

JORGE ALBERTO CONDORI APFATA

**PHYSIOLOGICAL AND METABOLIC ANALYSIS OF *Arabidopsis thaliana* WITH LOW EXPRESSION OF 2-OXOGLUTARATE DEHYDROGENASE SUBUNITS**

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Plant Physiology Graduate Program for obtention of the degree of *Doctor Scientiae*

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## RESUMO

CONDORI APFATA, Jorge Alberto, D. Sc., Universidade Federal de Viçosa, fevereiro do 2016. **Análise fisiológica e metabólica de *Arabidopsis thaliana* com baixa expressão das subunidades da 2-oxoglutarato desidrogenase.** Orientador: Adriano Nunes Nesi. Coorientadores: Wagner L. Araújo e Marcelo Rogalski

Uma função do ciclo dos ácidos tricarboxílicos (TCA) em plantas é a produção de 2-oxoglutarato (2-OG) necessário para a assimilação do nitrogênio. No ciclo TCA, o isocitrato desidrogenase e a 2-oxoglutarato desidrogenase (2-OGDH) estão envolvidos na síntese e consumo do 2-OG respectivamente. O complexo 2-OGDH é formado por três subunidades responsáveis pela descarboxilação do 2-OG a succinil CoA com a consequente redução do NAD<sup>+</sup>. Notavelmente a 2-OGDH tem uma função essencial na atividade metabólica geral em plantas, limitante na respiração e importante na interação carbono-nitrogênio. Em *Arabidopsis thaliana*, as subunidades E<sub>1</sub> e E<sub>2</sub> são codificadas cada uma por dois genes. Neste trabalho foram utilizados duas linhagens mutantes caracterizadas pela inserção do T-DNA para cada gene que codifica a subunidade E<sub>1</sub> e E<sub>2</sub> da 2-OGDH. As linhagens mutantes para as duas subunidades da 2-OGDH apresentaram uma diminuição substancial na respiração. A fotossíntese também foi alterada nas plantas com baixa expressão da subunidade E<sub>1</sub>. Muitas mudanças foram observadas para os metabólitos primários, diminuição dos níveis dos principais metabólitos que contem nitrogênio e aumento dos metabólitos relacionados com o metabolismo do carbono, culminando em alterações no crescimento vegetal e na produção de sementes. Embora as duas subunidades E<sub>1</sub> e E<sub>2</sub> são codificadas cada uma por dois genes, estes genes apresentam funções parcialmente redundantes no metabolismo e crescimento vegetal.

## ABSTRACT

CONDORI APFATA, Jorge Alberto, D. Sc., Universidade Federal de Viçosa, February, 2016. **Physiological and metabolic analysis of *Arabidopsis thaliana* with low expression of 2-oxoglutarate dehydrogenase subunits**. Adviser: Adriano Nunes Nesi. Co-advisers: Wagner L. Araújo and Marcelo Rogalski

One of the roles of the tricarboxylic acid (TCA) cycle in plants is the production of 2-oxoglutarate (2-OG) required for nitrogen assimilation. In this cycle, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (2-OGDH) is involved in synthesis and consumption of 2-OG, respectively. 2-OGDH complex is composed of three subunits responsible for decarboxylation of 2-OG to succinyl CoA with the consequent reduction of NAD<sup>+</sup>. Notably the 2-OGDH plays an essential role in overall metabolic activity in plants, being limited for respiration and playing important role in the carbon-nitrogen interactions. In *Arabidopsis thaliana*, E<sub>1</sub> and E<sub>2</sub> subunits are encoded each by two genes. Here, I used two T-DNA insertion mutant lines in each gene encoding E<sub>1</sub> and E<sub>2</sub> subunits of 2-OGDH. For both subunits of 2-OGDH mutant plants exhibited substantial reduction in respiration. The photosynthesis was also altered in plants with low expression of E<sub>1</sub> subunit. Several changes were observed for primary metabolites, with decreased levels of the main nitrogen containing metabolites and increase in metabolites related to carbon metabolism, culminated in alterations of plant growth and seed production. Although both E<sub>1</sub> and E<sub>2</sub> subunits each one are encoded by two genes, they display partial redundant roles in metabolism and plant growth.

## GENERAL INTRODUCTION

Respiration in plants consists of different metabolic pathways, namely glycolysis in the cytosol, TCA cycle into the mitochondrial matrix and electron transport chain together with oxidative phosphorylation taking place in the inner mitochondrial membrane (van Dongen et al., 2011). The TCA cycle has been extensively studied in the last decade (Araújo et al., 2012a; Nunes-Nesi et al., 2013). This cycle is composed of a set of eight enzymes localized in the mitochondrial matrix linking the oxidation of pyruvate and malate generated in the cytosol to CO<sub>2</sub> with the generation of NADH which is used as electrons source to the mitochondrial respiratory chain (Millar et al 2011). In addition, the TCA cycle is involved in the production of metabolic intermediates required for several other biosynthetic processes, such as nitrogen assimilation, metabolism of organic acids generated in other pathways and maintenance of redox homeostasis (Araújo et al., 2012a; Sweetlove et al., 2010). These other functions require the TCA cycle operating in a non-cyclic mode, and the balance between the cyclic and non-cyclic flow is highly dependent on the cell type and the physiological context (Sweetlove et al., 2010).

The role of the TCA cycle in illuminated leaves is of particular interest since it remains controversial, by the apparent paradox of reduced flow cycle in the light due to inhibition of complex pyruvate dehydrogenase (Araújo et al., 2013a; Tovar-Mendez et al., 2003), and the rapid export of TCA cycle intermediates out of the mitochondria for nitrogen assimilation (Nunes-nesi et al., 2007b; Hodges et al., 2002).

To understand the importance of the TCA cycle in illuminated leaves, a characterization of a natural mutant in *Solanum pennellii* Aco-1 indicated that aconitase is extremely important for carbon and nitrogen metabolism as well as photosynthesis (Carrari et al., 2003). Afterwards a systematic suppression of TCA cycle enzymes through reverse genetic was adopted to better understanding the function of this pathway in illuminated tissues (Nunes-Nesi et al., 2011). The antisense inhibition of the mitochondrial malate dehydrogenase in tomato enhanced the rate of carbon dioxide assimilation (Nunes-Nesi et al., 2005). The antisense inhibition of the iron-sulphur subunit of succinate dehydrogenase (SDH) in both tomato and *Arabidopsis* culminated in higher photosynthesis rate (Araújo et al., 2011; Fuentes et al 2011). By contrast, the CO<sub>2</sub> assimilation rate in tomato plant deficient in fumarase was reduced (Nunes-nesi et al., 2007a). The apoplastic organic levels in SDH and fumarase antisense plants revealed a negative correlation between the levels of both malate, fumarate and stomatal conductance (Araújo et al., 2011b). The enhanced rates of photosynthesis in mitochondrial malate dehydrogenase antisense plants appears to be regulated by changes in redox status (Nunes-Nesi et al., 2005), most likely relayed by ascorbate, throughout the L-galactono-1,4-lactone dehydrogenase (GLDH), the terminal biosynthetic enzyme of the ascorbate biosynthesis which was previously associated with the mitochondrial cytochrome pathway (Bartoli et al., 2000). Recently, has been proposed a role for GLDH during complex I formation, which is based on its binding to specific assembly intermediates (Schertl et al., 2012). However, despite the clear impact in the photosynthesis that changes in mitochondrial

metabolism might cause, the relationship between respiration and other metabolic processes in the light remain to be fully elucidated (Araújo et al., 2014).

Study using proteomics approaches in combination with affinity chromatography have indicated that several TCA cycle enzymes are *in vitro* targets of thioredoxins (Balmer et al., 2004). Thus, this study suggested that thioredoxin participates in the regulation of TCA cycle. Recently, it has been shown that thioredoxin-dependent activation of citrate synthase is most likely an important regulatory mechanism for regulation of TCA cycle *in vivo* (Schmidtman et al., 2014). Furthermore, thioredoxin may deactivate both SDH and fumarase, acting as a direct regulator of carbon flow through the TCA cycle (Daloso et al., 2015). In addition, novel insights about the regulation of the TCA cycle have also been provided by metabolic control analysis, which indicated that much of the control through this pathway is resident in fumarase, malate dehydrogenase, and 2-OGDH, suggesting that these enzymes would be sensitive targets for flux regulation (Araújo et al., 2012a; Nunes-Nesi et al., 2013).

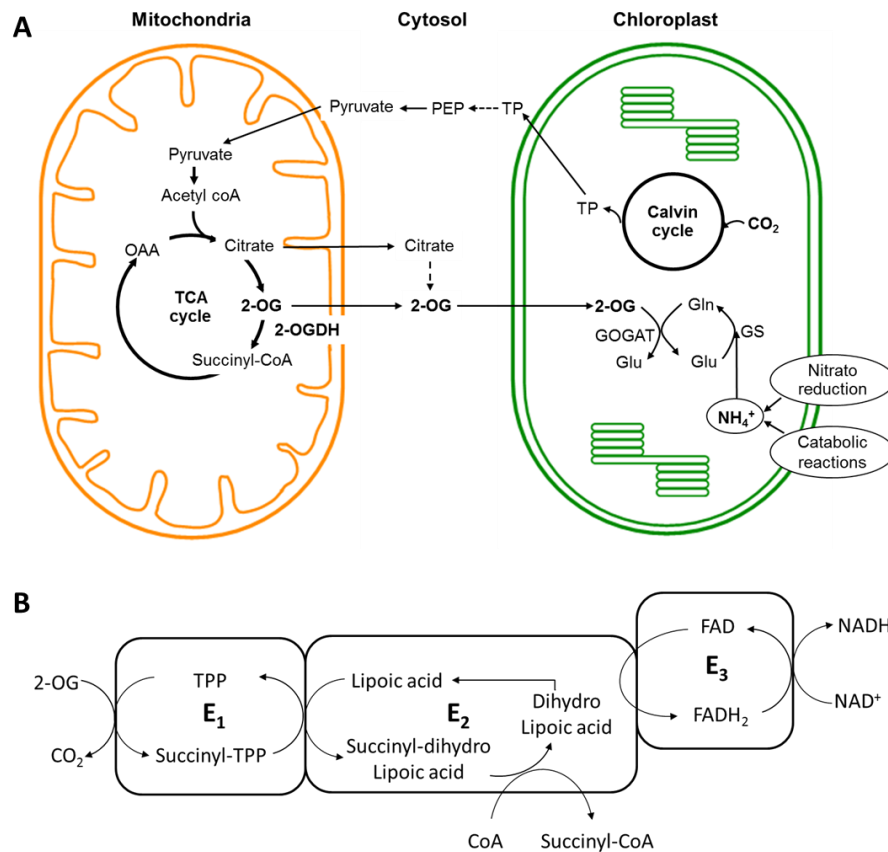
The reaction catalyzed by the 2-OGDH (Figure 1B) represents a metabolic branch point connecting the TCA cycle with nitrogen assimilation. For nitrogen assimilation 2-OG, substrate of 2-OGDH, represents the carbon skeleton needed for nitrogen incorporation. This compound is either irreversibly degraded by the 2-OGDH or is exported from mitochondria for nitrogen assimilation (Figure 1A) (Hodges et al., 2002). Thus 2-OG plays a role as a signal metabolite in plants (Feria Bourrellier et al., 2009). This role is, however, largely based on analogy to the role it plays in conjuncture with

the plastid PII protein in plants which may regulate a small number of enzyme systems in plants including N-acetyl-glutamate kinase (Feria Bourrellier et al., 2009; Ferrario-Méry et al., 2006) and plastid acetyl-CoA carboxylase (Feria Bourrellier et al., 2010). In this context, other enzymes are also important for nitrogen metabolism.

The enzyme isocitrate dehydrogenase produces 2-OG from isocitrate in the TCA cycle in the mitochondrial matrix and different isoforms are localized in the cytoplasm (Lemaitre et al., 2007). Other enzyme is aspartate aminotransferase that produce 2-OG and aspartate for reversible transfer of amino group of glutamate to oxaloacetate (Hodges et al., 2002).

The chemical inhibition of 2-OGDH in potato tubers (Araújo et al., 2008) and leaves in *Arabidopsis thaliana* (Araújo et al., 2012b) resulted in a dramatic reduction in respiratory rate. In addition, changes in the level of TCA cycle intermediates and important amino acids for the assimilation of nitrogen were observed. The inhibition by an antisense of the E<sub>1</sub> subunit of 2-OGDH in tomato plants reduced the respiratory rate, altered development and modified the metabolism of carbon and nitrogen (Araújo et al., 2012c)

The antisense inhibition of succinyl CoA ligase in tomato plants, (Studart-Guimarães et al., 2007) and the antisense inhibition of the 2-OGDH in tomato plants (Araújo et al., 2012c) revealed the completion of supply of succinate by an alternative pathway known as "GABA-shunt". This pathway makes a bypass of two steps in the TCA cycle, the conversion of 2-OG to succinyl CoA and subsequent conversion of succinyl CoA to succinate, catalyzed by a cytosolic enzyme glutamate decarboxylase and two mitochondrial enzymes, GABA transaminase and succinic semialdehyde



**Figure 1.** A simplified scheme showing carbon (C) and nitrogen (N) flow between three subcellular compartments.

**(A)** N metabolism is linked to C metabolism by the requirement C-skeletons. This involves several major metabolic processes including photosynthesis, the Calvin cycle, glycolysis, and the tricarboxylic acid (TCA) cycle that are carried out in several different subcellular compartments. The 2-OG may either be further degraded by 2-OGDH in the TCA cycle with energy production or provide the C-skeleton for inorganic nitrogen assimilation via glutamate biosynthesis. The ammonium is assimilated in the chloroplasts by the action of the GS/GOGAT pathway. For net glutamate production, the GOGAT requires C skeletons in the form of 2OG by two pathways. In one pathway, 2-OG is synthesized in the mitochondria by the isocitrate dehydrogenase (IDH) and exported to the cytosol. In the second pathway, citrate is exported from the mitochondria to the cytosol. Here it can either be stored in the vacuole or used by a cytosolic aconitase to generate the isocitrate required for the synthesis of 2-OG by the cytosolic ICDH. In both cases, 2-OG is imported into the chloroplast. **(B)** 2-OGDH is a multienzyme system comprising of the three subunits. The E<sub>1</sub> subunit is responsible for the initial decarboxylation of 2-OG. This step involves the critical thiamine pyrophosphate cofactor. Next, E<sub>2</sub> subunit catalyzes the transfer of the substrate from thiamine pyrophosphate to lipoic acid, and then to CoA. This creates the product, succinyl-CoA. Finally, E<sub>3</sub> subunit catalyzes the transfer of electrons from lipoic acid to NAD<sup>+</sup>, using FAD as a cofactor. This step reoxidizes lipoic acid for continued action in the E<sub>2</sub> subunit and forms the NADH that will later be essential in the electron transport chain. Abbreviations: PEP, phosphoenol pyruvate; TP, triose phosphate; OAA, oxaloacetate; 2-OG, 2 oxoglutarate; Gln, glutamine; Glu, glutamate; GOGAT; glutamate synthase; GS, glutamine synthetase; TPP, thiamine pyrophosphate.

dehydrogenase (Bouché et al., 2003; Michaeli and Fromm, 2015; Michaeli et al., 2011; Studart-Guimarães et al., 2007). This compensatory response alter cell pool of amino acids and nitrate (Araújo et al., 2012b, 2012c).

It has been demonstrated that the inhibition of 2-OGDH, via specific chemical inhibitor in heterotrophic and autotrophic tissue, limits the respiration (Araújo et al., 2008, 2012b), and the antisense inhibition of 2-OGDH in tomato led to alterations in whole plant development that were linked to reductions in total amino acids and nitrate pools (Araújo et al., 2012c). However, the physiological role and metabolic impact of reduction in the expression of genes encoding subunits of 2-OGDH are unknown. In *Arabidopsis thaliana* two genes encode the E<sub>1</sub> subunit and other two genes encode the E<sub>2</sub> subunit of 2-OGDH. Thus, in this work, my aim was to evaluate individually the function of these genes by the uses of T-DNA insertion mutant lines in genes encoded the E<sub>1</sub> and E<sub>2</sub> subunits of 2-OGDH, in order to determine their physiological and metabolic roles under optimal environmental conditions for *A. thaliana*.

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## CHAPTER I

## **Down regulation of 2-oxoglutarate dehydrogenase E<sub>1</sub> subunit impacts plant growth and seed production in *Arabidopsis thaliana***

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### **ABSTRACT**

The tricarboxylic acid (TCA) cycle enzyme 2-oxoglutarate dehydrogenase (2-OGDH) converts 2-oxoglutarate (2-OG) to succinyl-CoA concomitant with NAD<sup>+</sup> reduction. Notably 2-OGDH has an essential role in plant metabolism, it is limiting factor for respiration and has important role in carbon-nitrogen interactions. Although the role of the 2-OGDH has been previously demonstrated in heterotrophic and autotrophic plant tissues using specific inhibitor, here we used knockout mutant plants in each gene encoding the E<sub>1</sub> subunit of 2-OGDH of *Arabidopsis thaliana*. The mutant lines exhibited substantial reduction in respiration and photosynthesis. These mutant lines displayed also alterations in the levels of chlorophyll, protein, nitrate, sucrose and starch. In addition, the mutant lines displayed different responses in terms of plant growth and seed production. Our data confirm the importance of each isoforms of E<sub>1</sub> subunit in the TCA cycle in photosynthetically active tissues and suggest that the isoforms of E<sub>1</sub> subunit are not functionally redundant in plant growth of *Arabidopsis thaliana*. These results are discussed in the context of the importance of the two genes encoding 2-OGDH E<sub>1</sub> subunit for both metabolic and developmental process.

**Key words:** 2-oxoglutarate, nitrogen metabolism, respiration, TCA cycle

## INTRODUCTION

Respiration is comprised by different metabolic pathways, glycolysis, present in the cytosol, TCA cycle, which is located in the mitochondrial matrix, the electron transport chain and oxidative phosphorylation, both in the mitochondrial inner membrane. These four steps represent the major components of aerobic respiration (van Dongen et al., 2011). Mitochondrial metabolism supports several light-associated processes including photosynthesis, photorespiration, nitrogen metabolism, reductant transport and maintenance of photosynthetic redox balance (Araújo et al., 2012a). In addition to ATP production, mitochondria is the organelle where various compounds are produced, which includes vitamins, co-factors and several other intermediates essential for fundamental metabolic processes during growth and maintenance of the cell (Araújo et al., 2012a).

The TCA cycle is a metabolic pathway composed by a set of eight enzymes primarily linking oxidation of pyruvate and malate generated in the cytosol to  $\text{CO}_2$  with the generation of reducing equivalents, NADH and  $\text{FADH}_2$  that support ATP synthesis. At the same time TCA cycle is enclosed in a wider metabolic network that allows its activity to contribute to other aspects of metabolism and to provide carbon skeletons for biosynthetic processes (Fernie et al., 2004; Sweetlove et al., 2010; Cavalcanti et al., 2014).

The reaction catalyzed by 2-OGDH represents a metabolic branch point connecting the TCA cycle with nitrogen assimilation in which the substrate 2-OG either is irreversibly degraded by 2-OGDH or provides carbon skeletons for nitrogen assimilation (Hodges et al., 2002). Recently,

it was demonstrated that the inhibition of 2-OGDH, via specific chemical inhibitors in heterotrophic tissues and in photosynthetically active tissue, limits the respiration and additional results suggested that the enzyme plays an important role in carbon-nitrogen interactions (Araújo et al., 2008, 2012b). Moreover, 2-OG is an metabolite regulator of enzymes such as cytosolic pyruvate kinase and phosphoenolpyruvate carboxylase, mitochondrial citrate synthase, and alternative oxidase, each of them involved in sugar oxidation and/or organic acids flux and redox control between cytosol and mitochondria (Hodges et al., 2002). In addition, 2-OG plays a role as a signal metabolite in plants (Feria Bourrellier et al., 2009). This role is however, largely based on analogy to the role it plays in conjuncture with the plastid PII protein in plants which may regulate a small number of enzyme systems in plants including N-acetyl-glutamate kinase (Feria Bourrellier et al., 2009; Ferrario-Méry et al., 2006) and plastid acetyl-CoA carboxylase (Feria Bourrellier et al., 2010). 2-OG is also a substrate in a range of oxidative reactions catalyzed by 2-OG dependent dioxygenases, and these enzymes are widely spread in nature and are involved in several important biochemical processes like the rice dioxygenase for auxin oxidation (*DAO*) gene, which encodes a 2-OG Fe (II) dioxygenase responsible for catalyzing the irreversible oxidation of IAA to OxIAA (Zhao et al., 2013). In *A. thaliana*, the salicylic acid 3-hydroxylase is a 2-OG-dependent dioxygenase and is responsible for the inactivation of salicylic acid to 2, 3 dihydroxybenzoic acid in the presence of ferrous iron, ascorbate, 2-OG, and catalase (Zhang et al., 2013). In pumpkin (*Cucurbita maxima*) the final steps of biosynthesis of gibberellin are catalyzed by 2-OG-

dependent dioxygenases, the GA7-oxidase and the GA20-oxidase (Lange, 1997).

The 2-OGDH is a multienzyme complex comprising three catalytic components: Subunit E<sub>1</sub>, oxoglutarate dehydrogenase; subunit E<sub>2</sub>, dihydrolipoyl succinyl transferase, and subunit E<sub>3</sub>, dihydrolipoyl dehydrogenase (Millar et al., 1999). Consecutive action of the component involves several cofactors: thiamine diphosphate, Mg<sup>2+</sup>, lipoic acid and FAD<sup>+</sup> (Bunik and Fernie, 2009). The subunit E<sub>1</sub> and E<sub>3</sub> are regulated by their substrates and effectors include co-operative interactions of the active sites. Besides, product inhibition of all the enzyme components is known and allosteric regulation of the subunit E<sub>1</sub> by the product of the subunit E<sub>3</sub>, NADH, has been demonstrated (Strumilo, 2005). Increased ratios of NADH/NAD<sup>+</sup> and succinyl-CoA/CoA inhibit the 2-OGDH, whereas, Ca<sup>2+</sup> and AMP allosterically activate the subunit E<sub>1</sub> at sub-saturating concentrations of 2-OG by increasing the subunit E<sub>1</sub> affinity to its substrate (Bunik and Fernie, 2009; Strumilo, 2005).

There is now compelling evidence suggesting that the TCA cycle plays an important role in modulating flux rate from 2-OG to amino acid metabolism (Araújo et al., 2012c). The inhibition of 2-OGDH in potato tubers via application of a chemical inhibitor confirmed that this enzyme play an important role in nitrate assimilation as well as in amino acid metabolism (Araújo et al., 2008). Antisense inhibition of the 2-OGDH in tomato plants led to alterations in plant development that were linked to reductions in total amino acids and nitrate pools despite unaltered photosynthesis (Araújo et al., 2012c). Furthermore, it was observed that down regulation of 2-OGDH

E1 subunit increases GABA-shunt flux, presumably in compensation for decreased succinate production via the TCA cycle (Araújo et al., 2012c). *In silico* transcription analysis of these genes during plant development showed differential expression in different stages of development and in different plant tissues. Despite that, the physiological role of 2-OGDH in plants remains relatively poorly characterized. Nevertheless, it has been demonstrated that the inhibition of 2-OGDH, via specific chemical inhibitor in heterotrophic and autotrophic tissue, limits the respiration (Araújo et al., 2008, 2012b). In *A. thaliana* two genes encode E<sub>1</sub> and E<sub>2</sub> subunits of 2-OGDH and it is still unknown the physiological role of each isoform in this specie. Thus, in this work, we evaluated individually the function of the two genes encoding E<sub>1</sub> subunit in *A. thaliana* in order to investigate their physiological and metabolic roles in autotrophic and heterotrophic tissues under optimal environmental conditions.

## **MATERIAL AND METHODS**

### ***Isolation of T-DNA insertion mutants and genotype characterization***

Arabidopsis T-DNA-insertion lines for *E1-OGDH1* (At3g55410) and *E1-OGDH2* (At5g65750) genes that encode the subunit E<sub>1</sub> of the 2-OGDH complex were obtained from the Salk collection (Salk Institute for Biological Studies, La Jolla, EUA). For *E1-OGDH1* gene the lines *e1-ogdh1-1* (Salk\_088518) and *e1-ogdh1-2* (Salk\_072343), and for *E1-OGDH2* gene, lines *e1-ogdh2-1* (Salk\_122458) and *e1-ogdh2-2* (Salk\_055824) were isolated. For genotyping the T-DNA insertion lines, leaves of each plant were collected separately, and genomic DNA was extracted for PCR analysis. PCR reaction resulted in a genomic fragment of the target gene

using left primer (LP) and right primer (RP) and the T-DNA insertion using a T-DNA specific left border primer (LBb1.3). The primer used were: for *e1-ogdh1-1*, LP (ACAGGGCAGTGAAGAAAGTTTATGAACAA) and RP (GTGTATCTGCAACATGAGTGACATAAAGAA); for *e1-ogdh1-2*, LP (CTTGTTTTTCACAGCTCACAGTAAACTGAC) and RP (GAAAGGACAAA GCTGTTCAACTCTACAG); for *e1-ogdh2-1*, LP (GCTGCTGTGAGAAGAA CTTAGTCTC) and RP (CAGGTCTACTATGAGCTTGACGAAGA); for *e1-ogdh2-2*, LP (CGGAATTCGATGATGTTAAAGGACATCCT) and RP (GACGGAAACACTAGAGTGAAGAAAGTGAA); and primer specific for the T-DNA, LBb1.3 (GATTTTGCCGATTTCGGAACCACCAT). After initial screening, the knockout lines were isolated and homozygous plants were selected for further analyses.

#### ***Growth conditions and evaluation of biometric parameters***

Seeds were surface-sterilized and incubated for four days at 4°C in the dark on agar plates containing Murashige and Skoog media 0.5X (Murashige and Skoog, 1962) supplemented with sucrose 1%. Seeds were subsequently germinated and grown *in vitro* under short-day conditions (8 h/16 h of light/dark) with irradiance of 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 22°C and 20°C in the light and dark, and 60% relative humidity. After ten days under these conditions, the seedlings were transferred from plates to commercial substrate and grown in growth chamber under the same conditions. During the fourth week after transplanting, morphological and physiological analyzes as well as samples collection in liquid nitrogen for biochemical analyzes were performed.

For phenotyping reproductive tissues, the seedlings were transferred to commercial substrate and were kept in growth room at  $22 \pm 2$  °C, 60% relative humidity, irradiance of  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , with a photoperiod of 12 h light and 12 h dark to seed production. Siliques from wild-type and mutant plants were collected and cleared with 0.2N NaOH and SDS (Sodium dodecyl sulfate) 1% solution to remove chlorophyll according to Yoo et al., 2012. Cleared siliques were scored for length and number of seeds under a dissecting microscope (Stemi 2000-C, Zeiss). Six plants per genotype were used for the analysis and ten siliques were sampled from each plant.

To evaluate the root length, surface-sterilized seeds were plated on MS 0.5X medium, with 0.8% agar, incubated at 4°C for 48 h, and then grown vertically at 22 °C with a photoperiod of 12 h light and 12 h dark. Seedlings were examined and photographed. Root hair length from digital images was measured using ImageJ software (Abràmoff et al., 2005).

### ***Gene expression analysis***

After four weeks of growth leaf samples were collected and total RNA was extracted and purified using TRIzol® reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The RNA quality and integrity was monitored by spectrophotometer and by agarose gel electrophoresis. The total RNA was treated with DNaseI to remove possible contaminating genomic DNA in the samples. Two micrograms of RNA were used as template for first-strand cDNA synthesis using ImProm-II™ Reverse Transcriptase (Promega) and an oligo (dT) primer. qRT-PCR amplification of At3g55410 cDNA specific sequence was performed with a

forward primer (CCATCGGAAAGGAACCCATC) and a reverse primer (ATCACCCAAAGTTCTTATTTCAAAG). Similarly, qRT-PCR amplification of At5g65750 cDNA specific sequence was performed with a forward primer (GCTCTTCAACCTGACCCCATC) and a reverse primer (TTTCCAGTGACACTCTTTGGTAAC). qRT-PCR amplification of the cDNA encoding actin of *Arabidopsis thaliana* with a forward primer (CTTGACACCAAGCAGCATGAA) and a reverse primer (CCGATCCAGACACTGTACTTCCTT) served as control to normalize the transcripts of all samples.

### ***Gas exchange and chlorophyll fluorescence measurements***

Gas exchange parameters were determined simultaneously with chlorophyll a fluorescence measurements using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Instantaneous gas exchange parameters were measured after 1 h illumination during the light period under 1000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The reference  $\text{CO}_2$  concentration was set at 400  $\mu\text{mol CO}_2 \text{mol}^{-1} \text{air}$ . All measurements were performed using the 2  $\text{cm}^2$  leaf chamber at 25 °C, and the leaf-to-air vapor pressure deficit was kept at 1.3 to 2.0 kPa, while the blue light was set to 10% of the total irradiance to optimize stomatal aperture. Dark respiration ( $R_d$ ) was measured using the same gas exchange system as described above after 1 h in the dark period. Rate of photorespiration were calculated according to the model proposed by Sharkey (1988).

### ***Stomatal density and stomatal index***

After 2 h of illumination in the light/dark cycle, leaf impressions were taken from the abaxial surface of a fully expanded leaf with dental resin imprints (Berger and Altmann, 2000) and the images were taken with a digital camera (Axiocam MRc) attached to a microscope (Zeis, model AX10, Jena, Germany). All the measurements were performed on the obtained images. Stomatal density and stomatal index (the ratio of stomata to stomata plus other epidermal cells) were determined in at least six fields of 0.09 mm<sup>2</sup> per leaf from four different plants.

### ***Determination of metabolite levels***

Whole rosettes were harvested along the 8h/16h light/dark cycle in the start, middle and end of light period. Additionally, we harvested samples in the middle and end of dark period. Rosettes were flash frozen in liquid nitrogen and stored at -80 °C until further analyzes. The levels of starch, sucrose, fructose, and glucose in the leaf tissues were determined exactly as described previously (Ferne et al., 2001). Malate and fumarate levels were determined exactly as detailed by Nunes-Nesi et al. (2007). Proteins and amino acids were determined as described previously (Cross et al., 2006). The levels of nitrate were determined as described previously (Fritz et al., 2006). Photosynthetic pigments were determined exactly as described before (Porra et al., 1989).

### ***Phylogenetic Analysis***

Amino acids sequences were retrieved from the Gen-Bank through the BLASTp algorithm using At3g55410 and At5g65750 amino acids sequence as query. Sequences were aligned using the ClustalW software

package (Higgins and Sharp, 1988) using default parameters. Maximum Likelihood phylogenetic tree were constructed with MEGA5.2 software (Tamura et al., 2011). Distances were calculated using pair-wise deletion and Poisson correction for multiple hits; bootstrap values were obtained with 500 pseudo replicates.

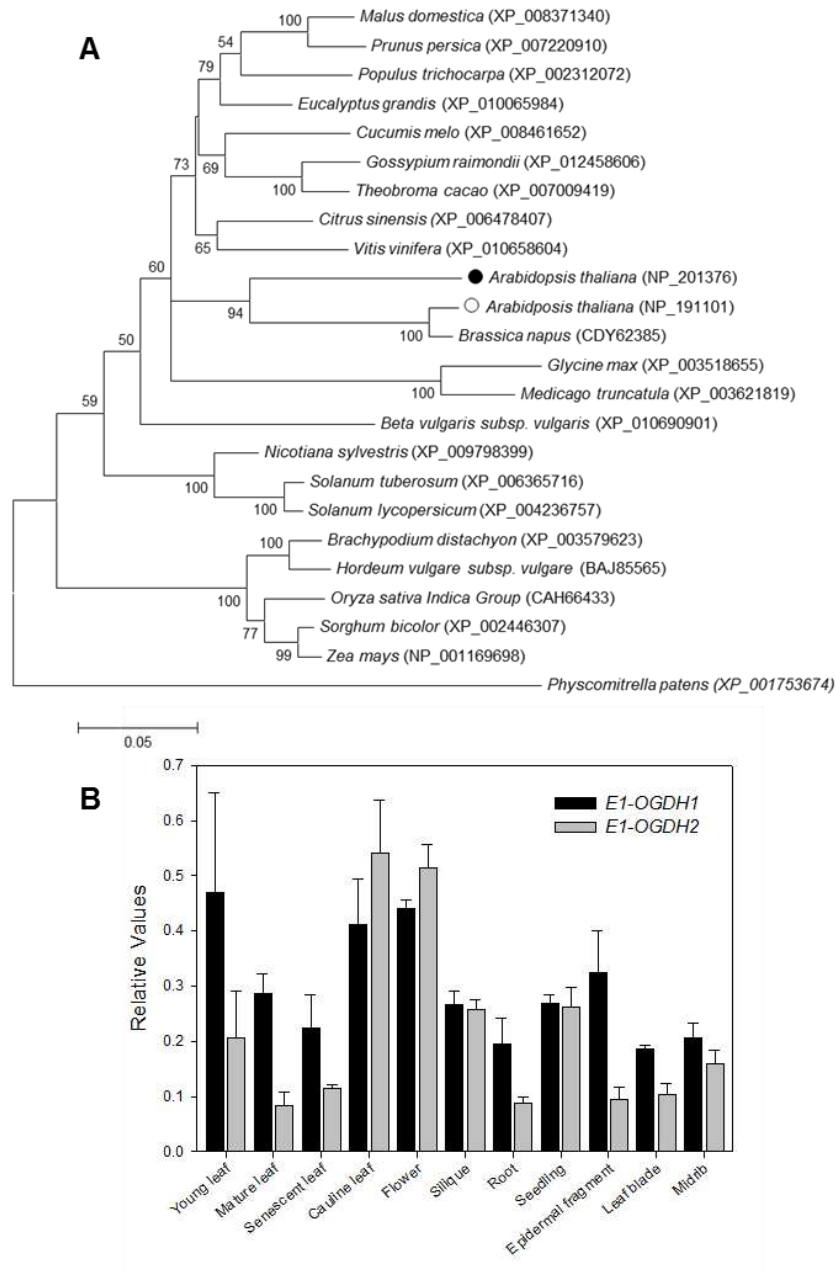
### ***Statistical Analysis***

The *t* tests have been performed using the algorithm embedded into Microsoft Excel (Microsoft, Seattle). The term significant is used in the text only when the change in question has been confirmed to be significant ( $P < 0.05$ ) with the *t* test.

## **RESULTS**

### ***Expression analysis of genes encoding 2-OGDH E<sub>1</sub> subunit***

The E<sub>1</sub> subunit of 2-OGDH is encoded by two genes, *E1-OGDH1* (At3g55410) and *E1-OGDH2* (At5g65750) that encodes a protein of 1017 and 1025 amino acids, respectively, with 87,02% of identity. These proteins consist of two conserved domains including the 2-OGDH domain and the transketolase-like domain. Both *E1-OGDH1* and *E1-OGDH2* bear characteristics of a mitochondrial transit peptide sequence, indicating a mitochondrial location for the proteins encoded by the *E1-OGDH1* and *E1-OGDH2*. The phylogenetic tree of amino acids of E<sub>1</sub> subunit of 2-OGDH indicates a very close relationship with homologues of plants and displays

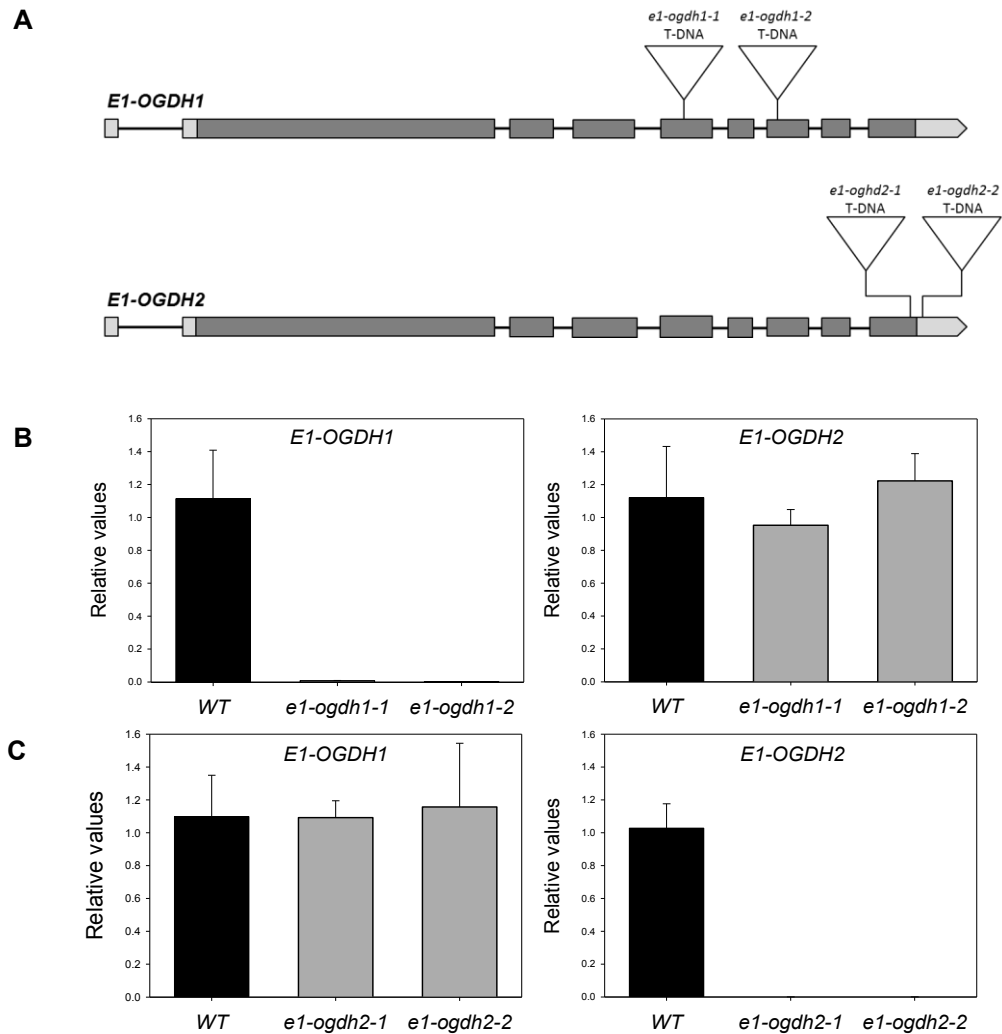


**Figure 1.** Phylogenetic analysis and characterization of expression of *E1-OGDH1* and *E1-OGDH2* isoforms in *Arabidopsis thaliana* wild type (Col-0). (A) Dendrogram of 2-OGDH  $E_1$  amino acid sequences, the protein accession numbers are given between brackets. The sequences retrieved from *Arabidopsis thaliana* are highlight with circles. The empty circle corresponds to *E1-OGDH1* and the black circle corresponds to *E1-OGDH2*. (B) Relative transcript abundance of the *E1-OGDH1* and *E1-OGDH2* in leaves of different phenological stages, cauline leaf, flower, silique, roots, seedlings, guard cell-enriched epidermal fragment, leaf blade and midrib. The gene expression was calculated by using the  $2^{-\Delta CT}$  method and actin was used to normalize the transcripts of all samples. Values are presented as means  $\pm$  SE of four individual plants.

two cluster, one group with monocots plants and other with dicots plants (Figure 1A). The amino acid sequence of *E1-OGDH1* of *Arabidopsis* (NP\_191101) revealed 98% identity to *Brassica napus* (CDY62385), 86% identity to *Nicotiana sylvestris* (XP\_009798399), 86% identity to *Solanum tuberosum* (XP\_006365716). The amino acid sequence of *E1-OGDH2* of *Arabidopsis* (NP\_201376) revealed 87% identity to *Brassica napus* (CDY62385), 86% identity to *Nicotiana sylvestris* (XP\_009798399), and 85% identity to *Solanum tuberosum* (XP\_006365716). Interestingly *E1-OGDH1* and *E1-OGDH2* revealed lower identity with sequence of *Physcomitrella patens* (XP\_001753674), 75% and 74% respectively.

To determine the degree of expression of both *E1* encoding genes we collected different tissues and organs of *A. thaliana* wild type plants and performed quantitative RT-PCR analysis. For that, samples from leaves at different phenological stages, flowers, siliques, roots and seedlings were analyzed. Additionally, the transcript levels in guard cell-enriched epidermal fragment, leaf blade and midrib were also analyzed (Figure 1B). The *E1-OGDH1* showed higher expression levels than *E1-OGDH2* in roots, young, mature and senescent leaves, and lower expression than *E1-OGDH2* in cauline leaf and flowers (Figure 1B).

Both genes displayed the same expression level in siliques and seedling. In addition, transcripts levels of *E1-OGDH1* and *E1-OGDH2* were analyzed in guard cell-enriched epidermal fragments, leaf blade and midrib. The results revealed similar expression patterns observed in mature leaf (Figure 1B).



**Figure 2.** Characterization and expression of *Arabidopsis* *E1-OGDH* mutant lines.

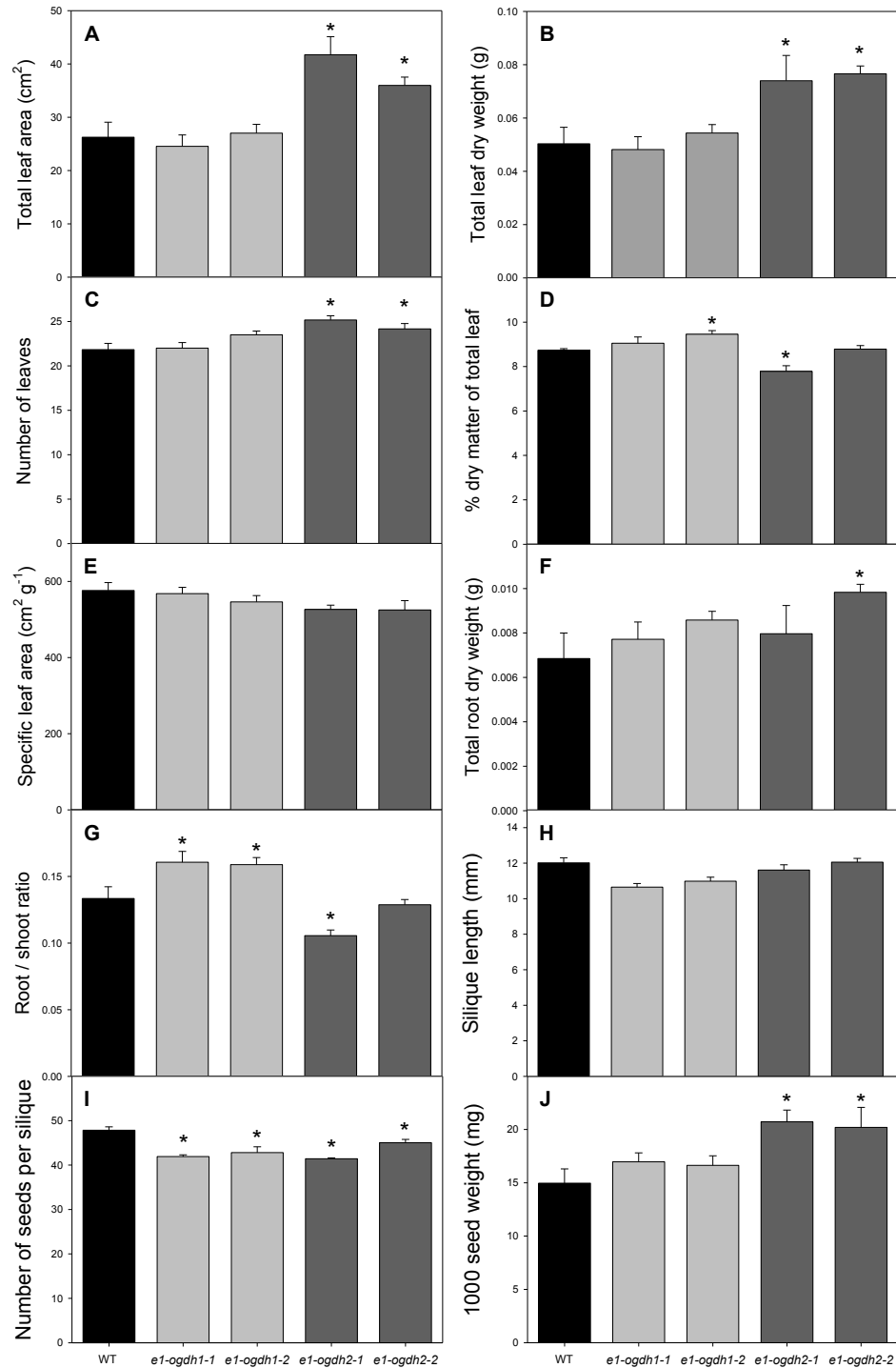
(A) Schematic representation of the *E1-OGDH1* and *E1-OGDH2* gene, the mutant lines obtained by PCR screening of a T-DNA mutant collection. (B) Expression analysis of *E1-OGDH1* and *E1-OGDH2* in mutant lines *e1-ogdh1-1* and *e1-ogdh1-2*. (C) Expression analysis of *E1-OGDH1* and *E1-OGDH2* in mutant lines *e1-ogdh2-1* and *e1-ogdh2-2*. Values are presented as means  $\pm$  SE of four individual plants and actin was used to normalize the transcripts of all samples.

We next studied the loss of function of E<sub>1</sub> in two independent T-DNA insertion line mutants for each gene. For that, a collection of *Arabidopsis* T-DNA insertion mutants was screened by PCR using oligonucleotides anchored in the gene *E1-OGDH1* and *E1-OGDH2*.

Two T-DNA mutant lines containing a T-DNA element inserted in the gene *E1-OGDH1* were isolated, The lines were named *e1-ogdh1-1* and *e1-ogdh1-2* (Figure 2A). Likewise, for the gene *E1-OGDH2*, two lines were isolated and named *e1-ogdh2-1* and *e1-ogdh2-2* (Figure 2A). The expression of both genes was assessed by quantitative RT-PCR analysis. In both mutant lines, *e1-ogdh1-1* and *e1-ogdh1-2*, the expression of *E1-OGDH1* was null in comparison with the wild-type, while the expression of *E1-OGDH2* was kept to the wild type level (Figure 2B). The mutant lines *e1-ogdh2-1* and *e1-ogdh2-2* also displayed clear reduction in the expression of *E1-OGDH2* in comparison with wild-type, but kept similar level in expression of *E1-OGDH1* as compared to wild type (Figure 2C).

#### ***Specific phenotypes of plants displaying lower expression of 2-OGDH E<sub>1</sub> subunit isoforms***

After four weeks growth, the plants of *e1-ogdh1-1* and *e1-ogdh1-2* lines were apparently very similar to the wild type plants. However, plants from *e1-ogdh2-1* and *e1-ogdh2-2* lines appeared to have faster growth than wild type plants (Supplemental Figure 2). In fact, the *E1-OGDH1* lines did not display significant changes in terms of total leaf area and leaf number as compared to wild type plants (Figure 3A, 3C). In addition, the total leaf dry weight did not differ from the wild type (Figure 3B), but the total root dry weight showed a mild increase although not statistically significant (Figure



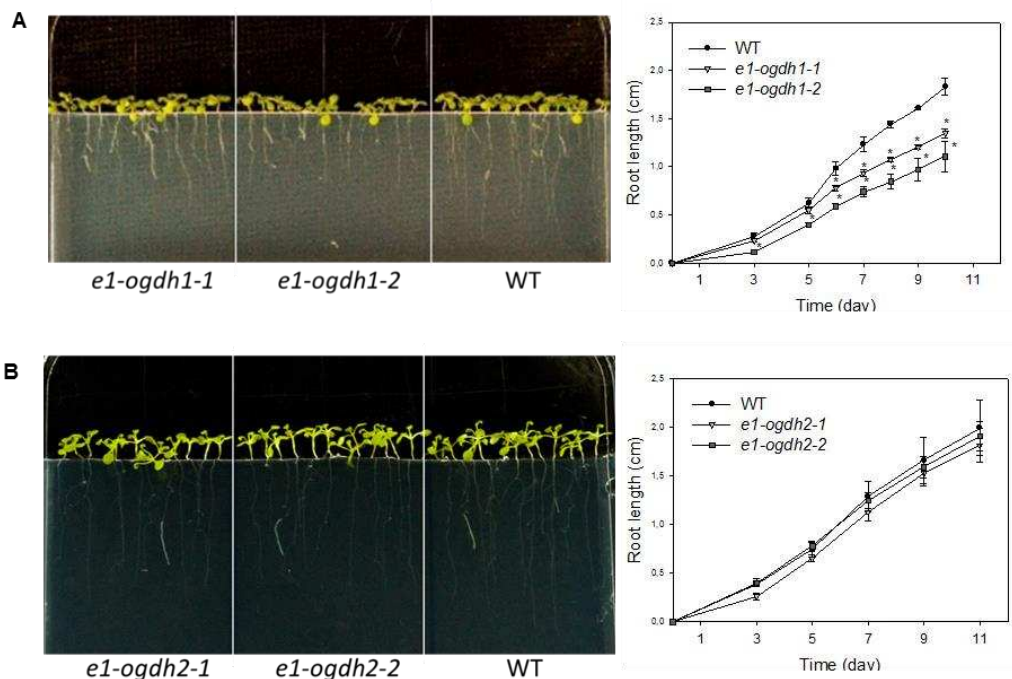
**Figure 3.** Growth phenotype of *Arabidopsis* *E1-OGDH* mutant lines. (A) Total leaf area, (B) Total leaf dry weight, (C) ) Number of leaves, (D) % dry matter, (E) Specific leaf area, (F) Total root dry weight, (G) Root/shoot ratio. (H) Silique length, (I) Number of seeds per silique, and (J) Weight of 1000 seeds. The lines used were as follows: the wild type, black bars; *E1-OGDH1* mutant lines, light gray bars; *E1-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of six individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0, 05$ ) from the wild type.

3F). Despite that, a significant increase in the root/shoot ratio was observed (Figure 3G). Additionally, it was observed that specific leaf area was not altered in the *e1-ogdh1* mutant plants (Figure 3E). However, plants from *E1-OGDH1* mutant lines showed a tendency of higher percentage of leaf dry matter (Figure 3D).

On the other hand, the plants from *E1-OGDH2* mutant lines displayed increase in total leaf area as compared to wild type plants (Figure 3A). The increase in the leaf area was accompanied by an increase in the total leaf dry weight and in the total leaf number (Figure 3B, 3C). Despite that, a decrease in percentage of leaf dry matter was observed (Figure 3D). Additionally, an increase in total root dry weight and a decrease in root/shoot ratio were observed (Figure 3F, 3G). Surprisingly, no significant change in terms of specific leaf area was observed (Figure 3E).

To study the role of *E1-OGDH1* and *E1-OGDH2* in reproductive tissues, silique length, number of seeds per silique and weight of 1000 seeds were quantified. Apparently, both *E1-OGDH1* and *E1-OGDH2* are of great importance in reproductive tissues. The reduction in expression of 2-OGDH E<sub>1</sub> subunit decreased the number of seeds per silique in all mutant lines (Figure 3I). In addition, the silique length was not significantly altered (Figure 3H and I). *E1-OGDH2* mutant lines were apparently also affected in processes related to reserve accumulation, increasing seed weight (Figure 3J) with a smaller number of seeds per silique. Interestingly, seeds of both set of mutant lines did not show alterations in the germination rate in comparison with wild type (Supplemental Figure 1).

In order to determine the importance of both *E1-OGDH1* and *E1-OGDH2* in the subsequent seedling establishment, we evaluated the initial root growth of seedlings. Apparently, the *E1-OGDH1* is important during early stages of root development as seedlings from mutant lines presented shorter roots (Figure 4A). However, on the *E1-OGDH2* mutant lines seedlings did not show significant changes in relation to wild type (Figure 4B).



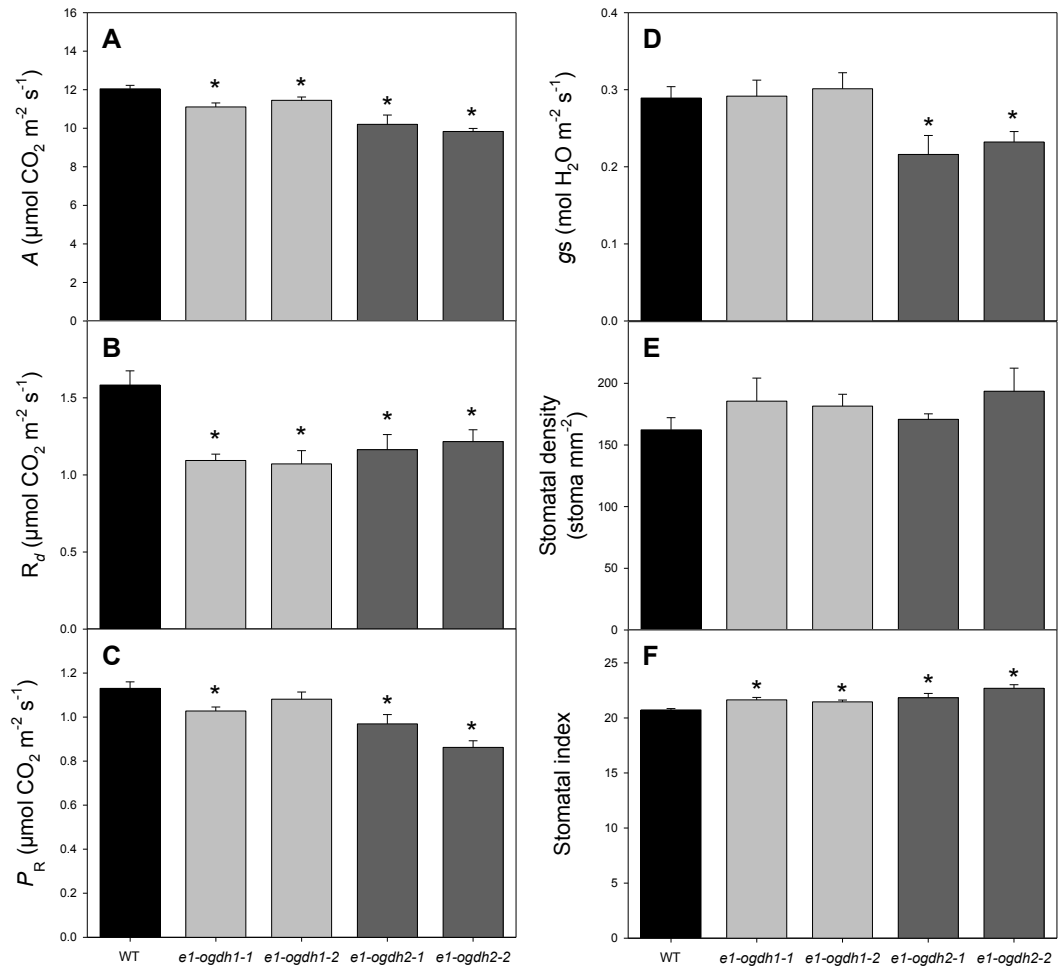
**Figure 4.** Phenotypic characterization of *Arabidopsis E1-OGDH* homozygous mutant.

Root growth of *Arabidopsis thaliana* of the wild type (WT), *E1-OGDH1* (A) and *E1-OGDH2* (B) mutant lines. The plants were growth on MS 0.5X medium for 11 days after germination. Values are presented as means  $\pm$  SE of four individual plate. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0, 05$ ) from the wild type.

### ***Analysis of photosynthetic parameters***

In order to gain insight into the effect of the reduction in the expression of 2-OGDH E<sub>1</sub> subunit, gas exchange analysis were measured in 4-week-old plants of the *E1-OGDH1* and *E1-OGDH2* mutant lines. Interestingly plants from *E1-OGDH1* and *E1-OGDH2* lines showed reduction in the CO<sub>2</sub> assimilation when compared to wild type plants (Figure 5A). Plants from *E1-OGDH1* mutant lines did not differ from the wild type in terms of stomatal conductance (Figure 5D) or internal CO<sub>2</sub> concentration (Supplemental table 1). However, *E1-OGDH2* mutant lines displayed a decrease in stomatal conductance (Figure 5D). Additionally, the stomatal density in *E1-OGDH1* and *E1-OGDH2* mutant lines did not show changes but the stomatal index increased significantly. Dark respiration and photorespiration were reduced in all mutant lines (Figure. 5B and 5C).

In addition to gas exchange analysis, we simultaneously performed chlorophyll a fluorescence analysis. It was observed that the photochemical events were minimally affected by the reduction in the expression of *E1-OGDH* genes. Fv/Fm ratio, which expresses the maximum PSII photochemical efficiency, showed also no changes, independent of the mutant lines. Fv'/Fm', the NPQ and qP parameters were also not affected. However, the mutant line *e1-ogdh2-2* was the one to show a decrease of ETR (Supplemental Table 1). All these traits varied minimally across the treatments, and photochemical factors are therefore unlikely to have prominent impacts on the differences observed in assimilation rate of CO<sub>2</sub>.



**Figure 5.** Effect of reduction in the expression of 2-OGDH E<sub>1</sub> subunit on photosynthetic and respiratory parameters.

(A) CO<sub>2</sub> assimilation rate, (B) Dark respiration. (C) Photorespiration. (D) Stomatal conductance. (E) Stomatal density. (F) Stomatal index. The lines used were as follows: the wild type, black bars; *E1-OGDH1* mutant lines, light gray bars; *E1-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of six individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type.

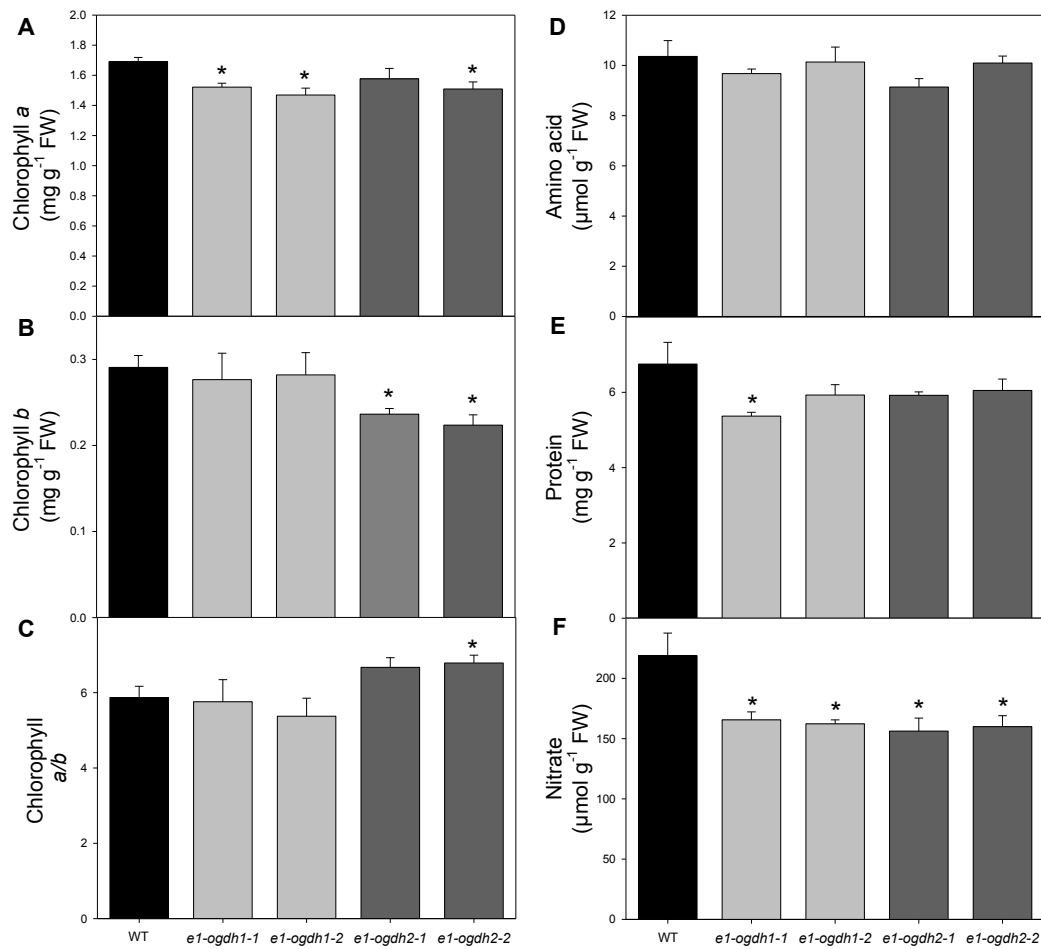
### ***Analysis of nitrogen metabolism***

Given the considerable changes in photosynthesis and respiration (Figure 5A and 5B) and the recognized link between mitochondrial metabolism and associated carbon/nitrogen interactions (Nunes-nesi et al., 2010a), we next evaluated the levels of the photosynthetic pigments, since these compounds have often been reported as important indicators of nitrogen deficiencies (Gauze et al., 2007). Analysis of pigment content in *E1-OGDH* mutant lines revealed that chlorophyll *a* was significantly decreased in both mutant lines (Figure 6A). However, the levels of chlorophyll *b* were significantly decreased only in *E1-OGDH2* mutant lines (Figure 6B). The chlorophyll *a/b* ratio was the same in *E1-OGDH1* mutant lines when compared with wild type plants. However, *E1-OGDH2* mutant lines displayed increased chlorophyll *a/b* ratio (Figure 6C).

The total levels of amino acids in leaves from 4 weeks old plants showed a tendency to decrease (Figure 6D). Likewise, protein levels decreased in all mutant lines, but significantly only in the line *e1-ogdh1-1* (Figure 6E). However all mutant lines displayed significant decrease in the levels of nitrate (Figure 6F).

### ***Analysis of carbon metabolism***

Given the changes in nitrogen metabolism, we next performed measurements to determinate the main carbohydrates content in fully expanded leaves from 4-week-old plants, harvested in the middle of the light period. The *E1-OGDH* mutant lines were characterized by significant increases in the levels of sucrose (Supplemental Figure 3C), without



**Figure 6.** Effect of decreased expression of 2-OGDH E<sub>1</sub> subunit on metabolite levels of the main nitrogen related compounds. (A) Chlorophyll a; (B) Chlorophyll b; (C) Ratio Chlorophyll a/b; (D) Total amino acids; (E) Protein; (F) Nitrate. Metabolite levels were determined in 4-week-old fully expanded leaves harvested in the middle of the light period. The lines used were as follows: the wild type, black bars; *E1-OGDH1* mutant lines, light gray bars; *E1-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type.

changes in the levels of glucose and fructose (Supplemental Figure 3A and 3B).

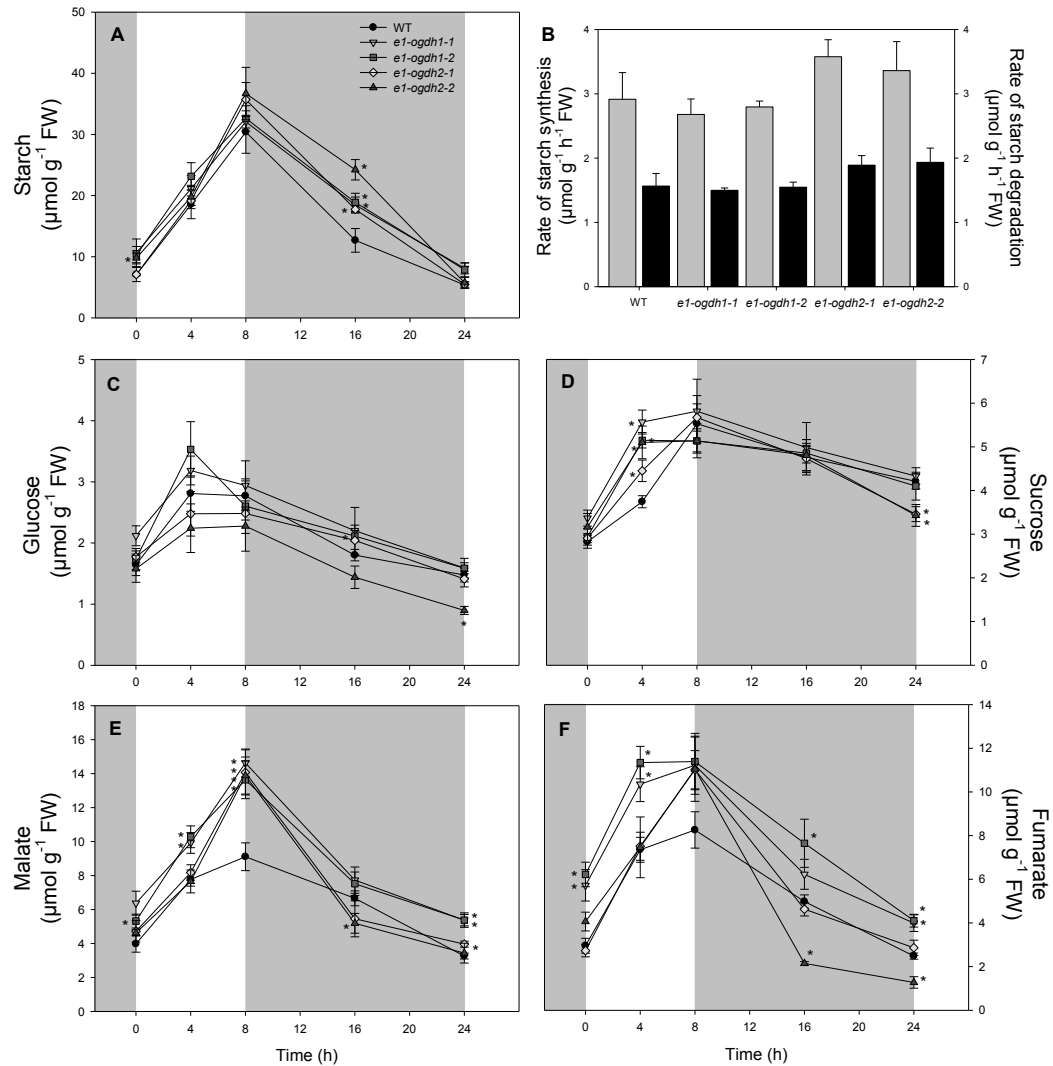
In addition, the starch levels increased in all mutant lines, being significant in the mutant line *e1-ogdh1-2* and *e1-ogdh2-1* (Supplemental Figure 3D). Additional analysis revealed that the levels of malate and fumarate in the *E1-OGDH1* mutant lines were increased significantly. However, in the *E1-OGDH2* mutant lines, the levels of these metabolites remained unaltered (Supplemental Figure 3E and 3F).

For a more detailed characterization of the mutant lines, biochemical analyzes were performed on fully expanded leaves of plants harvested at different time points. We first determined the levels of starch, sucrose, glucose and organic acids malate and fumarate at the beginning, middle and end of light period. Additionally, we also measured at the middle and end of dark period. Regarding the starch levels, the *E1-OGDH2* mutant lines showed higher levels at the end of the light period, although no significant change was observed. However, the higher levels of starch in all mutant lines was confirmed in the middle of dark period (Figure 7A). Interestingly, it could be noted an increase in the rate of starch synthesis in the *E1-OGDH2* mutant lines (Figure 7B). Additionally, the rate of starch degradation was unaltered in the *E1-OGDH1* mutant lines compared to wild type plants, but it was increased in the *E1-OGDH2* mutant lines (Figure 7B). Glucose levels showed the same behavior that wild type plants (Figure 7C) but sucrose levels were higher in the middle of light period in all mutant lines (Figure 7D). Both organic acids analyzed in this study, malate and fumarate, increased significantly in the middle of light period in the *E1-OGDH1* mutant

lines. Interestingly, at the end of light period, all mutant lines displayed high levels of malate and fumarate when compared to wild type plants. During the dark period the levels of malate and fumarate showed the same behavior that wild type plants (Figure 7E, 7F).

## **DISCUSSION**

In the present work, we have characterized the metabolic impact of reduced expression of E<sub>1</sub> subunit of 2-OGDH complex. According to the responses observed by chemical inhibition in heterotrophic and autotrophic tissue (Araújo et al., 2008, 2012b) and antisense inhibition of E<sub>1</sub> subunit of 2-OGDH in tomato (Araújo et al., 2012c), we confirmed the important role of 2-OGDH subunit E<sub>1</sub> in plants. We also demonstrated by expression analysis as well as by physiological and metabolic characterization that both isoforms of E<sub>1</sub> subunit plays distinct roles. The level of expression of each gene in mutant plants does not alter the expression level of the other, suggesting that there are no compensatory effects between the two isoforms at the expression level. Furthermore, consistent with data from previous experiments with inhibitors (Araújo et al 2008) and genetic approaches (Araújo et al 2012), the deficiency in E<sub>1</sub> subunit impacted severely dark respiration, reducing 29% (*E1-OGDH1*) and 22% (*E1-OGDH2*) the rate of respiration, which was also in agreement with computer analysis suggesting that 2-OGDH has a higher flux control coefficient in the respiration (Nunes-Nesi et al., 2012).



**Figure 7.** Diurnal change of the main carbon related compounds in leaf of *Arabidopsis thaliana* *E1-OGDH* mutant lines. (A) Starch. (B) Rate of starch synthesis, light gray bars; Rate of starch degradation, black bars. (C) Glucose. (D) Sucrose. (E) Malate. (F) Fumarate. The plants were harvest in the start, middle and end of light period, and in the middle and end dark period. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type. The average rates of starch synthesis and degradation were estimated as the difference between starch at end day and end night, divided by the length of the light period, or the night, respectively.

In this study, we observed that *E1-OGDH1* mutant plants did not show any changes in leaf area unlike the *E1-OGDH2* mutant plants, which increased leaf area, accompanied by an increase in leaf dry weight together with an increase in leaf number. This alteration in the aerial part displayed by the *E1-OGDH2* mutant plants resulted in a significant decrease in the root/shoot ratio. Although *E1-OGDH1* mutant plants showed no alterations in the aerial part, they displayed a significant increase in the root/shoot ratio. In tomato, down regulation of 2-OGDH E<sub>1</sub> subunit by antisense approaches accelerated plant development, which was observed by early flowering, accelerated fruit ripening, and a markedly earlier onset of leaf senescence (Araújo et al., 2012c). Thus, we conclude that, similar to tomato, lower expression of *E1-OGDH2* might lead to accelerated development in *Arabidopsis* plants.

It has been shown that alterations in the activities of mitochondrial enzymes led to defects in floral development, cytoplasmic male sterility and modified stamen phenotypes without affecting the vegetative plant phenotype (Bentolila and Stefanov, 2012; Geisler et al., 2012). The iron-sulfur *SUCCINATEDEHYDROGENASE1* (*SDH1*) subunit of complex succinate dehydrogenase, involved in both TCA cycle and respiratory electron transport chain, is essential for gametophyte development (León et al., 2007). However, the *SDH2-3* subunit is specifically expressed in the embryo maturation and have important role in the germination and is not essential for seed set and viability (Roschztardt et al., 2009). Moreover inhibition of citrate synthase in potato displayed ovaries disintegrated during flower development (Landschütze et al., 1995). According to the role of TCA

cycle in reproductive tissues, *E1-OGDH* mutant lines displayed a reduction in the number of seeds per silique. The regulation of TCA cycle is resident in the *2-OGDH*, and during megagametogenesis that comprises three successive rounds of nuclear division, resulting in a seven-cell embryo sac and during male gametophyte development, pollen grains showed a very high metabolic activity. Thus it is expected that a reduction in respiratory rate controlled by *2-OGDH* could affect these processes. Thus, the *E1-OGDH1* and *E2-OGDH2* are essential for seed set. Interestingly, the *E1-OGDH2* mutant lines were apparently affected in the process of accumulation of reserves during seeds development, increasing the final weight of the seed. However, despite the higher weight, seeds of both set of mutant lines *E1-OGDH1* and *E1-OGDH2* did not show lower germination rate, suggesting compensatory mechanisms for the lack of *E1-OGDH* in these tissues to prioritize the supply of nutrients to reproductive tissues. These results are in agreement with previous studies on tomato antisense plants for *E<sub>1</sub>* subunit (Araújo et al., 2012). Surprisingly, in that work, no alterations in fruit yield, seed production and germination were observed, suggesting that pathways of energy metabolism are tightly inter regulated at the whole-plant level in a manner that allows the plant to prioritize reproductive organs during senescence.

Previous results from *in silico* analysis suggest that the control of TCA cycle is greatly shared between fumarase, malate dehydrogenase and *2-OGDH* (Nunes-Nesi et al., 2012). Here, our results clearly indicate that loss of *E1-OGDH* function impacts respiratory rates, by 26% in comparison to WT. This result suggests that *2-OGDH E<sub>1</sub>* subunit isoforms contribute

substantially to the control of respiration in *Arabidopsis*. Previous studies have shown that both chloroplast functions and mitochondria are extremely coordinated and exhibit a strong interaction through intracellular metabolite pools (Nunes-Nesi et al., 2011). *E1-OGDH* mutant plants showed a severe decreased respiration, and additionally a decrease in the rate of carbon assimilation. The decrease in the photosynthetic performance is probably not caused by alterations in the photochemical events since the parameters related to chlorophyll fluorescence did not show significant alterations. Additionally, *E1-OGDH2* mutant lines displayed a decrease in *gs* (Figure 5D), without changes in *Ci* (Supplementary table 1). Moreover, both, *E1-OGDH1* and *E1-OGDH2* mutant lines did not show significant changes in stomatal density, but have an increased stomatal index (Figure 5E and 5F). Thus, these results indicate that the reduction in the photosynthesis was not limited by carbon uptake in *E1-OGDH1* mutant lines. Taken together the results presented here indicate differential role in stomatal function for each *E<sub>1</sub>* isoforms. The importance of mitochondrial metabolism in photosynthesis in the illuminated leaves has received much attention in the form of reverse genetic studies (Nunes-nesi et al., 2008). The antisense inhibition of fumarase in tomato showed impaired photosynthesis, which resulted in an increased concentration of malate and, to a lesser extent, of fumarate and, in turn, promoted stomatal closure (Nunes-nesi et al., 2007a). In contrast, the antisense inhibition of succinate dehydrogenase in tomato, exhibit an enhanced rate of photosynthesis due to decreased apoplastic levels of malate and fumarate (Araújo et al., 2011b). Thus, this result suggested the importance of the TCA cycle intermediates with respect to photosynthesis

and specifically with respect to their role in the regulation of stomatal aperture. The reduced rate of CO<sub>2</sub> assimilation in mutant lines in the genes encoding the E<sub>1</sub> subunit of 2-OGDH were accompanied with high levels of malate and fumarate especially the *E1-OGDH1* mutant lines which showed high levels of malate and fumarate in the middle of the light period, and at the end of the light period, all mutant lines showed high levels of both metabolites. Interestingly, the *E1-OGDH2* mutant lines showed low *gs* suggesting the role of malate and fumarate in stomatal function in low E<sub>1</sub> subunit expressing plants.

Interestingly all mutant lines in the genes encoding the E<sub>1</sub> subunit showed a severe decrease in total chlorophyll, especially chlorophyll *a*. In illuminated leaves there is a reduction of TCA cycle activity and an increase in demand for carbon skeletons for nitrogen assimilation (Nunes-Nesi et al., 2007; Foyer et al., 2011). Most of the assimilated nitrogen is invested in the photosynthetic apparatus, particularly Rubisco and light-harvesting complex (Nunes-nesi et al., 2010a). However, mild reductions in the activity of NAD-dependent isocitrate dehydrogenase in transgenic plants of tomato exhibited few changes in photosynthetic parameters and decreased levels of amino acid and photosynthetic pigments, but increased levels of nitrate and protein, unchanged levels of sucrose and reduction in starch levels (Sienkiewicz-Porzucek et al., 2010). The reduction in mitochondrial citrate synthase activity displayed few changes in the photosynthetic parameters, but increased rate of respiration, and led to slight decreases in the levels of photosynthetic pigment, with increased levels of nitrate, amino acids and starch (Sienkiewicz-Porzucek et al., 2008). The reduced rate of carbon

assimilation in *E1-OHDG1* and *E2-OGDH2* mutant plants were characterized by decreased levels of the main nitrogen metabolites but increased levels of carbon metabolites. The increased levels of starch and soluble carbohydrates and the decreased levels of photosynthetic pigments are diagnostic of a reduced rate of nitrate assimilation (Fritz et al., 2006; Gaude et al., 2007). The low nitrogen availability induces carbohydrate accumulation in leaf cell, which often causes suppression of photosynthesis, and the excess of carbohydrates would be preferentially respired by the non-phosphorylating pathways, such as the alternative oxidase and uncoupling protein (Noguchi and Terashima, 2006). During the photosynthesis, CO<sub>2</sub> and inorganic phosphate (Pi) are converted to triose phosphate in the chloroplast. The triose phosphate is converted to sucrose in the cytosol or starch in the chloroplast (Sharkey et al., 1986). The limitation of the utilization of triose phosphate would lower stromal Pi. Thus, anything that restricts triose phosphate utilization could effectively limit the photosynthesis, in particular the photophosphorylation which is very sensitive to Pi concentration (Paul and Foyer, 2001). Interestingly, *E1-OGDH1* and *E1-OGDH2* mutant plants display high levels of sucrose in the middle of the light period and a tendency to accumulate starch at the end of the light period. This suggests that a limitation in the triose phosphate usage would be limiting photosynthesis in these mutant lines.

Despite the lack of expression in the of E<sub>1</sub> subunits isoforms, without apparent compensatory effects, the mild metabolic phenotypes in terms of nitrogen metabolism suggested that flux through TCA cycle is only partially affected in the mutant lines, suggesting that bypasses supply succinate to

TCA cycle. Studies on the function of Succinyl-Coenzyme A ligase and E<sub>1</sub> subunit of 2-OGDH in tomato suggested that down regulation of these two enzymes activates the GABA-shunt, making a bypass of the enzyme 2-OGDH and Succinyl CoA ligase. This alternative pathway ensures the supply of succinate to maintain the electron transport chain (Araújo et al., 2012c; Studart-Guimarães et al., 2007). This was probably the case in *E1-OGDH1* and *E1-OGDH2* mutant plants. The activation of GABA-shunt could be decreasing the supply of 2-OG to the chloroplast where it is used to fix nitrogen. Here, all mutant lines showed a significant decrease in nitrate and total chlorophyll and a tendency to decrease amino acids and proteins. Even more, all studied mutant lines have high values of sucrose and it is proposed that mitochondria during photosynthesis provide ATP to maintain high rates of sucrose synthesis. Nevertheless, this could confirm that the activation of the GABA-shunt to maintain the supply of succinate and ensure the operation of the electron transport chain. Interestingly the *E1-OGDH1* mutant lines have elevated levels of fumarate in leaves collected at midday and end of the light period. Thus, all mutant lines showed high levels of fumarate, increasing the supply of succinate by the GABA-shunt could raise levels of fumarate catalyzed by the enzyme succinate dehydrogenase.

In the light, photosynthesis provides energy and carbon to support growth, whilst, at night, metabolism and growth depend on carbon reserves that accumulated in preceding light periods (Zeeman et al., 2010). Many plants use starch as their major transient carbon reserve (Sulpice et al., 2014). Starch accumulates in the light and is remobilized at night to support metabolism and growth (Zeeman et al., 2010). The rate of thiamin

biosynthesis, a cofactor of Pyruvate dehydrogenase (PDH) and 2-OGDH, directs their activity. The high thiamin availability during the dark period enhances the activity of the thiamine-requiring enzymes, like 2-OGDH, which in turn increases the carbon flux through the TCA cycle. This would exhaust the starch at the beginning of light period (Bocobza et al., 2013). Interestingly, all *E1-OGDH* mutant lines exhibit a reduced rate of respiration and displayed high levels of starch in the middle of the dark period, suggesting a reduction of carbon flux through the TCA cycle. Additionally, *E1-OGDH2* mutant plants accumulated more starch at the end of the light period, although no significant difference, with a higher rate of starch synthesis and during the dark period, a higher rate of starch degradation, which could explain the greater development of these plants. Thus, *E1-OGDH* mutant plants exhibit high levels of sucrose in the middle of light period and high levels of starch at the end of light period, that could suggests a limitation of the utilization of triose phosphate and limit the photosynthesis.

## CONCLUSIONS

The lack in the expression of genes encoding the 2-OGDH E<sub>1</sub> subunit reduces the rate of respiration and affect the photosynthesis, confirming the important role of 2-OGDH enzyme in controlling the respiration. In addition, both genes encoding the 2-OGDH E<sub>1</sub> subunit have important role in the plant metabolism. The mutation of these genes lead to alteration in the carbon and nitrogen metabolism that result in different responses in plant growth and seed production. It was also clear to suggest that each isoforms of E<sub>1</sub> have different physiological roles in the plant development. Thus, we conclude that by expression analysis as well as by physiological and

metabolic characterization that both isoforms of E<sub>1</sub> subunit play distinct roles in plant growth and there are no compensatory effects between the two isoforms.

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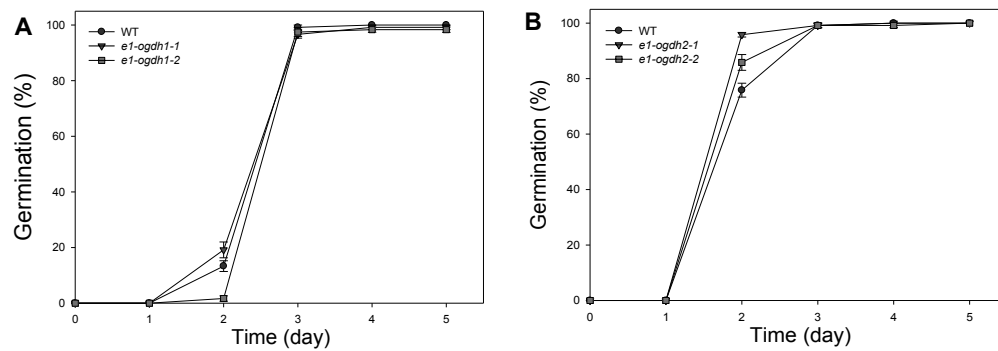
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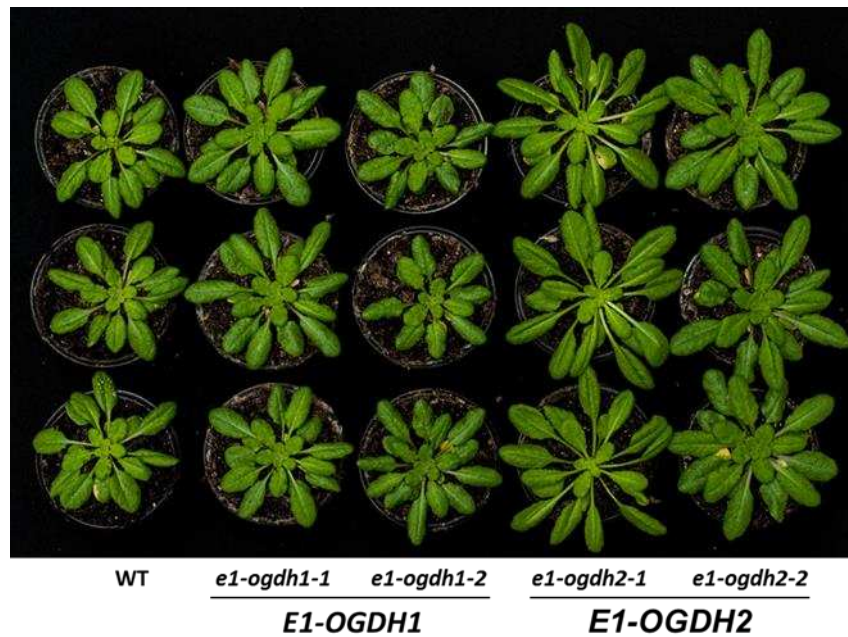
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## SUPPLEMENTAL DATA



**Supplemental Figure 1.** Germination of *Arabidopsis* wild type (WT) seeds, (A) E1-OGDH1 mutant seeds and (B) E2-OHDH2 mutant seeds. Values are means  $\pm$  SE of four plates containing 30 seeds.

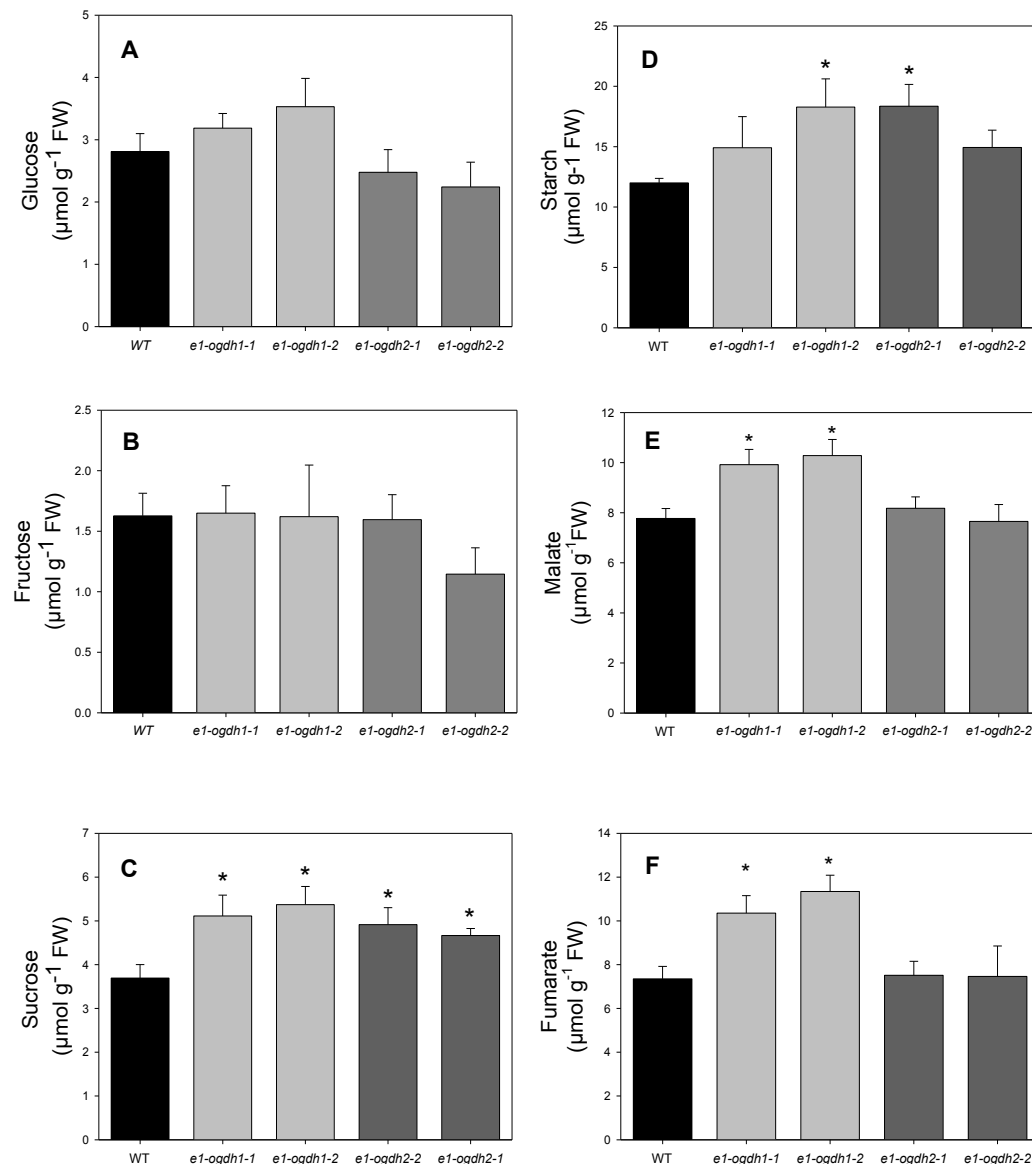


**Supplemental Figure 2.** Growth phenotype of *Arabidopsis* *E1-OGDH* mutant lines after four weeks of cultivation. The lines used were as follows: the wild type, *E1-OGDH1* mutant lines, and *E1-OGDH2* mutant lines.

**Supplemental Table 1.** Gas-exchange and chlorophyll fluorescence measurements.

| Parameters                                            | WT                | e1-ogdh1-1                         | e1-ogdh1-2        | e1-ogdh2-1        | e1-ogdh2-2                         |
|-------------------------------------------------------|-------------------|------------------------------------|-------------------|-------------------|------------------------------------|
| $C_i$ ( $\mu\text{mol CO}_2 \text{ mol}^{-1}$ )       | 313.93 $\pm$ 3.75 | 319.85 $\pm$ 4.09                  | 319.94 $\pm$ 4.10 | 303.78 $\pm$ 6.62 | 314.35 $\pm$ 3.84                  |
| $E$ ( $\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$ ) | 3.70 $\pm$ 0.18   | 3.70 $\pm$ 0.15                    | 3.77 $\pm$ 0.26   | 2.84 $\pm$ 0.27   | 3.10 $\pm$ 0.23                    |
| $WUE_i$ ( $A/g_s$ )                                   | 42.22 $\pm$ 2.29  | 38.95 $\pm$ 2.52                   | 38.91 $\pm$ 2.69  | 49.62 $\pm$ 4.33  | 43.05 $\pm$ 2.46                   |
| $F_v/F_m$                                             | 0.79 $\pm$ 0.001  | <b>0.78 <math>\pm</math> 0.003</b> | 0.78 $\pm$ 0.002  | 0.79 $\pm$ 0.001  | 0.79 $\pm$ 0.001                   |
| $F_v'/F_m'$                                           | 0.54 $\pm$ 0.004  | 0.54 $\pm$ 0.009                   | 0.55 $\pm$ 0.005  | 0.54 $\pm$ 0.012  | 0.55 $\pm$ 0.008                   |
| $\phi\text{PSII}$                                     | 0.206 $\pm$ 0.004 | 0.204 $\pm$ 0.009                  | 0.204 $\pm$ 0.005 | 0.203 $\pm$ 0.006 | 0.183 $\pm$ 0.008                  |
| NPQ                                                   | 1.86 $\pm$ 0.10   | 1.83 $\pm$ 0.08                    | 1.84 $\pm$ 0.13   | 1.74 $\pm$ 0.15   | 1.62 $\pm$ 0.14                    |
| qP                                                    | 0.382 $\pm$ 0.010 | 0.379 $\pm$ 0.018                  | 0.372 $\pm$ 0.013 | 0.375 $\pm$ 0.012 | 0.331 $\pm$ 0.019                  |
| ETR                                                   | 102.78 $\pm$ 1.99 | 102.02 $\pm$ 4.72                  | 102.13 $\pm$ 2.72 | 101.71 $\pm$ 3.16 | <b>90.86 <math>\pm</math> 4.24</b> |

$C_i$ : internal  $\text{CO}_2$  concentration;  $E$ : transpiration rate;  $WUE$ : intrinsic water-use efficiency;  $F_v/F_m$ : maximum PSII photochemical efficiency;  $F_v'/F_m'$ : actual PSII photochemical efficiency;  $\phi\text{PSII}$ : quantum yield of PSII; NPQ: non-photochemical quenching; qP: photochemical quenching; ETR: electron transport rate. Values are presented as means  $\pm$  SE of six individual plants per line. Values in bold indicated by Student's t test to be significantly different ( $P \leq 0.05$ ) from the wilt type.



**Supplemental Figure 2.** Metabolite levels of the main carbon related compounds of *Arabidopsis* *E1-OGDH* mutant lines.

Glucose (A), fructose (B), sucrose (C), starch (D), malate (E) and fumarate (F) levels were measured using leaf material harvested in the middle of the light period from 4-week-old plants. The lines used were as follows: the wild type, black bars; *E1-OGDH1* mutant lines, light gray bars; *E1-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's t test to be significantly different ( $P < 0.05$ ) from the wild type.

## CHAPTER II

## **Down regulation of 2-OGDH E<sub>2</sub> subunit impacts plant growth and seed production in *Arabidopsis thaliana***

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### **ABSTRACT**

The 2-oxoglutarate dehydrogenase (2-OGDH) is a multimeric complex composed of three subunits, whose combined catalytic activities promote the oxidative decarboxylation of 2-oxoglutarate (2-OG) to succinyl coenzyme A. The role of the 2-OGDH has previously been studied by antisense inhibition in tomato plant however the physiological role of this enzyme and its subunits still need further investigation. Here, we used T-DNA insertion mutant lines to specifically reduce the expression of the genes encoding E<sub>2</sub> subunit of 2-OGDH of *Arabidopsis thaliana*. The mutant plants exhibited substantial reduction in the rate of respiration and unaltered photosynthetic rate. The mutant plants also displayed changes in the levels of amino acids and nitrate but unaltered levels of chlorophyll and proteins. Additionally, changes in the levels of sucrose and organic acids were observed in the mutant lines. Each of the genes seems to have similar responses in plant growth and storing reserves in seeds. Our results indicate that each gene encoding the E<sub>2</sub> subunit have similar functions in the metabolism of carbon-nitrogen and plant growth. These results are discussed in the context of the importance of the two genes encoding 2-OGDH E<sub>2</sub> subunit for both metabolic and developmental process.

**Key words:** 2-oxoglutarate, respiration, TCA cycle

## INTRODUCTION

Mitochondrial metabolism plays important roles in many fundamental cellular processes such as photosynthesis, photorespiration, nitrogen metabolism, redox regulation and signaling (Nunes-Nesi et al., 2013). The tricarboxylic acid (TCA) cycle is composed by a set of eight enzymes primarily linking the product of the oxidation of pyruvate and malate, generated in the cytosol, to CO<sub>2</sub> with the generation of NADH for the oxidation by the mitochondrial respiratory chain (Nunes-Nesi et al., 2013). Moreover, at the same time, TCA cycle is clearly embedded in a wider metabolic network that allows its activity to contribute to other aspects of metabolism (Cavalcanti et al., 2014; Sweetlove et al., 2010). The function of the TCA cycle in illuminated leaves is still not fully understood and its operation in the light remains fragmented (Nunes-Nesi et al., 2007b). The antisense inhibition of the succinate dehydrogenase (SDH) in tomato plants (Araújo et al., 2011b) and SDH-deficient *Arabidopsis* (Fuentes et al., 2011) displayed higher photosynthesis as well as increased whole plant biomass. In addition, these plants displayed increased stomatal aperture and density (Araújo et al., 2011b; Fuentes et al., 2011). By contrast, antisense inhibition of fumarase lead decreased photosynthesis and total plant biomass (Nunes-Nesi et al., 2007a). Measurements of apoplastic organic acids levels in SDH and fumarase antisense plants, revealed a negative correlation between the levels of both malate and fumarate and stomatal conductance (Araújo et al., 2011a).

Hints to the regulation of the TCA cycle have been provided by a recent *in silico* metabolic control analysis which show that much of the

control through this pathway is resident in fumarase, malate dehydrogenase and 2-oxoglutarate dehydrogenase (Araújo et al., 2012a). The 2-OGDH complex occupies a central point in cellular metabolism within the TCA cycle and belongs to an important branch of the central metabolism of carbon and nitrogen (Araújo et al., 2013; Bunik and Fernie, 2009). The 2-OG may either be further degraded by 2-OGDH in the TCA cycle with energy production or provide the skeleton for inorganic nitrogen assimilation via glutamate biosynthesis (Araújo et al., 2013). 2-OG is used as an obligatory substrate in a range of oxidative reactions catalyzed by 2-OG dependent-dioxygenases (Kawai et al., 2014) and is involved in several important biochemical processes not only in nitrogen metabolism but also glucosinolate, flavonoid alkaloid and GA metabolism (Araújo et al., 2014b; Farrow and Facchini, 2014). In addition, 2-OG is a direct regulator of enzymes, such as cytosolic pyruvate kinase and PEP carboxylase, mitochondrial citrate synthase, and alternative oxidase, each involved in sugar oxidation and/or organic acid flux and redox control between cytosol and mitochondria (Hodges et al., 2002). Moreover, 2-OG plays a role as a signal metabolite in plants (Feria Bourrellier et al., 2009).

The 2-OGDH catalyzes the oxidative decarboxylation of 2-OG to form succinyl-CoA and NADH by the sequential operation of three enzymes: 2-oxoglutarate dehydrogenase ( $E_1$  subunit), dihydrolipoamide succinyl transferase ( $E_2$  subunit) and dihydrolipoamide dehydrogenase ( $E_3$  subunit) (Millar et al., 1999), with the consecutive action of several cofactors such as thiamine, diphosphate,  $Mg^{2+}$ , lipoic acid and  $FAD^+$  (Strumilo, 2005). 2-OGDH exists as a polymeric structure that comprises an  $E_2$  core of 24

subunits, to which are attached E<sub>1</sub> and E<sub>3</sub> homodimers (Millar et al., 1999). The two E<sub>3</sub> polypeptides found in potato mitochondria are shown to be associated with both 2-OGDH and pyruvate dehydrogenase complex (PDH) (Millar et al., 1999). Only one E<sub>3</sub> gene containing mitochondrial targeting sequences has been isolated from pea; the predicted polypeptide that it encodes has been proposed to participate in the catalytic function of PDH, 2-OGDH, glycine decarboxylase complex and branched-chain 2-oxoacid dehydrogenase complex (Millar et al., 1999; Timm et al., 2015). The regulation of 2-OGDH including allosteric responses to second messengers and metabolic indicator, such as Ca<sup>2+</sup>, ATP/ADP, SH/-S-S (thiol/disulfide), NADH/NAD<sup>+</sup>, Acetyl-CoA/CoA (Bunik and Fernie, 2009; Strumilo, 2005). The regulation of the dimeric component E<sub>1</sub> and E<sub>3</sub> by their substrates and effectors include co-operative interactions of the active site and allosteric regulations of E<sub>1</sub> by the product of E<sub>3</sub>, NADH (Bunik and Fernie, 2009). Allosteric effectors not directly involved in the reaction, such as AMP and Ca<sup>2+</sup>, exert their regulatory influence in a highly interactive manner, by increasing the E<sub>1</sub> affinity to their substrate (Strumilo, 2005). The 2-OGDH also catalyzes several side reactions of potential biological significance. These are the reactive oxygen species (ROS) producing activity of 2-OGDH, which was shown to contribute to ROS generated by neurons in response to glutamate (Zündorf et al., 2009). When the ratio of the 2-OGDH substrate and/or products promotes excessive formation of the dihydrolipate intermediate, 2-OGDH catalyzes oxidation of the later by molecular oxygen with the formation of the superoxide anion radical and thiyl radical of the complex-bound lipoate (Bunik and Fernie, 2009).

It has been demonstrated by the chemical inhibition of 2-OGDH in potato tubers that this enzyme plays an important role in nitrogen assimilation as well as in amino acid metabolism (Araújo et al., 2008). In addition the same inhibitor of 2-OGDH were used in leaf of *Arabidopsis thaliana* showing reduced respiration rates that was associated with imbalance in carbon-nitrogen metabolism and cell homeostasis (Araújo et al., 2012b). Moreover, the antisense inhibition of the 2-OGDH in tomato plants did not result in major changes in photosynthesis or growth rates but had greater impact on the respiration rate and indicated that 2-OGDH plays an important role in modulating the rate of flux from 2-OG into amino acid metabolism (Araújo et al., 2012c). Despite that, there are not direct genetic studies on the role of 2-OGDH in plants. The E<sub>2</sub> subunit of 2-OGDH in *Arabidopsis thaliana* is encoded by two genes and their physiological roles remains poorly characterized. In this work we evaluated the function of the two genes encoding the E<sub>2</sub> subunit of 2-OGDH in *A. thaliana* in order to investigate their physiological and metabolic roles in autotrophic and heterotrophic tissues under optimal environmental conditions.

## **MATERIAL AND METHODS**

### ***Isolation of T-DNA insertion mutants and genotype characterization***

*Arabidopsis* T-DNA insertion lines for At4g26910 (*E2-OGDH1*) and At5g55070 (*E2-OGDH2*) genes encoding E<sub>2</sub> subunit of the 2-OGDH complex were obtained from the Salk Institute for Biological Studies collection. For both genes, two lines were isolated: lines *e2-ogdh1-1* (Salk\_005851) and *e2-ogdh1-2* (Salk\_084620) for At4g26910 gene, and lines *e2-ogdh2-1* (Salk\_207269) and *e2-ogdh2-2* (Salk\_054508) for

At5g55070 gene. For genotyping the T-DNA insertion lines, leaves of each plant were collected separately, and genomic DNA was extracted. PCR analyses were performed for the target gene using left primer (LP) and right primer (RP) and the T-DNA insertion using a T-DNA specific left border primer (LBb1.3). The primer used were: for *e2-ogdh1-1*, LP (CGACATACATCATTGGTCTAGGCACTA) and RP (TGATATGACATCTAACAACACTGCAGGTT); for *e2-ogdh1-2*, LP (GATCATACGTAAGTGCGACATACATCATT) and RP (TGATATGACATCTAACAACACTGCAAGGTT); for *e2-ogdh2-1*, LP (CTGAGATGCTGTTTTAGATGGTTGCCT) and RP (CGAATCAAACACTAACCGAAGCAGAAG); for *e2-ogdh2-2*, LP (GATTCCTCTTCTCTGTGTATTGTATCCC) and RP (GTGTTGTATTGCTCCTTTGAAATCGTCCA); and primer specific for the T-DNA, LBb1.3 (GATTTTGCCGATTTTCGGAACCACCAT). The mutant lines were then selected and homozygous plants were isolated for further analysis.

### ***Growth conditions and evaluation of biometric parameters***

Seeds were surface-sterilized and incubated for 4 days at 4°C in the dark on agar plates containing MS 0.5X media (Murashige and Skoog, 1962). Seeds were subsequently germinated and grown *in vitro* under short-day conditions (8 h/16 h of light/dark) with irradiance of 150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 22°C and 20°C in the light and dark respectively, and 60% of relative humidity. After ten days, the seedlings were transferred from plates to substrate and grown in growth chamber under the same conditions. After four weeks growth after transplanting, morphological and physiological analyzes as well as collection of samples in liquid nitrogen for biochemical analyzes were performed.

For phenotyping reproductive tissues, the seedlings were transferred to commercial substrate and were kept in growth chamber at  $22 \pm 2$  °C, 60% relative humidity and irradiance of  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , with a photoperiod of 12 h light and 12 h dark for seed production. The siliques, collected from wild-type and mutant plants, were cleared with 0.2N NaOH and 1% SDS solution to remove pigments. The cleared siliques were then scored for length and number of seeds. Ten siliques were sampled from each plant and six plants were used for the analysis.

To evaluate root length, surface-sterilized seeds were plated on MS 0.5X medium, with 0.8% agar, incubated at 4°C for 48 h, and then grown vertically at 22 °C with a photoperiod of 12 h light and 12 h dark. Seedlings were examined, and photographed. Root hair length from digital images was measured using ImageJ software (Abràmoff et al., 2005).

### ***Gene expression analysis***

After four weeks of growth leaf samples were collected and total RNA was isolated and purified using TRIzol® reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The RNA quality and integrity were monitored by spectrophotometer and by agarose gel electrophoresis. The total RNA was treated with DNaseI to remove possible contaminating genomic DNA in the samples. Two micrograms of RNA were used as template for first-strand cDNA synthesis using ImProm-II™ Reverse Transcriptase (Promega) and an oligo (dT) primer. qRT-PCR amplification of At4g26910 cDNA specific sequence was performed with a forward primer (TGTCAAGGATGTTGTGGAGGATC) and a reverse primer (CGCAATACTCGGGAACTGTAAAG). In the same way, qRT-PCR

amplification of At5g55070 cDNA specific sequence was performed with a forward primer (AGGTAAAACCATACGGATACTGC) and a reverse primer (AAACTCAATACACCGATGCTTTCC). qRT-PCR amplification of the actin encoding gene of *A. thaliana* with a forward primer (CTTGCACCAAGCAGCATGAA) and a reverse primer (CCGATCCAGACACTGTACTTCCTT) served as control to normalize the transcripts of all samples.

### ***Stomatal density and stomatal index***

After 2 h of illumination in the light/dark cycle, leaf impressions were taken from the abaxial surface of the first fully expanded leaf with dental resin imprints (Berger and Altmann, 2000). The measurements were performed on the images. Stomatal density and stomatal index (the ratio of stomata to stomata plus other epidermal cells) were determined in at least six fields of 0.09 mm<sup>2</sup> per leaf from four different plants.

### ***Gas exchange and chlorophyll fluorescence measurements***

Gas exchange parameters were determined simultaneously with chlorophyll a fluorescence measurements using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Instantaneous gas exchange was measured after 1 h illumination during the light period under 1000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . The reference CO<sub>2</sub> concentration was set at 400  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ . All measurements were performed using the 2 cm<sup>2</sup> leaf chamber at 25 °C, and the leaf-to-air vapor pressure deficit was kept at 1.3 to 2.0 kPa, while the amount of blue light was set to 10% PPFD to optimize stomatal aperture. Dark respiration (R<sub>d</sub>) was measured using the same gas exchange system as described above

after at least 1 h in the dark period. Rate of photorespiration were calculated according to the model proposed by Sharkey (1988).

### ***Determination of metabolite levels***

The entire rosette was harvested in different times along of the light/dark cycle in the start, middle and end of light period. Additionally, we harvested samples in the middle and end of dark period. Rosettes were flash frozen in liquid nitrogen and stored at -80 °C until further analyzes. The levels of starch, sucrose, fructose, and glucose in the leaf tissues were determined exactly as described previously (Ferne et al., 2001). Malate and fumarate were determined exactly as detailed by Nunes-Nesi et al. (2007). Proteins and amino acids were determined as described previously (Cross et al., 2006). The levels of nitrate were determined as described previously (Fritz et al., 2006) and photosynthetic pigments determined exactly as described before (Porra et al., 1989).

### ***Phylogenetic Analysis***

Amino acids sequences were retrieved from the Gen-Bank through the BLASTp algorithm using At4g26910 and At5g55070 amino acids sequence as query. Sequences were aligned using the ClustalW software package (Higgins and Sharp, 1988) using default parameters. Maximum Likelihood phylogenetic tree were constructed with MEGA5.2 software (Tamura et al., 2011). Distances were calculated using pair-wise deletion and Poisson correction for multiple hits; bootstrap values were obtained with 500 pseudo replicates.

### **Statistical Analysis**

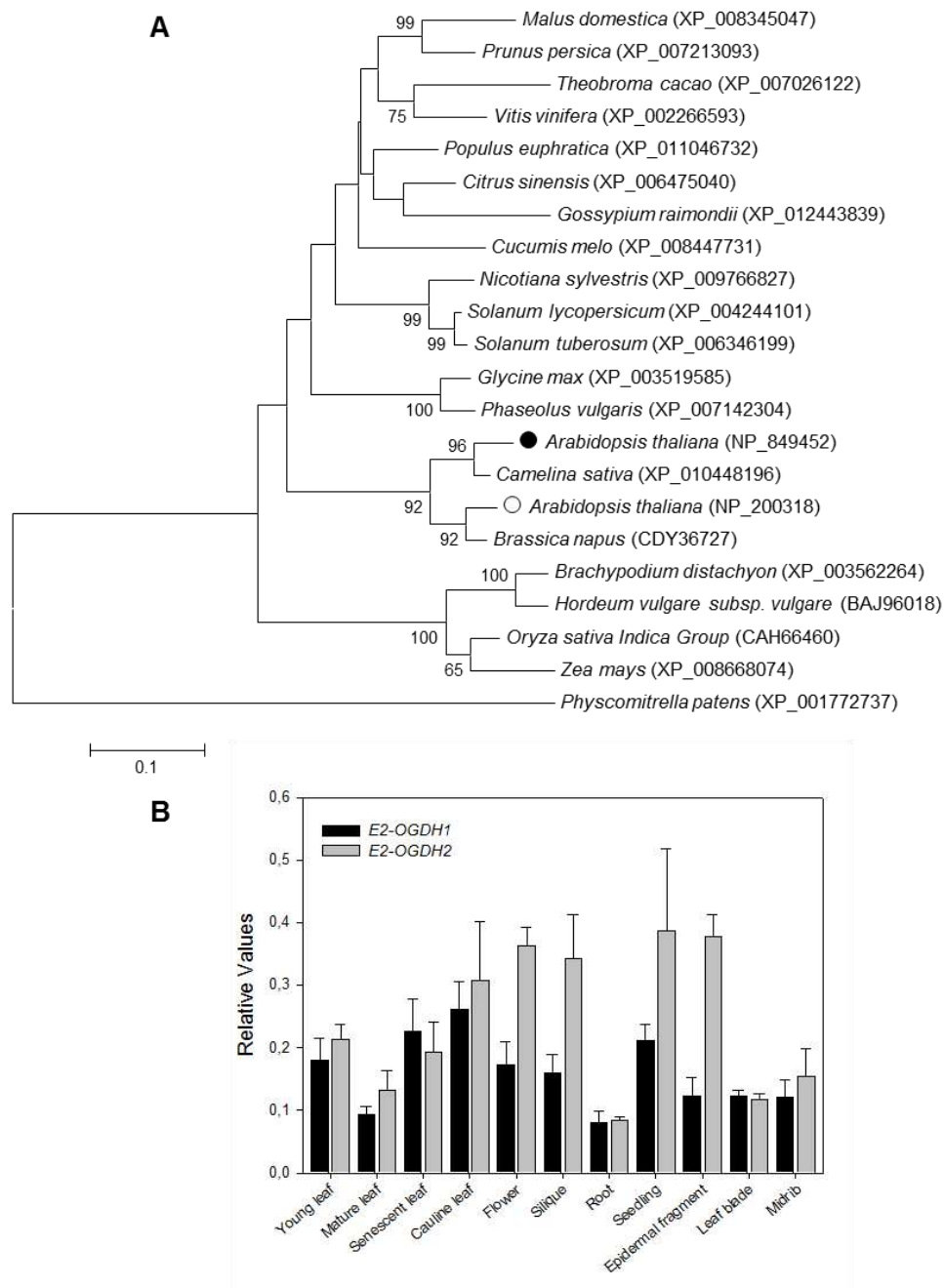
The *t* tests have been performed using the algorithm embedded into Microsoft Excel (Microsoft, Seattle). The term significant is used in the text only when the change in question has been confirmed to be significant ( $P < 0.05$ ) with the *t* test.

## **RESULTS**

### ***Expression analysis by qRT-PCR of genes encoding E<sub>2</sub> subunit of 2-OGDH complex***

The E<sub>2</sub> subunit of 2-OGDH is encoded by two genes, *E2-OGDH1* (At4g26910) and *E2-OGDH2* (At5g55070) that encodes a protein of 464 amino acids with 84,31% of identity. The proteins have two conserved domains including lipoyl domain of the dihydrolipoyl acyltransferase component and 2-oxoacid dehydrogenases acyltransferase domain, located exclusively in the mitochondria. The phylogenetic tree of E<sub>2</sub> subunit of 2-OGDH display two cluster, one cluster, represented by the monocot plants and other cluster by dicot plants, including the Orden *Brasicales* with *Arabidopsis thaliana*, *Brassica napus* and *Camelina sativa*. (Figure 1A).

The amino acids sequence of *E2-OGDH1* (NP\_849452) revealed 83% identity to *Brassica napus* (CDY36727), 71% identity to *Solanum lycopersicum* (XP\_004244101), 67% identity to *Brachypodium distachyon* (XP\_003562264), 69% identity to *Zea mays* (XP\_008668074). The amino acids of *E2-OGDH2* (NP\_200318) revealed 92% identity to *Brassica napus* (CDY36727), 71% identity to *Solanum lycopersicum* (XP\_004244101), 68% identity to *Brachypodium distachyon* (XP\_003562264), 69% identity to *Zea mays* (XP\_008668074).

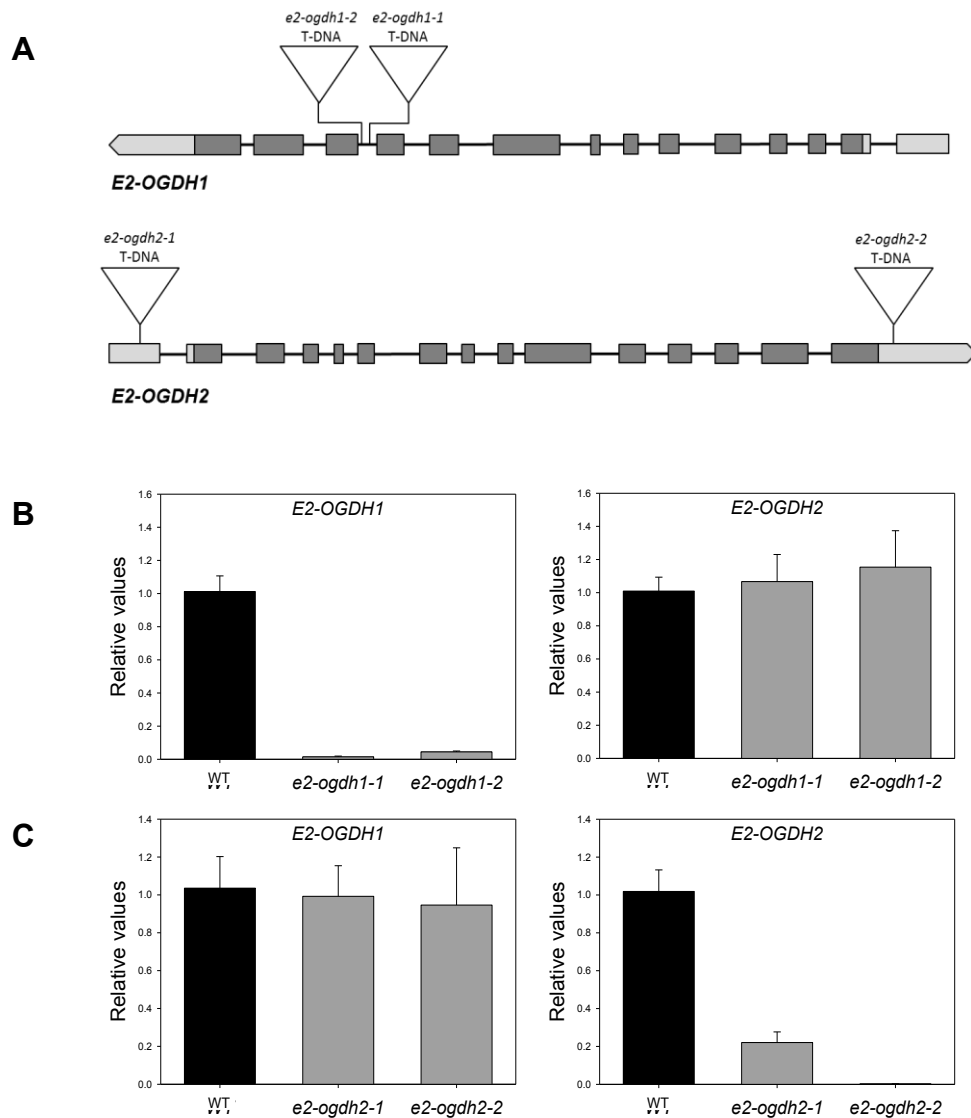


**Figure 1.** Phylogenetic analysis and characterization of expression of *E2-OGDH1* and *E2-OGDH2* isoforms in *Arabidopsis thaliana* wild type (Col-0). (A) Dendrogram of 2-OGDH E<sub>2</sub> amino acid sequences, the protein accession numbers are given between brackets. The sequences retrieved from *Arabidopsis thaliana* are highlight with circles. The empty circle corresponds to *E2-OGDH1* and the black circle corresponds to *E2-OGDH2* (B) Relative transcript abundance of the *E2-OGDH1* and *E2-OGDH2* in leaves of different phenological stages, cauline leaf, flower, silique, roots, seedlings, guard cell-enriched epidermal fragment, leaf blade and midrib. The gene expression was calculated by using the  $2^{-\Delta CT}$  method and actin was used to normalize the transcripts of all samples. Values are presented as means  $\pm$  SE of four individual plants.

To determine the expression levels of genes encoding E<sub>2</sub> subunit of 2-OGDH in different organs and tissues of *A. thaliana*, wild type (Col-0) plants were used for quantitative RT-PCR analysis. To this end, total RNA was isolated from leaves of different phenological stages, flowers, siliques, roots and seedlings. In addition, we also analyzed the expression of 2-OGDH E<sub>2</sub> subunit in guard cell-enriched epidermal fragments, leaf blade, and midrib (Figure 1B).

The gene *E2-OGDH2* has high expression in comparison to *E2-OGDH1* in young and mature leaves, whereas it was lower in senescent leaves (Figure 1B). In cauline leaf, flowers, siliques and seedlings, the expression of *E2-OGDH2* gene was higher than *E2-OGDH1*, whereas in root the same level of expression was observed (Figure 1B). The expression level of *E2-OGDH1* and *E2-OGDH2* genes was similar in leaf blade and midrib, but in epidermal fragment, the *E2-OGDH2* gene has higher expression in comparison to *E2-OGDH1* (Figure 1B).

Since *A. thaliana* has two genes encoding E<sub>2</sub> subunit of the complex 2-OGDH, we decided to study loss-of-function mutants of this gene to elucidate the role of each gene. For that, a collection of *Arabidopsis* T-DNA insertion mutants was screened and two mutant lines containing a T-DNA element inserted in the gene *E2-OGDH1* were isolated. These two lines were named *e2-ogdh1-1* and *e2-ogdh1-2*. Likewise, for the gene *E2-OGDH2*, we isolated two mutant lines, named *e2-ogdh2-1* and *e2-ogdh2-2* (Figure 2A).



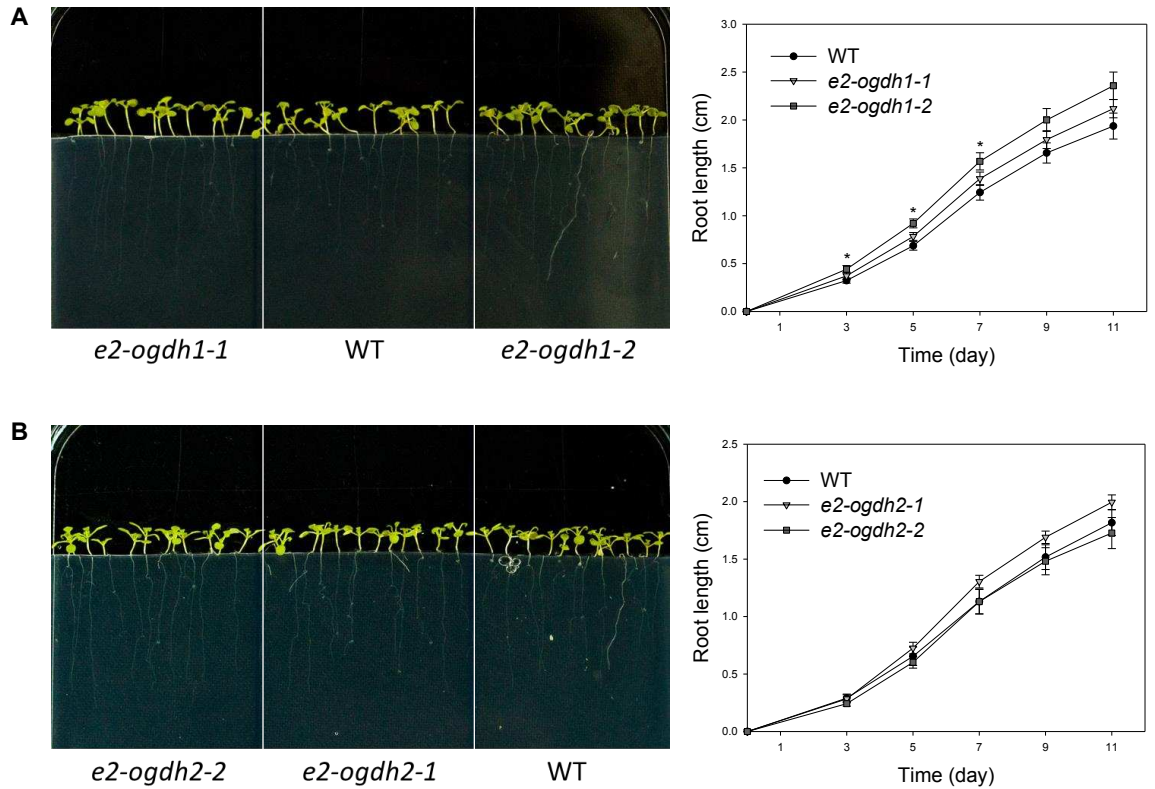
**Figure 2.** Characterization of *Arabidopsis* E2-OGDH mutant lines. (A) Schematic representation of the *E2-OGDH1* and *E2-OGDH2* gene, the mutant lines obtained by PCR screening of a T-DNA mutant collection. (B) Expression analysis of *E2-OGDH1* and *E2-OGDH2* in mutant lines *e2-ogdh1-1* and *e2-ogdh1-2*. (C) Expression analysis of *E2-OGDH1* and *E2-OGDH2* in mutant lines *e2-ogdh2-1* and *e2-ogdh2-2*. Values are presented as means  $\pm$  SE of four individual plants and actin was used to normalize the transcripts of all samples.

The mutant lines *e2-ogdh1-1* and *e2-ogdh1-2* displayed clear reduction in the expression of *E2-OGDH1* in comparison with the wild-type levels, while the same mutant lines displayed the same level in expression of *E2-OGDH2* (Figure 2B). The mutant lines *e2-ogdh2-1* and *e2-ogdh2-2* also displayed clear reduction in the expression of the target gene *E2-OGDH2* gene in comparison with wild-type levels. Accordingly, the same mutant lines displayed the same level in expression of *E2-OGDH1* gene (Figure 2C).

***Germination and seedling development of mutant plants of E<sub>2</sub> subunit of 2-OGDH***

In order to investigate the importance of both *E2-OGDH1* and *E2-OGDH2* in the physiology of seed, the germination rate and seedling establishment were analyzed.

Interestingly, seeds of both set of mutant lines, *E2-OGDH1* and *E2-OGDH2*, did not show alterations in the germination rate in comparison with wild type plants (Supplemental Figure 2). In contrast, seedling root growth was affected by the lower expression of the gene encoding *E2-OGDH1*, the root growth was accelerated, especially in mutant line *e2-ogdh1-2* (Figure 3A). In contrast, the *E2-OGDH2* mutant seedlings did not show significant changes in relation to wild type (Figure 3B).



**Figure 3.** Phenotypic characterization of *Arabidopsis* *E2-OGDH* homozygous mutant

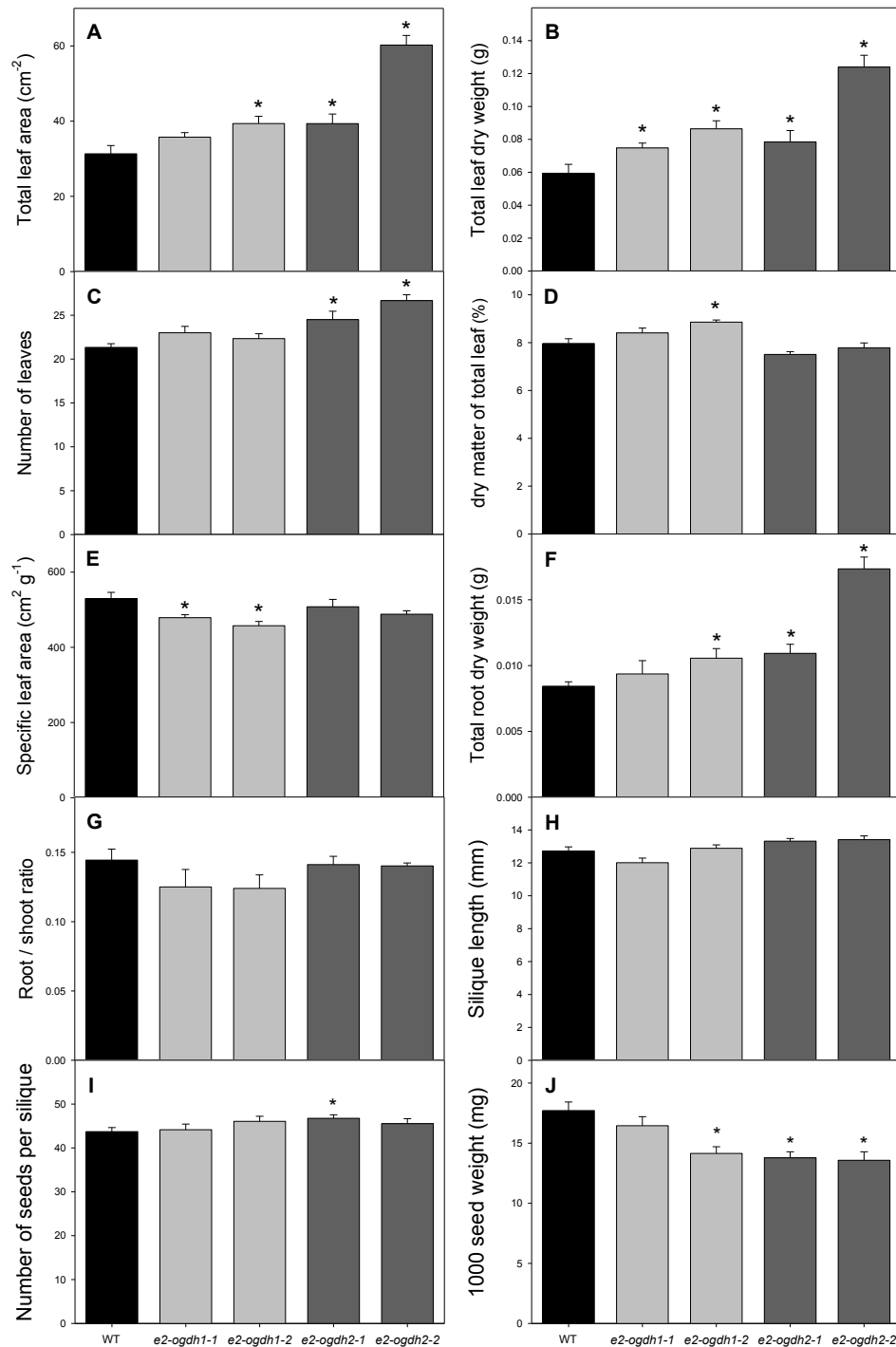
Root growth of *Arabidopsis thaliana* wild-type (WT), *E2-OGDH1* mutant lines (A) and *E2-OGDH2* mutant lines (B). The plants were growth on MS 0.5X medium for 11 days after germination. Values are presented as means  $\pm$  SE of four individual plate. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type.

### ***Phenotypes of plants with lower expression of E<sub>2</sub> subunit of OGDH***

After four weeks of cultivation, plants from the mutant lines of *E2-OGDH1* gene appeared to have a faster development than wild type plants (Supplemental Figure 1). Effectively, the *E2-OGDH1* mutant lines displayed an increase in total leaf area, significant for *e2-ogdh1-2* mutant line (Figure 4A). Accordingly the two mutant lines showed an increase in the total leaf dry weight and in total root weight (Figure 4B, 4F), which resulted in a reduced root/shoot ratio in relation to wild type plants (Figure 4G). Interestingly, the number of leaves did not show changes as compared to wild type plants (Figure 4C). However, a significant reduction in the specific leaf area was observed in the two mutant lines (Figure 4E).

Regarding *E2-OGDH2* mutant lines, a significant increase in total leaf area as compared to wild type plants was observed (Figure 4A). This increase in the leaf area was accompanied by an increase in the total leaf dry weight and in the number of leaves (Figure 4B, 4C). Additionally, an increased in root weigh was observed without changes in root/shoot ratio as compared to wild type plants (Figure 4F, 4G). Surprisingly, the specific leaf area showed no changes as compared to wild type plants (Figure 4E).

To study the role of *E2-OGDH1* and *E2-OGDH2* in reproductive tissues, we scored for silique length, seed number per silique and weight of 1000 seeds. The mutant lines of *E2-OGDH1* and *E2-OGDH2* genes showed no changes for silique length (Figure 4H). Minor increase in the number of seeds per silique was observed in all four mutant lines, significant only for *e2-ogdh2-1* (Figure 4I). Interestingly, the seed weight was significantly reduced in *e2-ogdh1-2*, *e2-ogdh2-1* and *ogdh2-2* (Figure 4J).

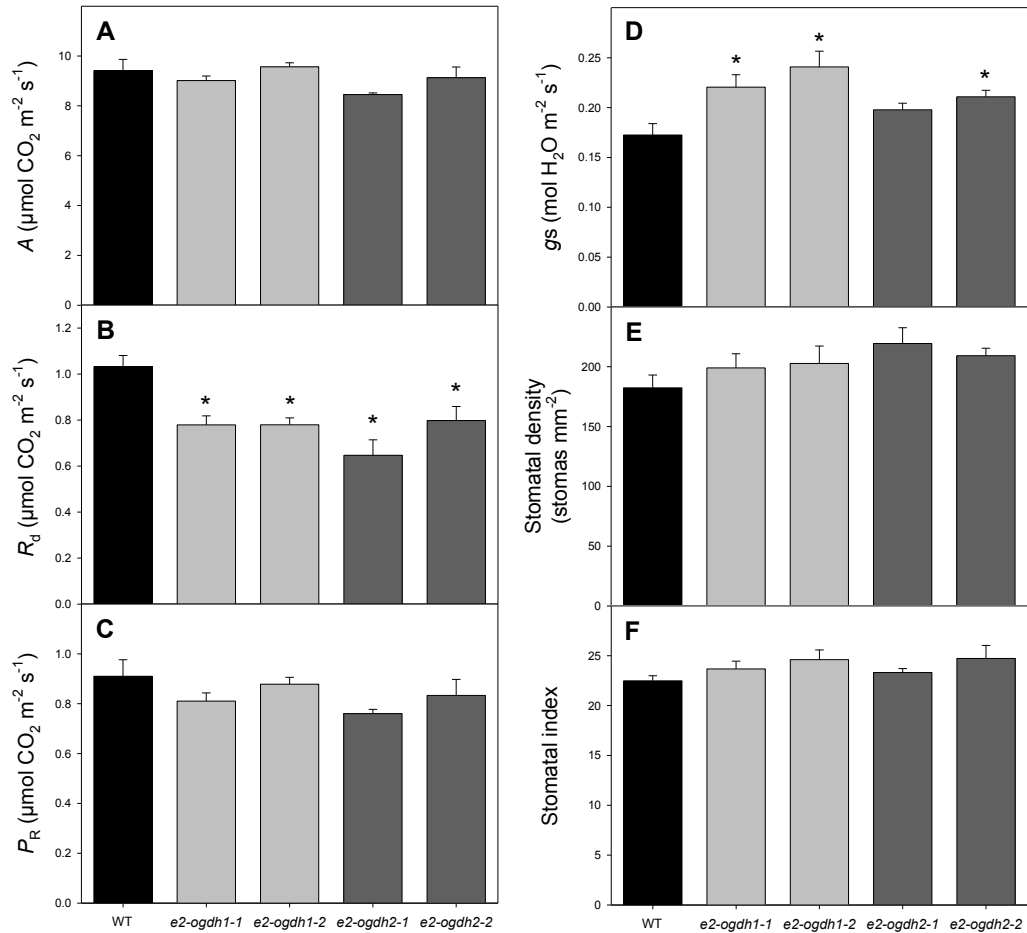


**Figure 4.** Growth phenotype of *Arabidopsis* *E2-OGDH* mutant lines. (A) Total leaf area, (B) Total leaf dry weight, (C) ) Number of leaves, (D) % dry matter, (E) Specific leaf area, (F) Total root dry weight, (G) Root/shoot ratio. (H) Silique length, (I) Number of seeds per silique, and (J) 1000 seed weight. The lines used were as follows: the wild type, black bars; *E2-OGDH1* mutant lines, light gray bars; *E2-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of six individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0, 05$ ) from the wild type.

### ***Analysis of photosynthetic parameters***

In order to gain insight into the impact of the reduction in the expression of 2-OGDH subunit E<sub>2</sub>, gas exchange analysis were performed in 4-week-old plants of the *E2-OGDH1* and *E2-OGDH2* mutant lines. Interestingly, the rate of carbon assimilation was unaltered when compared with wild type plants (Figure 5A). Additionally, all mutant lines showed an increase in the stomatal conductance (*g<sub>s</sub>*), being significantly in *E2-OGDH1* mutant lines (Figure 5D). This increase in *g<sub>s</sub>* resulted in a higher internal CO<sub>2</sub> concentration in all mutant lines and increased transpiration rates, being significant in the mutant lines of *E2-OGDH1*. Accordingly a significant decreased of the intrinsic water-use efficiency was observed in all mutant lines (Supplemental Table 1). Additionally, the stomatal density and the stomatal index did not show alterations in all mutant lines (Figure 5E, 5F). Interestingly, all mutant lines showed significant reduction in dark respiration (Figure 5B) and a tendency to decrease the rate of photorespiration (Figure 5C).

The photochemical events were minimally affected, the  $F_v/F_m$  ratio, which expresses the maximum PSII photochemical efficiency showed a decreased in the mutant lines *e2-ogdh1-2* and *e2-ogdh2-1*; the  $F_v'/F_m'$  displayed an increase in all mutant line, but significantly only in the mutant line *e2-ogdh-1-1*. The  $\phi$ PSII, NPQ, qP and ETR parameters were not affected (Supplemental Table 1). All these traits varied minimally across the mutant lines, and photochemical factors are therefore unlikely to have prominent effects in CO<sub>2</sub> assimilation rate.



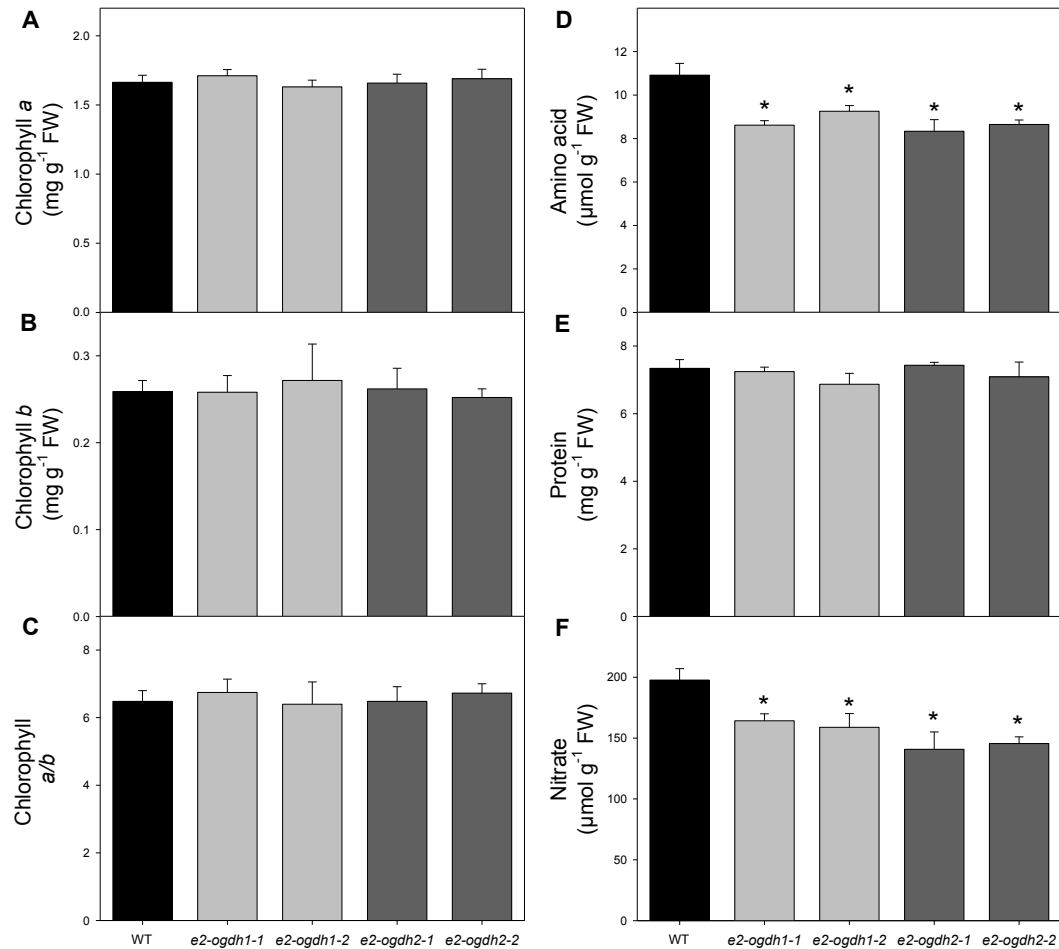
**Figure 5.** Effect of reduction in the expression of 2-OGDH E<sub>2</sub> subunit on photosynthetic and respiratory parameters.

(A) CO<sub>2</sub> assimilation rate, (B) Dark respiration. (C) Photorespiration. (D) Stomatal conductance. (E) Stomatal density. (F) Stomatal index. The lines used were as follows: the wild type, black bars; *E2-OGDH1* mutant lines, light gray bars; *E2-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0, 05$ ) from the wild type.

### **Biochemical analysis**

Given the considerable changes in respiration rates (Figure 5B) and the recognized link between mitochondrial metabolism and associated carbon/nitrogen interactions (Nunes-nesi et al., 2010a), we next decided to evaluate the levels of the main nitrogen containing compounds. First, we quantified the photosynthetic pigments, since these compounds have often been reported as important indicators of nitrogen deficiencies. This analysis revealed that in leaves of the mutant lines *E2-OGDH1* and *E2-OGDH2* the levels of chlorophyll *a* and *b* were not altered (Figure 6A, 6B) as well as chlorophyll *a/b* ratio, when compared to wild type plants (Figure 6C). Furthermore, we quantified the levels of total cellular amino acids in leaves of plants which were significantly reduced in all mutant lines (Figure 6D). Surprisingly, the levels of protein remained unaltered in all mutant lines (Figure 6E). However all mutant lines displayed significant decrease in the levels of nitrate (Figure 6F).

Given the changes in nitrogen metabolism, we next determined the levels of the main carbohydrate in leaves harvested in the middle of the light period. The *E2-OGDH1* and *E2-OGDH2* mutant lines were characterized by no changes in the levels of glucose and fructose (Supplemental Figure 3A, 3B). However, the levels of sucrose in the *E2-OGDH2* mutant lines were decreased (Supplemental Figure 3C). In addition, the levels of starch remained unaltered in all mutant lines. (Supplemental Figure 3D). Since malate and fumarate, intermediates of TCA cycle, plays important role in the regulation of stomata aperture, we next determined the levels of these

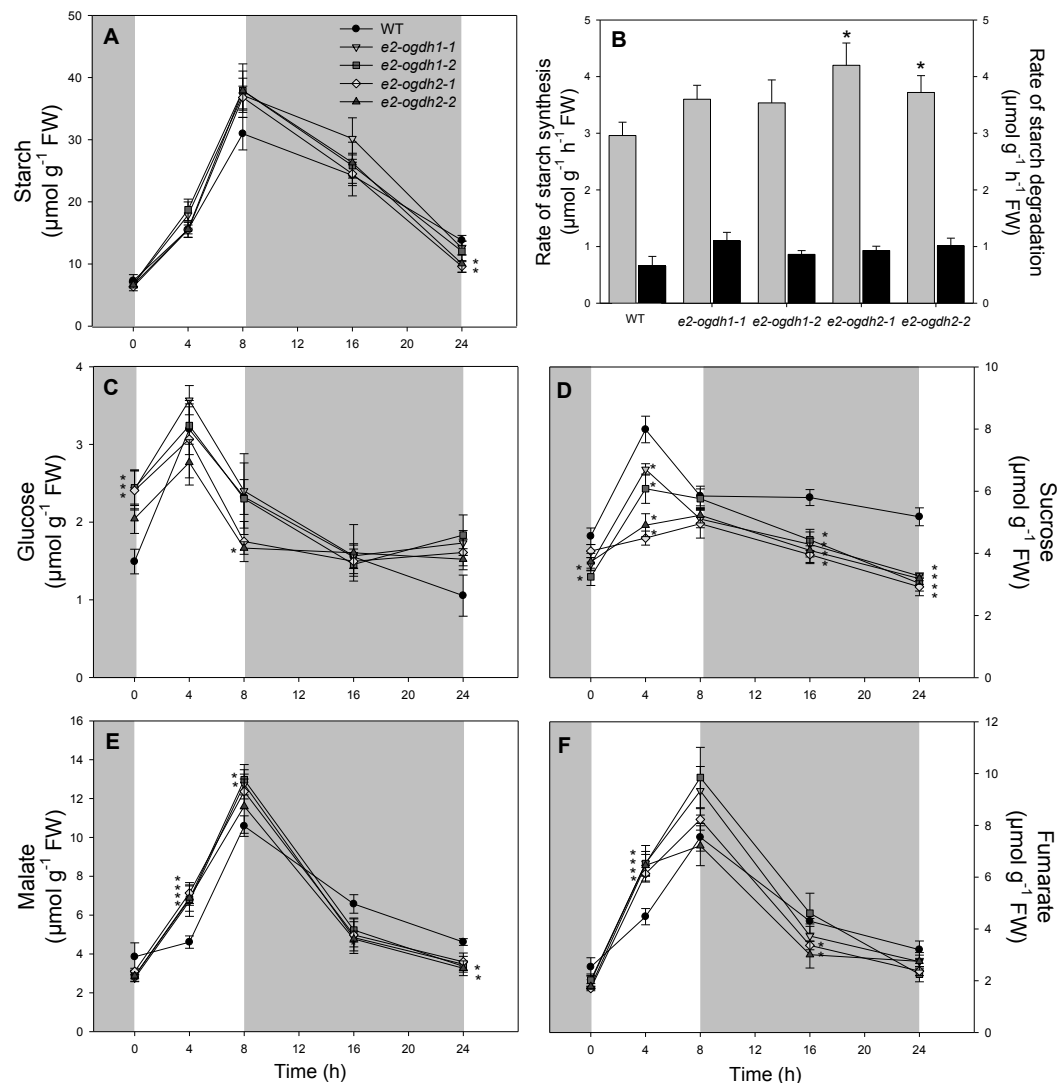


**Figure 6.** Effect of decreased expression of 2-OGDH  $E_2$  subunit on metabolite levels of the main nitrogen related compounds. (A) Chlorophyll *a*. (B) Chlorophyll *b*. (C) Chlorophyll *a/b* ratio. (D) Total amino acid. (E) Protein. (F) Nitrate. Metabolite levels were determined in 4-week-old fully expanded leaves harvested in the middle of the light period. The lines used were as follows: the wild type, black bars; *E2-OGDH1* mutant lines, light gray bars; *E2-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type.

organic acids. The levels of both malate and fumarate increased in all mutants lines significantly (Supplemental 3E, 3F).

For a more detailed characterization of the function of *E2-OGDH1* and *E2-OGDH2*, we quantified the main carbohydrates in leaves at different time points during the diurnal cycle. We determined the levels of starch, sucrose, glucose, and organic acids malate and fumarate in the start, middle and end of light period. Additionally, we analyzed these metabolites in the middle and end of dark period. Regarding the starch levels, in the light period, all mutant lines showed similar levels of starch as wild type plants, but in the end of the light period all mutant plants showed higher starch levels in comparison to wild type. Interestingly, at the end of the dark period the starch level was lower than in wild-type plants (Figure 7A). Additionally, we calculated the rates of starch synthesis and degradation for all genotypes, which were increased in all mutant lines (Figure 7B).

The glucose levels were higher at the end of dark period in all mutant lines when compared to wild type plants (Figure 7C). In contrast, sucrose levels were lower at the middle and end of dark period in all mutant lines and the same response was observed in the middle of light period (Figure 7D). The levels of both organic acids, malate and fumarate, decreased at the end of dark period in all mutant lines of *E2-OGDH1* and *E2-OGDH2*. Interestingly all mutant lines displayed high levels of malate and fumarate in the middle of light period when compared to wild type plants (Figure 7E, 7F).



**Figure 7.** Diurnal changes of the main carbon related compounds in leaf of *Arabidopsis thaliana* E2-OGDH mutant lines. (A) Starch. (B) Rates of starch synthesis (gray bars) and degradation (black bars). (C) Glucose. (D) Sucrose. (E) Malate. (F) Fumarate. The plants were harvest in the start, middle and end of light period, and in the middle and end dark period. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's *t* test to be significantly different ( $P < 0.05$ ) from the wild type. The average rates of starch synthesis and degradation were estimated as the difference between starch at end day and end night, divided by the length of the light period, or the night, respectively.

## DISCUSSION

In the present work we provided evidences about the physiological roles of the E<sub>2</sub> subunit of 2-OGDH. In general, the low expression of the genes encoding this subunit resulted in several changes in plant metabolism, which led to alteration of plant growth and seed production. In addition, we demonstrated by expression analysis in addition to physiological and biochemical analysis that both isoforms of E<sub>2</sub> subunit play similar roles in *Arabidopsis*. The level of expression of each gene in the mutant plants does not alter the expression level of the other, suggesting that compensation effects by the other isoform plays minor role or do not exist between the two isoforms. As would be expected based on previous reports (Araujo et al., 2008, 2012) lower expression of E<sub>2</sub> subunits lead to decrease, 25% (*E2-OGDH1*) and 30% (*E2-OGDH2*) reduction, in respiration rate. This results are in agreement with *in silico* metabolic control analysis for the TCA cycle enzymes which indicated that much of the flux control through the TCA cycle is 2-OGDH (Araújo et al., 2012a).

Furthermore, we observed that the *E2-OGDH1* and *E2-OGDH2* mutant plants showed increased leaf area accompanied by an increase in leaf dry weight (Figure 4A, 4B). Surprisingly the lack in expression of *E2-OGDH1* isoform showed a decrease in specific leaf area and no alterations in the number of leaf, whereas *E2-OGDH2* isoform showed no alterations in the specific area and increased leaf number. Taken together these results indicate that *E2-OGDH2* might be involved in plant development as suggested in tomato (Araújo et al., 2012) and lower expression of this isoform lead to accelerated growth in *Arabidopsis* plants.

Although the mitochondrion has its own genome and machinery for its replication the majority of mitochondrial polypeptides are encoded by the nuclear genome and the proteins produced in the cytosol and then imported into the mitochondria (Kuhn et al., 2009; Unseld et al., 1997). The importance of the mitochondrial function in the physiology and development of higher plants is demonstrated by the fact that mutations on the mitochondrial genome frequently lead to cytoplasmic male sterility (Kubo et al., 2011; Bentolila and Stefanov, 2012). Furthermore dysfunctions of delta-subunits of ATP synthase is associated with deficiencies in gametophyte development (Geisler et al., 2012). The succinate dehydrogenase, involved in both TCA cycle and the respiratory electron transport chain, is essential for gametophyte development (León et al., 2007). Moreover inhibition of the citrate synthase in potato, the first enzyme of the TCA cycle displayed ovaries disintegrated during flower development (Landschütze et al., 1995). In the present study, lower expression of *E2-OGDH1* and *E2-OGDH2* isoforms did not alter the number of seeds per silique neither silique length. Nevertheless, a clear reduction in seed weight, especially in mutant plants for *E2-OGDH2*, was observed. These results suggest that E<sub>2</sub> subunit does not play a role during gametophyte development. However, it seems to be necessary in processes related to seed maturation. This function seems to be more important for *E2-OGDH2* because decreased seed weight was observed in mutant lines for this isoform (Figure 4J). Interestingly, lower expression of *E2-OGDH1* and *E2-OGDH2* did not impact seed germination, suggesting compensatory mechanisms for the lack of *E2-OGDH* in these tissues, like the GABA-shunt, as previously observed in inhibitor-treated

potato tuber (Araújo et al., 2008) as well upon the inhibition of the reaction catalyzed by succinyl-CoA ligase (Studart-Guimarães et al., 2007) and by 2-OGDH in tomato (Araújo et al., 2012c) to prioritize the supply of nutrients to reproductive tissues.

The regulation of the TCA cycle have been provided by a recent metabolic control analysis which show that much of the control through this pathway is resident in 2-OGDH (Araújo et al., 2012a). In agreement with the chemical inhibition of the 2-OGDH in heterotrophic tissue (Araújo et al., 2008) and autotrophic tissue (Araújo et al., 2012b) and antisense inhibition of 2-OGDH in tomato (Araújo et al., 2012c), the mutant plants of each gene encoding the E<sub>2</sub> subunit of 2-OGDH displayed a considerably reduced rate of respiration.

Increasing number of evidences suggesting an important role for mitochondrial metabolism in essential physiological processes (Nunes-Nesi et al., 2013), and recently have been shown that the components of the mitochondrial electron transport chain are essential for the proper maintenance of intercellular redox gradients, to allow considerable rates of photorespiration and in turn efficient photosynthesis (Araújo et al., 2013a). In the present work, the lack in the expression of the two genes encoding the E<sub>2</sub> subunits did not alter the CO<sub>2</sub> fixation (Figure 5A). However, the mutant lines displayed high stomatal conductance (Figure 5D), together with increased C<sub>i</sub> (Supplemental table 1). *E2-OGDH1* and *E2-OGDH2* seem to be important in the regulation of stomatal aperture. Surprisingly malate levels in the middle and in the end of the light period were very high. Furthermore, high levels of fumarate in the middle of the light period were

observed. Measurements of organic acids levels in SDH antisense plants showed decreased apoplastic levels of malate and fumarate (Araújo et al., 2011b) and fumarase antisense plants displayed increased apoplastic levels of both metabolites (Nunes-nesi et al., 2007a), revealed a negative correlation between the levels of malate and fumarate and stomatal conductance. Apparently, the high levels in malate and fumarate in *E2-OGDH* mutant plants do not represent the levels of this organic acids in the apoplast but confirm the important role of mitochondrial in the triggering stomatal movement by controlling organic acid levels in both the vacuole and apoplast leading to a relative control of CO<sub>2</sub> assimilation (Araújo et al., 2014a). Additionally, the high levels of malate and fumarate in light period may be generated by non-cyclic TCA cycle operating in opposing directions, the PEPCase supplying oxaloacetate molecules to feed both malate and fumarate accumulation (Cheung et al., 2014; Lee et al., 2010; Sweetlove et al., 2010; Tcherkez et al., 2009).

One of the proposed roles for the mitochondrial metabolism in illuminated tissues is the production of a large proportion of the ATP required to sustain the high rates of sucrose synthesis (Nunes-Nesi et al., 2011). Despite the fact that the levels of the main carbon containing metabolites (glucose, fructose and starch) were not altered in the middle of the light period, the sucrose levels were reduced. This may suggest a high consumption of sucrose, the sucrose synthesis is the main consumer of photosynthesis products; the use of triose phosphate recycles Pi and increases the rate of ribulose 1,5 bisphosphate regeneration and carboxylation so that phosphorylation and photosynthesis can continue

(Sharkey et al., 1986; Stitt, 1986). Additionally, the mutant lines of both isoforms of E<sub>2</sub> subunit display no changes in rates of photosynthesis, by the contrast with mutant lines of both isoforms of E<sub>1</sub> subunits that display high levels of sucrose and the rate of photosynthesis was decreased. However, the levels of starch in the end of the light period were high in all mutant lines, and the rate of starch synthesis were very high especially in the *E2-OGDH2* mutant lines accompanied by a high rate of starch degradation, in agreement with the alterations in plant growth displayed by this line.

Mitochondria metabolism is linked with nitrogen assimilation, amino acid, carbon and redox metabolism (Szal and Podgórska, 2012). The TCA cycle also provides carbon skeletons to biosynthetic processes, which includes amino acids biosynthesis and thus involved in nitrogen assimilation (Foyer et al., 2011a; Noguchi and Yoshida, 2008; Nunes-nesi et al., 2010; Szal and Podgórska, 2012). Reductions in the activity of NAD-dependent isocitrate dehydrogenase in transgenic plants of tomato, exhibited few changes in photosynthetic parameters and decreased levels of amino acid and photosynthetic pigments, but increased levels of nitrate and protein, unchanged levels of sucrose, but significant reduction in the level of starch (Sienkiewicz-Porzucek et al., 2010). The reductions in mitochondrial citrate synthase activity displayed few changes in the photosynthetic parameters, but increased rate of respiration, and slight decreases in the levels of photosynthetic pigment but increased levels of nitrate amino acids and starch (Sienkiewicz-Porzucek et al., 2008). The reduced activity of NAD-dependent isocitrate dehydrogenase in mutant plants of Arabidopsis exhibit reduction of certain free amino acids and is not limiting for nitrogen

assimilation (Lemaitre et al., 2007). The reduction in expression of subunit E<sub>2</sub> did not alter the levels of chlorophylls, however it clearly reduced amino acids and nitrate levels (Figure 6D, 6F). These results corroborates with previous results indicating the importance of 2-OGDH for nitrogen metabolism (Araújo et al., 2014b; Hodges et al., 2002).

Previous studies showed that antisense inhibition of the E<sub>1</sub> subunit of 2-OGDH in tomato increased the levels of 2-OG and activated an alternative pathway supplying intermediates to TCA cycle. In this work, we hypothesize that the GABA-shunt is able to bypass the 2-OGDH complex and Succinyl CoA ligase and supplies succinate to maintain the electron transport chain according to previous work in tomato (Araújo et al., 2012c). The E<sub>1</sub> subunit of 2-OGDH catalyzes the initial stage of the reaction, the 2-OG is decarboxylated and bound to cofactor thiamin pyrophosphate, the succinyl group is transferred to the lipoyl domain of E<sub>2</sub> subunit where it is carried to the active site and transferred to coenzyme A, forming succinyl-CoA (Bunik and Fernie, 2009; Millar et al., 1999). In *E2-OGDH* mutant plants, it may be happening only the decarboxylation of 2-OG and then it maintains the succinyl group attached to the E<sub>1</sub> subunit in the absence of the multimeric complex formed by the subunit E<sub>2</sub>. The predicted sequence of E<sub>2</sub> subunit of *Arabidopsis*, identified by similarity to the potato N-terminal sequences, lacks the E<sub>1</sub>/E<sub>3</sub>-binding motif (Millar et al., 1999). The mammalian *E2-OGDH* also lacks this motif and it is proposed that *E1-OGDH* is responsible for binding the E<sub>3</sub> component of OGDH (Rice et al., 1992). Moreover, a high affinity interaction has been demonstrated between N-terminus of E<sub>1</sub> and E<sub>3</sub> subunit of bovine OGDH, which forms a stable sub

complex (McCartney et al., 1998). The purified form of E<sub>1</sub> subunit of *E. coli* retains decarboxylase activity (Frank et al., 2007). In the mutant plants, the 2-OG could be suffering an initial decarboxylation catalyzed by the E<sub>1</sub> subunit of 2-OGDH. Thus the partial oxidation would maintain low levels of 2-OG, which would affect the GABA-shunt and decrease the availability of 2-OG to be used in nitrogen assimilation by GS/GOGAT. However, additional metabolite analysis are required to support this hypothesis.

## **CONCLUSIONS**

In these work, we confirmed the important role of 2-OGDH enzyme, by reducing the expression of two genes encoding E<sub>2</sub> subunit. By silencing the expression of the two genes, a strong reduction in the respiration occurred without altering photosynthesis. The lack in the expression of both E<sub>2</sub> subunit isoforms leads to changes in carbon and nitrogen metabolism. This suggests that E<sub>2</sub> subunit plays critical regulatory role in the rate of respiration and, consequently, plant metabolism and growth. Furthermore, alterations in stomatal conductance and the associated changes in malate and fumarate levels makes us tempting to speculate that 2-OGDH E<sub>2</sub> subunit is also involved in the regulation of stomatal aperture as suggested for other TCA cycle enzymes.

We also conclude that both isoforms of E<sub>2</sub> subunit have similar roles in plant growth and therefore no compensatory effects between the two isoforms were observed.

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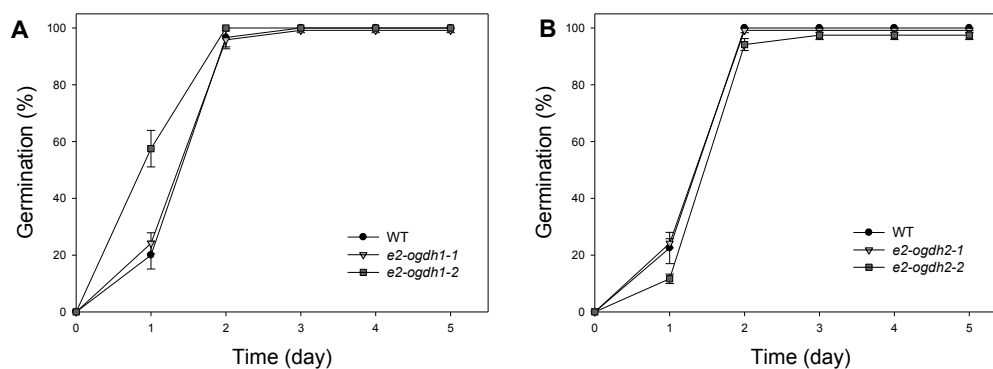
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**Zündorf G, Kahlert S, Bunik VI, Reiser G** (2009) Alpha-ketoglutarate dehydrogenase contributes to production of reactive oxygen species in glutamate-stimulated hippocampal neurons in situ. *Neuroscience* **158**: 610–616

## SUPPLEMENTAL DATA



**Supplemental Figure 1.** Growth phenotype of *Arabidopsis* E2-OGDH mutant lines after four weeks of cultivation. The lines used were as follows: the wild type, E2-OGDH1 mutant lines, and E2-OGDH2 mutant lines.

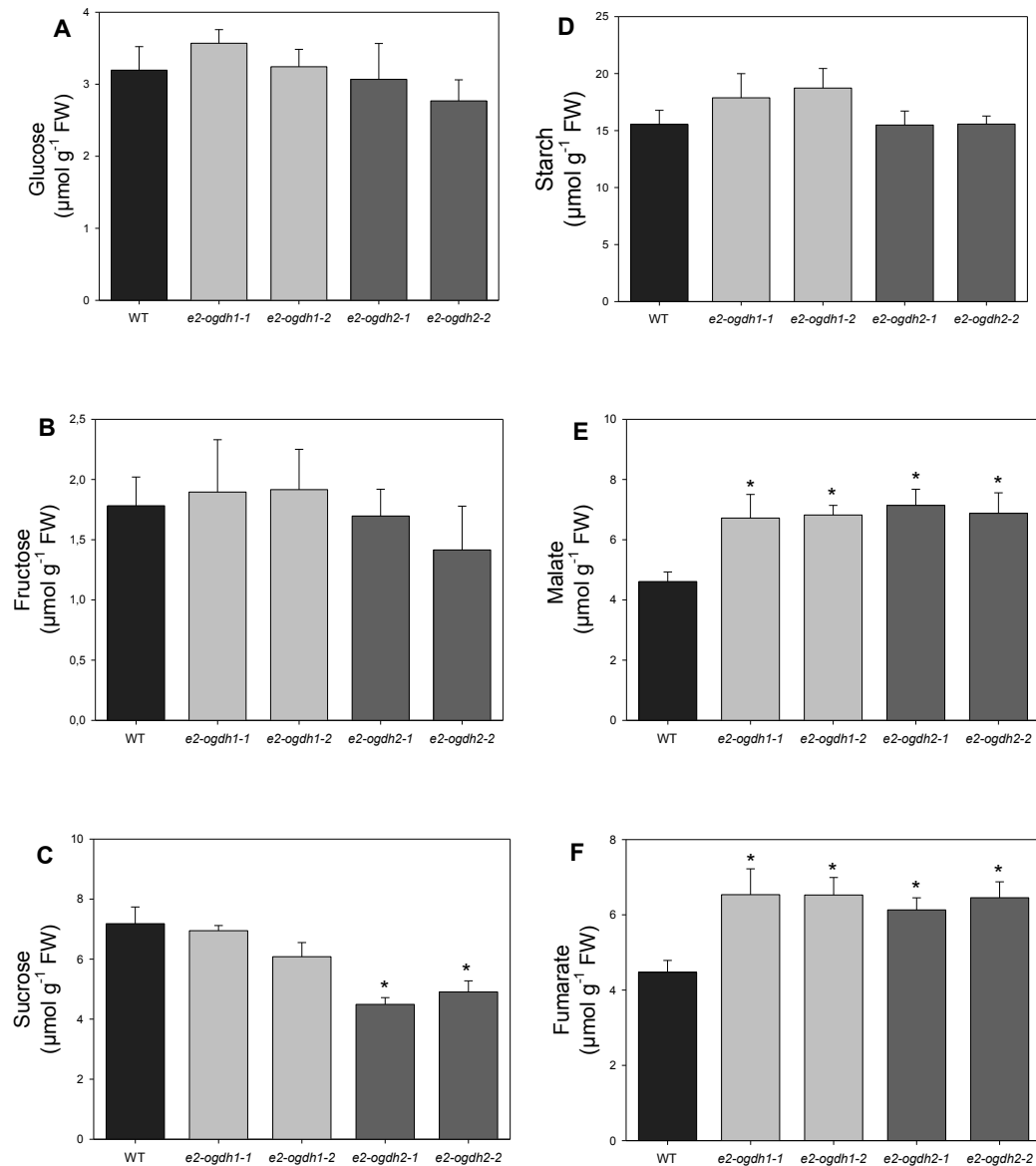


**Supplemental Figure 2.** Germination of *Arabidopsis* wild type (WT) seeds, (A) e2-ogdh1 mutant seeds and (B) e2-ogdh2 mutant seeds. Values are means  $\pm$  SE of four plates containing 30 seeds.

**Supplemental Table 1.** Gas-exchange and chlorophyll fluorescence measurements of Col-0 *Arabidopsis* plant and E2 2-OGDH mutants plants.

| Parameters                                            | WT                | e2-ogdh1-1                          | e2-ogdh1-2                          | e2-ogdh2-1                          | e2-ogdh2-2                          |
|-------------------------------------------------------|-------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| $C_i$ ( $\mu\text{mol CO}_2 \text{ mol}^{-1}$ )       | 296.49 $\pm$ 3.96 | <b>318.89 <math>\pm</math> 4.72</b> | <b>319.60 <math>\pm</math> 3.74</b> | <b>316.94 <math>\pm</math> 2.47</b> | <b>315.91 <math>\pm</math> 2.50</b> |
| $E$ ( $\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$ ) | 2.23 $\pm$ 0.11   | <b>2.59 <math>\pm</math> 0.14</b>   | <b>2.79 <math>\pm</math> 0.13</b>   | 2.36 $\pm$ 0.05                     | <b>2.56 <math>\pm</math> 0.06</b>   |
| $WUE_i$ ( $A/g_s$ )                                   | 55.08 $\pm$ 2.44  | <b>41.52 <math>\pm</math> 3.02</b>  | <b>40.37 <math>\pm</math> 2.51</b>  | <b>42.91 <math>\pm</math> 1.45</b>  | <b>43.31 <math>\pm</math> 1.55</b>  |
| $F_v/F_m$                                             | 0.78 $\pm$ 0.001  | 0.77 $\pm$ 0.003                    | <b>0.77 <math>\pm</math> 0.003</b>  | <b>0.77 <math>\pm</math> 0.003</b>  | 0.78 $\pm$ 0.002                    |
| $F_v'/F_m'$                                           | 0.51 $\pm$ 0.004  | <b>0.53 <math>\pm</math> 0.005</b>  | 0.52 $\pm$ 0.007                    | 0.52 $\pm$ 0.006                    | 0.52 $\pm$ 0.005                    |
| $\phi\text{PSII}$                                     | 0.179 $\pm$ 0.006 | 0.169 $\pm$ 0.003                   | 0.180 $\pm$ 0.005                   | 0.169 $\pm$ 0.004                   | 0.168 $\pm$ 0.007                   |
| NPQ                                                   | 1.83 $\pm$ 0.13   | 1.79 $\pm$ 0.09                     | 1.88 $\pm$ 0.09                     | 2.00 $\pm$ 0.09                     | 1.75 $\pm$ 0.08                     |
| qP                                                    | 0.350 $\pm$ 0.015 | 0.317 $\pm$ 0.007                   | 0.348 $\pm$ 0.009                   | 0.325 $\pm$ 0.011                   | 0.323 $\pm$ 0.015                   |
| ETR                                                   | 89.54 $\pm$ 3.23  | 84.70 $\pm$ 1.60                    | 90.21 $\pm$ 2.39                    | 84.46 $\pm$ 1.85                    | 84.18 $\pm$ 3.72                    |

$C_i$ : internal  $\text{CO}_2$  concentration;  $E$ : transpiration rate;  $WUE$ : intrinsic water-use efficiency;  $F_v/F_m$ : maximum PSII photochemical efficiency;  $F_v'/F_m'$ : actual PSII photochemical efficiency;  $\phi\text{PSII}$ : quantum yield of PSII; NPQ: non-photochemical quenching; qP: photochemical quenching; ETR: electron transport rate. Values are presented as means  $\pm$  SE of five individual plants per line. Values in bold indicated by Student's t test to be significantly different ( $P \leq 0.05$ ) from the wilt type.



**Supplemental Figure 3.** Metabolite levels of the main carbon related compounds of *Arabidopsis* *E2-OGDH* mutant lines.

Glucose (A), fructose (B), sucrose (C), starch (D), malate (E) and fumarate (F) levels were measured using leaf material harvested in the middle of the light period from 4-week-old plants. The lines used were as follows: the wild type, black bars; *E2-OGDH1* mutant lines, light gray bars; *E2-OGDH2* mutant lines, dark gray bars. Values are presented as means  $\pm$  SE of five individual plants per line. Asterisks indicated by Student's t test to be significantly different ( $P < 0, 05$ ) from the wild type.

## GENERAL CONCLUSIONS

By using T-DNA insertion mutant lines for E<sub>1</sub> and E<sub>2</sub> subunits of 2-OGDH in *Arabidopsis thaliana*, I confirmed the importance of 2-OGDH in plant metabolism. In all lines analyzed, it was observed that the enzyme indeed exhibits strong control over leaf respiration rate, and this control was independent of the subunit. However, I observed specific effects of reduced expression of each gene encoding subunits of 2-OGDH indicating that its isoforms play partial-redundant functions in *Arabidopsis*. In addition to metabolic and physiological changes, I observed that the reduction in the expression of 2-OGDH isoforms lead to alterations morphological features, such as leaf area, number of leaves, root size, seed per silique and weight of seeds. In summary, I presented here compelling evidence of the importance of individual isoforms of the 2-OGDH subunits in plant growth, metabolism and, at some extent, plant development. All four isoforms of subunits exhibited greater influence on respiration rate similar to what have been observed previously. It suggests that 2-OGDH E<sub>1</sub> and E<sub>2</sub> subunits play critical regulatory role in the rate of respiration and consequently, plant metabolism and growth.