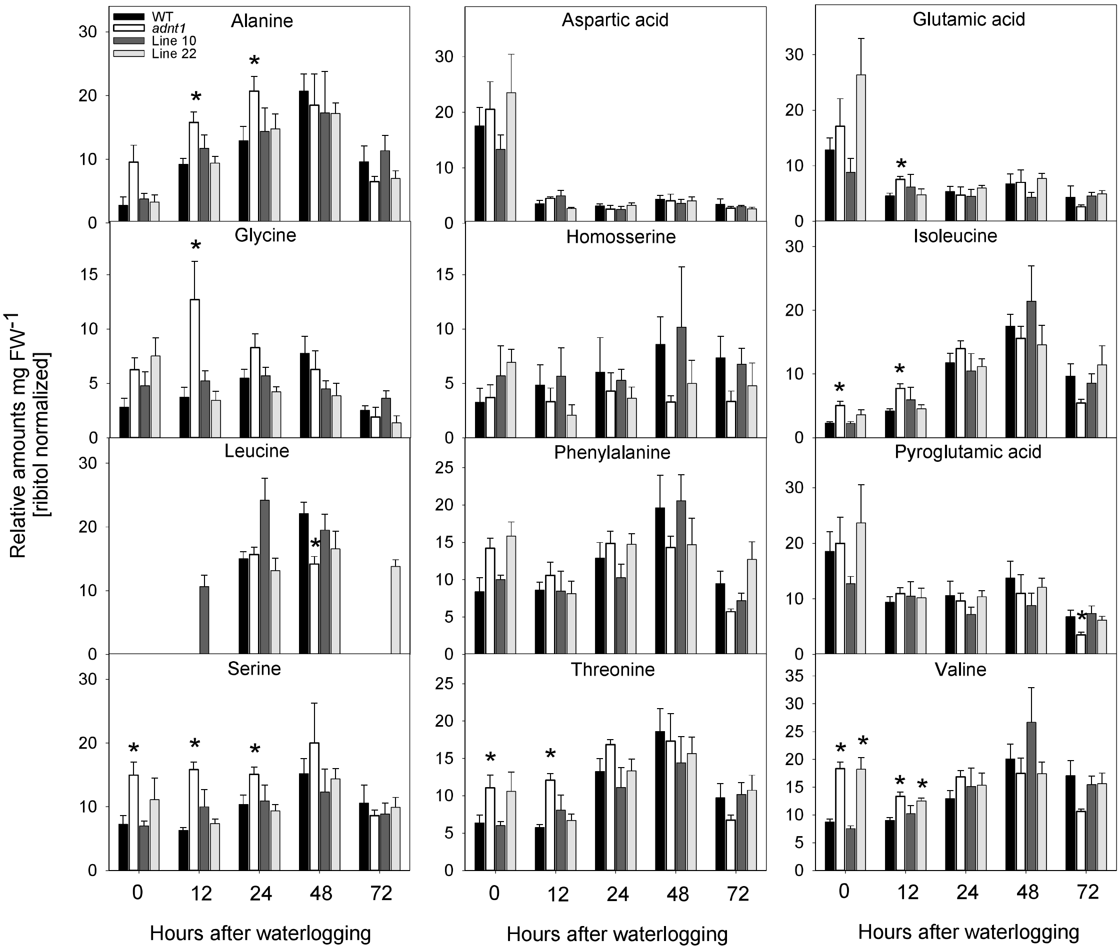
Supplemental Figure 2. Amino acids extracted from leaves of 4 weeks plants submitted to waterlogging conditions in times 0, 12, 24, 48, and 72h were analyzed by GC-MS. Chromatograms and mass spectra were evaluated using TagFinder 4.0 (Luedemann et al., 2008). The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples, asterisks indicate statistically significant differences as compared to wild type by t-test (*$P < 0.05$).



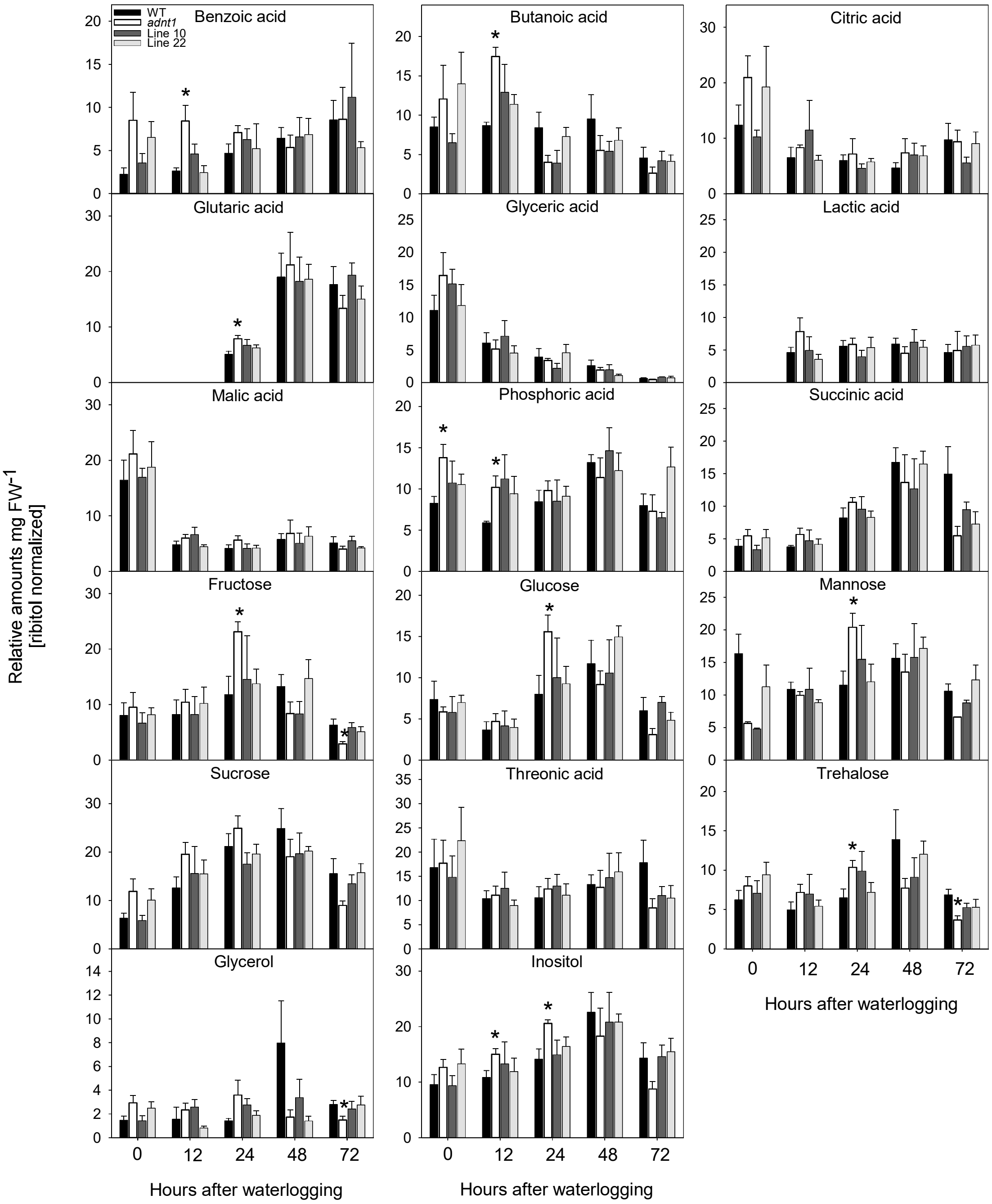
Supplemental Figure 3. Sugars and alcohol sugars extracted from leaves of 4 weeks plants submitted to waterlogging conditions in times 0, 12, 24, 48, and 72h were analyzed by GC-MS. Chromatograms and mass spectra were evaluated using TagFinder 4.0 (Luedemann et al., 2008). The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples, asterisks indicate statistically significant differences as compared to wild type (WT) by t-test (**P* < 0.05).



Supplemental Figure 4. Organic acids and other metabolites extracted from leaves of 4 weeks plants submitted to waterlogging conditions in times 0, 12, 24, 48, and 72h were analyzed by GC-MS. Chromatograms and mass spectra were evaluated using TagFinder 4.0 (Luedemann et al., 2008). The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples, asterisks indicate statistically significant differences as compared to wild type (WT) by t-test (**P* < 0.05).



Supplemental Figure 5. Amino acids extracted from roots of 4 weeks plants submitted to waterlogging conditions in times 0, 12, 24, 48, and 72h were analyzed by GC-MS. Chromatograms and mass spectra were evaluated using TagFinder 4.0 (Luedemann et al., 2008). The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples, asterisks indicate statistically significant differences as compared to wild type (WT) by t-test (*$P < 0.05$).



Supplemental Figure 6. Organic acids, sugars, and alcohol sugars extracted from roots of 4 weeks plants submitted to waterlogging conditions in times 0, 12, 24, 48, and 72h were analyzed by GC-MS. Chromatograms and mass spectra were evaluated using TagFinder 4.0 (Luedemann et al., 2008). The data were normalized by fresh weight (FW), internal control (Ribitol). Values are means ± standard error of six independent samples, asterisks indicate statistically significant differences as compared to wild type (WT) by t-test (**P* < 0.05).

CHAPTER III

FUNCTIONAL CHARACTERIZATION OF THE APC1 MITOCHONDRIAL CARRIER IN *Arabidopsis thaliana*

ABSTRACT

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, March, 2021. **Functional characterization of the APC1 mitochondrial carrier in *Arabidopsis thaliana***. Adviser: Adriano Nunes Nesi.

The three isoforms of the mitochondrial ATP-Mg/P_i transporter in *A. thaliana* (APC1, APC2, and APC3), were initially predicted by sequence similarity with the already identified mitochondrial ATP-Mg / Pi carrier in humans. Unlike other mitochondrial adenylate carriers, they transport ATP without the antiporter of another adenylate (ADP or AMP), the antiport is made by inorganic phosphate (Pi). The CRISPR/Cas9 system was used through the vector pKAMA-ITACHI (pKI1.1R) to obtain mutant plants to characterize this transporter in *A. thaliana*. After two generations we obtained eleven Cas9 free T2 lines with alteration in the gene sequence. Four generated lines (Line 8.7, 10.2, 10.9, and 11.2) were selected because they exhibited different mutations between them. We observe that APC1 mutant plants exhibit reduced silique length and a lower number of seeds per silique. Additionally, our results indicate that defective APC1 protein leads to the production of short roots and reduced fresh weight in seedlings growing under a sucrose-free medium. These results indicate that disruption of APC1 can generate seeds with compromise reserves, which explains the reason for root growth and seedling fresh weight is recovered in presence of sucrose. Together, our results suggest that APC1 carrier perform important roles in reproductive organs, affecting mainly silique size and seed production.

Keywords: ATP, Mitochondrial Carrier Family (MCF), silique length, CRISPR/Cas9 system.

RESUMO

NERI-SILVA, Roberto, D.Sc., Universidade Federal de Viçosa, março de 2021. **Caracterização funcional do transportador mitocondrial APC1 em *Arabidopsis thaliana***. Orientador: Adriano Nunes Nesi.

As três isoformas do transportador mitocondrial ATP-Mg / Pi em *A. thaliana* (APC1, APC2 e APC3), foram inicialmente preditas por similaridade de sequência com o transportador mitocondrial ATP-Mg / Pi já identificado em humanos. Ao contrário de outros transportadores de adenilatos mitocondriais, eles transportam ATP sem o antiporte de outro adenilato (ADP ou AMP), o antiporte é feito por fosfato inorgânico (Pi). O sistema CRISPR / Cas9 foi utilizado por meio do vetor pKAMA-ITACHI (pKI1.1R) para obter plantas mutantes para caracterizar este transportador em *A. thaliana*. Após duas gerações, obtivemos onze linhagens T2 sem a presença de Cas9 com alteração na sequência do gene. Quatro linhas (Linha 8.7, 10.2, 10.9 e 11.2) foram selecionadas por exibirem mutações diferentes entre si. Nós observamos que as plantas mutantes APC1 apresentam um menor comprimento de síliquas e um menor número de sementes por síliquas. Além disso, nossos resultados indicam que a proteína APC1 defeituosa leva à produção de raízes curtas e redução do peso fresco em plântulas em meio sem sacarose. Esses resultados indicam que a inativação de APC1 pode gerar sementes com reservas comprometidas, o que explica o motivo do crescimento prejudicado da raiz e o fato do peso fresco das plântulas ser recuperado na presença de sacarose. Juntos, nossos resultados sugerem que o transportador APC1 desempenha um papel importante nos órgãos reprodutivos, afetando principalmente o tamanho da síliquas e a produção de sementes.

Palavras chave: ATP, Família de Transportadores Mitocondriais, comprimento de síliquas, Sistema CRISPR/Cas9.

INTRODUCTION

Respiratory metabolism, present in all eukaryotic organisms that perform aerobic respiration, can be divided into three main stages: glycolysis, tricarboxylic acid (TCA) cycle, and electron transport chain (ETC) (Millar et al., 2011). The main function of respiration is to convert organic compounds into chemical energy in the form of adenosine triphosphate (ATP) (Fernie et al., 2004). Besides, the respiration stages provide carbon skeletons for biosynthetic processes (van Dongen et al., 2011) connecting the mitochondria directly or indirectly, with all the organelles of the plant cell (Sweetlove et al., 2007). This connection is made by transmembrane proteins located in the inner membrane of the mitochondria (Palmieri et al., 2011b). Most of these transporters belong to a mitochondrial carrier family (MCF) (Picault et al., 2004b; Monné and Palmieri, 2014). Currently, 58 MCF members were identified in the *Arabidopsis thaliana* genome (Toleco et al.; Nunes-Nesi et al., 2020). The MCF is characterized by having a conserved structure of six transmembrane domains, composed of three repetitive modules, with about 100 amino acids in length each (Haferkamp and Schmitz-Esser, 2012). Although MCF members have a large part of their structure highly conserved in different organisms (Palmieri et al., 2011b), they also exhibit high variability in the mode of transport, substrate, and specificity (Palmieri et al., 2008a). Among them are the group of transmembrane proteins called adenylate transporters that are present also in plastid, peroxisome, endoplasmic reticulum, and plasma membrane (da Fonseca-Pereira et al., 2018).

The adenylates (ATP, ADP, and AMP) are adenine-based nucleosides linked to phosphate groups playing a vital role in all living organisms, providing energy to various metabolic processes necessary for the maintenance of cellular homeostasis, both in optimal growth conditions and under stress conditions (da Fonseca-Pereira et al., 2018a). Among the adenylates, the ATP molecule releases a greater amount of energy during the hydrolysis of the phosphate groups, the value of the variation of free energy ($\Delta G'^{\circ}$) is -30.5 kJ/mol for hydrolysis of ATP in ADP and -45.6 kJ/mol for hydrolysis of ATP in AMP (Lehninger et al., 2014).

In heterotrophic eukaryotic cells, ATP is regenerated mainly in the mitochondrial matrix through the binding of ADP + Pi catalyzed through ATP synthase complex that is coupled to mitochondrial ETC via oxidative phosphorylation (Kühlbrandt, 2015). In plants, ATP is also produced in large quantities in the chloroplast, through ATP synthase complex that is coupled to chloroplast ETC, through the photochemical reaction of the photosynthetic process (Gardeström and Igamberdiev, 2016). Most of the ATP produced in the chloroplast is used to fix and reduce CO_2 via the Calvin-Benson cycle, and at night the plant cell relies on the production of mitochondrial ATP (Gardeström and Igamberdiev, 2016). This confirms once again the function of a powerhouse of mitochondria even in plant cells that have a second ETC in the chloroplast, and, therefore, the importance of efficient ATP transport from mitochondria to sustain the metabolism of the plant cell. Considering that, ATP is generated mainly in the mitochondrial matrix and chloroplast stroma but is used throughout the cell, the adenylate carriers are essential for the whole-cell metabolism (Geigenberger et al., 2010).

Until now, 15 adenylate carriers have been identified in plants, and only nine have been functionally characterized (Arai et al., 2008; Kirchberger et al., 2008; Leroch et al., 2008; Palmieri et al., 2008b; Yin et al., 2010; Rieder and Neuhaus, 2011; Gigolashvili et al., 2012; Hoffmann et al., 2013). The remaining six, which were characterized only in terms of transport mode and substrate specificity are named AAC1-3 and APC1-3 (Haferkamp et al., 2002; Lorenz et al., 2015). The three isoforms of the mitochondrial ATP-Mg/P_i transporter in *A. thaliana* (APC1, APC2, and APC3), were initially predicted by sequence similarity (Palmieri et al., 2011b) with the already identified mitochondrial ATP-Mg / Pi carrier in humans (Traba et al., 2009b). Unlike other mitochondrial adenylate carriers, they transport ATP without the antiporter of another adenylate (ADP or AMP), the antiport is made by inorganic phosphate (Pi), which is then transported back to the cytosol by the mitochondrial phosphate transporter (MPT) (Zhu et al., 2012). Another particular characteristic of these carriers is the existence of Ca^{2+} binding domains at the N-terminal end, which can be sites for regulating the transport of these proteins since Ca^{2+} acts as an important secondary messenger in eukaryotes (Wagner et al., 2016). The three isoforms of the APC carrier were characterized in terms of localization and expression in Arabidopsis plants (Monné et al., 2015). These analyzes indicated that the *At*APC1 isoform is the most expressed in all analyzed tissues, particularly in vascular tissues, seedlings, filaments of stamens, and developing siliques (Monné et al., 2015). Nevertheless, the physiological role of *At*APC1 carrier remains unknown. For this reason, in this study, we investigated the physiological role of *Arabidopsis* *At*APC1 from the early stages of development to the adult plant.

MATERIAL AND METHODS

Obtaining mutant plants using CRISPR/Cas9 System

To obtain mutant plants, the CRISPR/Cas9 system was used through the vector pKAMA-ITACHI (pKI1.1R) (Tsutsui and Higashiyama, 2017). Guide RNAs (gRNA) were designed using the online platform CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) (Liu et al., 2017), with twenty base pairs that have the PAM sequence (NGG) which is the Cas9 endonuclease recognition site (Jinek et al., 2012). Guide RNAs directed preferentially to the first exon were used to increase the chances of generating early stop codons in the sequence of genes, as well as avoiding gRNAs that have off-targets, especially if they are from the other transporter isoforms.

Transformation of *E. coli* and *A. tumefaciens* cells using thermal shock

After the construction of the vector containing the gRNAs, high-efficiency *E.coli* bacteria (DH5α) were transformed by thermal shock (Inoue et al., 1990). The selection of transformed colonies was performed by plating the aliquot used in the transformation in Petri dishes containing 20 mL of solid LB medium containing 100 µg mL^{-1} of Spectinomycin. The plates were kept in an incubation chamber at 37°C for 24 hours for colony growth. Then, the selection of isolated colonies was carried out. The selected colonies were transferred to falcon tubes containing 3 mL of liquid LB medium with Spectinomycin in the same concentration. The tubes were kept in a 37°C incubation chamber with an agitation of 90 rpm for 24 hours. After this period, plasmid DNA extraction from these colonies was performed through mini-preparation and, then a PCR (Polymerase Chain Reaction) was performed to confirm the transformation of these colonies. After that, the plasmid DNA of the confirmed colonies was used to transform *A. tumefaciens* cells using the thermal shock method. These cells went through the same selection process mentioned for *E. coli* cells, with the difference that these cells were incubated at 28°C for a period of 48 to 72 hours at each stage. After confirmation of the transformed colonies by colony PCR, they were used to transform plants of *A. thaliana* (ecotype Col-0) by the method of floral dip (Clough and Bent, 1998).

Genotype characterization and selection of nullizygote plants

The seeds from the plants used for transformation were collected and placed to germinate in a selective MS medium (Murashige T und Skoog F, 1962) containing 50 μg mL^{-1} of Hygromycin. The selected seedlings were transferred to 80 ml pots containing commercial substrate Carolina Soil and grown in a growth chamber, under light radiation of 150 μmol of photons m^{-2} s^{-1}, an average temperature of 21ºC, 60% humidity, and photoperiod of 8h light and 16h dark. After four weeks leaf material was collected for DNA extraction to confirm the mutant plants by PCR using specific primers for the transformation vector sequence. The plants confirmed by PCR were selected for sequencing the gene of interest. The results of the sequencing were analyzed with the aid of the CLC Main Workbench 8 program. The seeds from the mutant were evaluated in a stereomicroscope (model SZX16, Olympus) with X-Cite 120LED (Excelitas Technologies). The seeds without red fluorescence were selected, indicating that these seeds do not have the transformation cassette as described by Tsutsui and Higashiyama (2017). The selected seeds were germinated and placed to grow in a growth chamber, under light radiation of 150 μmol of photons m^{-2} s^{-1}, an average temperature of 21ºC, 60% humidity, and a photoperiod of 8h light and 16h dark.

Plant material and establishment of experimental conditions

The seeds from wild-type (Col-0) and mutants lines were sterilized in 1 mL of 70% ethanol for one minute. Then, the ethanol was removed and the seeds were soaked in 1 mL of 2.5% sodium hypochlorite for 15 min. The dead seeds and the supernatant were discarded and the sterilized seeds were washed at least five times in sterile water. After sterilization, the seeds were distributed in sterile Petri dishes on solid MS medium, containing half of the salts and vitamins MS supplemented with 1% sucrose [w/v], and soaked for 48 hours in the dark, at 4°C. The plates containing the seeds were transferred to a growth chamber under short-day conditions (8 h light/16 h dark) at 150 μmol photons m^{-2} s^{-1}, 21°C, and 60% relative humidity, (RH). Then, 14-day-old seedlings were transferred to plastic pots containing commercial substrate Carolina Soil Padrão EC 0.7 - 8 Kg (Eucatex, São Paulo, Brazil). After transplantation, the plants were kept in a growth chamber under the same conditions mentioned above.

Seed germination and growth analysis

The seeds collected from the mutant and wild-type plants were sterilized and grown in a growth chamber, under light radiation of 150 µmol of photons m^{-2} s^{-1}, an average temperature of 21ºC, 60% humidity, and photoperiod of 8h light and 16h dark. Six repetitions were carried out with 50 seeds for each genotype. The following growth parameters were analyzed in 4-week-old plants: rosette dry mass (RDW), rosette leaf area (RLA), number of leaves (LN), total leaf area (TLA). RLA and TLA were determined by the digital imaging method, where the sheets were digitized with a coupled scanner (Hewlett Packard Scanjet G2410, Palo Alto, California, USA). The images were processed in the ImageJ software (Schneider et al., 2012).

Determination of photosynthetic parameters

The fluorescence emission assessments of chlorophyll *a* and gas exchange were carried out on fully expanded leaves of the mutant and wild plants, with 4-week-old plants. The fluorescence of chlorophyll *a* was assessed using a modulated pulse amplitude fluorometer (Mini-PAM, H. Walz, Effeltrich, Germany). After being adapted to the dark, for 30 minutes, leaf tissues were initially exposed to a weak pulse of distant red light (0.03 µmol m^{-2} s^{-1}) to determine the initial fluorescence. Then, a pulse of saturating light with an irradiance of 6000 µmol photons m^{-2} s^{-1} and a duration of 0.8 s, was applied to estimate the maximum fluorescence emitted. The maximum photochemical efficiency of the FSII, the quantum electron transport efficiency (qFSII), the efficiency of excitation energy capture by the open reaction centers of the FSII (Fv '/ Fm'), and the electron transport rate (ETR) was also estimated. (Damatta et al., 2002; Lima et al., 2002).

The net assimilation rate of carbon (*A*), stomatal conductance (g_s), sub-stomatic CO_2 concentration (C_i), the ratio between the sub-stomatic concentration and CO_2 environment (C_i/Ca), and leaf transpiration (*E*) were evaluated in a system open, under artificial light equivalent to that of the environment and partial CO_2 pressure of 38 Pa, with an infrared gas analyzer (Li 6400XT, Li-Cor, Lincoln, USA).

Determination of metabolite contents

Leaf samples were collected at three time points, beginning, middle, and end of the light period. Aliquots of 50-60 mg frozen and soak leaf material of 4-week-old plants were homogenized in liquid nitrogen and subsequently, the metabolites were extracted with a mixture of chloroform–methanol-water according to Lisec et al., (2006). The soluble fraction and the precipitated material resultant from the extraction were stored at -20ºC. The soluble fraction was used to quantify the contents of chlorophylls *a* and *b*, soluble carbohydrates (glucose, fructose, and sucrose) (Fernie et al., 2001a), total soluble amino acids (Cross et al., 2006), and malate and fumarate (Nunes-Nesi et al., 2007). The ethanol insoluble fraction was used to quantify the levels of starch (Fernie et al., 2001a) and proteins (Bradford, 1976).

Characterization of the reproductive stage

To characterize the reproductive organs, the seedlings were transferred to the commercial substrate Carolina Soil and kept in a growth room at 22°C ± 2, 60% RH, the irradiance of 150 µmol of photons m^{-2} s^{-1}, with a photoperiod of 12 h of light and 12 h of dark for twelve weeks (for seed production). Siliques from wild and mutant plants were collected and clarified with a solution of 0.2N NaOH and 1% sodium dodecyl sulfate [w/v] to remove chlorophyll (Yoo et al., 2012). Ten clarified siliques were used to analyze the length, width, and the number of seeds per silique under a dissecting microscope (Stemi 2000-C, Zeiss) with an adapter (SOLIGOR tube for Canon A650 IS B52 Wide) for CANON 12.1 megapixels camera (Power Shot A650 IS). The number of fertile and siliques branches per plant was also quantified. Six plants per genotype were used for the analyses. Also, ten seeds from ten different siliques from each plant were evaluated, using six replicates per genotype, in terms of length and diameter using the aforementioned devices. Besides, the weight of a thousand seeds and the weight of all seeds produced by the same plant were quantified. The biometric data obtained from the photographic record was evaluated using the program ImageJ version 1.51j8 for Windows (University of Wisconsin-Madison).

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. The data source is retrieved from Schmid et al., (2005).

Experimental design and statistical analysis

The experiments were conducted in a completely randomized design, with six replications. To determine the difference between genotypes. *t*-test was performed using the algorithm incorporated in Microsoft Excel (Microsoft Corporation, Seattle, WA, USA). Uneven variances were assumed and taken into account in the calculations. Values with $P<0.05$ with the *t*-test were considered significant.

RESULTS

We performed a bioinformatic analysis with the data of expression of all seven mitochondrial adenylate carriers in different organs and tissues (Fig. 1) using publicly available data from the expression browser tool at the Bioarray Resource (BAR) (Waese and Provart, 2017). We can see that the AAC's carriers are the genes with the high expression signal from all. Also, the AAC1 is the isoform with the greatest expression level follow by AAC2 and AAC3. The expression pattern of AAC1 and AAC2 is more similar, since the tissue with high expression is mature pollen, and in AAC3 is dry seed, which is the lower expression data in AAC1 and AAC2. ADNT1 has a relatively stable expression level in all organs and tissues analyzed, with mature pollen, dry and imbibed seed exhibiting greater values.

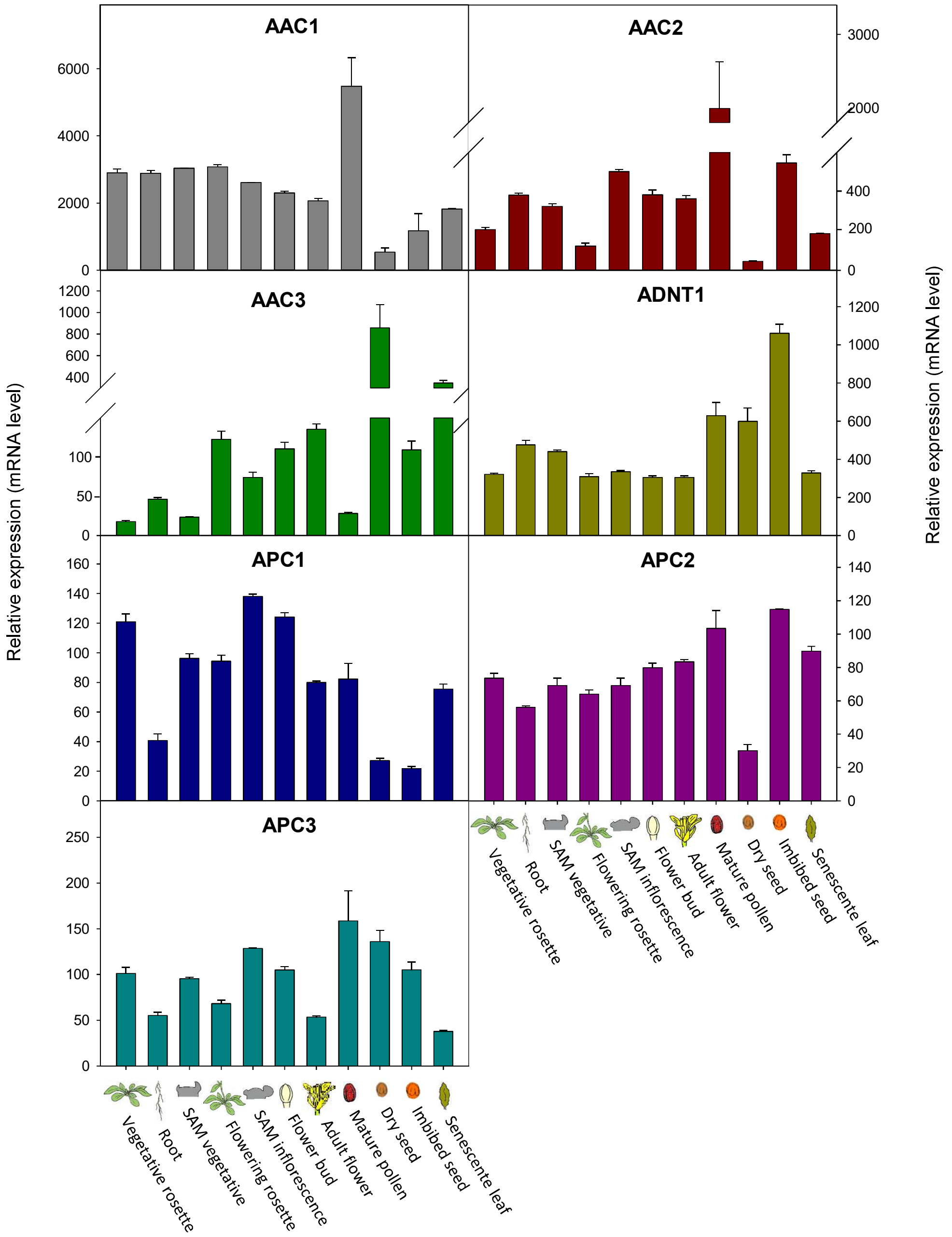


Figure 1. Expression profile of gene encoding mitochondrial adenylates carriers in different organs and developmental stages of *Arabidopsis thaliana*. The expression data corresponding to mRNA levels retrieved from the expression browser tool at the Bioarray Resource (BAR) (http://www.bar.utoronto.ca).

The APC's carrier shows a very similar intensity in expression signal, although it has already been confirmed by RT-qPCR and histochemical analysis (GUS) that APC1 is the isoform with a high expression (Monné et al., 2015). The APC1 exhibits a high expression level in the vegetative rosette, Shoot Apical Meristem (SAM) inflorescence, and flower bud. In the APC2 the great expression level is mature pollen, imbibed seed, and senescent leave. In last, APC3 shows high expression values in SAM inflorescence, mature pollen, and dry seed.

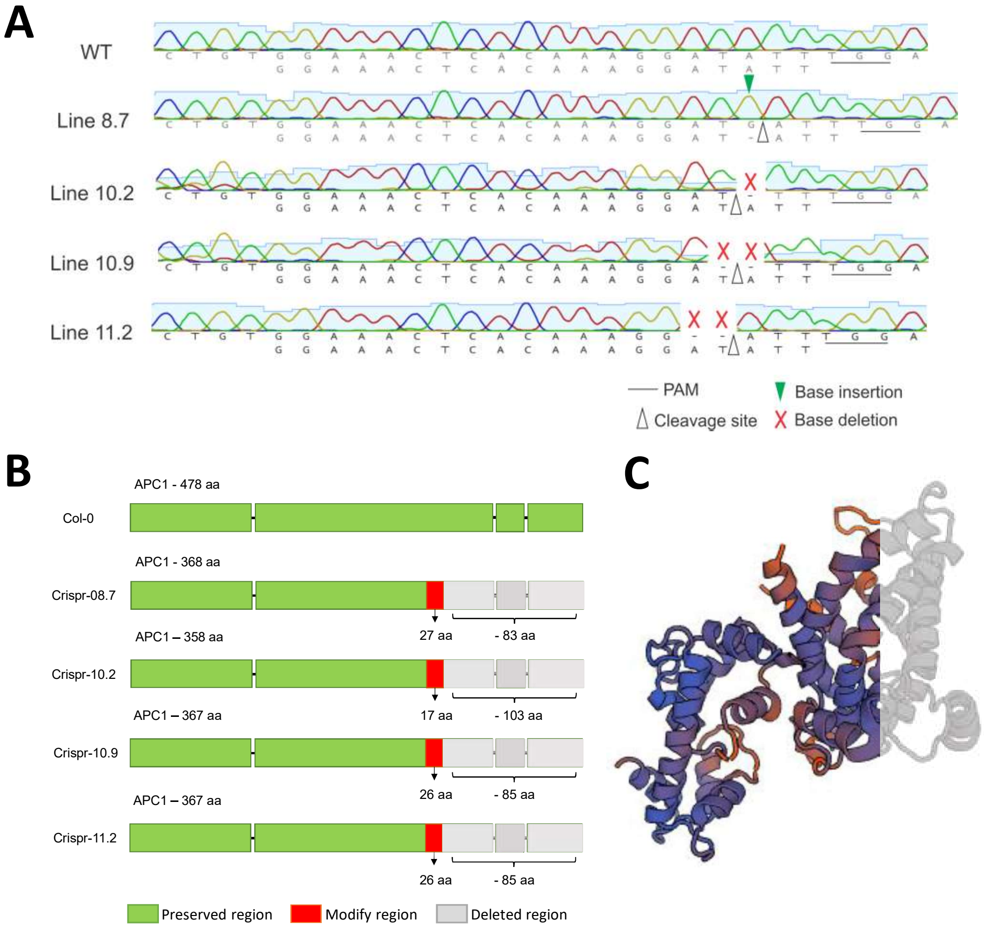


Figure 2. Representation of alterations in the Cas9-free T_2 lines. Sequencing results of lines analyzed in software Geneious Prime 2020 showing the modified region by Cas9 activity (**A**). The models represent the proteins with the respective domains, in green represent the region with no alterations in amino acids sequence, in red is the region with modifications in amino acids sequence in comparison with the wild-type protein, and in the region in light gray is the region where the amino acids were deleted from the final protein (**B**). 3D structure of APC1 protein showing in the erased region the area of barrel where the amino acids were deleted in the mutant lines (**C**).

To characterize the function of APC1 in plants, the CRISPR/Cas9 system was used to obtain mutant lines, in T1 generation were obtained five lines with alterations in the gene sequence. After two generations we obtained eleven Cas9 free T2 lines with alteration in the gene sequence. Four generated lines were selected because they exhibited different mutations between them, being that line 8.7 have an insertion of one guanine (G), the line 10.2 present a deletion of one adenine (A), line 10.9 exhibit a deletion of one thymine (T) and one adenine (A), and for last the line 11.2 present a deletion of one adenine (A) and one thymine (T) in this order on gene sequence (Fig. 2A). The deleted bases affected the reading frame of the gene, creating an early stop-codon, compromising the second, third, and forty domains of the protein (Fig. 2B). The compromised region of protein corresponds to the barrel of the APC1 carrier which is essential for its function creating, as well as, a defected protein (Fig. 2C).

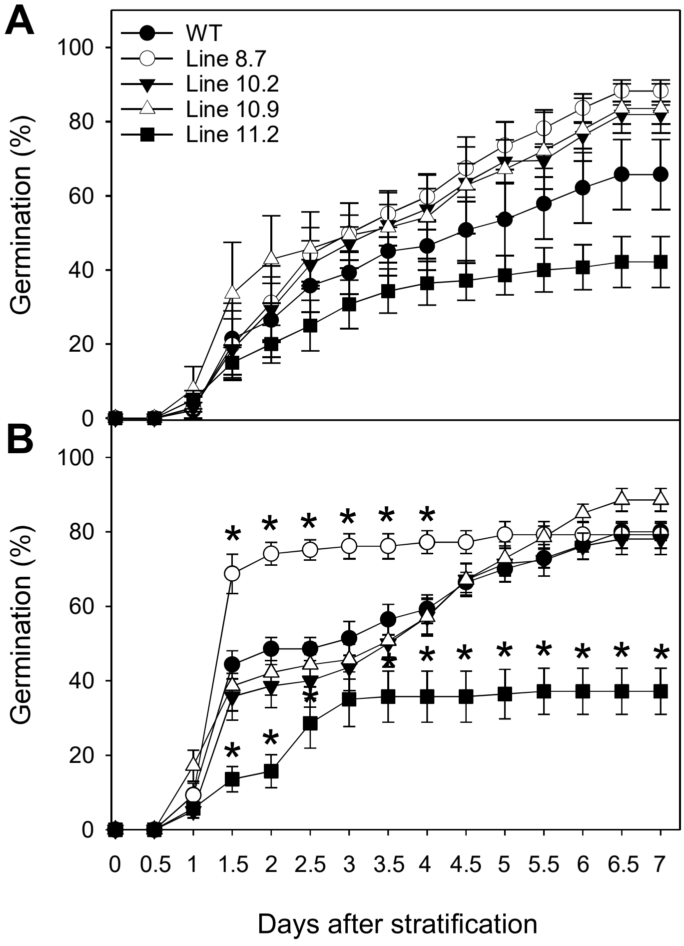


Figure 3. The seed germination rate of APC1 Cas9-free Arabidopsis mutant lines on agar plates containing half-strength Murashige and Skoog (MS) medium plus 1% (w/v) (**A**) sucrose or (**B**) sucrose-free. Radicle emergence was used as a morphological marker for germination.

Values are means ± SE of five independent biological replicates. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

The APC1 carrier has a strong expression in flowers, as well as in young siliques (Monné et al., 2015). Thus, to evaluate the APC1 role during seedling development, we performed a germination test in the presence and absence of sucrose in the medium. On seeds germinated in presence of sucrose (Fig. 3A), we did not observe any difference among the genotypes over the time course of the germination process, although in absence of sucrose (Fig. 3B) line 8.7 displayed a higher germination rate from a time of 1.5 to 4 days, while line 11.2 showed opposite result with a reduced germination rate from a time 1.5 to 7 days.

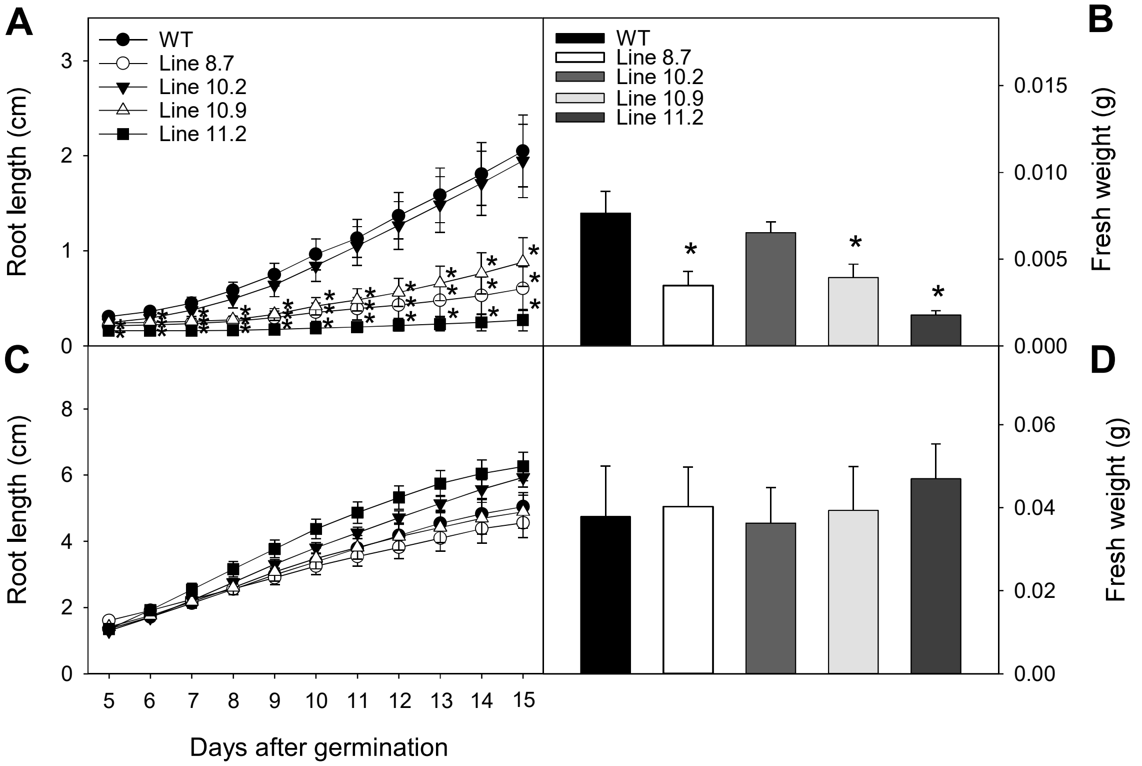


Figure 4. Root length of APC1 Cas9-free Arabidopsis mutant lines seedlings grown on agar plates containing half-strength Murashige and Skoog (MS) medium sucrose-free (**A**) or plus 1% sucrose (w/v) (**C**). Fresh weight of seedling with fifteen days grown on half-strength MS medium sucrose-free (**B**) or plus 1% sucrose (w/v) (**D**). Values are means ± SE of five independent biological replicates. Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05).

Likewise, we investigated the impact of APC1 loss on root growth, on the same conditions of a germination test. We observed a clear phenotype in plants from three lines (8.7, 10.9, and 11.2) that exhibited a reduced root length in comparison with wild-type plants in absence of sucrose (Fig. 4A). Additionally, the same lines showed a reduced fresh weight on 15 days old seedlings (Fig. 4B). Although in presence of sucrose the root length and fresh weight don’t present a difference among the genotypes (Fig. 4C, 4D).

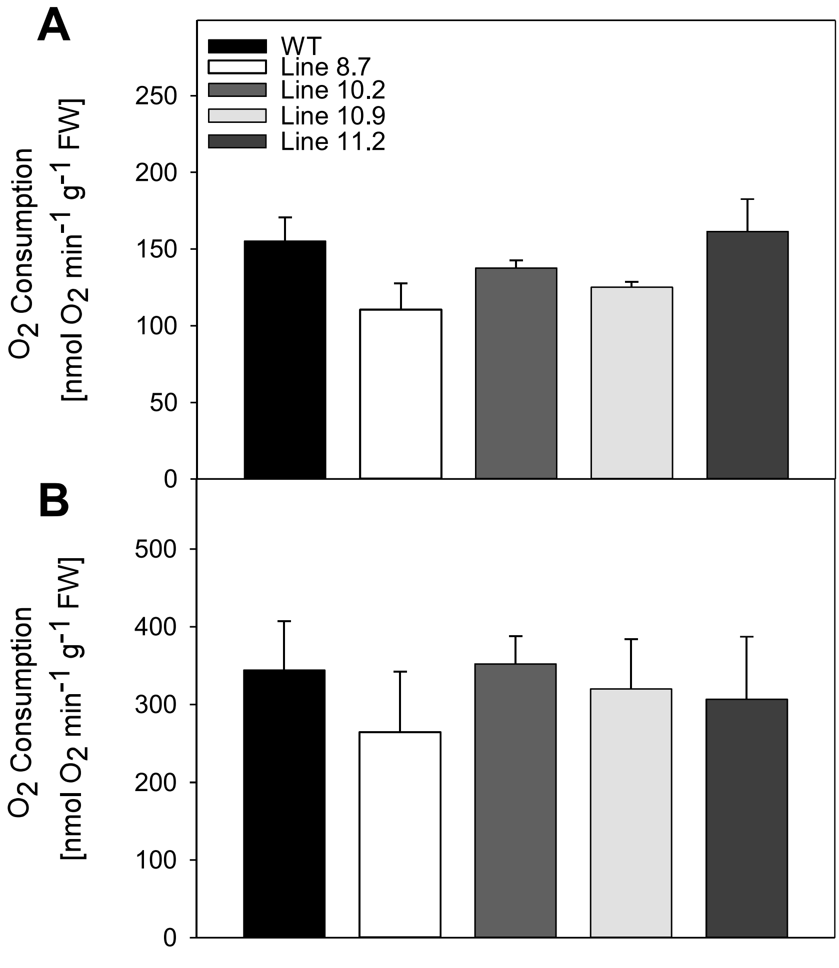


Figure 5. Oxygen consumption in leaves (**A**) and roots (**B**) of three-weeks-old APC1 Cas9-free Arabidopsis mutant lines. Values are means ± SE of four individual determinations per genotype.

To evaluate if the small root length in APC1 deficient plants is due to the reduced respiratory rate we analyzed the oxygen consumption by leaves and roots tissues of three-week-old plants. Both organs did not exhibit any differences between all genotypes (Fig. 5).

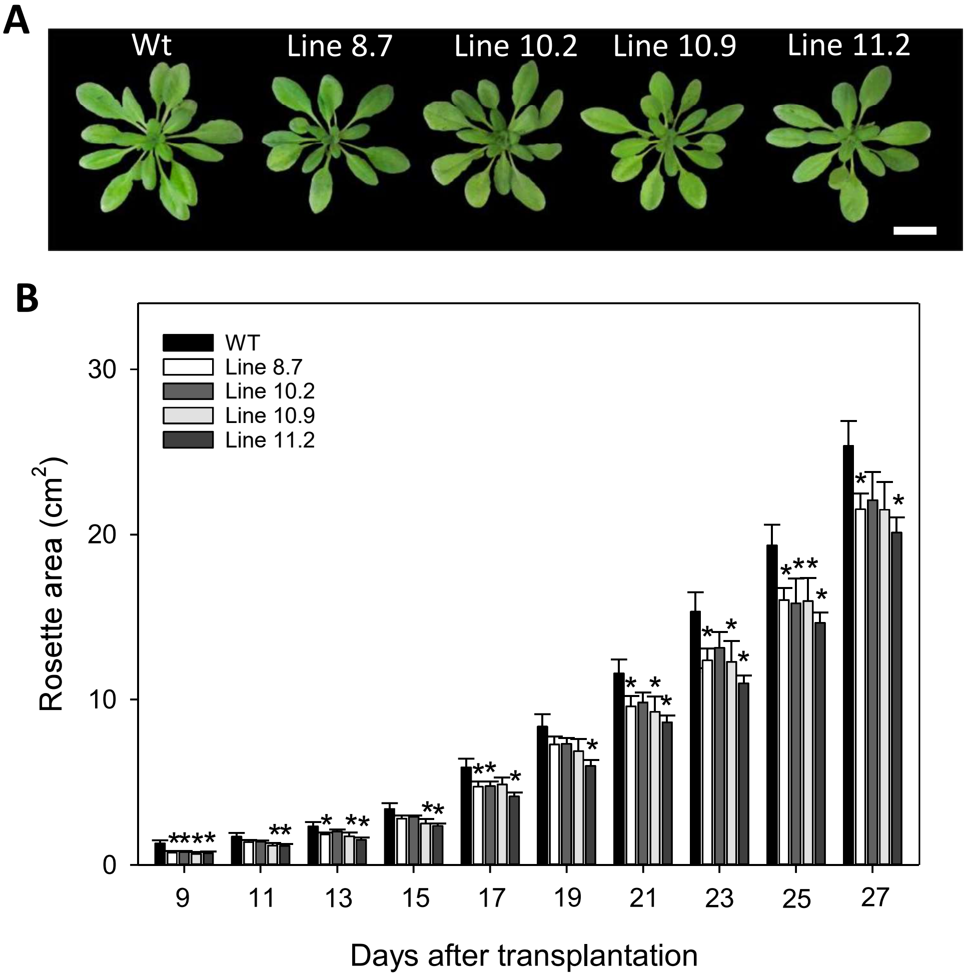


Figure 6. Growth phenotype of APC1 Cas9-free Arabidopsis mutant lines. Wild-type (WT) and *APC1* mutant lines 8.7, 10.2, 10.9, and 11.2 after four weeks of cultivation (**A**), and rosette area over time in short-day conditions (**B**). The white bar represents 2 cm. Values are means ± SE of ten independent biological replicates**.** Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$).

Furthermore, to evaluate if the APC1 plays a role during vegetative growth, we monitored the increase of rosette area in an almost three-week period. Similar to root growth, we noticed in the rosette of mutant plants, a reduced growth over the whole period evaluated (Fig. 6B). Additionally, we analyze other biometric parameters to understand the extension of APC1 role in morphological aspects in vegetative and reproductive phases. We observed a clear reduction in almost all parameters analyzed, such as total leaf area (Fig. 7B), number of leaves (Fig. 7C), shoot fresh weight (Fig. 7D), silique length (Fig. 7E), and number of seeds per silique (Fig. 7F). In general, all mutant lines exhibited a reduction in cited parameters, but the lines 10.9 and 11.2 that possess a deletion of 2 bases pair (Fig. 2A) in gene sequence exhibit the strongest phenotype.

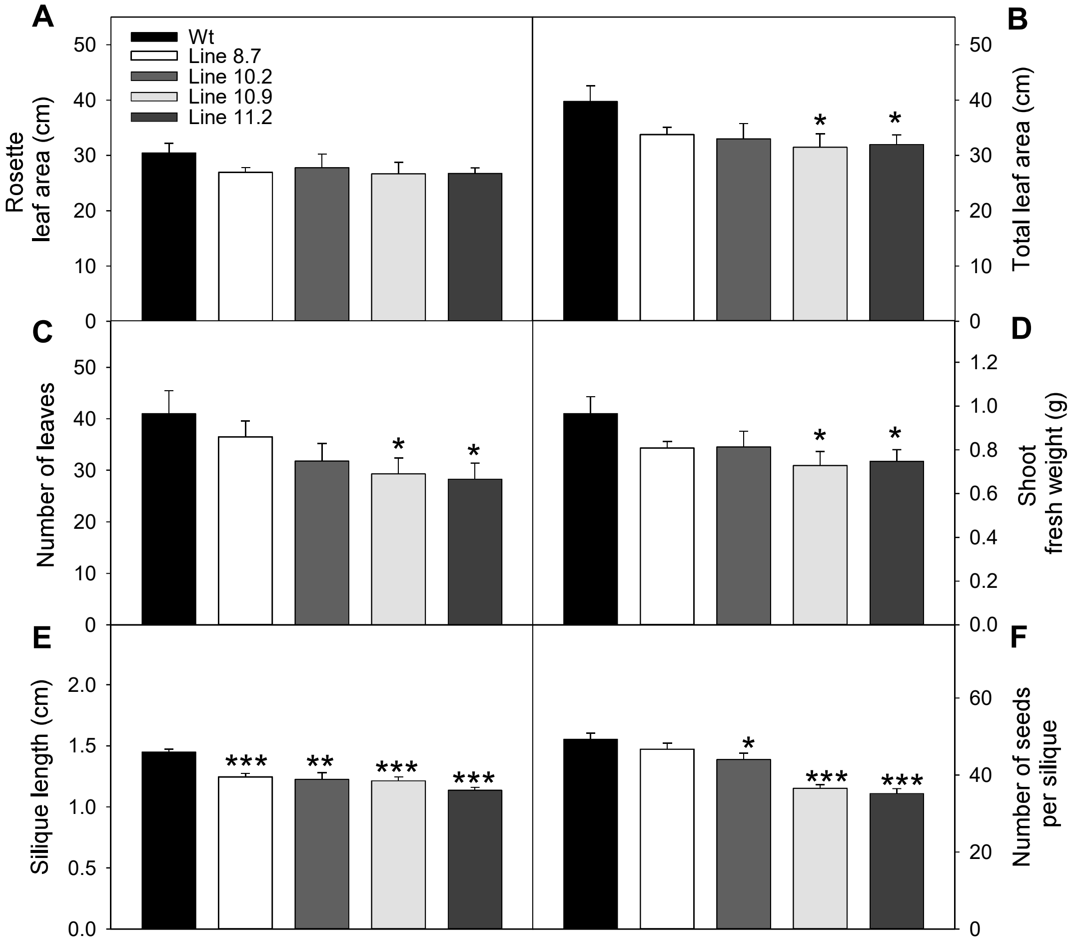


Figure 7. Biometric parameters of plants from APC1 Cas9-free Arabidopsis mutant lines with four-week-old plants grown under short-day conditions. Rosette leaf area (**A**), total leaf area (**B**), number of leaves (**C**), shoot fresh weight (**D**), silique length (**E**), and number of seeds per silique (**F**). Values are means ± SE of ten independent biological replicates**.** Asterisks indicate values that were determined by Student's t-test to be significantly different (*P* < 0.05*, 0,01**, and 0,001***).

In addition to morphological results, we performed physiological analyses to investigate the impact of APC1 disruption in autotrophic tissues. In all analyzed parameters we did not observe any difference among the genotypes (Fig. 8). These results indicate that APC1 is more important in organs and tissues with rapid growth and heterotrophic, and its deficiency does not have a great impact on mature leaves.

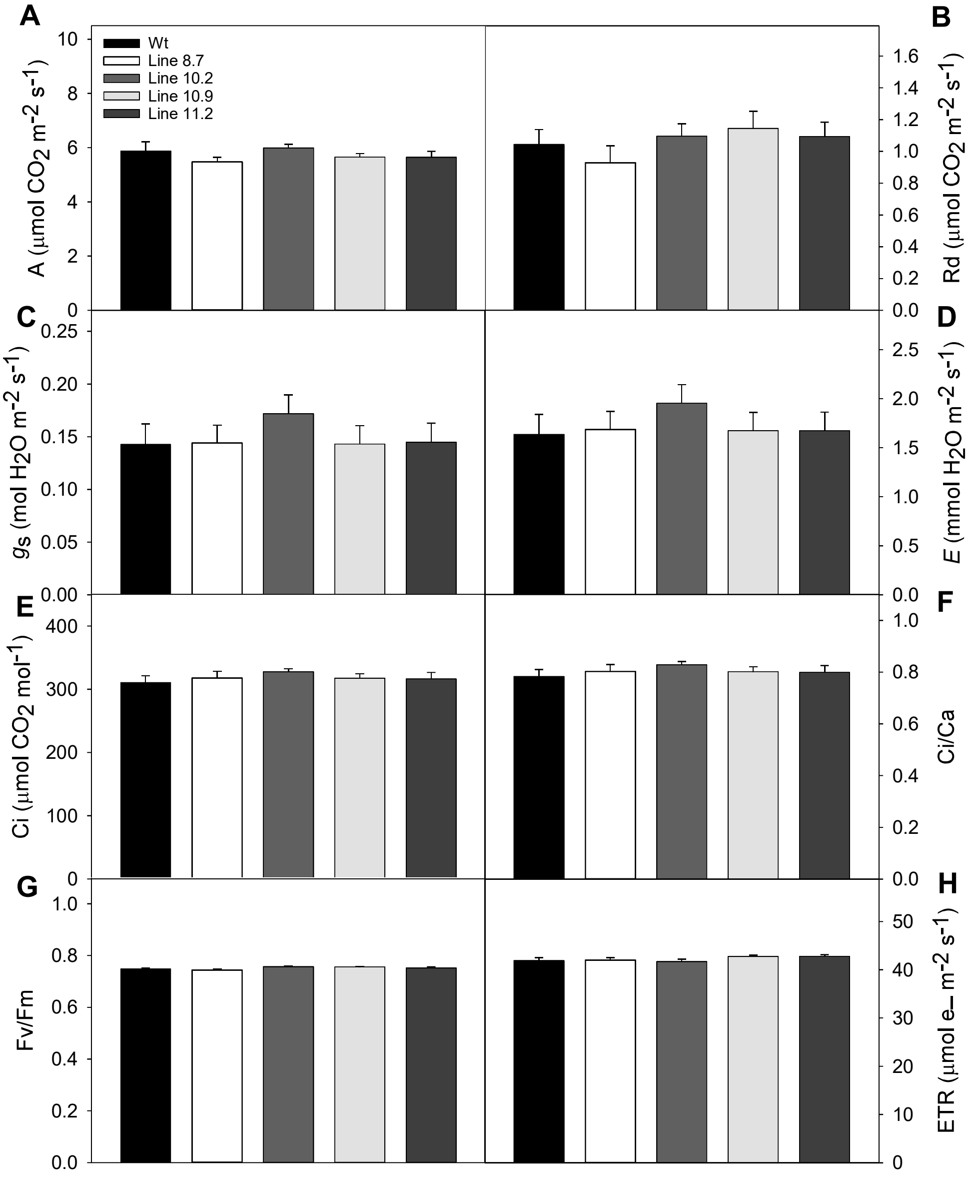


Figure 8. Gas exchange parameters of plants from APC1 Cas9-free Arabidopsis mutant lines. The measurements were performed in four-week-old plants grown under short-day conditions. Photosynthesis (**A**), dark respiration (**B**), stomatal conductance (**C**), transpiration (**D**), internal concentration of CO_2 (**E**), Ci/Ca ratio (**F**), Fv/Fm (**G**), and electron transport rate (**H**). Values are means ± SE of ten independent biological replicates.

To further evaluate the role of the APC1 carrier in leaves, we performed a metabolic analysis. First of all, we quantified the levels of photosynthetic pigments in full expanded leaves of four-week-old plants. All metabolites evaluated including, chlorophyll *a*, chlorophyll *b*, chlorophyll *a*/*b* ratio, and total chlorophyll content did not exhibit any difference between the genotypes (Fig. 9).

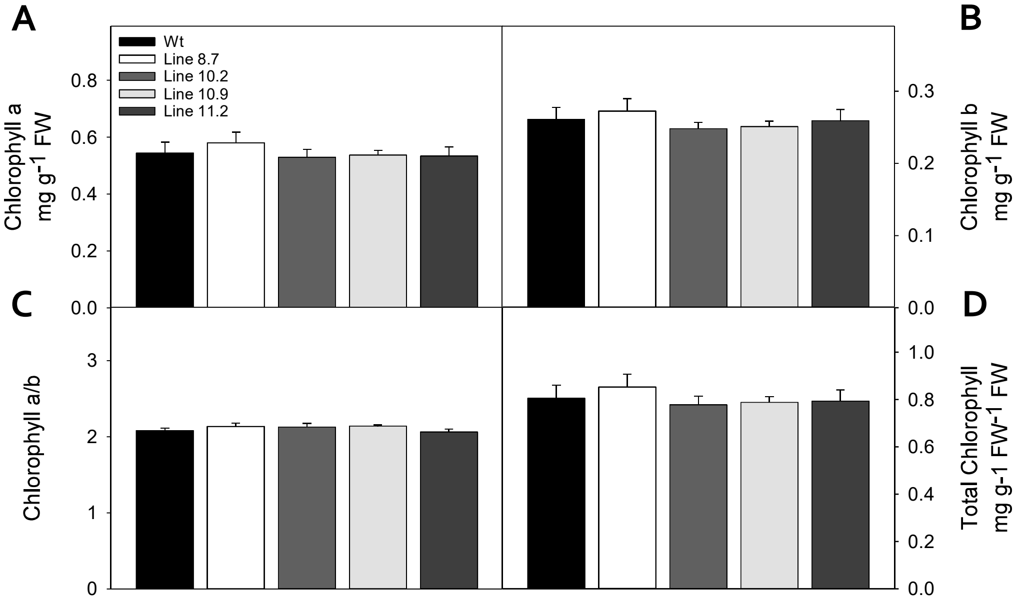


Figure 9. Variation in the levels of photosynthetic pigments in leaves of plants from APC1 Cas9-free Arabidopsis mutant lines, grown under short-day conditions. Chlorophyll *a* (**A**), chlorophyll *b* (**B**), the ratio of chlorophyll *a*/*b* ratio (**C**), and total chlorophyll content (**D**). Values are means ± SE of six independent biological replicates.

Surprisingly, no difference was observed between genotypes in all metabolites quantified. Metabolites related to nitrogen metabolism (protein, total amino acids) (Fig. 10A, and B), and carbon metabolism (starch, glucose, fructose, sucrose) (Fig. 10C, D, E, and F). Additionally, it was quantified the levels of organic acids (malate and fumarate) (Fig. 10G, and H). Together these results suggest that the absence of APC1 carrier does not affect the metabolism of mature leaves.

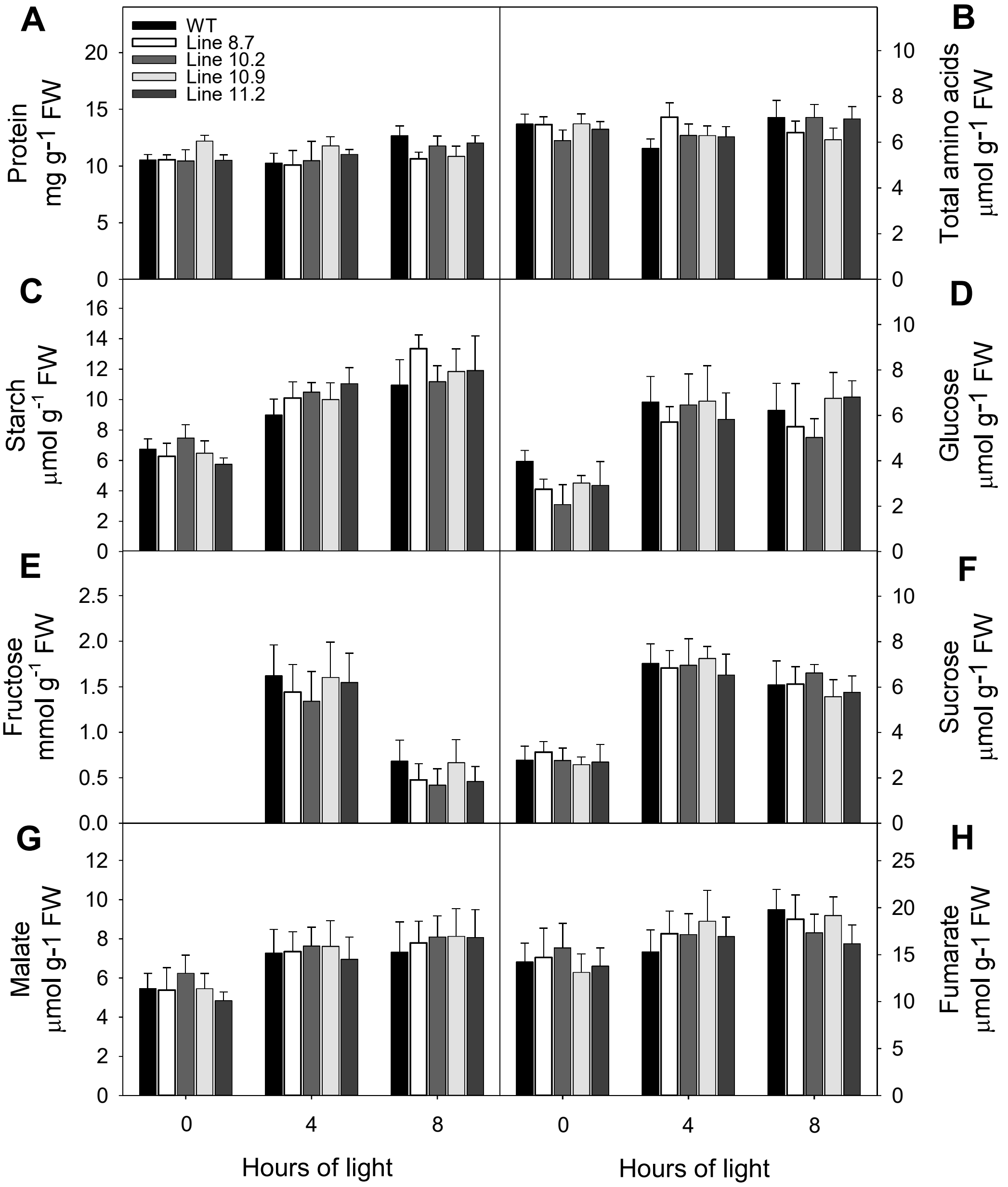


Figure 10. Variation of the main carbon and nitrogen-related compounds in leaves from plants of APC1 Cas9-free Arabidopsis mutant lines, grown under short-day conditions. The identification of 0, 4, and 8 hours represents the beginning, middle and final of the light period. Protein (**A**), total amino acids (**B**), starch (**C**), glucose (**D**), fructose (**E**), sucrose (**F**), malate (**G**), and fumarate (**H**). Values are means ± SE of six independent biological replicates**.**

DISCUSSION

APC1 carrier plays an important role during fruit development and seed production

The development and production of reproductive organs is a high energy demand process, which requires a proper activity of mitochondria and ATP transport across adenylates carriers(Xu et al., 2008). Also, floral organs have high levels of mitochondrial transcripts (McCabe et al., 2000), showing the great importance of mitochondria metabolism in the development of the reproductive stage. During the growth and development of floral organs, also occur an increase in the number of mitochondria to sustain the need for energy and intermediates (Millar et al., 2011). APC1 is the most expressed isoform of APC's carriers, also is highly expressed in flowers (Monné et al., 2015) and flower buttons (Fig. 10). This expression pattern is in agreement with results from APC1 mutant plants which exhibit reduced silique length and a lower number of seeds per silique (Fig. 6E, and F). It is valid to assume that, these organs present development and growth impaired by the inefficient ATP transport by the absence of APC1 carrier. Otherwise, other transporters could replace APC1 function, nevertheless, it is known that some carriers and some isoforms are more needed in certain organs and situations (Fig.1). For example, the AAC1 is more expressed in mature pollen while ADNT1 have higher expression levels in imbibed seed (Fig. 1).

APC1 carrier is necessary for normal seedling growth and development

It has been shown that *APC1* is the isoform more expressed between the APC's carriers, being highly expressed in seedlings leaves (Monné et al., 2015). Our results indicate that defective APC1 protein leads to the production of short roots and reduced fresh weight in seedlings growing under sucrose-free medium (Fig. 3 A, B), this indicates that exists a problem with carbon supply. These can be explained both by a delay in reserves mobilization by Glyoxylate Cycle or a deficit in seed reserves, which due to an expression pattern is more probably be the last case. Although the root growth is reduced in APC1 deficient lines, the expression pattern of APC1 doesn't suggest that the absence of this carrier can cause this phenotype, because the expression level in roots is lower when compared with the other tissues. The rapidly growing tissues like a seedling, require a large energy supply to sustain their growth. The absence of a mitochondrial adenylate carrier like APC1 affects the proper ATP transport to the cytosol, which can directly affect the ATP supply to important organelles in this process, such as, peroxisome and endoplasmic reticulum (ER). Additionally, we observe an impairment in silique size and seed production with *APC1* expression pattern. These results can indicate that disruption of APC1 can generate seeds with compromise reserves, which explains also the reason for root growth and seedling fresh weight is recovered in presence of sucrose.

Does APC1 carrier have an important role in adult plants under optimal growth conditions?

Despite the great importance of mitochondrial metabolism in the development and growth of autotrophic organs, defective APC1 carrier doesn’t display any remarkable physiological and metabolic effect. Although APC1 and the other mitochondrial adenylate carrier exhibit high levels of expression in leaves, in photosynthetic tissues the ATP also can be delivered by chloroplast (Gardeström and Igamberdiev, 2016). This in part explains why disruption of mitochondrial adenylate carries doesn’t demonstrate an impact on leaf development (Palmieri et al., 2008b). Also, the function of the APC1 carrier can be compensated by the activity of the isoforms or the other mitochondrial adenylate carriers such as AAC1 and ADNT1, indicating that your role is more specific to certain organs and environmental conditions. Also exist the possibility that APC1 performs a great function in these organs during unfavorable conditions. Given that this gene show an up-regulation and mainly the large number of co-expressed genes between all adenylates carriers related stress responses under a series of unfavorable situations (da Fonseca-Pereira et al., 2018a).

CONCLUSIONS

The vector pKI1.1R utilized to obtain the Cas9-free T_2 lines produced mutations guided by gRNA that modify the reading frame, creating an early stop codon, resulting in a disruption of APC1 protein. Together, our results suggest that APC1 carrier plays an important role during the development of reproductive organs, affecting mainly silique size and seed production. Likewise, disruption of APC1 carrier protein affects the development of seedlings and shoot growth during the vegetative phase, although without remarkable changes in physiologic and metabolic parameters in fully expanded leaves of adult plants. Otherwise, is necessary to investigate the role of the other isoforms of APCs (APC2, and APC3), to understand the contribution and specific function of the APC carrier family in the development of *A. thaliana* plants.

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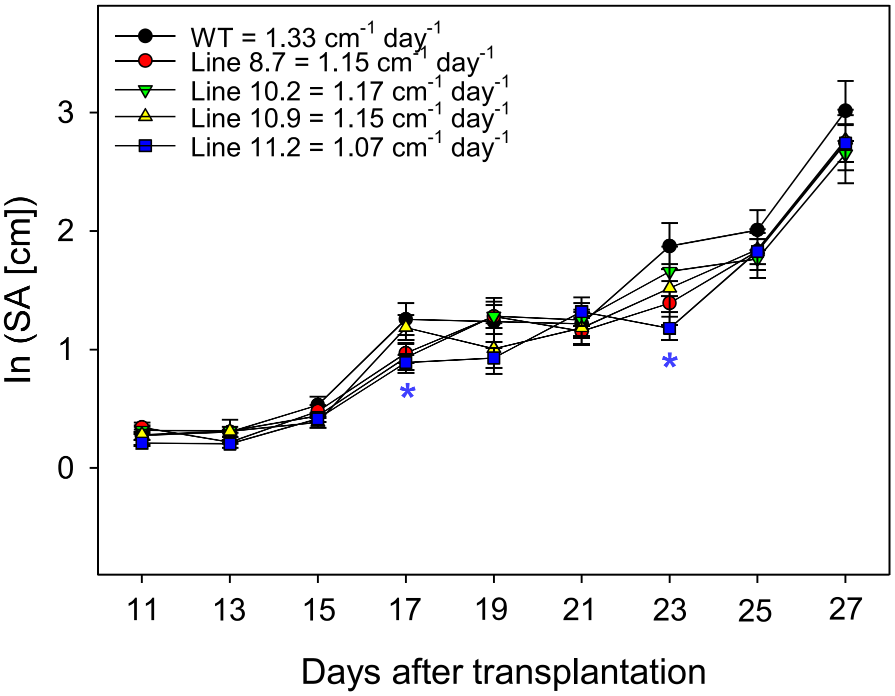
SUPPLEMENTAL DATA



Supplemental Figure 1. The relative growth rate over vegetative development of Arabidopsis *APC1* Cas9-free T_2 plants. Values are means ± SE of ten independent biological replicates**.** Asterisks indicate values that were determined by Student's t-test to be significantly different ($P < 0.05$).

GENERAL CONCLUSION

The experiments and data discussed here demonstrated the importance of adenylate carriers in plant metabolism and physiology. Acting not only as energy transport within the cell, but also as a player in a highly complex network displayed during stress responses. Additionally, we demonstrated in waterlogging conditions that the downregulation of ADNT1 expression seems to compromise the adenylates balance leading to an early “hypoxic status” in ADNT1 deficient plants. Besides, APC1 exhibited an impact in the growth and development of reproductive and rapidly growing organs, silique, and seedlings respectively. These results indicate a vast field of study to elucidate the contribution of these carriers to the development of plants under optimal conditions, and to stress responses. Thus, we hypothesize that mitochondrial adenylates carriers are potential targets for plant breeding using new and efficient techniques like CRISPR/Cas9 in the near future.