MARCEL VIANA PIRES

ON THE ROLE OF BRANCHED-CHAIN AMINO ACIDS IN *Arabidopsis thaliana* SUBJECTED TO WATER STRESS CONDITIONS

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

VIÇOSA
MINAS GERAIS – BRAZIL
2012

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

P667o
2012

Pires, Marcel Viana, 1983-
On the role of branched-chain amino acids in *Arabidopsis thaliana* subjected to water stress conditions / Marcel Viana Pires. – Viçosa, MG, 2012.
x, 101f. : il. (algumas color.) ; 29cm.

Inclui anexos.
Orientador: Raimundo Santos Barros
Tese (doutorado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 69-94

1. Plantas - Metabolismo. 2. *Arabidopsis thaliana*. 3. Aminoácidos. 4. Plantas - Efeito da seca. I. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-Graduação em Fisiologia Vegetal . II. Título.

CDD 22. ed. 572.4

MARCEL VIANA PIRES

ON THE ROLE OF BRANCHED-CHAIN AMINO ACIDS IN *Arabidopsis thaliana* SUBJECTED TO WATER STRESS CONDITIONS

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Pos-Graduate Program in Plant Physiology for obtention of the degree of *Doctor Scientiae*

APPROVED: December 17th, 2012

Camila Caldana
(Member)

Dimas Mendes Ribeiro
(Member)

Adriano Nunes-Nesi
(Co-advisor)

Wagner L. Araújo
(Co-advisor)

Raimundo Santos Barros
(Advisor)

ACKNOWLEDGMENTS

I would like to thank CAPES (Coordination for Scientific Support for Post-Graduate Level Training), FAPESB (Foundation for Research Support of the State of Bahia) and DAAD (Deutsche Akademische Austausch Dienst) for the scholarships conceded. I also thank the Universidade Federal de Viçosa (UFV), the Graduate Program in Plant Physiology of UFV, and the Max-Planck-Institut für Molekulare Pflanzenphysiologie (MPIMP), Potsdam-Golm, Germany.

I am indebted to my supervisor Prof. Raimundo Santos Barros for his guidance and support and for always been willing to meet and help me in all instances.

Thanks are also due to Dr. Alisdair R. Fernie, my supervisor during the sandwich-training at the MPIMP, for the amazing opportunity and for his valuable suggestions. I also would like to thank the Max-Planck Society for funding this research.

Thanks to Prof. Wagner L. Araújo for his support and expertise on all aspects of this research, for his suggestions and comments provided on scientific writing of this thesis.

Thanks to my defense committee members, Dra. Camila Caldana, Dr. Dimas Ribeiro and Prof. Adriano Nunes-Nesi, for their knowledgeable perspectives that contributed to this research.

I am also indebted and would like to thank to all members of my working group at the MPIMP (Central Metabolism), Dr. Toshihiro Obata, Dra. Do Thi Phuc, Dra. Tamar Avin-Wittenberg, Dra. Sandra Witt, Dr. Takayuki Tohge, Phuong Anh

Pham, Danilo de Menezes Daloso, Alexandra Bottcher, Sebastian Moschen, Alexandra Florian, Regina Wendenburg, Saleh Alseekh, Ilse Balbo, Susan Bergmann, and Ina Krahnert, for all help with my research and for being a wonderful laboratory/office mates. I also would like to express my gratitude to Josef Bergstein for the excellent photography of the plants and to Karin Koehl and the Greenteam of the MPIMP for their assistance with plant growth.

Thanks are also necessary to my friends in Germany, Nadia Martins, Carla Antônio, Ana Licuco, Laís Scattolon, Maria Benina, Angelika Lüscher, and Regina Dettmann, for the friendship and so many pleasant moments.

Many thanks also go to my mother, Rita, my father, Nilton, my sister, Ana Cláudia, and my brother, Luciano, for all the encouragement.

I also would like to thank all members of my working group at UFV (Plant Growth and Development), Izabel Chaves, Eduardo Medina, Priscila Oliveira, and Amana Obolari, for the really nice atmosphere in the laboratory.

I also would like to thank all my friends in Viçosa, specially Dênis Cunha, Giuliana Soares, Mirelle Abreu, Josiane Guimarães, Leandro Elias, Samuel Martins, Alice Pita, and Rafael Barcellos, for the friendship, support and for the good moments we have shared.

BIOGRAPHY

Marcel Viana Pires, son of Nilton Barros Pires and Rita de Cassia Viana Pires, was born in Jequié (Bahia State, Brazil), in October 24th, 1983.

In April 2001, he started the undergraduate course in Biology at Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia State, and achieved the bachelor degree in October 2005. He achieved the Master degree in Crop Production at the same University in February 2008.

In March 2009 he started this PhD studies at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State. He spent one year as a guest PhD student at the Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm, Germany, under the supervision of Dr Alisdair R. Fernie before finishing his studies in Plant Physiology at UFV.

TABLE OF CONTENTS

RESUMO vii
ABSTRACT ix
1. INTRODUCTION 1
1.1. Objectives 5
2. LITERATURE REVIEW 6
2.1. The pathways of mitochondrial plant respiration 6
2.2. Respiration and drought tolerance 10
3. MATERIAL AND METHODS 16
3.1. Plant material and experimental conditions 16
3.2. Water loss measurements and relative water content 18
3.3. Determination of chlorophyll *a* fluorescence parameters 18
3.4. Biochemical characterization 19
3.4.1. Basic analysis 19
3.4.1.1. Extraction of plant materials 19
3.4.1.2. Determination of chlorophyll content 19
3.4.1.3. Determination of total soluble proteins 20
3.4.1.4. Determination of starch 20
3.4.1.5. Determination of total amino acids 21
3.4.1.6. Determination of nitrate 21
3.4.2. Gas chromatography associated with mass spectrometry 21
3.4.2.1. Extraction of plant material for metabolic profiling 22
3.4.2.2. Derivatization for metabolic profiling 22

3.4.2.3. Data analysis for metabolic profiling 23
3.5. Quantitative Real-Time PCR..... 23
3.6. Germination assays..... 26
3.7. Statistical analysis 26
4. RESULTS 27
4.1. Phenotype of *Arabidopsis* mutants under water deficit conditions..... 27
4.2. Phenotype of *Arabidopsis* mutants after irrigation recovery..... 30
4.3. Metabolic characterization of *Arabidopsis* mutants under water deficit conditions 32
4.4. Gene expression of *Arabidopsis* mutants under water deficit conditions 42
4.5. Germination assays of *Arabidopsis* mutants under osmotic and salt stresses . 44
5. DISCUSSION 47
5.1. Future perspectives for improving the understanding of the linkages between mitochondrial respiration and drought tolerance..... 64
6. CONCLUSIONS..... 67
REFERENCES..... 69
SUPPLEMENTAL DATA..... 95

RESUMO

PIRES, Marcel Viana, D.Sc., Universidade Federal de Viçosa, dezembro de 2012. **Papel dos aminoácidos de cadeia ramificada em *Arabidopsis thaliana* submetidas à condições de limitada disponibilidade hídrica**. Orientador: Raimundo Santos Barros. Co-orientadores: Adriano Nunes-Nesi e Wagner L. Araújo

Estudos recentes demonstram que o metabolismo vegetal e particularmente a respiração são alterados sob condições de estresses ambientais. Ademais, sob tais condições, vias alternativas são induzidas para suprir o processo respiratório com substratos alternativos. Uma dessas rotas envolve o complexo protéico flavoproteína de transferência de elétrons/ flavoproteína de transferência de elétrons oxidoredutase da ubiquinona (ETF/ETFQO), e é responsável pela doação alternativa de elétrons à cadeia de transporte mitocondrial. Abordagens recentes demonstraram que as enzimas desidrogenase do 2-hidroxiglutarato (D2HGDH) e desidrogenase do isovaleril-CoA (IVDH) atuam na doação de elétrons para o *pool* de ubiquinona via complexo ETF/ETFQO. Entretanto, o papel desempenhado por essa rota na resposta das plantas ao défice hídrico ainda não foi completamente elucidado. O presente trabalho fornece evidências fenotípicas, fisiológicas, metabólicas e moleculares de que vias alternativas respiratórias, e particularmente as enzimas ETFQO, IVDH e D2HGDH, desempenham um papel significativo nos mecanismos de tolerância à seca em *Arabidopsis thaliana*. As plantas mutantes *etfqo-1*, *d2hgdh-2* e *ivdh-1* mostraram-se mais sensíveis à seca em comparação ao tipo selvagem e aos mutantes superexpressando *D2HGDH*, apresentando sintomas de senescência mais acentuados, tais como murcha e clorose foliar. Além disso, foram observadas diminuições nos

valores de alguns parâmetros relacionados à senescência nesses mutantes, tais como teor relativo de água, teores de clorofilas e eficiência fotoquímica máxima do fotossistema II. Plantas *etfqo-1*, *d2hgdh-2* e *ivdh-1* não conseguiram recuperar o crescimento vegetativo após a retomada da irrigação, indicando que o complexo ETF/ETFQO seria um importante mecanismo de resistência à seca. O défice hídrico induziu uma extensa reprogramação metabólica em todos os genótipos analisados, culminando com o aumento do teor de aminoácidos totais, bem como diminuições nos teores de proteínas, amido e nitrato. Em adição, o perfil metabólico permitiu a identificação de uma série de compostos envolvidos na tolerância ao défice hídrico. Particularmente, incrementos nos níveis de aminoácidos de cadeia ramificada (BCAA), isoleucina, leucina e valina, parecem estar relacionados ao aumento da utilização dos mesmos como fonte alternativa de elétrons para a cadeia de transporte mitocondrial, sob condições de estresse hídrico. Análises de expressão gênica, por sua vez, revelaram, simultaneamente, um inesperado baixo nível de fotorrespiração, bem como uma possível manutenção da operação do ciclo dos ácidos tricarboxílicos durante o estresse hídrico. Por fim, o complexo ETF/ETFQO, assim como o catabolismo de BCAA, parecem desempenhar um papel relevante nos mecanismos de tolerância ao estresses salino e osmótico em eventos germinativos em *Arabidopsis*. Em conjunto, esses dados indicam que o metabolismo mitocondrial alternativo pode ser altamente eficaz na tolerância à seca.

ABSTRACT

PIRES, Marcel Viana, D.Sc., Universidade Federal de Viçosa, December 2012. **On the role of branched-chain amino acids in *Arabidopsis thaliana* subjected to water stress conditions.** Advisor: Raimundo Santos Barros. Co-Advisors: Adriano Nunes-Nesi and Wagner L. Araújo

Recent evidence has demonstrated that under stress situations plant metabolism and particularly respiration are reorganized and alternative pathways are induced in order to provide substrates for the respiratory process. One of these enzymatic pathways involves the electron-transfer flavoprotein/ electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETFQO) complex, which provides an alternative input of electrons into the mitochondrial electron transport chain (mETC). It has been also recently demonstrated that both isovaleryl-CoA dehydrogenase (IVDH) and 2-hydroxyglutarate dehydrogenase (D2HGDH) act as electron donors to the ubiquinol pool via ETF/ETFQO pathway under stress situations, such as dark-induced senescence. However, the role of this pathway in response to water deficit remains as yet unclear. Here phenotypical, physiological, metabolical, and molecular evidences indicate that alternative pathways of respiration and more specifically ETFQO, IVDH and D2HGDH enzymes seem to play a significant role in drought-tolerance mechanisms in *Arabidopsis thaliana*. Plants of *etfqo-1*, *d2hgdh-2* and *ivdh-1* knockout (KO) mutants were more sensitive to drought than wild type (WT) and lines overexpressing *D2GHDH*. Those mutants were characterized by more pronounced symptoms of senescence, such as wilting and chlorosis. In addition, it was observed decreased values of some senescence-related parameters in KO plants,

such as relative water content, chlorophylls and maximum photochemical efficiency of photosystem II. KO mutants were less able to restore vegetative growth after the irrigation recovery indicating that the ETF/ETFQO pathway is important for plants to withstand drought as well as to recover growth after re-watering. Drought stress induced an extensive metabolic reprogramming in all genotypes leading to increases in total amino acids levels, as well as decreases in protein, starch and nitrate contents. Furthermore, metabolite profile allowed the identification of a range of compounds involved in drough-tolerance. Particularly, the enhanced levels of branched-chain amino acids (BCAA), isoleucine, leucine and valine, seem to be related to increased usage of those amino acids as alternative source of electrons to mETC under water stress conditions. Gene expression analyses revealed both unexpected low rates of photorespiration and a possible maintenance of tricarboxylic acid (TCA) cycle operation during water stress. Finally, the ETF/ETFQO pathway as well as BCAA catabolism seems to play a relevant role in both salt- and osmotic-tolerance mechanisms in *Arabidopsis* germination events. In summary compelling evidence indicates that alternative mitochondrial metabolism is likely highly effective in drought tolerance.

1. INTRODUCTION

Water scarcity is considered one of the main environmental factors limiting plant growth and yield. Predictions have recently suggested that in the near future climate changes will most likely be associated with even worse problems, leading to frequent periods of drought as well as threats to both natural and agricultural ecosystems (Gornall et al., 2010). Accordingly, water deficit leads to several morphological and physiological changes in plants. Such alterations occur at both temporal (*e.g.* vegetative and reproductive stages are distinctly affected) and spatial scales (*e.g.* distinct organs and tissues present different behaviors) (Chaves et al., 2002). Those changes include reduction in shoot growth (Tardieu et al., 2000), while root growth is maintained (Sharp and Davies, 1979), decreases in photosynthesis and transpiration rates (Boyer, 1982), accumulation of abscisic acid (ABA) and several osmolytes (Morgan, 1992), changes in signaling pathways (Chaves et al., 2003), activation of detoxification processes (Hare et al., 1998), reduction in tissue water potential, and transcriptional and post-transcriptional regulation of genes (Bray, 2004; Xue et al., 2008).

It is well recognized that the plant productivity largely depends on the balance between photosynthesis and respiration (Krömer et al., 1993; Raghavendra and Padmasree, 2003; Nunes-Nesi et al., 2005, 2007). Surprisingly, although the effects of water stress on photosynthesis have been largely documented (Bartoli et al., 2005; Ribas-Carbo et al., 2005; Flexas et al., 2006) the impact of water limitation on respiration at the physiological level remains largely unknown (Flexas et al., 2005). Not surprisingly the effects of water limitation on mitochondrial respiration

have also strong impacts on agricultural yield (Flexas et al., 2005; Atkin and Macherel, 2009). This is most likely because respiratory process involves a variety of physiological functions including ATP synthesis, supply of carbon (C) skeletons for biosynthetic processes as well as regulation of cellular redox potential (Scheibe et al., 2005; Bauwe et al., 2010; van Dongen et al., 2011). Moreover respiration plays an important role in the adaptive responses of plants to several abiotic stresses by being able to eliminate reactive oxygen species (ROS) (Lambers et al., 2005; Pastore et al., 2007; Atkin and Macherel, 2009; Dinakar et al., 2010). Additionally cell respiration is one extremely flexible process being characterized by: (*i*) the possibility to use several compounds as substrate, such as starch, sucrose, lipids, protein, and amino acids (Ishizaki et al., 2005, 2006; Araújo et al., 2010); (*ii*) the interaction among diverse cell compartments and distinct metabolic pathways including glycolysis (Plaxton and Podesta, 2006), photosynthesis (Nunes-Nesi et al., 2011), photorespiration (Timm et al., 2008), nitrogen metabolism (Noguchi and Terashima, 2006), reductant transport and the maintenance of photosynthetic redox balance (Rasmusson and Escobar, 2007; Nunes-Nesi et al., 2008); (*iii*) the possible operation of non-cyclic modes of the carboxylic acid (TCA) cycle pathway in response to metabolic limitations, *e.g.* under hypoxic conditions (Rocha et al., 2010; Sweetlove et al., 2010); and (*iv*) the presence of alternative non-phosphorylating enzymes, such as alternative oxidases (AOX) (Rasmusson et al., 2009), uncoupling protein (UCP) (Sweetlove et al., 2006; Vercesi et al., 2006), and external NAD(P)H dehydrogenases (Rasmusson et al., 2008). Despite being such a fundamental and robust process, its flexibility and several other factors associated with it make respiration highly sensitive to environmental changes. In this way, given that photosynthesis can be limited by a range of environmental conditions and occur only in specific organs containing chlorophyll whilst respiration occurs continuously in each cell of every plant organ, it is reasonable to assume that respiration plays an important role as a mechanism for controlling yield, especially when photosynthesis is affected, for example, under limited water availability conditions (Flexas et al., 2005).

Compelling evidences has recently demonstrated that, although plant respiration is mainly dependent on carbohydrates oxidation (Plaxton and Podesta, 2006), under stress conditions (which affects carbohydrates supply), the respiratory metabolism is altered and other pathways are induced in order to provide alternative substrates for the respiratory processes (Ishizaki et al., 2005, 2006; Araújo et al.,

2010). In mammals, the electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO), a nuclear-encoded protein located in the inner mitochondrial membrane, accepts electrons from the electron transfer flavoprotein (ETF) which is localized in the mitochondrial matrix, and then reduces ubiquinone (Ruzicka and Beinert, 1977; Beckmann and Frerman, 1985; Zhang et al., 2006). In these species, the ETF/ETFQO complex is essential for the catabolism of fatty acid, diverse amino acids, and choline, supplying the mitochondrion with alternative respiratory substrates to sucrose (Frerman, 1988; Frerman and Goodman, 2001). Similarly to the situation observed in mammal cells, the ETF/ETFQO complex was identified in plants being located in the mitochondrial membrane (Heazlewood et al., 2004). This complex has shown to be highly induced at a transcriptional level during dark-induced senescence (Buchanan-Wollaston et al., 2005) and oxidative stress (Lehmann et al., 2009). Using a combination of enzymatic, metabolic, and isotopic labeling approaches in *Arabidopsis thaliana* mutants it has been recently demonstrated that products derived from the ETF/ETFQO pathway (mainly aromatic and the branched-chain amino acids – BCAA, isoleucine, leucine, and valine) are potential alternative electron donors at the mitochondrial level (Ishizaki et al., 2005, 2006; Araújo et al., 2010). This donation occurs either directly, with the transfer of electrons to the mitochondrial electron transport chain (mETC) via ETF complex, or indirectly, by the direct feeding of catabolic products into the TCA cycle (Araújo et al., 2010).

The current knowledge concerning the enzymes capable of performing this alternative donation of electrons to mETC still remains limited. Thus, it was only recently that the enzyme 2-hydroxyglutarate dehydrogenase (D2HGDH) was characterized (Engqvist et al., 2009). Similar to the situation observed in mammal cells (Achouri et al., 2004) it catalyzes the conversion of 2-hydroxyglutarate to 2-oxoglutarate, providing the ETF/ETFQO complex with electrons by using aromatic amino acids and lysine as substrates (Araújo et al., 2010). Furthermore, isovaleryl-CoA dehydrogenase (IVDH) also plays a fundamental role in supplying the ETF/ETFQO complex with electrons, being involved in lysine, aromatic amino acids and BCAA degradation (Araújo et al., 2010). Interestingly it was similarly observed in *Arabidopsis* plants lacking the genes encoding D2HGDH and IVDH (*d2hgdh-2* and *ivdh-1*, respectively) a strong accumulation of aromatic amino acids and BCAA as well as isovaleryl-CoA during dark-induced senescence (Araújo et al., 2010). During these conditions the phenotype of *d2hgdh-2* and *ivdh-1* mutants were similar

to those observed in *Arabidopsis* plants with mutations in genes encoding ETF and ETFQO (Ishizaki et al., 2005, 2006). Altogether these results indicate that both D2HGDH and IVDH are able to donate electrons to the ubiquinone pool via ETF/ETFQO complex (Araújo et al., 2010). It is equally important to note that those T-DNA mutants also presented early senescence phenotype under other more common physiological circumstances, such as cold (13°C), short days (8 h light/16 h dark), and continuous light (24 h light/0 h dark) (Araújo et al., 2010). Despite the fact that those enzymes are encoded by unique genes and operate in two separate albeit functionally similar pathways, IVDH seems to be of more relevance in supplying ETF/ETFQO with electrons than D2HGDH, since the first seems to use more potential substrates (Araújo et al., 2010). However it is important to note that although only those two enzymes are currently known to supply the ETFQO complex with electrons in *Arabidopsis* (Araújo et al., 2011a), the remarkable phenotypic changes observed in those mutants illustrate the importance of such metabolic pathway in plants.

Although the role of alternative substrates under dark-induced senescence seems to be well accepted, it is important to note that the physiological role of alternative substrates during other stress situation such as water shortage still remains largely unknown. Bearing that in mind, studies aiming to fully understand the role of the BCAA as alternative substrate supplying electrons to the mETC under water deficit conditions are of great significance. It is not surprisingly that osmotic adjustments can also contribute to the maintenance of cell turgor, which is a requirement underlying plant growth during water stress (Bartels and Sunkar, 2005). Nevertheless, although much is known about the osmoprotectant function of some metabolites (*e.g.* proline), the role of osmoprotection mechanisms under situations in which the mitochondrial metabolism is genetically altered remains mostly uncharacterized.

The understanding of respiratory process in plants under stress conditions seems to be a very useful tool for the augmentation of our current comprehension of adaptive mechanisms underlying stress responses as well as for studies of gene function for genetic engineering programs targeting, among others, water-stress tolerance. Despite recent advances in our understanding of plant respiration (van Dongen et al., 2011; Araújo et al., 2012) little is currently known about the relative importance of metabolic pathways involved in respiration under stress conditions.

Accordingly a more detailed analysis of plants with genetically altered respiratory activity, mainly through the manipulation of alternative respiratory pathways would help us to understand this intriguingly connection. Such analysis would allow a better understanding of mechanisms associated to acclimatization as well as to those involved in the interaction between respiration and photosynthesis under water deficit conditions.

1.1. Objectives

The main goal of this study was to elucidate the physiological role of enzymes involved in the alternative supply of electrons to the mETC in *Arabidopsis thaliana* plants under limited water conditions.

More specifically, it aims to:

i) investigate molecular and metabolic responses in *Arabidopsis* plants deficient in the expression of IVDH, D2HGDH, and ETFQO enzymes under water deficit conditions;

ii) understand the role of BCAA as potential alternative substrates for the donation of electrons to the mETC under limited water conditions;

iii) analyze the role of BCAA metabolism during the recovery of irrigation;

iv) evaluate osmoprotectant mechanisms in *Arabidopsis* plants in situations in which the mitochondrial metabolism has been genetically modified.

2. LITERATURE REVIEW

2.1. The pathways of mitochondrial plant respiration

Mitochondrion presents two membrane systems that functionally limit them into four regions. The outer membrane is not osmotically active and contains relatively few proteins. The inner membrane is osmotically active and impermeable to most solutes. It presents several proteins including specific transporters for substrates, ions, and macromolecules as well as most of the componentes of the mETC. The intermembrane space, in turn, contains relatively few but essential proteins. For example, cytochrome *c* is a soluble protein that interacts with other protein complexes, transfering electrons between the components of the mETC located in the inner membrane. Moreover, the inner membrane encloses the matrix compartment, which contains several enzymes not only from the TCA cycle but also from amino acid metabolism (Rasmusson et al., 2004).

The reducing equivalents that are produced throught the TCA cycle activity are further used by the mETC to power the synthesis of ATP. Interestingly the electron transport chain in the inner membrane of both plant and animal mitochondria shares the same basic design (Rasmusson et al., 2004). Thus the classical pathway of mETC involves the flow of electrons from either the TCA cycle NADH or succinate dehydrogenases via four inner-membrane protein complexes to oxygen, with the concomitant translocation of protons into the inter-membrane space. Dehydrogenases reduce ubiquinone to ubiquinol that is oxidized by the cytochrome chain, to finally reduce oxygen to water. The commonly referred

dehydrogenases, which are responsible for the donation of electrons to the ubiquinone pool of the mETC, are the rotenone-sensitive NADH dehydrogenase (Complex I), which oxidizes matrix NADH, and succinate dehydrogenase (Complex II), which oxidizes the TCA cycle intermediate succinate to fumarate (Figure 1). Ubiquinone reduced by these protein complexes is then oxidized by the cytochrome chain that consists of the *bc1* complex (Complex III), soluble cytochrome *c* in the intermembrane space, and cytochrome *c* oxidase (Complex IV). The electron transport activity of complex I, III and IV is coupled to extrusion of protons across the inner membrane. The resultant potential across the inner membrane is finally used to drive ATP synthesis by the mitochondrial ATP synthase (Complex V) (Fernie et al., 2004; Rasmusson et al., 2004, 2008; van Dongen et al., 2011).

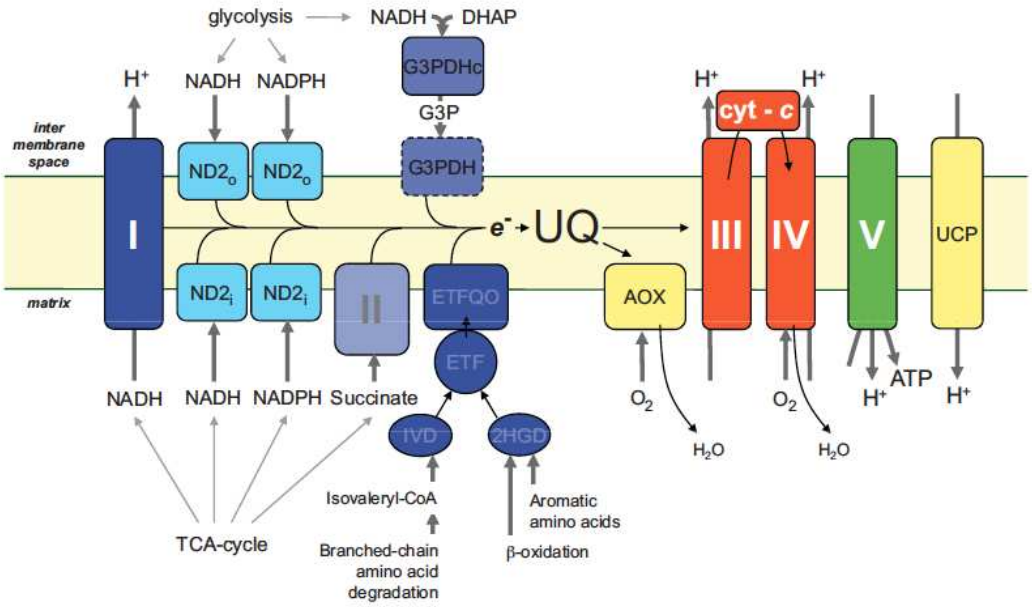


Figure 1. Schematic overview of the components involved in classical and alternative pathways of the mETC. Abbreviations: I, NADH dehydrogenase; II, succinate dehydrogenase; III, cytochrome *c* (cyt-c) reductase; IV, cytochrome *c* oxidase; V, ATP synthase; 2HGD, 2-hydroxyglutarate dehydrogenase; AOX, alternative oxidase; DHAP, dihydroxyacetone phosphate; ETF, electron-transfer flavoprotein; ETFQO, electron-transfer flavoprotein: ubiquinone oxidoreductase; G3P, glycerol-3-phosphase; G3PDH, G3P dehydrogenase; G3PDHc, cytosolic G3P dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; ND2i, internal type II NAD(P)H dehydrogenase; ND2o, external type II NAD(P)H dehydrogenase; UCP, plant uncoupling protein; UQ, ubiquinone pool. Source: van Dongen et al. (2011)

Recent efforts into plant mitochondrial electron transport has, however, focussed on alternative components that serve as non-phosphorylating by-passes (Figure 1). These include alternative non-phosphorylating enzymes, such as alternative oxidases (AOX) (Rasmusson et al., 2009), uncoupling protein (UCP) (Sweetlove et al., 2006; Vercesi et al., 2006), and external NAD(P)H dehydrogenases (Rasmusson et al., 2008). These alternative mechanisms will be discussed in details in the following section. In addition, several other electron entry points have been characterized in plants, such as L-galactono-1,4-lactone (GalL) dehydrogenase (Valpuesta and Botella, 2004), glycerol-3-phosphate (G3P) dehydrogenase (Shen et al., 2003, 2006), and finally ETF/ETFQO pathway (Ishizaki et al., 2005, 2006; Araújo et al., 2010). This has substantially increased the number of branches and thus the complexity of mETC in plants (Rasmusson et al., 2008). The importance of such by-passes have been expertly reviewed elsewhere (Rasmusson et al., 2008; van Dongen et al., 2011; Jacoby et al., 2012) and with the exception of the ETF/ETFQO pathway will not be discussed here.

The ETF/ETFQO complex is probably the most central of several additional electron donors in plants and exhibits high similarity to its homologous complex in animal systems (van Dongen et al., 2011). In mammals it has been observed that the ETF/ETFQO complex is involved in the oxidation of fatty acids, choline and various amino acids (Frerman, 1987, 1988) thereby providing an alternative substrate to feed electrons into the mETC. The presence of the ETF/ETFQO complex in plant mithocondria was first reported by Heazlewood and Millar (2005) who identified the ETF system by liquid chromatography–mass spectrometry proteomics in *Arabidopsis*. Subsequently, Buchanan-Wollaston et al. (2005) observed that this protein complex was induced at the transcription level during dark-induced senescence, while Lehmann et al. (2009) demonstrated that oxidative stress induces the expression of this complex.

The physiological role of both ETF and ETFQO was investigated via a characterization of TDNA knockout mutants of the respective proteins in *Arabidopsis* (Ishizaki et al., 2005, 2006). The authors observed accumulation of isovaleryl-CoA and phytanoyl-CoA, two intermediate products of amino acid metabolism and fatty acid oxidation, respectively, during dark-induced C starvation of ETF and ETFQO knockout mutants (Ishizaki et al., 2005, 2006). Moreover, strong evidences for the involvement of isovaleryl- CoA dehydrogenase (IVDH) in

transferring electrons from aromatic amino acids, BCAA, and lysine to the ETF/ETFQO complex, whereas the enzyme 2-hydroxyglutarate dehydrogenase (D2HGDH) does so using aromatic amino acids and lysine as substrate (Araújo et al., 2010), and that both pathways are linked to protein degradation (Araújo et al., 2011c) was recently provided (Figure 2). Interestingly, knockout mutants of both enzymes display early senescence under several growth conditions, such as cold, continuous light and short day (Araújo et al., 2010). However, the understanding of regulation of the responses of this alternative pathway of respiration is still limited and further efforts are needed to, for example, characterize the linkage between protein degradation, amino acid turnover and alternative respiration (Araújo et al., 2011c)

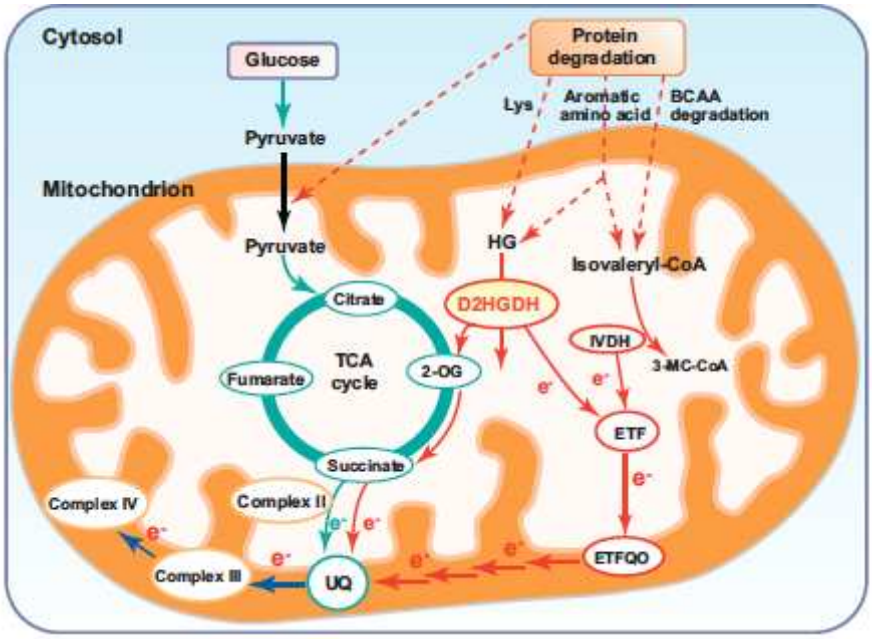


Figure 2. The ETF/ETFQO complex feeding electrons to the mETC. Dotted arrows represent possible transport processes and multi enzymatic reactions. Abbreviations: ETF, electron-transfer flavoprotein; ETFQO, electron-transfer flavoprotein: ubiquinone oxidoreductase; HG, hydroxyglutarate; Lys, lysine; 3-MC-CoA, 3 methylcrotonyl-CoA; 2-OG, 2-oxoglutarate; UQ, ubiquinone. Source: Araújo et al. (2011c)

2.2. Respiration and drought tolerance

The percentage of fixed C released through respiration is, generally, higher in plants under water deficit (Zagdanska, 1995; Atkin and Macherel, 2009), despite the lack of consensus amongst researchers on the extent of this comparison. For instance, Bartoli et al. (2005) verified increased respiratory rates in wheat plants under water deficit, while Gonzalez-Meler et al. (1997) observed a decrease respiration in beans and pepper, and Ribas-Carbo et al. (2005) have not found any alterations in soybean plants. While the maintenance of respiratory rates in plants under stress may have a negative impact in the overall C balance, due to the continuous loss of CO_2 to the atmosphere, this maintenance can play a significant role in both plant development and survival under water deficit conditions. In good agreement many studies have already suggested that mitochondria and chloroplasts are organelles intimately connected by metabolic and signaling pathways and, thus, photosynthesis would also be intimately dependent on mitochondrial functions (Nunes-Nesi et al., 2005, 2007; Sweetlove et al., 2006; Araújo et al., 2011b).

Changes induced by water deficit in the respiratory flux may reflect variations in (*i*) respiratory enzyme activities, (*ii*) substrate availability (by decreasing C fixation and, consequently, production of sugars) and (*iii*) demand for ATP (associated with growth, maintenance of cellular metabolism, and ion transport) (Atkin and Macherel, 2009). It is well known that under optimum temperature conditions, the respiratory rate in well hydrated plants is rarely limited by the enzymatic capacity (Atkin et al., 2005). Although relatively little is known about the role of the enzymatic capacity in limiting respiratory flux under water deficit conditions, Herppich and Peckmann (2000) have observed that the *in vitro* activity of a number of respiratory enzymes were not altered by water deficit, in two species with crassulacean acid metabolism (CAM). Moreover, the velocity of drought-induced alterations in the respiratory flux suggest that changes in enzymatic capacity *per se* are not likely to be the main cause of variations in respiratory rates (Atkin and Macherel, 2009).

Limitations in the respiratory substrates under water deficit commonly are result from first, decreased photosynthetic rates (Lawlor and Cornic, 2002), second, changes in C allocation to the synthesis of compounds involved in osmotic adjustment (Dekankova et al., 2004), and third, decreased sugar concentration

resulting from water stress (Lawlor and Fock, 1977). However, it should be pointed out that a decrease in photosynthesis is not necessarily followed by a decrease in respiratory rates (Bartoli et al., 2005; Ribas-Carbo et al., 2005). In this way, it has been demonstrated that soluble sugar concentration might decrease (Lawlor and Fock, 1977), increase (Dekankova et al., 2004), or keep unaltered (Ghashghaie et al., 2001) during the onset of water deficit. Thus, decreased substrate availability does not seem to be the only factor responsible for limiting respiratory rates under water deficit conditions, at least in the short-medium run (Atkin and Macherel, 2009).

Under suitable water supply mitochondria provide to chloroplasts C skeletons (*e.g.* citrate) required for nitrogen (N) assimilation, as well as ATP, which are fundamental to sucrose synthesis, phloem transport, and maintenance of cellular processes, such as protein turnover and maintenance of ionic gradients (Bouma et al., 1994; Hoefnagel et al., 1998; Atkin and Macherel, 2009). By sharp contrast, under water deficit a decreased provision of C skeletons from mitochondria to chloroplasts is observed, mainly due to the fact that *de novo* N assimilation, especially at nitrate reductase level, is decreased (Fresneau et al., 2007). It is important to highlight, however, that the provision of ATP to chloroplasts is maintained mainly via chloroplast nucleotides (Reinhold et al., 2007), which, at least partially, can compensate for energetic damages occurred during oxidative phosphorylation in chloroplasts under water deficit (Atkin and Macherel, 2009). It has been demonstrated that in *Arabidopsis* two genes encoding chloroplast nucleotide transporters *(*AtNTT1 and AtNTT2) had increased expression levels under water deficit or osmotic stress situations (Toufighi et al., 2005; Winter et al., 2007), which seems to corroborate the hypothesis that mitochondrial ATP may be imported by chloroplasts under such stressful conditions.

The relationship between mitochondria and chloroplasts is even more evident when the oxidation is analyzed through mETC (Noctor et al., 2007), which reduces equivalents produced via photochemical reactions in chloroplasts, thus allowing for the maintenance of photosynthetic rates (Nunes-Nesi et al., 2008). Such reducing equivalents may be exported to the mitochondria via either the photorespiratory cycle or the malate/oxaloacetate shuttle, and thereafter oxidized through the mETC, resulting in either the production of ATP or the dissipation of energy as heat (Atkin and Macherel, 2009). These processes, however, are intrinsically dependent on the functioning of the TCA cycle, which requires an elevated ATP/NADPH ratio,

derived from non-cyclic photophosphorylation. It seems reasonable to assume therefore that part of the reducing equivalents generated in chloroplasts under water deficit should be reoxidized via other processes in the plastids (*e.g.* antioxidant systems) or even in other cellular compartments (Krömer, 1995).

Plant mitochondria exhibit large flexibility in electron transfer and energy dissipation, which is of critical importance in stressed environmental conditions (Atkin and Macherel, 2009). In addition to the classical oxidative phosphorylation machinery, plant mitochondria contain non-phosphorylating respiratory bypasses of electron transport and of proton-coupled ATP synthesis (Jacoby et al., 2012). The specific enzymes branche from the common electron path and allow the mETC to adapt to different cellular and metabolic requirements. Recently, the known number of plant respiratory chain dehydrogenases has increased, including both components specific to plants and those with mammalian counterparts (Rasmusson et al., 2008). For instance, NAD(P)H dehydrogenases proteins (Escobar et al., 2004; Rasmusson et al., 2004) and alternative oxidase enzymes (AOX) (Vanlerberghe and McIntosh, 1997) allow for deviation in electron transfer, while the proton gradient may be dissipated by mitochondrial uncoupling proteins (UCPs) (Sluse et al., 2006) or by parallel activity of K^+/H^+ antiporters and K^+ symporter channels (Pastore et al., 1999) (Figure 3). Moreover, substantial increases in some mitochondrial transporters activities (Fratianni et al., 2001) as well as mitochondrial protein import have been reported under water deficit conditions, suggesting a relevant capability of biogenesis and repair (Taylor et al., 2003). These systems allow the reoxidation of substrates via mitochondria as well as prevent the accumulation of reactive oxygen species (ROS) derived from the over-reduction of the mETC (Bartoli et al., 2004; Atkin and Macherel, 2009).

An increased AOX expression has been extensively observed in plants under several stress situations, such as low temperatures (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992), reduced phosphate availability (Parsons et al., 1999; Juszczuk et al., 2001), application of inhibitors of mitochondrial protein synthesis (Day et al., 1996), and herbicides inhibiting BCAA synthesis (Aubert et al., 1997; Gastón et al., 2003). Interestingly this increase is especially observed under conditions in which the accumulation of ROS is stimulated (Wagner and Krab, 1995). Accordingly water deficit increases the risk of oxidative stress by increasing the production of ROS in different cellular compartments (Bartoli et al., 2004).

Under such conditions, an alternative pathway via AOX seems to play a role in preventing the formation of ROS as well as in sustaining respiration under circumstances in which the cytochrome *c* pathway seems to be limited (Lambers et al., 2005) (Figure 3).

Alterations in the *in vivo* activities of cytochrome *c* oxidase (COX) and AOX in plants under limited water availability conditions have also been frequently reported (Ribas-Carbo et al., 2005; Flexas et al., 2006; Pastore et al., 2007). Unlike the classical pathway, in which electrons flow via cytochrome *c* (responsible for the generation of the proton-motive force used in the synthesis of ATP), the alternative pathway streams electrons directly from the ubiquinone to O_2, via AOX enzyme (Figure 3). Accordingly Ribas-Carbo et al. (2005) have observed decreased COX activity in soybean leaves under water deficit while AOX displayed elevated activity under the same conditions. Similarly, Pastore and colleagues examined a variety of drought-tolerant wheat (*Triticum durum*) and have not only verified significant increased AOX activity but also elevated UCP activity and the existence of an ATP-sensitive K^+ channel suggesting that energy dissipation systems may decrease mitochondrial ROS production (Pastore et al., 2007). It has been also demonstrated that an AOX positive regulation in wheat plants (*Triticum durum*) under water deficit is important for the maintenance of photosynthetic capacity and, consequently, drought tolerance (Bartoli et al., 2005).

The physiological role of UCP is also related with the regulation of ROS production under a range of abiotic stress conditions (Fernie et al., 2004; Vercesi et al., 2006) including pathogen attacks (Maxwell et al., 2002), excess light, drought, temperature, and mechanical stress (Apel and Hirt, 2004). Thus although there is a growing body of evidence suggesting that UCPs play a role in alleviating stress caused mainly by ROS overproduction direct evidence of this role is still lacking (Sweetlove et al., 2006; Vercesi et al., 2006; van Dongen et al., 2011). These uncoupling proteins are located in the inner mitochondrial membrane and catalyze proton flux through the membrane dissipating the proton gradient as heat (Ricquier and Bouillaud, 2000; Krauss et al., 2005). It is currently accepted that UCPs are activated by, among other factors, products of lipid peroxidation, such as superoxide radical and aldehyde (Considine et al., 2003; Smith et al., 2004). Therefore, superoxide activation of UCP represent a mechanism by which ROS production seems to be regulated, given that the production of superoxide radical and other

ROSs via respiratory chain increases nonlinearly under situations of high membrane potential (*e.g.* water deficit) (Smith et al., 2004; Sweetlove et al., 2006) (Figure 3). For instance, it has been demonstrated that two genes encoding UCPs in *Arabidopsis* (*AtPUMP1* and *AtPUMP5*) are highly expressed under water deficit (Kreps et al., 2002; Seki et al., 2002), which brings the importance of these proteins to drought-tolerance mechanisms into evidence. Furthermore, higher levels of *AtUCP1* in tobacco improved tolerance to salt and drought stresses, and this protection was correlated with lower oxidative stress (Begcy et al., 2011).

Alternative NAD(P)H dehydrogenase proteins constitute another important mechanism related with electron transfer deviation and consumption of reducing power to reduce ubiquinone pool. These proteins do not fully span the mitochondrial membrane and the sides remain exposed either to the matrix or the intermembrane space (Finnegan et al., 2004; Rasmusson et al., 2008). Unlike the integral protein that constitutes the mETC complex I, alternative NAD(P)H dehydrogenases are not involved in proton transport from the matrix to the intermembrane space. Thus, these proteins do not contribute directly to the formation of proton-motive force or ATP synthesis (van Dongen et al., 2011). Intriguingly, under several stress situations, such as excess light, drought, or pathogen infection (Dodd et al., 2010), the supply of electrons to the mETC via NAD(P)H dehydrogenase is altered, suggesting that this protein plays an additional role under stressful situations (Figure 3). It is important to mention, however, that the role of these alternative dehydrogenases under limited water availability conditions has not yet been fully understood.

In an attempt to elucidate the physiological role of enzymes involved in the alternative supply of electrons to the mETC in *Arabidopsis thaliana* plants under limited water conditions a metabolic and physiological approach was undertaken by using a range of T-DNA insertion lines as well as overexpressor lines of enzymes known to donate electrons to the mETC cultivated under water limitation conditions. The results presented here revealed that the ETF/ETFQO pathway as well as BCAA catabolism seems to play a relevant role in the tolerance mechanisms to drought episodes. This pathway is equally important for both salt- and osmotic-tolerance mechanisms during *Arabidopsis* germination events. In summary, compelling evidence is provided suggesting that alternative mitochondrial metabolism is likely highly effective in drought tolerance. The consequences of this short-term water limitation were additionally monitored at the steady-state metabolite level as well as

gene expression level, with the combined data discussed in the context of the current models of metabolic regulation and importance of plant respiration as well its alternative pathways and BCAA metabolism in the illuminated leaf under water shortage.

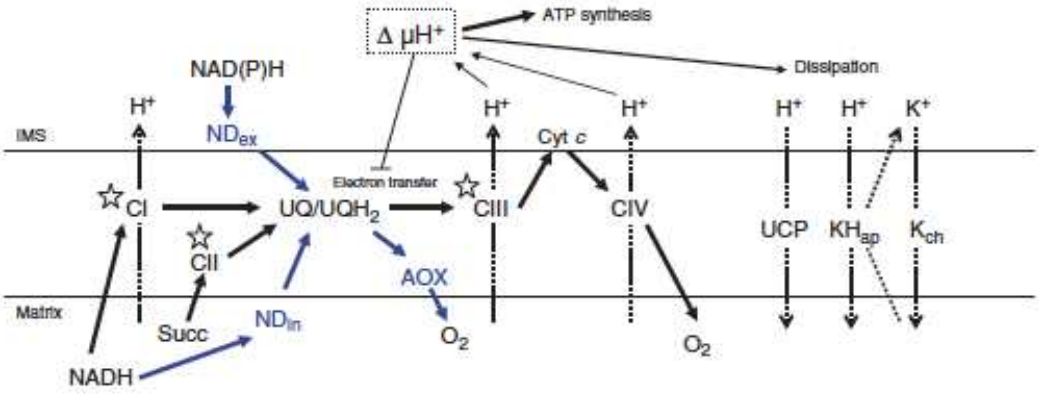


Figure 3. Energy dissipating systems in plant mitochondria membrane. The scheme shows the pathway of electrons from the substrates to oxygen via the different complexes (I-IV) of the mETC (bold arrows) and the coupled extrusion of protons (dotted arrows). Plant alternative dehydrogenases (NDin, NDex) and alternative oxidase (AOX) are indicated in blue. Stars indicate the major sites of ROS formation. The systems capable of dissipating the electrochemical proton gradient are the uncoupling protein (UCP), and the combination of the K^+/H^+ antiporter (KHap) with the potassium channel (Kch). Source: Atkin and Macherel (2009)

3. MATERIAL AND METHODS

3.1. Plant material and experimental conditions

All *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype (Col-0). The T-DNA mutant lines GK756G02 (*ivdh-1*, Araújo et al., 2010; Figure 4A), SAIL844G06 (*d2hgdh-2*, Araújo et al., 2010; Figure 4B) and SALK_007870 (*etfqo-1*, Ishizaki et al., 2005; Figure 4C) were handled exactly as previously described. Three different *D2HGDH* overexpressor lines (D2HGDH OX 6.3, 8.4 and 9.3) were also used. The Arabidopsis Genome Initiative locus numbers for the major genes discussed in this study were as follows: *IVDH*, At3g45300; *D2HGDH*, At4g36400; and *ETFQO*, At2g43400 (The Arabidopsis Genome Initiative; http://www.arabidopsis.org/). It is important to note that, under normal growth conditions (non-stressed conditions), all mutant lines present wild type like phenotype (Figure 4D).

Seeds were initially sterilized in 70% ethanol for 2 min. The ethanol was removed and seeds were soaked in a solution containing 1 mL of 5% sodium hypochlorite and Tween 20. Dead seeds and supernatants were removed and sterilized seeds were rinsed thoroughly with sterile distilled water. Finally, seeds were sown on one half concentrated MS medium (Murashige and Skoog, 1962) pH 5.7, 1% sucrose (*w/v*), solidified with 0.8% (*w/v*) agar and kept in darkness for 3 days at 4°C. The seeds were transferred to a growth chamber (150 µmol m^{-2} s^{-1}, 22°C, 70% relative humidity, RH) and after germination seedlings were cultivated under long-day conditions (16 h light/8 h dark). Later on, 7-to-10-day old seedlings at the

1.02 phenological growth stage (2 rosette leaves < 1 mm; Boyes et al., 2001) were transferred to plastic pots containing commercial substrate and cultivated in growth chamber (150 µmol m^{-2} s^{-1}, 22°C, 70% RH), under short-day conditions (8 h light/16 h dark) for two more weeks to stimulate rosette growth. Finally, plants were transferred to a greenhouse (150 µmol m^{-2} s^{-1}, 22°C, 70% RH), and cultivated under long-day conditions (16 h light/8 h dark).

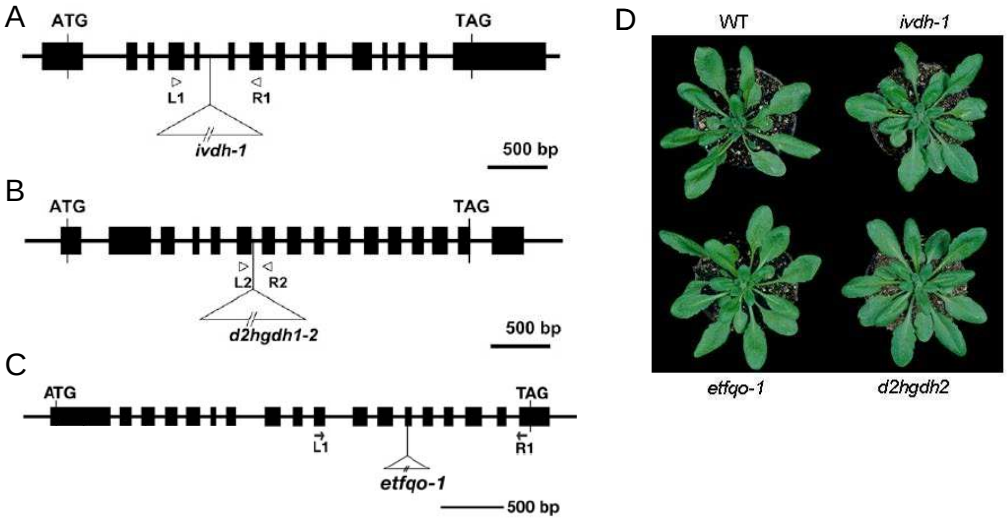


Figure 4. Squematic representation of the sites of T-DNA insertion and phenotype of mutants used in this study. Gene structure of *Arabidopsis* knockout mutants *ivdh-1* (A), *d2hgdh-2* (B) and *etfqo-1* (C). Arrowheads represent positions of primer used for genotyping of wild-type and mutant lines; closed boxes indicated exons. (D) Images of 4-week old *Arabidopsis* plants (wild type and mutant lines) cultivated in a greenhouse (150 µmol m^{-2} s^{-1}, 22°C, 70% RH) and under long-day conditions (16 h light/8 h dark), under normal growth conditions (non-stress conditions). WT: wild type. Source: A and B adapted from Araújo et al. (2010); C adapted from Ishizaki et al. (2005)

Four-week-old plants, at the 3.9 phenological growth stage (rosette growth complete; Boyes et al., 2001) were subjected to a progressive water deficit by suspension of irrigation and then given recovery irrigation aiming at analyzes the capacity of recovery vegetative growth of those plants. At days 0, 5, 7 and 10 of drought stress and following 1, 3 and 5 days of recovery irrigation samples were harvested for further analysis. Control plants were watered daily to maintain soil

water close to field capacity. The pots containing plants were daily weighed to assess the level of water in the soil. Whole rosettes of six independent samples by genotype were harvested, at each harvest day, and were immediately immersed in liquid nitrogen and stored at -80°C until further use. For the metabolic analysis, frozen leaf tissues were ground in a mixer mill (Retsch MM301, Retsch, Haan, Germany), in appropriate container with steel balls for 45s at 25 Hz. After grinding, samples were aliquoted and stored at -80°C until further use.

3.2. Water loss measurements and relative water content

For water loss measurements, the weight of detached leaves incubated abaxial side up under greenhouse conditions was obtained between 8 and 12h, at 20 min interval. Water loss was calculated as a percentage of the initial fresh weight (Araújo et al., 2011c).

Leaf relative water content (RWC) was assessed to monitor the status of leaf hydration at 0, 5, 7, and 10 days without watering as well as at 1, 3, and 5 days after the recovery of irrigation. One leaf from each replicate was excised and weighed in order to obtain the fresh weight (FW). Afterwards, leaves were hydrated for 2 hours in Petri dish containing distilled water, under greenhouse conditions, and weighed in order to obtain the turgid weight (TW). Finally, leaves were oven-dried at 72 ºC for 72 h and weighed in order to obtain the dry weight (DW). For the calculation of RWC, the following equation was used:

$$RWC\,(\%) = \frac{FW - DW}{TW - DW}$$

3.3. Determination of chlorophyll *a* fluorescence parameters

Chlorophyll *a* fluorescence was assessed using a pulse modulated chlorophyll fluorescence monitoring system (FMS2, Hansatech, Norfolk, United Kingdom). In dark-adapted (30 min) leaves, the leaf tissue was illuminated with weak modulated short pulse of far-red light (1-2 μmol m^{-2} s^{-1}), in order to determine the initial fluorescence (F_0). Saturating white light pulses of 6000 μmol m^{-2} s^{-1} were applied for 1.0 s to ensure for maximum fluorescence emissions (F_m), from which the variable-

to-maximum chlorophyll fluorescence ratios, F_v/F_m = [(F_m – F_0)/F_m)] were calculated. This ratio has been used as a measure of the potential photochemical efficiency of PSII (Oh et al., 1996).

3.4. Biochemical characterization

3.4.1. Basic analysis

3.4.1.1. Extraction of plant materials

About 20 mg of plant material were aliquoted in 1.5 *Eppendorf* tubes. For extraction, 250 μL 98% ethanol were added to the material and the tubes were shaken for 20 min at 80°C. Later on, tubes were centrifuged for 5 min at 14000 *g* and supernatant (S1) collected in a new 2 mL tube. The pellet was resuspended in 250 μL 80% ethanol, shaken for 20 min at 80°C, and centrifuged once more for 5 min at 14000 *g* in order to collect the supernatant (S2). The same procedure was repeated with 250 μL 50% ethanol in order to obtain the supernatant (S3). The three supernatant fractions (S1 + S2 + S3) were pooled and the tubes were kept in ice to determine chlorophyll content. For further analysis, pellet was stored at -80°C and the mix of supernatants at -20°C. Chlorophyll content was determined exactly as described by Porra et al. (1989). The levels of starch in the leaf tissue were determined as described previously (Fernie et al., 2001). The levels of nitrate, total amino acids, and protein were measured as described by Sienkiewicz-Porzucek et al. (2008).

3.4.1.2. Determination of chlorophyll content

The content of chlorophyll (*a* and *b*) was determined immediately after ethanolic extraction using aliquots from the pooled supernatants (S1 + S2 + S3) and 98% ethanol placed on microplates. The absorbance was measured at 645 and 665 nm in a microplate reader (ELx808, BioTek Instruments Inc., Winooski, USA). The content of chlorophyll (*a* and *b*) were expressed in fresh weight (FW) basis. Based on the estimates, the content of chlorophyll *a* was determined following the equation:

chlorophyll *a* = (A665 x 5.48) – (A645 x 2.16). The content of chlorophyll *b* was determined accordingly to the equation: chlorophyll *b* = (A645 x 9.67) – (A665 x 3.04) (Arnon, 1949). Later, the total content of chlorophyll (*a* + *b*) as well as chlorophyll *a*/*b* ratio was determined.

3.4.1.3. Determination of total soluble proteins

In order to determine protein contents, the pellet was resuspended in 1 mL 100% ethanol and centrifuged at 14000 *g* for 10 min, at 4°C. The supernatant was discarded and tubes were centrifuged once more at 14000 *g* for 5 min, at 4°C. Afterwards, 400 µL KOH 0.2 M were added and tubes incubated for 60 min, at 95°C. Later on, tubes were let to cool down at room temperature and centrifuged at 14000 *g* for 5 min. Aliquots of supernatants were distributed on microplates in duplicates, as well as different concentrations of standard bovine serum albumin solutions (BSA) (0, 2, 4, 6, 8, 10 and 12 µg/µL of BSA in KOH 0.2 M). Afterwards, 250 µL of Bradford solution (1:5 dilution) were added and the microplate was incubated at room temperature, for 5 min. The absorbance was measured at 595 nm on the same microplate reader described above and the content of protein was calculated according to Bradford (1976).

3.4.1.4. Determination of starch

A commercial kit (R-Biopharm AG, Darmstadt, Germany) was used to measure starch levels. The pellet was neutralized in a solution of acetic acid 1 M and centrifuged at 14000 *g* for 10 min, at 4°C, after becoming homogenized with the KOH 0.2 M as described above. For the determination of starch content, 25 µL of the solution 1 of the kit (citrate buffer/amyloglucosidase), 3 µL of distilled water and 10 µL of neutralized supernatant were pipetted on the microplate and it was heated at 56 °C, for 60 min for the hydrolysis of starch to glucose molecules. Afterwards, the microplate was let cool down on ice for 2 min. After fast centrifugation (3000 *g* for 2 min), 250 µL of the solution 2 of the kit (TRA buffer/NADP/ATP) were added to the samples and absorbance measured at 340 nm on the same microplate reader described above. To start the reaction 3 µL of solution 3 of the kit (hexokinase and glucose-6-phosphate dehydrogenase enzymes suspension) were added and

absorbance measured for 60 min at 340 nm. The content of NADPH produced is stoichiometrically equivalent to the content of glucose, which was then used for the determination of the content of starch in samples.

3.4.1.5. Determination of total amino acids

To determine total amino acids contents, 50 μL of Na-Citrate buffer 1 M + 0.2% ascorbic acid, pH 5.2, was added to an aliquot of the supernatant mix (S1 + S2 + S3) and to 50 μL of ninhydrin solution were added. A standard curve of leucine solutions (1 mM leucine and 70% ethanol; 0, 2, 4, 8, 10, 20 and 50 nM) was also used. Tubes were covered and heated for 20 min, at 95°C. After fast centrifugation (10000 *g* for 10 s), the content was transferred, in duplicates, to a microplate and absorbance was measured at 570 nm on the same microplate reader described above.

3.4.1.6. Determination of nitrate

Nitrate levels were determined in a detection mixture composed of potassium phosphate buffer 1 M (pH 7.5), NADPH 50 mM in NaOH 5 mM and nitrate reductase (Roche 5U/mL in phosphate buffer 0.1 M). Distilled water, as a control, was used instead of nitrate reductase to determine the levels of nitrite present in samples. Different concentrations of potassium nitrate (KNO_3) were used as standard solutions (0, 0.4, 0.8, 1.6 and 3.2 mM). 95 μL of the detection mix and 5 μL of ethanolic extract were pipetted, in duplicates, on the microplate, and incubated for 30 min, at 25°C. Later, 15 μL of N-methyl-phenazine methyl-sulfate (PMS) were added to the plate and, once again, incubated for 20 min, at 25°C. Finally, 60 μL of sulfanilamide (1% w/v in phosphoric acid 3 M) and 60 μL of N-(1-naphthyl)-ethylenediamine dihydrochloride (NNEDA; 0.02% w/v) were added and the microplate was incubated again for 10 min, at 25°C. Absorbance was measured at 570 nm on the same microplate reader described above.

3.4.2. Gas chromatography associated with mass spectrometry

Metabolite profiling was performed using an established gas chromatography-mass spectrometry (GC-MS) (Lisec et al., 2006) for the separation

and identification of metabolites. This method allows for the detection of amino acids, organic acids, sugars, among other classes of compounds. The system consists of a gas chromatograph (6890N Aligent, Böblingen, Germany) connected to a mass spectrometer Pegasus III (Leco Instruments, St. Joseph, USA). The auto sampler (CTC Combi PAL, CTC Analytics AG, Zwingen, Switzerland) aspirates, with an injection syringe, over 1 μL of sample, which is then transferred to an injection port where it is vaporized at 230°C. Non-volatile components remain in the injector. Volatile substances are carried, via helium gas, to the chromatograph column (30m MDN-35 Machery-Nagel, Düren, Germany). This column is made of glass capillary, polyphenylmethylsiloxane-coated. This compound forms the stationary phase and is responsible for the separation of metabolites. Variations in analysis retention time are due to different compound affinities and to the column coating.

3.4.2.1. Extraction of plant material for metabolic profiling

Approximately 50 mg of homogenized plant materials were aliquoted in 2 mL *Eppendorf* tubes and extracted in 1400 μL of 100% methanol and 60 μL of internal standard (0.2 mg ribitol mL^{-1} water). Tubes were shaken for 15 min at 70°C and 950 *g* and next centrifuged at 14000 *g* for 10 min. The supernatant was transferred to glass tubes (Schott GL14, Schott AG, Mainz, Germany) and, afterwards, 750 μL of 100% chloroform and 1500 μL of distilled water were added. Glass tubes were centrifuged at 4000 *g* for 15 min. Finally, 150 μL of the upper phase of each sample were transferred to new 2 mL *Eppendorf* tubes and let to dry overnight in a vacuum centrifuge (SPD 111 V-230, Thermo Scientific, Waltham, USA). To avoid metabolite oxidation, tubes were filled, after dried, with argon gas and stored at -80°C until the derivatization procedure (Lisec et al., 2006).

3.4.2.2. Derivatization for metabolic profiling

Before derivatization, pellets were vacuum-centrifuged once more for over 20 min to secure the complete removal of condensed water. Afterwards, pellets were resuspended in 40 μL of methoxyamine hydrochloride (20 mg^{-1} mL of pyridine) and incubated for 60 min, at 37°C, and centrifugated at 950 *g*. After incubation, 70 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and 20 μL of a mixture of

fatty acid methyl esters (FAMES), which the retention times are already known (see Supplemental Table S1), were added to the samples. Tubes were incubated one more time for 30 min, at 37°C and 950 *g* and, finally, contents were transferred to specific CG-MS glass vials (1.1 STVG, 8-SC-ST15, Chromacol, Welwyn Garden City, United Kingdom).

3.4.2.3. Data analysis for metabolic profiling

GC-MS data obtained via Chroma TOF software were analyzed with Pegasus (LECO Instruments GmbH, Möchengladbach, Germany) and TagFinder v4.0 (Luedemann et al., 2008), based on the FAME MDN35 Library, from Golm Metabolome Database (http://gmd.mpimp-gol.mpg.de/download/). Metabolite peak heights were extracted and the retention time was calculated. The identification of compounds was done by means of comparison among mass spectrometry and retention times calculated with those obtained from reference compounds commercially available. The detection of peaks was performed through the identification of similarities among mass spectrometry in a time interval of about 0.25 min the retention time expected for each targeted metabolite. Metabolites were then identified through exact retention times and their corresponding mass spectrometry (Roessner et al., 2001; Lisec et al., 2006).

3.5. Quantitative Real-Time PCR

For the analysis of transcription levels of some genes related to amino acids and N metabolism, photorespiration and TCA cycle quantitative real-time PCR was performed exactly as described by Zanor et al. (2009). Analyses were performed in whole rosettes by harvesting and immediately snap-freezing leaf samples in liquid nitrogen. Extraction of total RNA (around 100 mg of leaf material, aliquoted in 2 mL *Eppendorf* tubes) was made in 1 mL of trizol reagent and 200 µL of 100% chloroform. Tubes were centrifuged at 12000 *g* for 15 min, at 4°C. About 600 µL of supernatant was transferred to new tubes and, afterwards, 600 µL of isopropanol was added. The tubes were incubated at room temperature for 10 min and then centrifuged again at 12000 *g* for 10 min, at 4°C. The supernatant was discarded and, after adding 1 mL 70% ethanol, the tubes were centrifuged once more at 9500 *g* for 5

min, at 4°C. The supernatant was discarded and the tubes were let to dry at room temperature for 10 min. The pellet was resuspended in 20 µL diethyl pyrocarbonate (DEPC)-treated H_2O. Then, total RNA was treated with DNAse I (RQ1 RNase free DNase I, Promega, Madison, WI, USA). The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a Nanodrop ND-1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA). Finally, 2 µg of total RNA were reverse transcribed using cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's recommendations.

For qRT-PCR, primers based on the cDNA sequence of genes were designed with Primer3 (http://frodo.wi.mit.edu/). The complete list of genes and primers that were used here are described in the Table 1. qRT-PCR was performed on an optical 384-well plate using the automated 7900 HT Fast Real-time PCR system (Applied Biosystems, Foster, CA, USA) as described by Caldana et al. (2007). Five microliters of reaction volume contained 0.5 µL of template (reverse transcribed, first-strand cDNA), 2.5 µL of SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA), and 2 µL of each mix of specific primer 0.5 µM were used. To normalize gene expression, the constitutively expressed polyubiquitin 10 (*UBQ10*) was amplified using the following primers: forward, 5'-AGCAGTTGGAGGATGGCAGAAC-3'; reverse, 5'-CGGAGCCTGAGAACAAGATGAAGG-3'. The reference gene was measured using two replicates in each PCR run, and their mean cycle threshold was used for relative normalized expression analyses.

Table 1. Primers used in the RT-PCR analyses performed in this study

Gene	Gene name	Locus	Forward primer	Reverse primer
AGPase1	ADP Glucose Pyrophosphorylase 1	At5g48300	5'-AGGGCTGTGTTATCAAGAACTGC-3'	5'-TATGCAGGAACGGAGTCCAACC-3'
AspAT1	Aspartate Aminotransferase 1	At2g30970	5'-TGCTGGGAGCACTTTCATGGAG-3'	5'-GGCAAGCTTCAATGTTAGGTCCAC-3'
CAT2	Catalase 2	At4g35090	5'-AAGTATCCAACTCCGCCTGCTG-3'	5'-TGGATGAATCGTTCTTGCCTCTC-3'
Fd-GOGAT	Ferredoxin-dependent Glutamate Synthase	At5g04140	5'-TGTGTAGTCGTGCTTGGAAAGGTG-3'	5'-TGCAGGCGCAGTTACTCTTTGG-3'
FUM1	Fumarase 1	At2g47510	5'-ACTCTTCCTCGCCTCTATCAGC-3'	5'-AGTGTTTAATCCTGTCCCAACCG-3'
GDCT	Aminomethyltransferase glycine decarboxylase	At1g11860	5'-TTGGTGGTCAATGCTGGCTGTAG-3'	5'-AAGGACCCTGAAGGGCAAGAAGAG-3'
GDH1	Glutamate Dehydrogenase 1	At5g18170	5'-TTGGTTGTGATCCTAGCAAGCTC-3'	5'-AAGAATCCAAGCCATTGTCTGAGG-3'
GS	Glutamine synthetase	At5g35630	5'-GGTGAAGTTATGCCTGGACAGTGG-3'	5'-AGCACACCAAACATGATCACCTG-3'
MDH1	Malate dehydrogenase 1	At1g53240	5'-TGAGCTTCCCTTCTTCGCATCG-3'	5'-TGCCTTCTCTAATCCCATCCTCTC-3'
NADP_ICDH	NADP-dependent isocitrate dehydrogenase	At1g54340	5'-TTGGACTGCCTAACCGCGATTTC-3'	5'-TCCCTAACACGAGCTTCATCCG-3'
PGLP1	2-phosphoglycolate phosphatase 1	At5g36790	5'-GCCACACAACAGCTTGAGAA-3'	5'-TCTCCCTTCCAAATCACACC-3'
RCA	Rubisco activase	At2g39730	5'-AGACCGTATCGGTGTCTGCAAG-3'	5'-CCCTCAAAGCACCGAAGAAATCG-3'
SHMT1	Serine hydroxymethyl transferase 1	At4g37930	5'-ACTATGCCCGCATCAGAAAGGTC-3'	5'-AGCATAGTCGAACGGTGAAGGG-3'
SHMT2	Serine hydroxymethyl transferase 2	At5g26780	5'-AGGCCTACCAGGATCAAGTTCTCC-3'	5'-TCCTTTCGCAAGCAAAGTCTCAG-3'
UBQ10	Polyubiquitin 10	At4g05320	5'-AGCAGTTGGAGGATGGCAGAAC-3'	5'-CGGAGCCTGAGAACAAGATGAAGG-3'
UCP	Mitochondrial uncoupling protein	At3g54110	5'-AAGGGCACCATTGACTGCTTCG-3'	5'-CCGGACATACTTCTTTGCCTGTTC-3'

3.6. Germination assays

For the germination assays, approximately 100 seeds of each genotype were sterilized (70% ethanol, 5% sodium hypochlorite, Tween 20, and distilled water), soaked in Petri dishes (n = 3) containing MS medium (Murashige and Skoog, 1962), one half strength, pH 5.7, 1% sucrose (control) and without sucrose (treatments, Table 2), and kept in the dark for 3 days at 4°C. The Petri dishes were transferred to a growth chamber (150 µmol m^{-2} s^{-1}, 22°C, 70% RH) under long-day conditions (16 h light/8 h dark). The assessment of seed germination started two days after transferring dishes to the growth chamber. Daily assessments occurred always at noon and germination (radicle emergence) was scored until seven days after stratification.

Table 2. Treatments used in experiments on seed germination

Treatments	Concentrations	Function
Control		
NaCl	50 and 100 mM	Salt stress inductor
Mannitol	200 mM	Osmotic stress inductor
Lysine	10, 50 and 100 mM	Amino acid
Valine	10, 50 and 100 mM	Brached-chain amino acid

3.7. Statistical analysis

The experiments were conducted in a completely randomized design with 3-6 replicates of each genotype. For the determination of chlorophyll *a* fluorescence parameters, a sample unit consisted of one plant per pot (n = 6). The experimental unit in the analysis of metabolites and gene expression consisted of 20-100 mg of plant material by reaction, with six biological and two technical replications by genotype. For the germination assay, a Petri dish containing approximately 100 seeds was used as a sample unit (n = 3). Data were statistically examined using analysis of variance and tested for significant ($P < 0.05$) differences using Student's t tests. All statistical analyses were performed using the algorithm embedded into Microsoft Excel.

4. RESULTS

4.1. Phenotype of *Arabidopsis* mutants under water deficit conditions

Arabidopsis knockout (KO) and overexpressor (OE) lines as well as their wild type (WT) background plants were cultivated under long-day conditions (16 h light/8 h dark) and did not show any aberrant phenotype during vegetative growth prior to the imposition of water deficit (Figure 5A). After the suspension of the irrigation, all phenotypes showed senescence symptoms, although it should be mention that clear differences were observed. The *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutant plants began to show symptoms of chlorosis and leaf wilting four-to-five days after the onset of water deficit. By contrast, dehydration symptoms in both WT and OE lines (D2HGDH OX 6.3, D2HGDH OX 8.4 and D2HGDH OX 9.3) began to become visible six-to-seven days without watering (Figure 5B). Interestingly at the end of the water deficit period (10 days without water), almost all KO mutant plants were either dead or completely dehydrated showing severe leaf necrosis, while both WT and OE lines presented higher survival rates, though showing intense signs of dehydration (Figure 5C).

In good agreement with the phenotype alterations observed (Figure 3), water loss observed in detached leaves from KO mutants was higher (30-32% after 180 min) than in both WT and OE lines (22-25%) after 180 min (Figure 6A). In addition, all genotypes studied showed similar values of RWC (approximately 93%) before the imposition of water stress (Figure 6B). After irrigation cessation, a reduced RWC was only observed after five days in *ivdh-1* (39%); after 7 days without watering KO

mutants showed smaller values of RWC (19-34%) significantly different from those observed in the WT and OE lines (60-69%). At the end of the treatment (10 days), all genotypes showed similar RWC values (15-24%). Accordingly decreases in RWC during the experimental period were compatible with the progressive phenotype alterations observed.

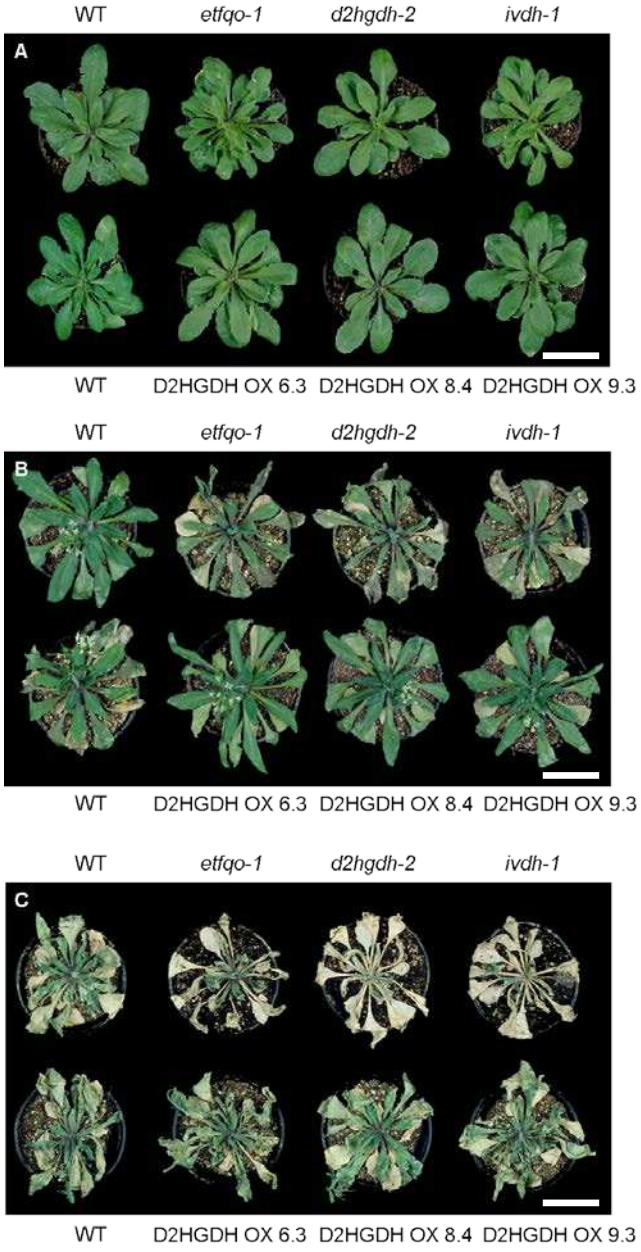


Figure 5. Phenotype of *Arabidopsis* mutants and wild type plants (WT) under drought stress treatment. Images of 4-week-old, long-day-grown *Arabidopsis* plants immediately (0 day, A) and after further treatment for 5 (B) and 10 days (C) without watering. Scale bar represents 3 cm

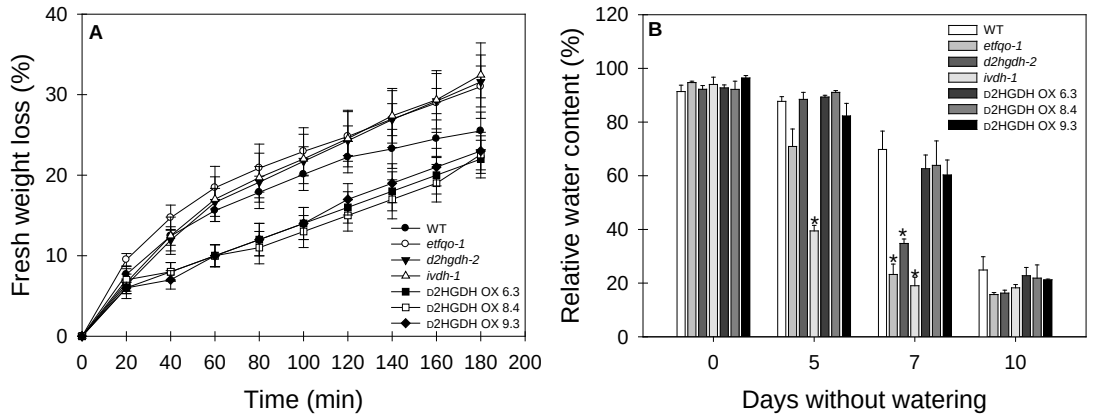


Figure 6. Fresh weight loss from detached leaves (A) and relative water content (B) of leaves of 4-week-old, long-day-grown, *Arabidopsis* plants after further treatment for 0, 5, 7 and 10 days without watering. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT)

In order to next evaluate senescence mechanisms, some parameters related to the function of chloroplasts and commonly associated with the diagnosis of leaf senescence were assessed (Oh et al., 1996). More specifically, chlorophyll contents and maximum photochemical efficiency of PSII (F_v/F_m) were evaluated. After irrigation cessation, total chlorophyll content (*a*+*b*) decreased more rapidly in KO mutants in comparison to both WT and OE lines (Figure 7A). Interestingly a trend for increased chlorophyll *a*/*b* ratio was observed during the first days without watering, followed by drop after five days, and a new increase at the end of the experiment (Figure 7B). Such increments in chlorophyll *a*/*b* ratio are a typical characteristic associated with chlorophyll degradation in *Arabidopsis* plants under senescence conditions (Pruzinská et al., 2005). Reductions in F_0 values were also observed, especially in KO mutants over the experimental period (Figure 7C). Moreover, a dramatic decrease in F_v/F_m values was observed after five days without watering in *ivdh-1* and after seven days in *d2hgdh-2* and *etfqo-1* mutants (Figure 5D). On the other hand, F_v/F_m values in the WT and OE lines remained practically constant over almost all the experiment showing decreases only after 10 days without watering (Figure 7D). When taken together, these results indicate that *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutants are likely more sensitive to water deficit than the WT and OE lines, and that alternative pathways of respiration and that more specifically

ETFQO, *IVDH* and *D2HGDH* seems to play a significant role in drought-tolerance mechanisms in *Arabidopsis*.

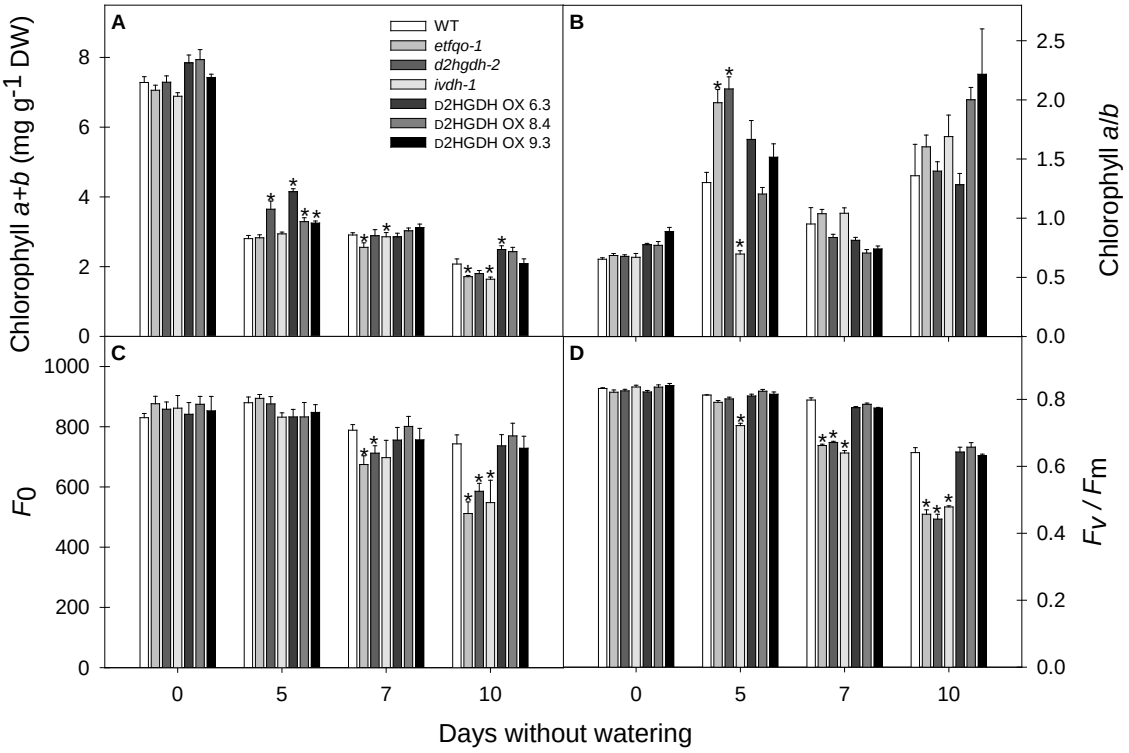


Figure 7. Total chlorophyll content (A), chlorophyll *a/b* ratio (B), initial fluorescence, F_0 (C) and F_v/F_m (D), the maximum quantum yield of PSII electron transport, of leaves of 4-week-old, long-day-grown, *Arabidopsis* plants after further treatment for 0, 5, 7 and 10 days without watering. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT). DW, dry weight

4.2. Phenotype of *Arabidopsis* mutants after irrigation recovery

In order to assess plant recovery capacity the irrigation was restored 10 days after the onset of water deficit. Accordingly it was observed that both WT plants and OE lines were able to recover both vegetative and reproductive growth, while KO mutants did not show any sign of recovery, even after a further five-days irrigation, prevailing among these lines characteristics of senescence, such as intense dehydration and necrosis (Figure 8).

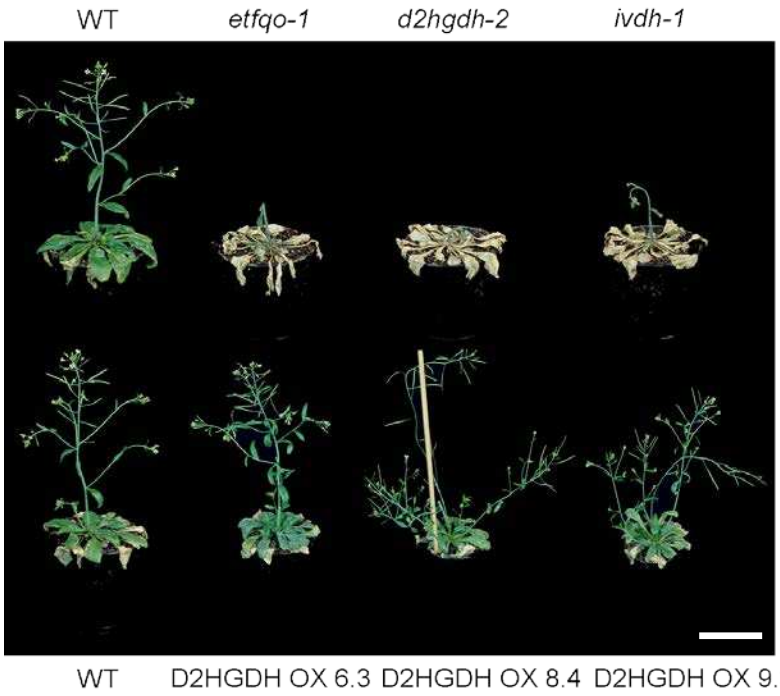


Figure 8. Phenotype of 6-week-old, long-day-grown *Arabidopsis* mutants and wild type plants (WT) 5 days after re-watering. Scale bar represents 5 cm

Increments of RWC were observed at three (68-72%) and five (88-92%) days after the reestablishment of the irrigation only in WT plants and OE lines. In contrast, all KO genotypes showed smaller RWC values (25-29%) after five days of recovery, differing significantly from the values observed in both WT plants and OE lines (Figure 9A). Similarly, WT and OE lines were able to recover F_v/F_m values (Figure 9B) observed at the beginning of the experiment (day 0, non-stress condition, Figure 7D), indicating that the water availably was enough to fully recover the photosynthetic machinery in those plants. Although after irrigation restoration KO mutants showed F_v/F_m values higher than the ones observed at the end of water deficit period (Figure 7D and Figure 9B), they were not able of recovering the initial values (day 0) as verified in both WT plants and OE lines. Concomitantly these results indicate that the damages occurred in chloroplasts, caused by dehydration, were associated with photosynthetic impairments and were most likely irreversible.

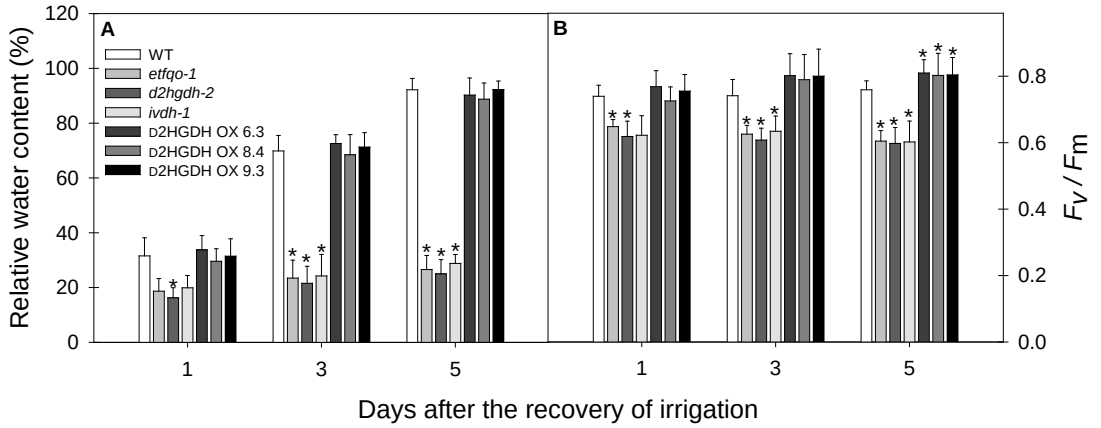


Figure 9. Relative water content (A) and the maximum quantum yield of PSII electron transport, F_v/F_m (B) of leaves of 6-week-old, long-day-grown, *Arabidopsis* plants after further treatment for 1, 3 and 5 days after re-watering. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT)

4.3. Metabolic characterization of *Arabidopsis* mutants under water deficit conditions

It is important to mention that all genotypes used in this study showed similar levels of total soluble proteins (Figure 10A), total amino acids (Figure 10B), nitrate (Figure 10C), and starch (Figure 10D) in samples harvested immediately prior to the start of the drought treatment (day 0), indicating that silencing of *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutants has minor, if any, impact on leaf primary metabolism of these plants under non-stress conditions. However, five days without irrigation culminated in decreases of over 55% of protein contents in all genotypes (Figure 10A). Significant increases in the levels of total amino acids were observed after 10 days without watering in KO mutants in comparison with the WT and OE lines (Figure 10B). Interestingly nitrate (Figure 10C) and starch (Figure 10D) levels were significantly reduced over the experimental period, mainly in KO mutants. After 10 days without watering a dramatic decrease in starch content in all genotypes was observed; more specifically, starch was not found in *d2hgdh-2* and *ivdh-1* mutants after 10 days without watering (Figure 10D).

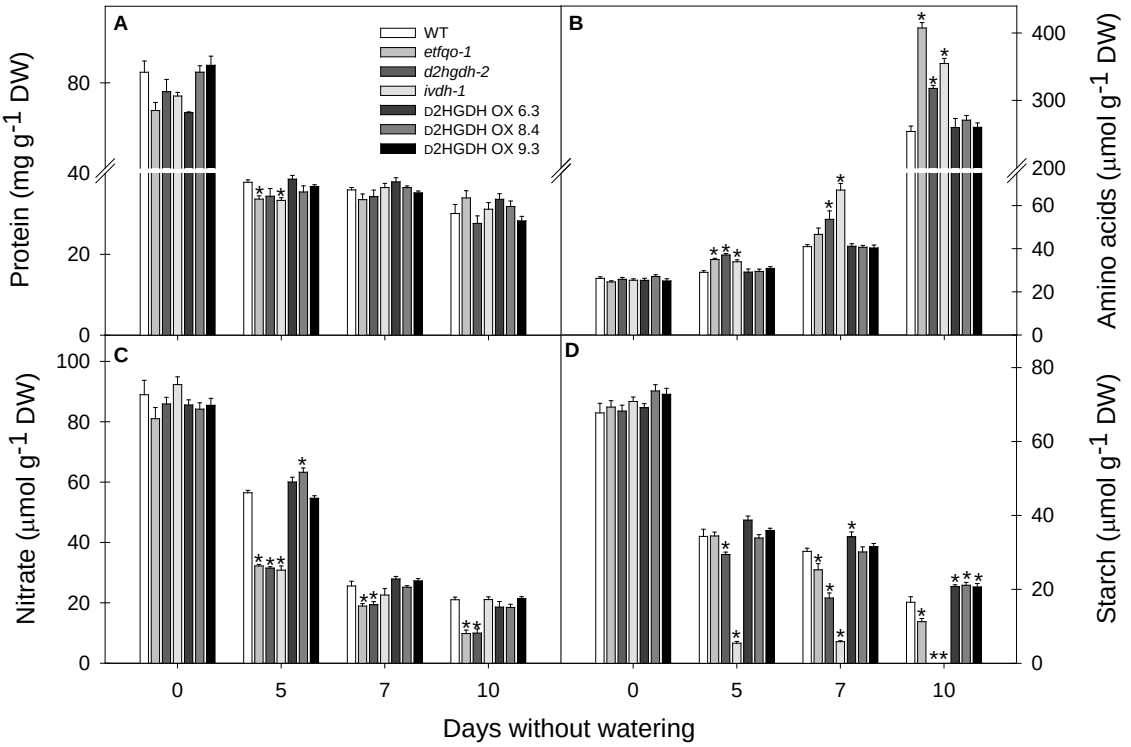


Figure 10. Protein (A), amino acids (B), nitrate (C) and starch content (D) of leaves of 4-week-old, long-day-grown *Arabidopsis* plants after further treatment for 0, 5, 7 and 10 days without watering. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT). DW, dry weight

Metabolic regulation is one of the main mechanisms involved in the maintenance of osmotic potential under water deficit (Bowne et al., 2012). Thus, an established GC-MS protocol for metabolite profiling (Lisec et al., 2006) was used allowing simultaneous identification and quantification of a total of 50 metabolites in KO mutants and WT plants, including 21 amino acids, 9 sugars, 13 organic acids, 5 sugar alcohols, 2 polyamines, and 1 fatty acid. Results obtained are summarized and displayed in false color as a heat map (Figure 11) in order to provide an easy overview of the metabolic changes and the relative values of specific metabolites were presented in Figure 12-14. The full data set is additionally provided as Supplemental Table S2.

In response to the imposed treatments, considerable changes in the levels of a wide range of organic acids, amino acids, and sugar were evident. Thus, the levels of most amino acids increased significantly, particularly after 10 days without watering

(Figure 12). The main differences were observed in the levels of aromatic amino acids (phenylalanine and tryptophan) and BCAA (isoleucine, leucine, and valine). The KO mutants showed significant increases in comparison to WT starting values in the levels of isoleucine (143-194 x-fold), leucine (136-186 x-fold) and valine (63-84 x-fold), as well as in the levels of GABA (82-84 x-fold), lysine (109-121 x-fold), and proline (~13 x-fold) after 10 days without water. Moreover, significant increases in the levels of alanine (~47 x-fold), phenylalanine (182-103 x-fold), ornithine (23-28 x-fold), and tryptophan (60-80 x-fold) were verified in *d2hgdh-2* and *ivdh-1*. By contrast, mild increases in the levels of glutamate (in all KO lines) and aspartate (*etfqo-1* and *ivdh-1*) were observed.

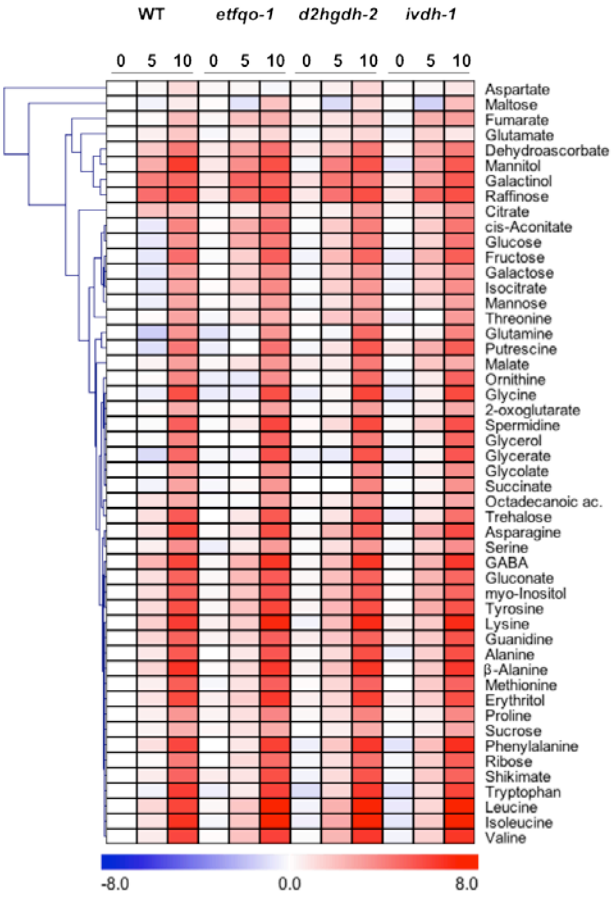


Figure 11. Heat map of metabolite levels in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Red colors represent increase in metabolite levels; blue colors highlight decreases using a false-color scale. Values are means of six independent samplings and are representative of the log2-transformed values. Data are normalized with respect to the mean response calculated for the control at day 0

Drought conditions also promoted significant increases in the levels of sugars from different classes (Figure 13). After five days without irrigation KO mutants plants showed significant increases in the levels of fructose (2.7-5 x-fold), galactose (2.5-2.9 x-fold), glucose (2.3-6 x-fold), mannose (1.5-2 x-fold), and ribose (2-2.8 x-fold). Moreover, after 10 days without watering it was verified significant increases in the levels of glucose (15-19 x-fold) in all KO mutants, fructose (~33 x-fold), maltose (3.6-4.2 x-fold), and ribose (24-33 x-fold) in *etfqo-1* and *ivdh-1*, mannose (7.4-8 x-fold) in *etfqo-1* and *ivdh-1*, as well as galactose (~10 x-fold) only in *etfqo-1*. Even though some values were higher than the ones observed at day 0, at the end of the experiment significantly lower values were observed in the levels of trehalose (only in *ivdh-1*), in comparison with the values found for WT plants (Figure 13).

Significant increases were found in the levels of organic acids (Figure 13). Regarding TCA cycle intermediates, levels of *cis*-aconitate (3-6 x-fold) and isocitrate (2.8-3.2 x-fold) were significantly increased in all KO mutants after five days without watering, while fumarate (3.5-5 x-fold) were significantly increased only in *etfqo-1* and *ivdh-1*. In addition, the levels of 2-oxoglutarate (6-7.7 x-fold), citrate (7-8.6 x-fold), and succinate (10-14 x-fold) increased significantly in all KO mutants after 10 days without watering. Moreover, increases in the levels of other TCA cycle intermediates, such as *cis*-aconitate, malate, and isocitrate were also observed (Figure 13). It was also observed after 10 days without watering increased levels of some sugar alcohols, such as erythritol (45-76 x-fold), glycerol (14-28 x-fold), and mannitol (37-74 x-fold), as well as polyamines such as putrescine (18-33 x-fold) and spermidine (32-55 x-fold) (Figure 14).

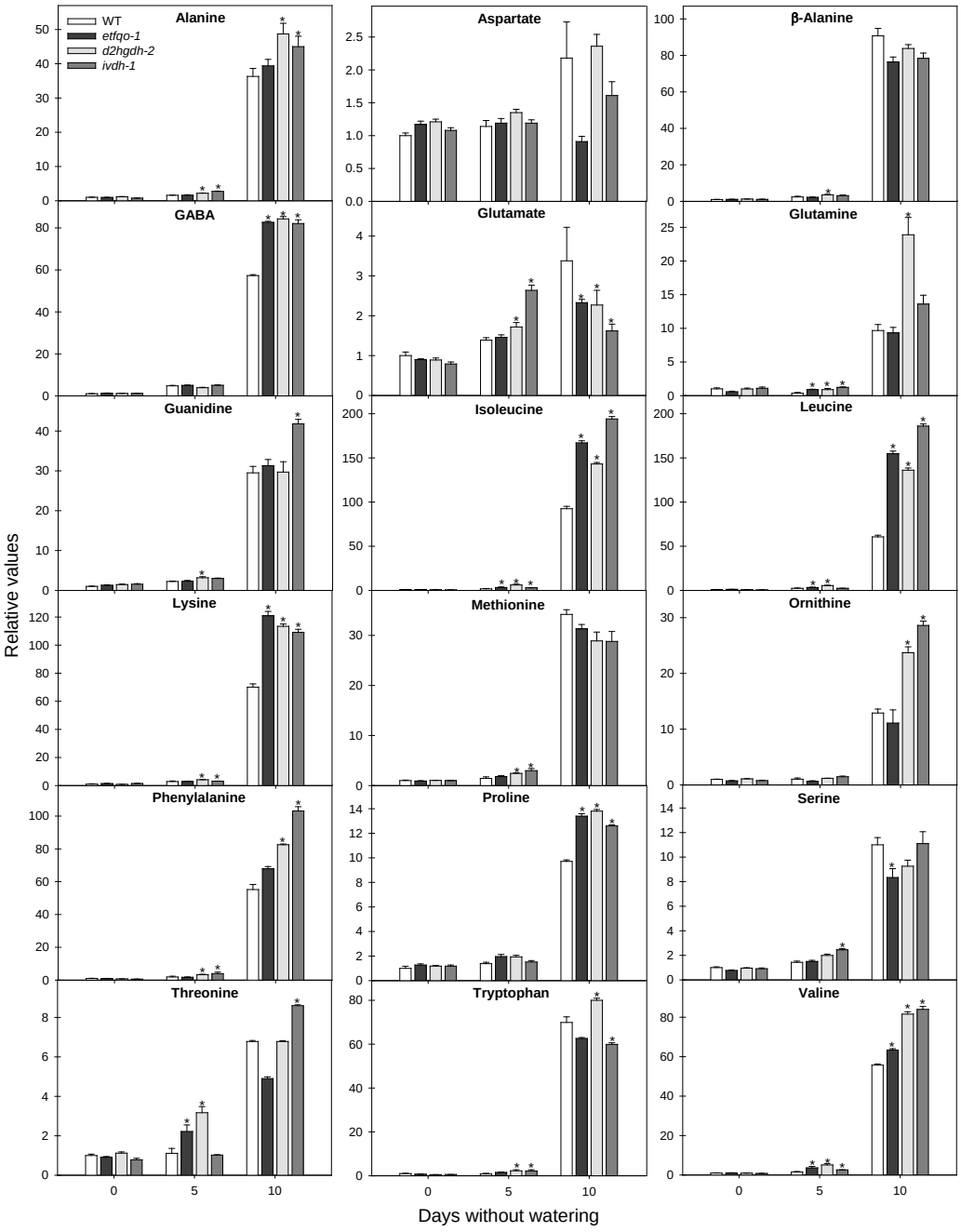


Figure 12. Relative levels of amino acids in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. The *y*-axis values represent the metabolite level to the wild type. Data were normalized to the mean response calculated for the 0 day drought-treated leaves of WT. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from WT

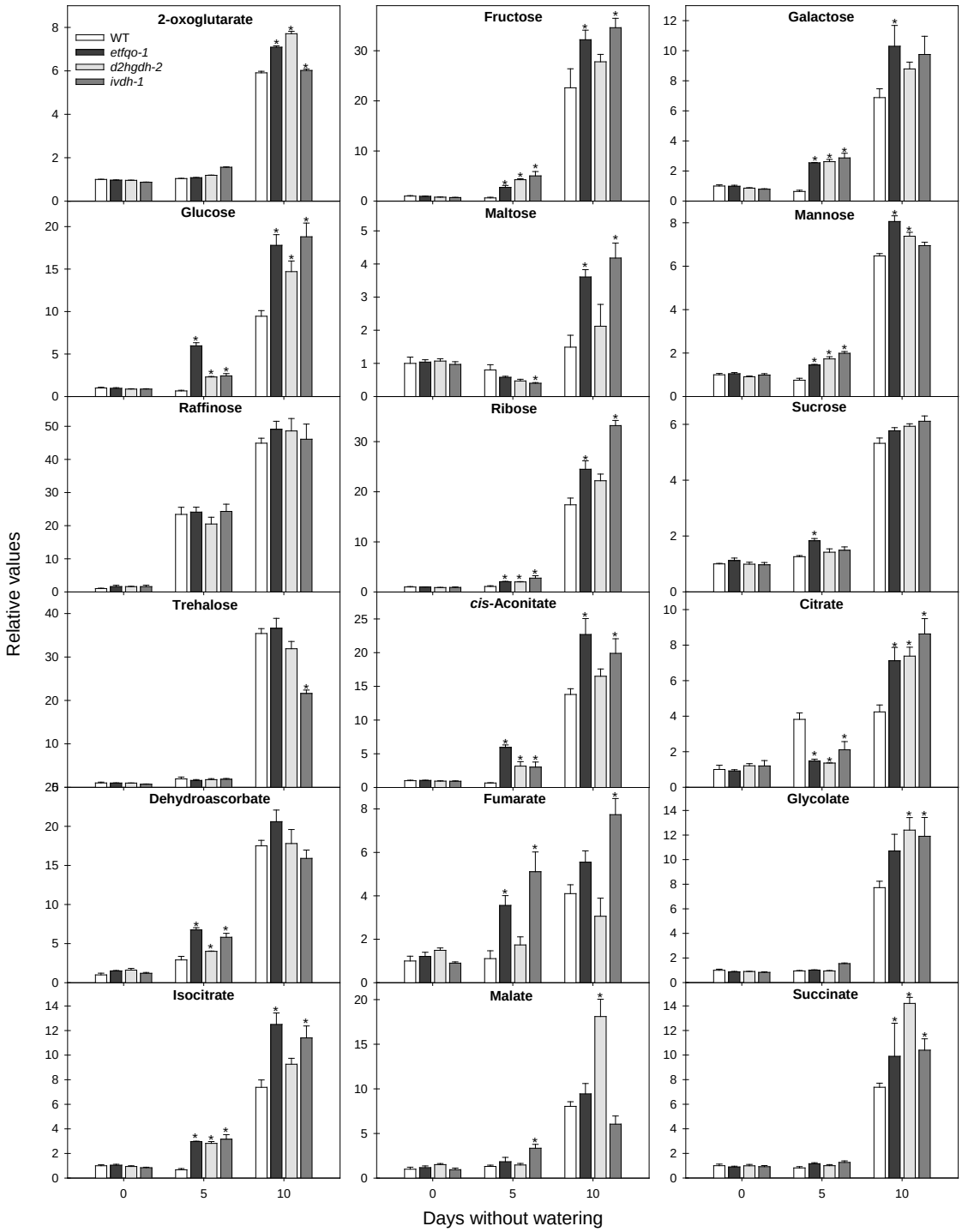


Figure 13. Relative levels of sugars and organic acids in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Levels of the indicated sugars and organic acids are presented as in Figure 12

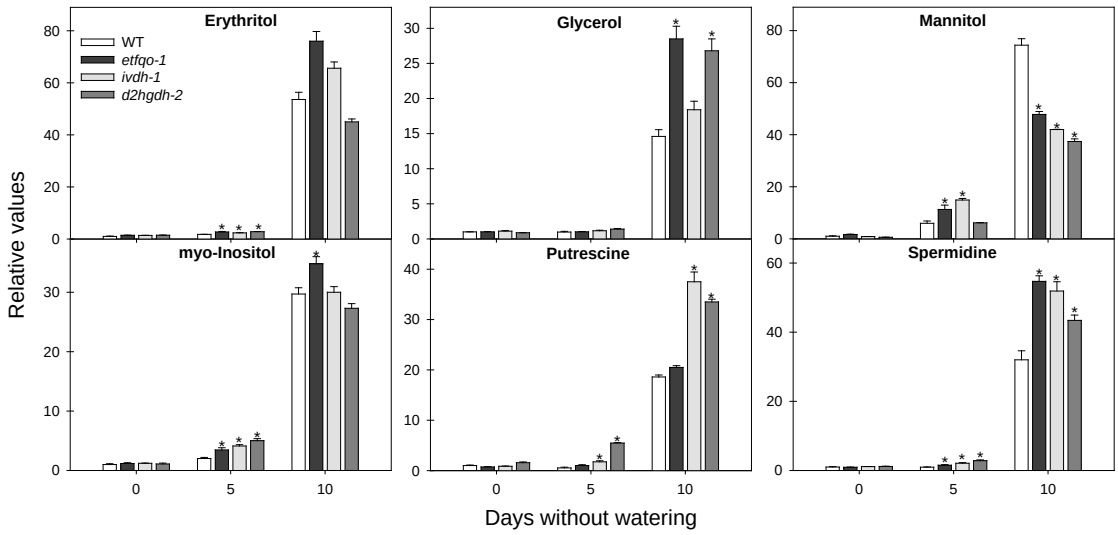


Figure 14. Relative levels of sugar alcohols and polyamines in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Levels of the indicated sugar alcohols and polyamines are presented as in Figure 12

Analyzing the metabolite profiling of OE lines the identification and quantification of a total of 47 metabolites, including 18 amino acids, 10 sugars, 11 organic acids, 5 sugar alcohols, 2 polyamines, and 1 fatty acid were possible. The data obtained are summarized and displayed in false color as a heat map (Figure 15) in order to provide an easy overview and the relative values of specific metabolites were presented in Figure 16-18. The full data set is additionally provided as Supplemental Table S3.

The levels of most of metabolites were increased 10 days after the irrigation suspension in OE lines. It should be mention, however, that the extension of changes in those lines was clearly lower than those observed in KO mutants. Moreover, a clear pattern of down regulation of levels of some metabolites in those lines (Figure 15) in comparison to those pattern verified in KO mutants (Figure 11) was clearly observed.

The OE lines showed significant increases in the levels of aspartate (1.3-3.2 x-fold), GABA (86-94 x-fold), guanidine (4-5.7 x-fold), ornithine (20-25 x-fold), proline (15-18 x-fold), threonine (7-8.4 x-fold), tryptophan (9-21 x-fold), and valine (87-104 x-fold). Significant increased levels of some sugars, such as maltose (2.6-4.4 x-fold), raffinose (14-20 x-fold), and sucrose (7.8-9.6 x-fold) were also observed in

all OE lines, while the levels of trehalose decreased at the end of experiment (Figure 17).

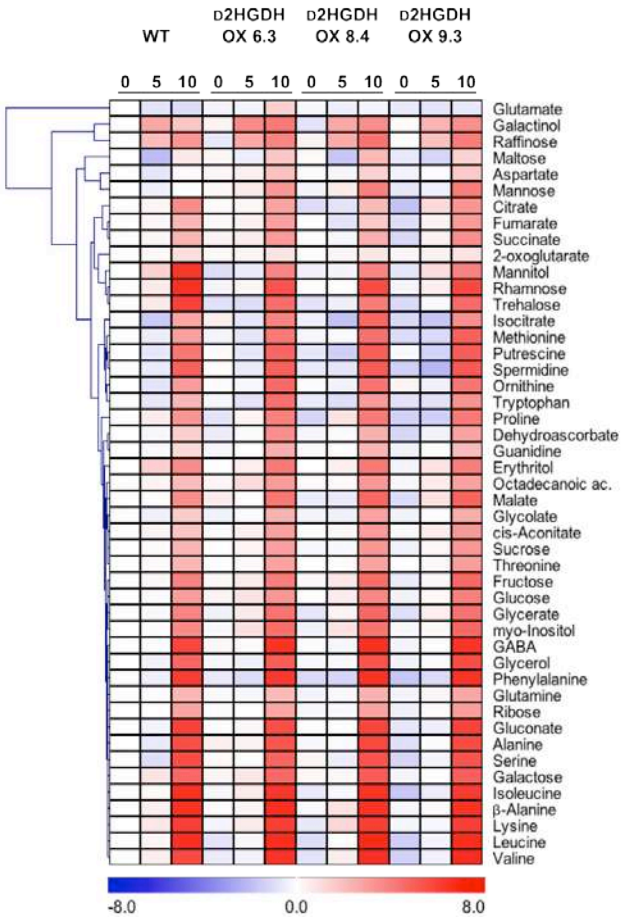


Figure 15. Heat map of metabolite levels in *Arabidopsis* overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Red colors represent increase in metabolite levels; blue colors highlight decreases using a false-color scale. Values are means of six independent samplings and are representative of the log2-transformed values

Interestingly the levels of the majority of organic acids in OE lines (Figure 17) increased after 10 days without watering as observed in KO mutants (Figure 13). In such case, increments in cis-aconitate, dehydroascorbate, glycolate, isocitrate, and malate were observed in all OE lines. Furthermore, similarly to the situation observed in KO mutants, water restriction promoted increases in sugar alcohols in OE lines, such as glycerol (33-51 x-fold) and myo-inositol (20-27 x-fold), as well as

polyamines, such as putrescine (28-34 x-fold) and spermidine (31-36 x-fold) (Figure 18).

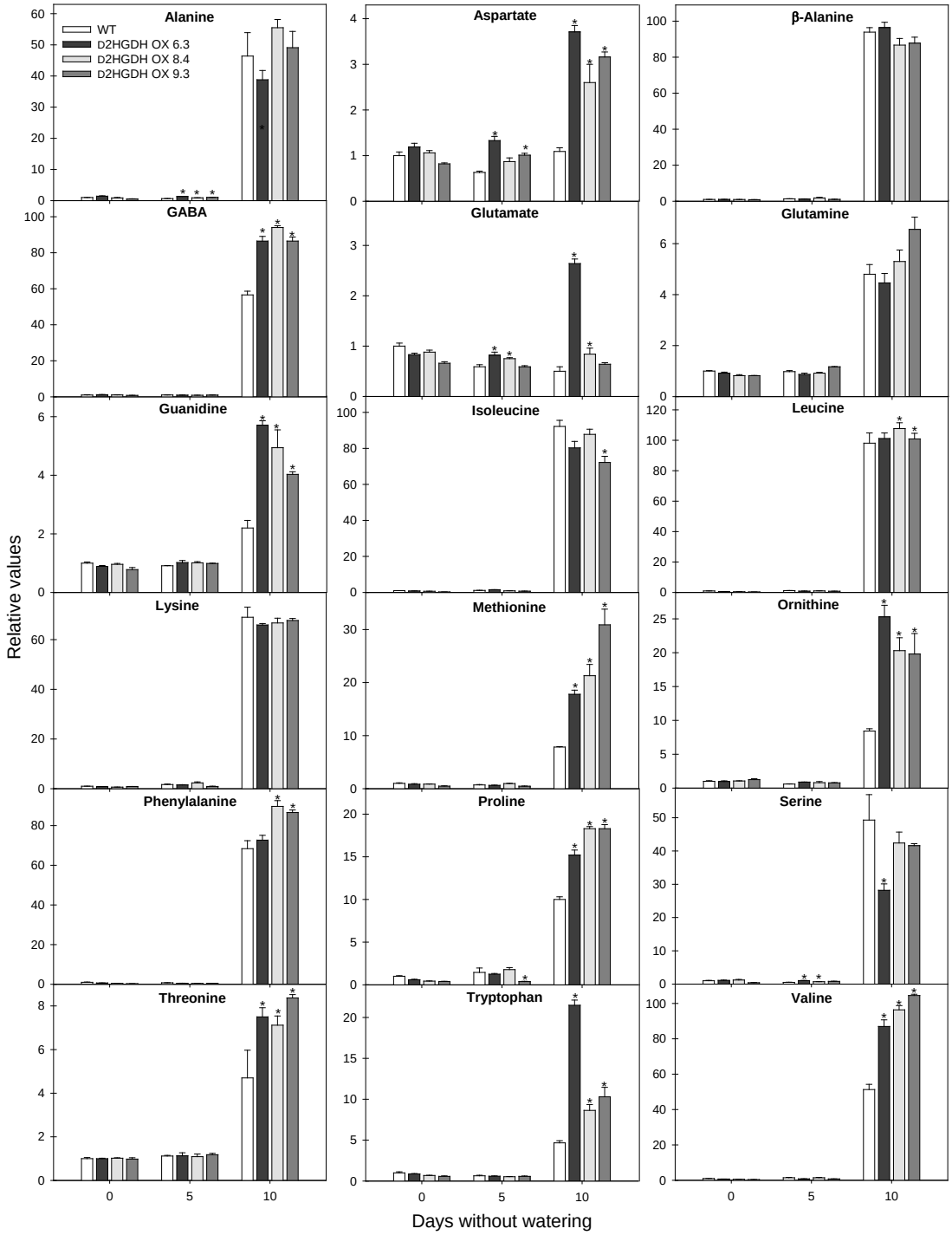


Figure 16. Relative levels of amino acids in *Arabidopsis* overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Levels of the indicated amino acids are presented as in Figure 12

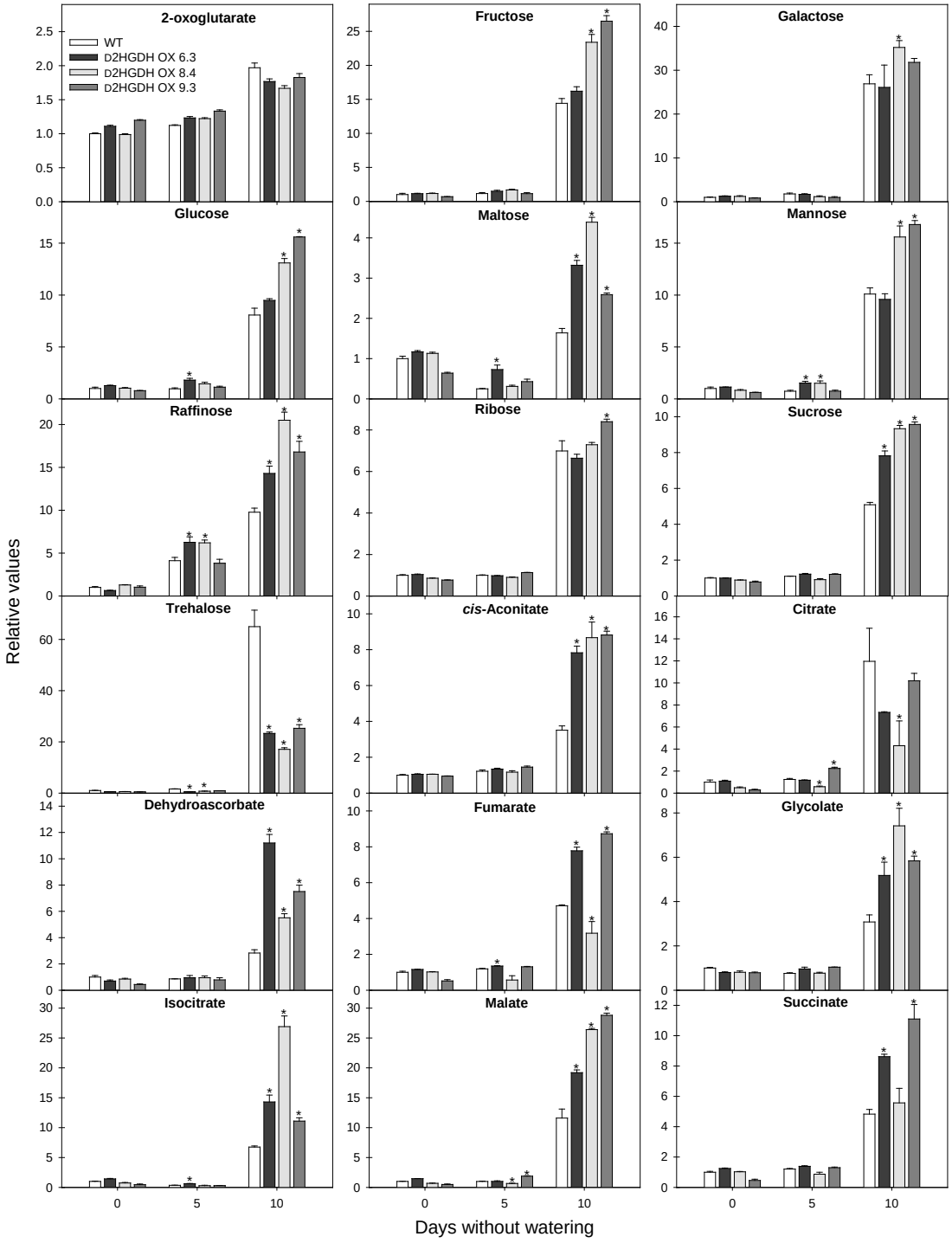


Figure 17. Relative levels of sugars and organic acids in *Arabidopsis* overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Levels of the indicated amino acids are presented as in Figure 12

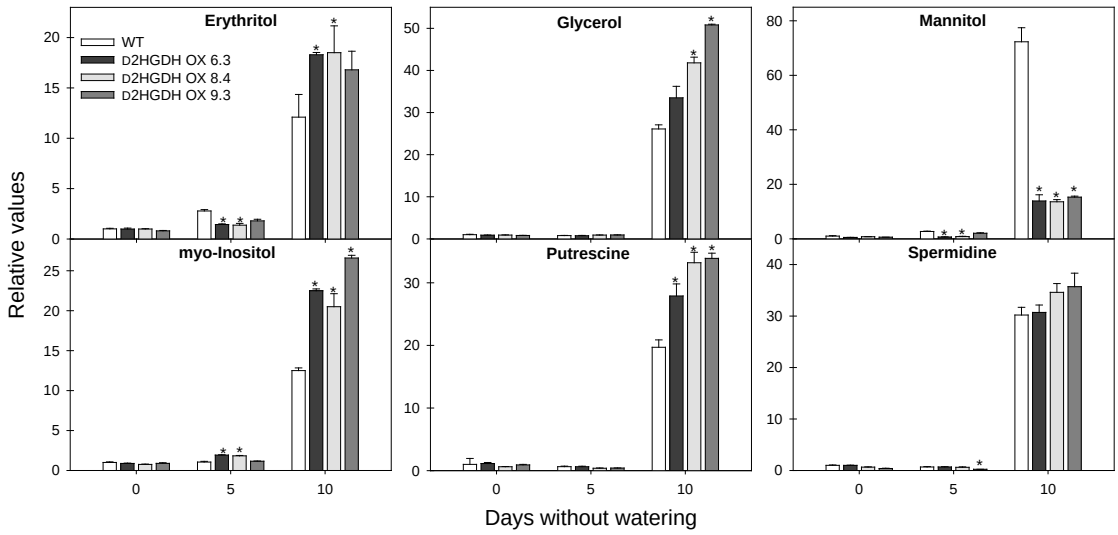


Figure 18. Relative levels of sugar alcohols and polyamines in *Arabidopsis* overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Levels of the indicated amino acids are presented as in Figure 12

4.4. Gene expression of *Arabidopsis* mutants under water deficit conditions

In order to evaluate changes in the gene expression at the beginning of the experiment and after withholding water for 5 and 10 days, the expression of selected genes of KO mutants and WT plants was also determined (Figures 19 and 20). First, a down regulation of photorespiration related genes, such as 2-phosphoglycolate phosphatase 1 (*PGLP1*) and both serine hydroxymethyl transferase 1 (*SHMT1*) and 2 (*SHMT2*) was observed. More specifically, transcript levels of *SHMT1* and *SHMT2* were not detected after 10 days without watering in all genotypes. Moreover, a dramatic decrease of expression of genes related to oxidative stress alleviation (peroxissomal catalase 2, *CAT2*), photosynthesis (rubisco activase, *RCA*), alternative respiration pathways (uncoupling protein, *UCP*), and metabolism of nitrogen and amino acids (ferredoxin-dependent glutamate synthase, *Fd-GOGAT*; glutamate dehydrogenase 1, *GDH1*; and glutamine synthetase, *GS*) was observed. Taken together, these results are in good agreement with the unexpected low levels of photorespiratory encoding genes observed in the present study. Additionally, minor changes in the expression of TCA cycle related genes, such as malate dehydrogenase

(*MDH*) and fumarase 1 (*FUM1*) were observed, most likely to maintain respiratory rates at adequate levels under drought.

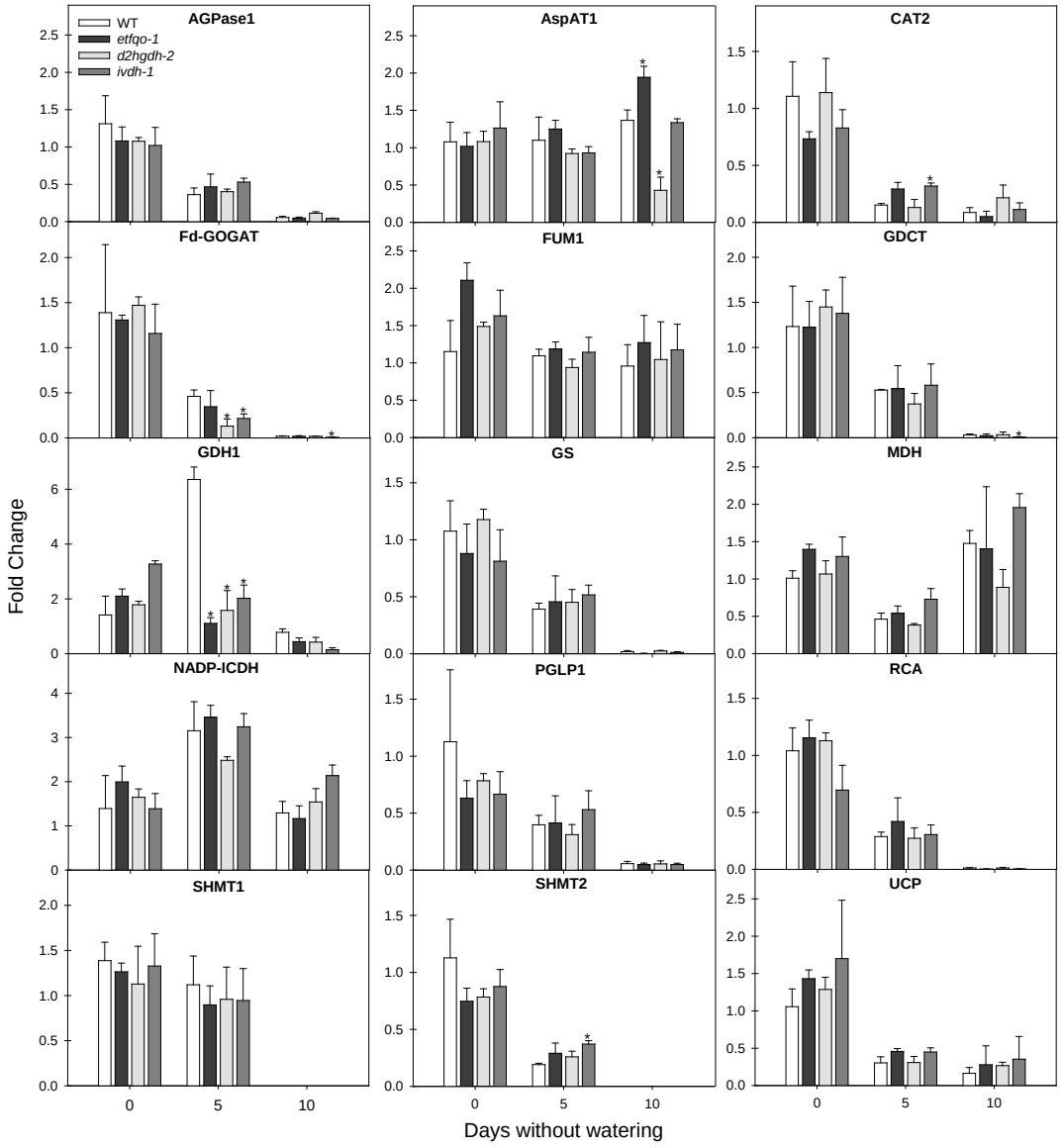


Figure 19. Transcript levels of genes related to amino acids metabolism, photorespiration and TCA cycle in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as quantified by qRT-PCR. The *y*-axis values represent the transcript level to the wild type. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type

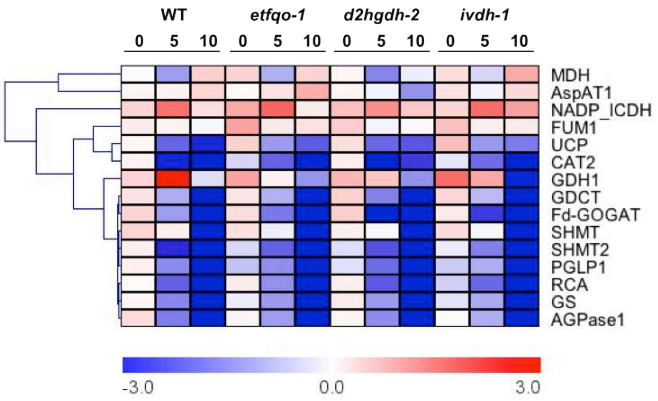


Figure 20. Heat map of transcript levels of genes related to amino acids metabolism, photorespiration and TCA cycle in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as quantified by qRT-PCR. Red colors represent increase in transcript levels; blue colors highlight decreases using a false-color scale. Values are means of six independent samplings and are representative of the log2-transformed values

4.5. Germination assays of *Arabidopsis* mutants under osmotic and salt stresses

Given the range of data obtained here suggesting impairment of metabolic process on drought it was next decided to characterize the germination efficiency and seedling establishment. For this end seeds of KO and OE mutants, as well as WT, were stratified by imbibing at 4°C in the dark for 3 days, and then germination was scored daily for seven days after stratification. As shown in Table 3 and Figure 21, germination rates of all mutants were similar to the values observed for WT seeds under the control treatment. By sharp contrast, osmotic stress induced by mannitol affected the germination efficiency in KO mutants and D2HGDH OX 9.3. Although the differences between OE lines may seem contradictory, the different sites of insertion of the OE construction and enzymatic activities (Engqvist et al., 2011) in those lines may explain, at least partially, such discrepancies observed. Furthermore, salt stress induced by NaCl affected the germination only in KO mutants.

Given the high accumulation of BCAA observed in the mutant lines it was next decided to evaluate the impact of lysine and valine on seed germination. Surprisingly, a dramatic decrease in germination of seeds of all genotypes was

observed after the further treatment with increasing concentrations of lysine (Table 3). More specifically, germination was not detected in KO mutants while a lower germination (around 6%) in both WT and OE lines was observed in presence of 100 mM of lysine. Finally, the treatment with the BCAA valine influenced the germination efficiency in a lower extension than the lysine treatment. This is in good agreement with previous studies demonstrating that the significant elevation of lysine levels in *Arabidopsis* seeds, by either enhancing its synthesis or blocking its catabolism, causes a retardation of germination (Zhu and Galili, 2003).

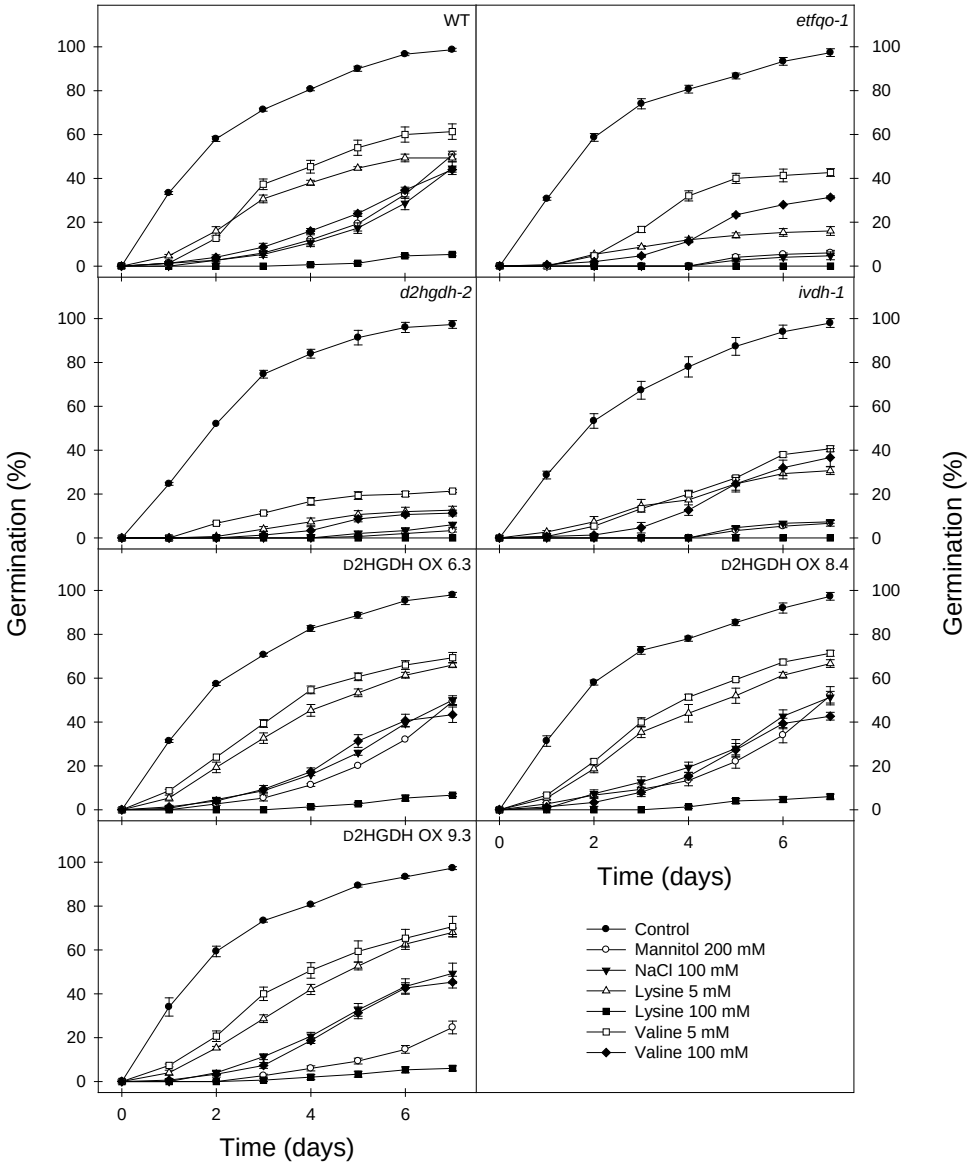


Figure 21. Germination of *Arabidopsis* mutants and wild type (WT) seeds. Values are means ± SE of three independent samplings

Table 3. Germination of *Arabidopsis* mutants and wild type (WT) seeds. Values are means ± SE of three independent samplings. Bold numbers indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT)

Treatments	**Genotypes**						
	WT	***etfqo-1***	***d2hgdh-2***	***ivdh-1***	**D2HGDH OX 6.3**	**D2HGDH OX 8.4**	**D2HGDH OX 9.3**
Control	98.7 ± 0.7	97.3 ± 1.8	97.3 ± 1.8	98 ± 2	98 ± 1.2	97.3 ± 1.8	97.3 ± 0.7
Mannitol 200 mM	50.7 ± 1.8	**6 ± 1.2**	**3.3 ± 0.7**	**6.7 ± 1.3**	49.3 ± 1.8	52 ± 4.2	**24.7 ± 2.9**
NaCl 50 mM	52 ± 1.2	**30.7 ± 2.9**	**6.7 ± 0.7**	**31.3 ± 1.3**	50.7 ± 0.7	52.7 ± 1.8	51.3 ± 1.8
NaCl 100 mM	44.7 ± 2.9	**4.7 ± 1.8**	**6 ± 1.2**	**7.3 ± 0.7**	50 ± 2	51.3 ± 2.7	49.3 ± 4.7
Lysine 10 mM	62.7 ± 1.3	**15.3 ± 2.4**	**17.3 ± 0.7**	**37.3 ± 3.3**	56 ± 2	59.3 ± 3.3	60.7 ± 1.8
Lysine 50 mM	26.7 ± 1.8	**3.3 ± 0.7**	**1.3 ± 0.7**	**4.7 ± 0.7**	25.3 ± 3.7	26 ± 2.3	24.7 ± 2.9
Lysine 100 mM	5.3 ± 0.7	**0 ± 0**	**0 ± 0**	**0 ± 0**	6.7 ± 0.7	6 ± 1.2	6 ± 1.2
Valine 10 mM	62.7 ± 0.7	**41.3 ± 2.7**	**27.3 ± 1.8**	**42 ± 3.1**	**50.7 ± 1.8**	54.7 ± 1.8	52.7 ± 3.7
Valine 50 mM	42.7 ± 2.4	**32.7 ± 1.8**	**16 ± 3.1**	35.3 ± 2.4	45.3 ± 2.9	44.7 ± 1.8	44.7 ± 1.3
Valine 100 mM	44 ± 1.2	**31.3 ± 0.7**	**11.3 ± 1.3**	36.7 ± 4.1	43.3 ± 3.5	42.7 ± 1.8	45.3 ± 2.7

5. DISCUSSION

Both plant growth and development are negatively affected by environmental stresses, such as drought. However, plants respond and adapt themselves to continuous environmental fluctuations with suitable physiological, developmental and biochemical changes in order to cope with these stress conditions. Interestingly, one of those adaptive mechanisms observed here was the growth arrest of all genotypes during water deficit, mainly observed in KO mutants (Figure 5). It should be mentioned, however, that arrest of plant growth during stress conditions largely depends on the severity and speed of the stress (Bartels and Sunkar, 2005), allowing cellular essential solutes to be diverted from growth requirements to stress-related functions (Yang et al., 2010). Mild osmotic stress leads rapidly to growth inhibition of leaves and stems, whereas roots may still continue to elongate (Nonami and Boyer, 1990; Spollen et al., 1993). In addition, drought-induced cessation of shoot growth is accompanied by the inhibition of leaf initiation and by accelerated senescence of older leaves (Vanková et al., 2012). Growth arrest can be viewed as a mechanism to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief (Bartels and Sunkar, 2005). Therefore, growth recovery after water supply which was only observed in WT and OE lines (Figure 8), suggest that the catabolism of BCAA might be of a high importance under stress situations, as previously suggested (Araújo et al., 2011d). The inhibition of shoot growth during water deficit is likely to contribute to solute accumulation, such as amino acids, sugars and polyamines (Figures 12-14), and thus eventually to osmotic adjustment (Osorio et al., 1998). Although root growth was not

evaluated here, the maintenance of root growth under drought stress is an adaptive mechanism that facilitates water uptake from deeper soil layers (Bartels and Sunkar, 2005) and should be considered in further studies.

In addition to the growth arrest detected another common mechanism observed in the present study was the increased water loss evaluated either by detached leaves (Figure 6A) or relative water content (Figure 6B) which was strongly observed in KO plants. Drought stress often leads to cellular dehydration, which causes osmotic stress and therefore removal of water from the cytoplasm to the extracellular space resulting in a reduction of both cytosolic and vacuolar volumes (Bartels and Sunkar, 2005). Additionally, water deficit can result in a concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradients, loss of turgor, disruption of membrane integrity, and denaturation of protein (Bray, 1997; Hoekstra et al., 2001; Yang et al., 2010; Alcázar et al., 2011; Chen and Murata, 2011). Moreover, complete loss of free water will result in desiccation or dehydration of whole plant. In agreement the results presented here indicate that the damages in membrane integrity as well as dehydration process were much more drastic in KO mutants when compared to WT and OE lines (Figure 5 and Figure 6).

Photosynthesis is generally reduced under water deficit situations through stomatal closure or metabolic impairment (Reddy et al., 2004). Accordingly stomatal closure under drought is most likely an avoidance strategy adopted by plants to save water and maintain cell turgor (Chaves and Oliveira, 2004; Skirycz and Inze, 2010), resulting in reduced rates of net C assimilation as well as transpiration. After five days without watering, mild drought affected the RWC (Figure 6B) and F_v/F_m ratio values (Figure 7D) only in KO mutants. On the other hand, under prolonged water restriction (10 days), all genotypes showed a drastic decrease in RWC associated to impairment in F_v/F_m values. F_v/F_m is commonly reported as a good indicator of the effect of environmental stress in photosynthesis. Thus under normal growth conditions F_v/F_m varied from 0.75 to 0.85, showing efficient conversion of light energy at PSII level (Baker, 2008). Altogether, the fact that RWC (Figure 6B), F_v/F_m ratio (Figure 7D), and even chlorophyll content (Figure 7A) decline abruptly during the water stress suggests an orchestrated down-regulation of the whole photosynthetic metabolism (Cornic et al., 1989; Xue et al., 2008). Taken together, those phenotypic and senescence-related results indicated that *ETFQO*, *IVDH* and

D2HGDH genes and therefore mitochondrial metabolism seem to play a significant role in drought-tolerance in *Arabidopsis*. Similar results observed for these genotypes in other common physiological circumstances experienced by most plants at some stages of their life cycle, such as extended darkness, cold, continuous light, and short days conditions (Ishizaki et al., 2005, 2006; Araújo et al., 2010) are highly indicative of the importance of this alternative pathway in the tolerance of plants to several stress situations. Moreover, recent evidences suggest the operation of this alternative respiratory pathway in the dark period of a normal light-dark cycle in photosynthetic tissues of *Arabidopsis* (Caldana et al., 2011; Engqvist et al., 2011). Thus it is reasonable to assume that the ETF/ETFQO pathway functions as an alternative electron donation pathway in plants under stress and is most likely an evolutionary and conservative mechanism of high importance particularly during environmental stress conditions. Additional evidence that mitochondrial metabolism is of pivotal important under stress situation has been recently discussed in detail elsewhere (Jacoby et al., 2011, 2012)

The recovery following re-irrigation was able to restore vegetative and reproductive growth in both WT plants and OE lines, while KO mutants did not show any sign of recovery (Figure 8). When water shortage is relieved, the plants need to restart growth as quickly as possible and this requires modulation of the normal senescence program (Vanková et al., 2012). Interestingly, OE lines exhibited also a mild delay in the senescence during the drought treatment as observed by the lower decline of F_v/F_m and a faster recovery of growth similarly to WT plants. Stress recovery is a highly complex process involving rearrangements not only transcriptional but also at protein and metabolite levels. The OE and WT leaves rapidly repaired their photosynthetic machinery after stress recovery, as observed by the increased F_v/F_m values (Figure 9B). This will most likely culminate in enhanced C assimilation enabling the re-establishment of plant growth. Furthermore, WT plants and OE lines were able to increase their RWC at the beginning of the stress recovery (Figure 9A). On the other hand, KO plants, in which senescence is advanced, were not able to restore growth. A general metabolic impairment is likely to be the main reason of this behavior in KO mutants under more severe stress. At this stage, photosynthesis recovery upon re-watering was incomplete, disabling those plants to recover growth. When considered together, these results indicated that impairment in electron transfer to mETC mediated by the ETF/ETFQO pathway is

important for plants to withstand drought as well as to recover growth after re-watering.

Drought stress induces an extensive metabolic reprogramming in plants (Alvarez et al., 2008; Charlton et al., 2008; Sanchez et al., 2011; Bowne et al., 2012; Witt et al., 2012). Not surprisingly some of these changes were clearly observed in this study, such as increase in total amino acids levels, as well as decreases in protein, starch and nitrate contents (Figure 10). It is well known that almost all organisms, ranging from microbes to animals and land plants, synthesize compatible solutes (*e.g.* amino acids, sugars) in response to water stress (Burg et al., 1996). On the other hand, drought induces metabolic changes related to protein turnover, such as increased protein degradation (Bray, 1997). In addition it is often reported that drought increases the risk of oxidative stress in plants by increasing the production of ROS in different cellular compartments (Bartoli et al., 2004). It has been also demonstrated that degradation of oxidatively damaged proteins will generated specific peptides that can act as secondary ROS messengers and could contribute to retrograde ROS signaling during stress (Møller and Sweetlove, 2010). Yet, the contribution of protein breakdown to the pool of amino acids during the relatively early response to abiotic stresses is generally more limited than that occurring in both senescence and programmed cell death, in which there is massive protein degradation (Less and Galili, 2008). Thus, protein degradation (Figure 10A) could be associated to the increase in amino acids levels, especially in KO mutants (Figure 10B). It is important to mention that the link between protein degradation and carbon metabolism during drought stress has been uncharacterized in plant systems. The results presented here suggest a metabolic strategy in which protein degradation can contribute to carbon utilization and plant growth maintenance during drought episodes. However, it should be pointed that protein is a less efficient respiratory substrate than carbohydrate in plants (Araújo et al., 2011d).

By contrast to the situation observed previously (Foyer et al., 1998; Bray, 2002), decreased levels of nitrate were observed in all genotypes, especially in KO mutants following 10 days without watering (Figure 10C). The decreased level of nitrate seems to be a consequence of protein breakdown and as such N is likely being reallocated for the synthesis of amino acids (Figure 10B). By assuming that nitrate was not excreted under conditions of water stress (Venekamp et al., 1989), the difference in nitrate content in the plant at the beginning and that at the end of the

water-withholding period may be caused by a conversion of nitrate into drought-induced amino acids, such as proline, aromatic amino acids and BCAA (Figures 12 and 16). Therefore, high levels of these amino acids were observed in all genotypes in this study. It is important to mention that KO plant lines used in the present study have impairments in the degradation of BCAA and therefore increased levels are in fact expected. When taken together these changes are highly consistent with what would be expected at a global level following restriction of energy metabolism and result in a co-ordinated regulation of many aspects of protein synthesis, amino acid metabolism, and N metabolism all of which would be anticipated to participate in the observed shifting of the cellular metabolism in response to water limitation.

In *Arabidopsis*, more than 50% of the photosynthate is stored as starch (Zeeman and Rees, 1999). The depletion of starch content during water stress (Figure 10D) is probably due to decreased photosynthesis rates. Intriguingly, starch was not detected in *d2hgdh-2* and *ivdh-1* mutants after 10 days without watering, indicating high damage in the photosynthetic apparatus of those plants. This is further supported by the lack of growth of those plants when the irrigation recovery takes place (Figure 8). Depletion in starch content in *Arabidopsis* under moderate drought conditions has been previously observed (Harb et al., 2010). The same authors reported an increase in mRNA levels of genes α- and β-amylase amongst others related to starch biodegradation (Rizhsky et al., 2004; Harb et al., 2010). Moreover, starch hydrolysis, which requires activities of hydrolytic enzymes, mostly results in increased soluble sugars contents in leaves, such as hexoses (Kaplan and Guy, 2004; Bartels and Sunkar, 2005; Basu et al., 2007; Kempa et al., 2008) in good agreement with the results also observed in the present study (Figures 12 and 17). On the other hand, some works have suggested that fumarate can act as an alternative and potentially flexible carbon sink for photosynthate similar to starch (Chia et al., 2000; Fahnenstich et al., 2008; Gibon et al., 2009; Araújo et al., 2011b). Thus, the increased levels of fumarate observed mainly in *etfqo-1* and *ivdh-1* following 5 days without watering (Figure 13) is likely an indicative of a strong stress-related switch in photosynthate storage in these mutant lines. In good agreement with the results presented here it remains highly possible that the engineering of fumarate metabolism may provide opportunities to improve not only plant growth but also plant performance under stress situations.

Plants can perceive abiotic stresses and elicit appropriate responses involving altered gene expression and metabolism as well as growth and development. The regulatory circuits include stress sensors and/or receptors, signaling pathways comprising a network of protein-protein interactions, transcription factors and promoters, and finally the output proteins or metabolites (Bartels and Sunkar, 2005). In the present study several changes in metabolite levels, such as amino acids, sugars, organic acids and polyamines (Figures 11-18) were observed. Accumulation of amino acids has been observed in many plants exposed to drought (Less and Galili, 2008; Urano et al., 2009; Lugan et al., 2010; Virlouvet et al., 2011; Bowne et al., 2012; Witt et al., 2012), although the current knowledge of the role of this effect in stress tolerance remains fragmentary. Amino acids are amongst the most important metabolites within living systems. Not only do they serve as the basic components of proteins, but they are also intermediates of metabolic pathways leading to the synthesis of multiple primary and secondary metabolites serving diverse functions including, amongst others, energy homeostasis and the response of plants to various abiotic and biotic stresses (Araújo et al., 2011d). The increase in amino acids might stem from amino acid synthesis and/or from enhanced stress-induced protein breakdown (Krasensky and Jonak, 2012). While the overall accumulation of amino acids upon stress might indicate cell damage in some species (Widodo et al., 2009), increased levels of specific amino acids should have a beneficial effect during stress acclimation (Krasensky and Jonak, 2012). Furthermore, it was recently shown that the survival of yeast cells when starved for various amino acids is correlated with oxidative stress response and the operation of non-respiratory mitochondrial functions (Petti et al., 2011). In sharp contrast to situation observed in yeast, the systems-level interactions of amino acid metabolism with other biological networks in plants are still largely unknown.

Under several abiotic stress conditions (*e.g.* cold, salt, and drought) proline accumulates and functions as an osmoprotectant (Apse and Blumwald, 2002; Zhu, 2002). However, proline did not accumulate to high levels in all genotypes in this study when compared to other amino acids such as GABA and BCAA (Figures 12 and 16). On the other hand, glutamine levels were specifically elevated in *d2hgdh-2* after 10 days without watering (Figure 12), suggesting that proline biosynthesis is likely inhibited and glutamate is converted to glutamine instead of proline during the stress. It has been suggested that plants that were engineered to overaccumulate

proline in order to enhance their tolerance to abiotic stress (Kishor et al., 1995; Nanjo et al., 1999; Nuccio et al., 1999; Rontein et al., 2002) might not be resistant to field conditions (Deuschle et al., 2001; Rizhsky et al., 2004), especially because proline can also be toxic to cells if it is not properly removed (Hellmann et al., 2000; Deuschle et al., 2001; Mani et al., 2002; Nanjo et al., 2003). Moreover, it has been reported that the effect of proline during dehydration tolerance may be smaller than that of some sugars (Hoekstra et al., 2001). It seems tempting to speculate that, at least in *Arabidopsis*, proline might not act as an osmoprotector. Further studies are clearly deserved to elucidate the role of this amino acid under stress situations in several plant species.

It is reasonable to assume that both aspartate and glutamate are mainly used as N and C donors for the biosynthesis of amino acids and organic acids which could explain, at least partially, their low levels particularly in KO mutants (Figure 12). Those amino acids are central regulators of C/N metabolism, interacting with multiple metabolic networks (Less and Galili, 2008). Accordingly recent studies have suggested interplay between the so-called GABA shunt and the TCA cycle through several bypasses (Fait et al., 2008). It is well known that GABA is mainly metabolized via succinic semialdehyde dehydrogenase to succinic acid, thus fueling the TCA cycle via the GABA shunt (Bown and Shelp, 1997; Studart-Guimarães et al., 2007). The metabolic profiling data presented here allows the speculation that the relatively small accumulation of proline coupled to decreases in glutamate level in KO mutants (Figure 12) promoted an activation of the GABA shunt under dehydration in response to an increased demand for TCA cycle intermediates to maintain the synthesis of secondary metabolites (Kinnersley and Turano, 2000). Furthermore, abiotic stress seems to increase cytosolic Ca^{2+} concentrations, which stimulates calmodulin-independent GABA decarboxylase (GAD) activity and further GABA synthesis (Bouche and Fromm, 2004). Although caution should be taken with this information evidence has demonstrated that among all five *GAD* homologs present in the *Arabidopsis* genome, *GAD4* was the most highly expressed gene under hypoxia in roots (Miyashita and Good, 2008) suggesting the importance of this pathway under abiotic stress. Further analysis of these metabolic pathways is clearly required although this could explain, at least partially, the increased GABA levels in OE lines (Figure 16), since it was observed minor changes in glutamate as well as glutamine levels in these genotypes.

It is important to note that aromatic amino acids, such as tryptophan, phenylalanine, and tyrosine, which are synthesized via the shikimic acid pathway, function as precursors of a great variety of secondary metabolites, such as glycosides, lignin and terpene precursors (Pichersky et al., 2006; Korkina, 2007; Tempone et al., 2007; Less and Galili, 2008). Surprisingly Witt et al. (2012) have observed that the levels of tryptophan and phenylalanine also increased in maize under water deficit. In this way, tryptophan seems to provide a buffer between ROS and proteins inside the chloroplast, thus functioning as an osmoprotectant under water deficit situations (Bowne et al., 2012). Moreover, several studies have suggested an important role of BCAA (*e.g.* leucine, isoleucine, and valine) in the metabolism of plants under water deficit conditions. For instance, Urano et al. (2009) have observed that in *Arabidopsis* the level of BCAA increased under drought conditions and that such increase seems to be regulated at a transcriptional level. Additionally the activities of some enzymes related to the catabolism of BCAA showed rapid increase in response to abiotic stresses (Less and Galili, 2008) and, therefore it is reasonable to suggest that those enzymes might play an important role in the metabolism of BCAA under stress situations. In addition, transgenic maize plants over-expressing a gene that encodes an ABA-, stress-, and ripening-induced protein (*ZmASR1*) showed higher tolerance to water deficit than its WT counterpart (Virlouvet et al., 2011). Remarkably, those plants presented significant decrease in the levels of BCAA under conditions of limited water availability. It was suggested that the increased biomass observed in those plants is related with the transcriptional regulation of genes involved in the biosynthesis of BCAA (Virlouvet et al., 2011), which might indicate that the degradation of such amino acids is intimately related with increased water-stress tolerance in those plants. Similar results were also observed in the present study and provide compelling evidence for a fundamental role of BCAA in stress tolerance. Notwithstanding whether this is mediated by either an osmoprotectant effect or increased usage of those amino acids as alternative source of energy remains to be fully elucidate.

Among the amino acids detected in metabolic profiling, BCAA were the most affected by drought, especially in KO mutants (Figure 12 and 16). Isoleucine biosynthesis is highly coordinated with both leucine and valine biosynthesis (Less and Galili, 2008). Although there is now evidence for a further mitochondrial BCAA transaminase (BCAT) (Kochevenko et al., 2012), the BCAA biosynthesis seems to

occur exclusively in plastids where valine and isoleucine are formed in two parallel pathways using four common enzymes, namely acetohydroxy acid synthase (AHAS), ketolacid reductoisomerase (KARI), dihydroxy-acid dehydratase (DHAD), and BCAT, while leucine synthesis branches off from 2-oxoisovalerate, the last intermediate of the valine biosynthetic pathway, to follow a three-step chain elongation catalyzed by isopropylmalate synthase (IPMS), isopropylmalate isomerase (IMPI), and isopropylmalate dehydrogenase (IPMDH), which ends with a transamination catalyzed by a BCAT (Binder et al., 2007; Joshi et al., 2010). Interestingly the transcripts of the *ZmAHAS1*, *ZmKARI1*, *ZmKARI2* and transaminase *ZmBCAT4* were up-regulated in maize under drought (Virlouvet et al., 2011), indicating a fine tuning of biosynthetic genes under the abiotic stress condition. This is in good agreement with the results observed in the present study and highlights the essential role of the BCAA (and protein degradation) under stress situations (Araújo et al., 2011d).

The biosynthesis of amino acids in plants is commonly regulated by end product feedback inhibition loops in which enzymes in a given amino acid biosynthesis pathway are feedback inhibited by the amino acid product that they synthesize (Galili, 1995; Radwanski and Last, 1995). Consequently, the potential roles of downstream catabolic enzymes that convert amino acids into other metabolites in the regulation of fluxes of amino acid metabolism under specific physiological conditions have been largely ignored (Less and Galili, 2008). However, it is important to mention that the mRNA levels of several catabolic enzymes of the amino acids are highly regulated by developmental, metabolic, and environmental cues, and seem to be stimulated much more frequently than biosynthetic genes (Galili et al., 2001; Galili, 2002; Mikkelsen et al., 2003; Stepansky and Galili, 2003; Jander et al., 2004; Less and Galili, 2008).

The branched-chain keto-acid dehydrogenase complex (BCKDC) is involved as an early step in the catabolism of the BCAA in mitochondria (Yeaman, 1989; Diebold et al., 2002). In this pathway, the three BCCA are firstly transaminated to their respective branched-chain keto acid by BCAT. This reversible reaction is also the final step of the biosynthesis of these amino acids (Taylor et al., 2004). After the transamination step, the keto acids are decarboxylated and esterified to CoA by the BCKDC. The CoA esters generated (*e.g.* isovaleryl-CoA) are then oxidized by an acyl-CoA dehydrogenase delivering electrons to the electron transfer flavoprotein

(ETF) that directly donates electrons into the mETC at ubiquinone, as previously demonstrated (Ishizaki et al., 2005, 2006; Araújo et al., 2010). The expression of the BCKDC *E2*, *E1α*, and *E1β* genes is induced by some stress conditions such as darkness, mannitol, photosynthetic inhibitors, and sugar starvation being also elevated during leaf senescence (Fujiki et al., 2000, 2001). Thus, BCAA promote their own catabolism mainly when plant cells are sugar starved and they may be alternative sources of respiratory substrates for the TCA cycle during severe plant stress, acting as alternative C sources and/or can act during detoxification process given that BCAA and their respective keto acids are toxic to cells (Taylor et al., 2004). In this way, BCAA can both replenish the TCA cycle and directly feed the mETC, to partly offset the lack of photosynthate under stress conditions (Caldana et al., 2011).

Dehydration-treated plants have a greater need to adjust osmotically, in order to alleviate the loss of cell turgescence. Accordingly several classes of sugar may act as osmolytes, such as monosaccharides (*e.g.* glucose), disaccharides (*e.g.* trehalose), oligosaccharides (*e.g.* raffinose), and some sugar alcohols (*e.g.* mannitol). Trehalose is commonly synthesized in non-vascular plants in response to water deficit, acting as a mechanism of protein and cellular membrane stabilization (Yang et al., 2010). However, the majority of angiosperms, except for those highly desiccation-tolerant, accumulate insignificant amounts of trehalose (Yang et al., 2010). Aiming to increase trehalose biosynthesis in rice (Garg et al., 2002; Jang et al., 2003) and tomato (Cortina and Culianez-Macia, 2005), transgenic plants with genes encoding key biosynthetic enzymes, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), were obtained under the control of tissue-specific, stress-responsive, or constitutive promoters. Garg et al. (2002) and Jang et al. (2003) have observed that, after a period of 8- or 12-days without watering, non-modified rice plants presented leaf wilting and curling symptoms, while transgenic plants showed vigorous vegetative growth and few stress-related visible symptoms. Surprisingly drought induced a dramatic accumulation of trehalose in WT plants in comparison to KO (Figure 13) and OE (Figure 17) lines, indicating that this metabolite seems not to be directly related to stress sensitivity in those mutants lines.

Fructose and glucose levels were increased by drought treatment especially in KO mutants (Figure 13) and this accumulation is likely due to increased hydrolysis of starch (Figure 10D). Those metabolites also present osmoprotection function and,

when related to elevated concentrations of raffinose, seem to jointly act in the dehydration tolerance of wheat seedlings (Bogdan and Zagdanska, 2006). Similarly, Urano et al. (2009) have also observed increased level of fructose in *Arabidopsis* plants under water deficit. On the other hand, sucrose can also replaces proline in plants as the major osmoprotectant during the more severe combined dehydration and heat-stress treatment (Rizhsky et al., 2004). However, sucrose has accumulated in low extension in the present study (Figures 13 and 17) indicating the conversion of this metabolite in some others compounds such as raffinose.

Oligosaccharide such as raffinose may act as water replacements in severe water stress situations, protecting membranes and proteins by forming a hydration shell around macromolecules (Hoekstra et al., 2001). As a result of the sugar interaction, a phase transition during drying is largely prevented, and this is thought to be of pivotal importance for desiccation tolerance by avoiding possible lateral phase separations of membrane components and excessive leakage during rehydration (Wolfe and Bryant, 1999; Hoekstra et al., 2001). Thus, the current accepted hypothesis is that sugars act either as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structures. Sugars are also thought to interact with polar headgroups of phospholipids in membranes so that membrane fusion is prevented. However, it is unknown whether sugars fulfill this function on their own characteristics or in conjunction with other molecules such as LEA proteins (Bartels and Sunkar, 2005).

Increased levels of organic acids as a consequence of water stress were observed here (Figures 13 and 17). In most studies, organic acids and TCA cycle intermediates increased in response to stress such as higher temperature or drought (Kaplan et al., 2004; Usadel et al., 2008; Urano et al., 2009). Interestingly, the highest levels of TCA intermediates (*e.g.* 2-oxoglutarate, citrate, succinate and others) were found in KO mutants (Figure 13), while only significant increases in *cis*-aconitate, isocitrate and malate levels were observed in OE lines (Figure 17). These results seems to indicate that there was an increased need for production of organic acids in drought-stressed plants which is likely to help the maintenance at basal levels of the respiration rates in KO mutants. Venekamp et al. (1989) reported that increases in concentrations of organic acids related to TCA cycle are most likely originated from induced synthesis by dehydration and are directly linked to the proline synthetic pathway, a mechanism which controls the cytoplasmic pH level.

However, the unexpectedly low levels of proline detected in all genotypes indicate that glutamate may be used for the synthesis of GABA instead of proline and that this production of GABA is directly linked to TCA cycle by succinate synthesis (Studart-Guimarães et al., 2007). The results presented here also indicate that during water stress conditions the TCA cycle might be working in a non-cyclic manner, as previously observed under other stress situations (Sweetlove et al., 2010).

Sugar alcohols, such as mannitol and galactinol, are also commonly associated with desiccation protection and as such accumulated in high levels during the water deficit, especially in KO mutants (Figure 14). Abebe et al. (2003) report that, in mild concentrations, mannitol may increase tolerance to both water deficit and salt stress in wheat plants by facilitating the development of biomass under stress conditions. On the other hand, a high accumulation of mannitol generally causes severe abnormalities such as sterility and stunted growth (Abebe et al., 2003; Yang et al., 2010). Interestingly, after 10 days without watering an increase in galactinol levels was observed (see Tables S2 and S3 in Supplemental material). In this case, galactinol might have been used in the synthesis of raffinose, which may thus be used as a source of C storage as well as a compatible solute (Hannah et al., 2006), given that high concentrations of this sugar have been observed under severe water deficit (Figures 13 and 17). Moreover, recent approaches suggests that galactinol plays a novel role in the protection of cellular metabolism, in particular, the photosynthesis, from oxidative damage caused by several types of abiotic stresses (Nishizawa et al., 2008).

Polyamines (*e.g.* putrescine, spermidine, spermine) are small-nitrogenized compounds also involved in plant response to several types of stress, such as water deficit (Alcázar et al., 2011). It was observed high levels of putrescine and spermidine after water deficit, especially in KO mutants (Figure 14), indicating the involvement of those metabolites in water deficit response. These increases were accompanied by increased levels of ornithine, a precursor of putrescine (see Tables S2 and S3 in Supplemental material). Polyamines are well known by their anti-senescence and anti-stress effects due to the neutralization ability and antioxidant properties, as well as the capability of stabilizing membranes and cell walls (Alcázar et al., 2011).

Several genes related to the metabolism of polyamines have been identified and expression profiles have been analyzed under different stress conditions (Alcázar

et al., 2006a). Many authors have reported the increased expression of arginine decarboxylase 2 (*ADC2*), spermidine synthase 1 (*SPDS1*) and spermine synthase (*SPMS*) genes in plants under limited water availability conditions (Urano et al., 2003; Alcázar et al., 2006b). Furthermore, transgenic rice plants over-expressing *ADC* and S-adenosylmethionine decarboxylase (*SAMDC*) genes presented significant increase in putrescine levels, the polyamine precursor of spermidine and spermine (Capell et al., 1998, 2004). Interestingly, increased expression of *ADC2*, *SPDS1* and *SPMS* genes after water deficit seems to be an ABA-dependent response (Alcázar et al., 2006b), once gene expression induction was not observed in *Arabidopsis* deficient (*aba2*) or insensitive (*aba1*) mutants to ABA. Furthermore, the same authors have also observed the presence of dehydration-responsive element-binding as well as ABA-responsive element-binding in promoter regions of *ADC2*, *SPDS1* and *SPMS* genes. Such results indicate that ABA regulates expression of many genes related to polyamine biosynthesis, particularly under environmental stress conditions.

Stress-tolerant plants have evolved certain adaptive mechanisms to display different degrees of tolerance, which are largely determined by genetic plasticity (Bartel and Sunkar, 2005). Molecular genetic studies have been performed with *Arabidopsis*, which does not display extreme drought tolerance, but shows several stress responses at the molecular level and has therefore been successfully used for a genetic dissection of stress response pathways (Zhu et al., 2002; Shinozaki et al., 2003; Demirevska et al., 2008; Hoekstra et al., 2011). In the present study, several genes related to amino acids and N metabolism, photorespiration and TCA cycle were evaluated by qRT-PCR and two different patterns in the gene expression levels could be observed (Figures 19 and 20). The first one focuses on down-regulation of some genes directly and indirectly related to photorespiratory pathway. For instance, the *PGLP1* gene encodes the chloroplastidic enzyme 2-phosphoglycolate phosphatase, which plays an important role in early steps of photorespiratory pathway by catalyzing the conversion of 2-phosphoglycolate and H_2O to glycolate and phosphate (Siedow and Day, 2000). Moreover, both isoforms of *SHMT*, *SHMT1* and *SHMT2*, are responsible for encoding the enzyme serine hydroxymethyltransferase, which catalyzes the reversible conversion of serine to glycine in mitochondria (Siedow and Day, 2000). The metabolism of glycine and serine occurs by several different pathways, one of which is strongly associated with photorespiration (Less and Galili, 2008). In summary, a dramatic decrease of the

transcript levels of those genes was observed during water deficit indicating the unexpected low levels of photorespiration (Figure 19). It should be mention, however, that the direct association between transcripts, proteins and metabolites should be taken with caution given that there are several reasons for the lack of association between these levels of organizations. Moreover, it is generally recognized that the genome, proteome, and metabolome interact non-linearly with environment (Fleury et al., 2010; Fernie and Stitt, 2012).

Catalase, a common enzyme found in almost all living organisms exposed to oxygen, catalyzes the decomposition of hydrogen peroxide (H_2O_2) to H_2O and oxygen and can be highly active in peroxisome during the photorespiration, due to overproduction of H_2O_2 by the conversion of glycolate to glyoxylate. Surprisingly, it was observed a decrease in transcript levels of *CAT2*, a peroxissomal gene that encode catalase, in all genotypes under severe drought (Figure 19), corroborating the hypothesis of low levels of photorespiration. Nevertheless, increased photorespiratory flux during drought could significantly increase the oxidative load on the photosynthetic cell (Noctor et al., 2002). Finally, it is noteworthy that the enzymatic antioxidant systems can be active only under conditions of sufficient water and that, in the dried state, only molecular antioxidants (*e.g.* glutathione, ascorbate, polyols, carbohydrates, proteins such as peroxiredoxin, and amphiphilic molecules such as tocopherol, quinones, flavonoids and phenolics) can alleviate oxidative stress (Vertucci and Farrant, 1995).

Although several studies emphasize the importance of the mitochondrial UCP enzyme to relieve oxidative stress in plants under drought conditions (Kreps et al., 2002; Seki et al., 2002; Apel and Hirt, 2004), it was found lower levels of the transcript of *UCP* after drought (Figure 19). However, there is compelling evidences indicating that lower levels of *UCP1* restricts photorespiratory flux as well as photosynthetic assimilation rate in an *Arabidopsis ucp1* mutant (Sweetlove et al., 2006). These limitations are likely caused by a restriction of RuBP in the Calvin-Benson cycle because of reduced recycling of 2-phosphoglycolate into 3-phosphoglycerate via the photorespiratory pathway (Bykova et al., 2005; Sweetlove et al., 2006). This suggests that the main role of *UCP1* in leaves is to maintain the redox poise of the mETC to facilitate photosynthesis (Sweetlove et al. 2006).

The low transcript levels of RuBisCO activase protein (*RCA*) during the water deficit (Figure 19) are also an indicative of impairments in both photosynthesis and

photorespiration. The activity of RuBisCO is regulated by the RCA that requires ATP for activation of RuBisCO but is inhibited by ADP (Portis, 2003). For instance, drought can cause both reversible and irreversible inactivation of RuBisCO. RCA can act releasing the tight binding inhibitors from RuBisCO; however, this removal may be impaired because of reduced concentrations of ATP during drought episodes (Tezara et al., 1999). In addition, RCA is also susceptible to high temperatures (Crafts-Brandner and Salvucci, 2000) that may be directly related to drought stress (Parry et al., 2002). A further consequence of decreased ATP synthesis under drought could be the presence of more RuBisCO inhibitors and fewer carbamylated RuBisCO catalytic sites (Lawlor, 2002; Lawlor and Tezara, 2009). These changes result from the decline in RCA activity caused by a drop in the ATP/ADP ratio. Inactivation of RuBisCO catalytic sites with increasing water deficit will decrease both carboxylation and oxygenation and, therefore, photosynthesis and photorespiration (Carmo-Silva et al., 2008). This is good agreement with the results obtained here and suggests an extensive metabolic reprogramming following water deficit. Whilst the precise nature of this metabolic reorganization could not be fully resolved here it remains an exciting topic for future research.

Another indicative of decline in photosynthetic assimilation of CO_2 was the decrease in *AGPase* transcript levels (Figure 19), which is related to low levels of starch after 10 days without watering (Figure 10D). AGPase enzyme catalyzes the first step of starch biosynthesis by generating the sugar nucleotide ADP-glucose and inorganic pyrophosphate from glucose 1-phosphate and ATP. ADP-glucose functions as the glucosyl donor for glucan synthesis by starch synthase (Dennis and Blakeley, 2000). Other effects of repressing *AGPase* expression were reported by Weigelt et al. (2009) in *AGPase* knockdown pea seeds, as well as by Kakumanu et al. (2012) in drought stressed maize ovaries.

The second pattern observed in the gene expression levels focus on minor changes of expression of TCA cycle related genes (Figure 19), such as *MDH* and *FUM1*. It has been shown that the TCA cycle is altered by several stresses such as drought in maize (Witt et al., 2012), salt stress in tobacco (Zhang et al., 2011) and by flooding stress in soybean (Rajjou et al., 2006) and *Lotus japonica* (Rocha et al., 2010). However, the metabolic profiling data alongside with gene expression results of TCA cycle related genes seem to indicate a possible maintenance of respiratory pathway under drought. Given that the TCA cycle has completely different modes

between light and dark respirations (Sweetlove et al., 2010) it is therefore likely to be acting different modes of regulation during water restrictions as well. The results presented here indicate that the TCA cycle is most likely working in a non-cyclic manner upon water shortage. Since the TCA cycle provides C skeletons for many biosynthetic pathways, a maintenance of the levels of those genes could provides rich C sources for diverse uses within a plant (Guo et al., 2009), especially in conditions of limited water availability.

A third inference derived from the work presented here may be related to genes involved in N metabolism (*GS*, *Fd-GOGAT* and *GDH*). Nitrogen assimilation is a fundamental biological process and the initial conversion of nitrate to nitrite by NR enzyme is followed by a reduction to ammonium (NH_4^+) by nitrite reductase. In the central N metabolic pathway the three-enzyme circuit (GS, Fd-GOGAT and GDH) assimilates NH_4^+ and produces two central intermediates, glutamine and glutamate which provide N for the synthesis of all other N-containing components. While GS enzyme catalyzes glutamine synthesis, glutamate can be synthesized by the action of either GS/GOGAT or GDH, respectively, with high or low affinity for NH_4^+ (Yan, 2007). Moreover, NH_4^+ can be formed as a co-product of photorespiratory pathway in mitochondria, by the conversion of glycine to serine catalyzed by SHMT enzyme. Interestingly transcript levels of genes (*GS*, *Fd-GOGAT* and *GDH* in N metabolism, as well as *SHMT1* and *SHMT2* in photorespiration) that encoding those enzymes were decreased by severe water deficit (Figure 19). Furthermore, it was observed a dramatic decrease in nitrate levels after water deficit especially in KO mutants (Figure 10C). It has been reported that total GS activity may decrease, increase or be unaffected by drought or salt stresses (Santos et al., 2004; Yan et al., 2005; Martinelli et al., 2007). Altogether these results indicated that low levels of the toxic compound NH_4^+ in drought-stressed plants resulted in low energetic requirements to invest in the GS/GOGAT system. In addition, these data corroborate with the indicative of low levels of photorespiratory rates discussed above. In agreement with the results observed for the *UCP1* in *Arabidopsis*, the results presented here indicate that alternative pathways of respirations are likely to be important for the maintenance of adequate photorespiratory rates. The combinatory application of new profiling techniques has great potential to identify as yet uncharacterized enzymes and compensatory mechanism involved in the photorespiration as well as how photorespiration

interacts, and to what extent, with other metabolic pathways (Fernie et al., 2012).

To investigate whether osmotic and salt stress as well as excess of lysine and valine can be toxic to seeds and affect the germination rates *Arabidopsis* seeds were sowed on plates that contained different concentrations of mannitol, NaCl and the amino acids cited above. First, dramatic decreases in germination rate after further treatment with high concentrations of mannitol and NaCl were observed (Table 3 and Figure 21). Salt stress could reduce germination either by limiting water absorption by the seeds (Dodd and Donovan, 1999), by affecting the mobilization of stored reserves (Bouaziz and Hicks, 1990; Lin and Kao, 1995; Prakash and Prathapasenan, 1988) or by directly affecting the structural organization or synthesis of proteins in germinating embryos (Ramagopal, 1990). Germination of KO mutant seeds was particularly inhibited by high concentrations of mannitol (200 mM) and NaCl (100 mM) indicating that these genotypes are extremely sensitive to osmotic and salt stresses. When considered together these data also indicate that *ETFQO, IVDH* and *D2HGDH* genes seem to play a relevant role in salt- and osmotic-tolerance mechanisms in *Arabidopsis* germination events and are in good agreement with the pivotal role of this alternative respiratory pathway in the tolerance of *Arabidopsis* plants to sub-optimal conditions.

Lysine can promote retardation of germination in *Arabidopsis* seeds. This is achieved by significant elevation of its levels through either enhancing its synthesis or blocking its catabolism (Zhu and Galili, 2003; Angelovici et al., 2009, 2011). It has been also reported that, at the metabolite level, an enhanced lysine metabolism negatively regulates the activity of the TCA cycle and, as a consequence, might lead to an energy limitation which is required for proper seedling establishment, induction of photosynthesis and further plant growth (Angelovici et al., 2009, 2011). In the present study it was observed a strong impairment of germination after treatment with lysine, especially in *d2hgdh-2* (Table 3 and Figure 21). D2HGDH enzyme is responsible for the donation of electrons to the ETF/ETFQO complex via the degradation of lysine, while IVDH is involved in degradation of the BCAA, phytol and lysine under C-limiting conditions (Araújo et al., 2010). Thus, *d2hgdh-2* seems to be related, under conditions of high lysine concentration, to decreased catabolism of this amino acid and the consequent reduction of the energy flow, leading to a more pronounced decrease in germination rate. In contrast, the overexpression of *D2HGDH* in the three lines studied was able to recover the WT phenotype, as

observed by similar germination rates. Valine, in turn, leads to a slightly decrease in germination rates in comparison to lysine (Table 3 and Figure 21). It has been reported that the loss of function mutation in *IVDH* causes increased soluble leucine and valine accumulation in *Arabidopsis* seeds (Gu et al., 2010). Although IVDH enzyme is able to metabolize BCAA such as valine in situations of C starvation (Araújo et al., 2010), the reduced germination of *ivdh-1* plants under high concentrations of valine was not significantly different from WT, indicating that this enzyme might be involved in catabolism of a range of compounds and not solely BCAA. These results indicate that catabolism plays an important role in regulating levels of BCAA in seeds. Thus, although the exact nature of this intriguingly metabolic feature could not be fully elucidated here it remains as an exciting research avenue to be followed and further experiments are clearly required.

5.1. Future perspectives for improving the understanding of the linkages between mitochondrial respiration and drought tolerance

Altogether the results presented in this work clearly demonstrated that plant respiration and the metabolic interactions within the mETC are more complex than previously thought. In addition, it provided novel insights into the role of alternative respiratory pathways and the metabolism of BCAA during water restriction episodes. Several enzymes involved in the biosynthesis (Mourad and King, 1995; Singh, 1999; Dumas et al., 2001) and degradation of BCAA (Däschner et al., 2001; Mooney et al., 2002; Schuster and Binder, 2005; Ishizaki et al., 2005, 2006; Araújo et al., 2010) have been already identified and characterized in *Arabidopsis*. However, thus far relatively little is currently known about transcriptional regulation of the genes coding for these enzymes. Since *Arabidopsis* and other plant species are capable of both *de novo* BCAA biosynthesis and degradation, these counteracting pathways have to be carefully balanced to maintain the functionally required homeostasis of this important group of amino acids, particularly under stress situations and finally energy deprivation conditions. Unraveling the underlying regulatory mechanisms of BCAA metabolic pathways will be one of challenges for future studies (Binder, 2010). Moreover, studies aiming to analyze the impacts of environmental stresses such as drought on the balance of biosynthesis and catabolism of BCAA are of pivotal importance to understand the relations of these pathways on plant-stress

adaptation.

Recent studies using TCA cycle antisense mutants have demonstrated the critical importance of the enzymes involved in the steps of the cycle not only within the TCA cycle itself, but also their importance for the delivery of organic acids for many other important physiological processes, such as photosynthesis, photorespiration, nitrogen assimilation and amino acid metabolism, and even stomatal function (Nunes-Nesi et al., 2005, 2007; Araújo et al., 2011c). Notwithstanding detailed insights into the assembly of mitochondrial machinery, the retrograde signaling by mitochondria during oxidative stress and the regulation of respiratory rate are still clearly required in order to maximize respiration for plant survival particularly in harsh environments as well as to minimize respiratory losses to enhance plant yields (Jacoby et al., 2012). The results presented here suggests that further analyzes of mitochondrial metabolism under stress situations are likely to identify additional components of the mETC with high importance under such situation. Taking our incomplete understanding of the respiration into consideration it is very likely that not only enzymes directly involved in this process but also transport processes will be of pivotal importance for plant tolerance to an increasing number of stress situations.

Furthermore, it was recently demonstrated that plants of rice and wheat employ a specific mechanism to trap and reassimilate photorespired CO_2. A continuous layer of chloroplasts covering the mesophyll cell periphery that is exposed to the intercellular air space creates a diffusion barrier for CO_2 exiting the cell. This facilitates the capture and reassimilation of photorespired CO_2 in the chloroplast stroma (Bush et al., 2012). However, more efforts are needed to clarify the overall significance of photorespiratory CO_2 trapping, particularly in scenarios of climate change such as increased temperature and decreased rainfall patterns. Thus the combinatory application of new and more sensitive profiling techniques coupled with the adaptation of global profiling techniques is likely to enable us to pursue new avenues of research in order to increase our current understanding of the complex networks governing the role of as yet uncharacterized enzymes and compensatory mechanism involved in the photorespiration as well as how photorespiration interacts, and to what extent, with other metabolic pathways (Fernie et al., 2012), particularly under stress situations.

It has been reported that additional components that serve as non-

phosphorylating bypasses of the mETC such as internal and external NAD(P)H dehydrogenases (Møller, 2001), the ETF/ETFQO complex (Ishizaki et al., 2005, 2006; Araújo et al., 2010), glycerol-3-phosphate dehydrogenase (Shen et al., 2003, 2006) and AOX (Vanlerberghe and McIntosh, 1997; Fiorani et al., 2005) as well as the membrane potential-dissipating UCPs (Vercesi et al., 2006; Sweetlove et al., 2006) can alter the gearing between the TCA cycle and oxidative phosphorylation to facilitate the anaplerotic function of plant mitochondria for organic acid provision to cellular biosynthetic pathways without the full operation of the TCA cycle (Jacoby et al., 2012). Elucidation of the magnitude of these processes under abiotic stress conditions and their relative contribution to maintenance of respiratory flux remains to be fully investigated.

Taken together, the results presented in this work highlight the pivotal importance of the alternative ETF/ETFQO complex pathway, not only during the severe stress imposed by dark induced senescence (Ishizaki et al., 2005, 2006; Araújo et al., 2010), but also under more physiological common situations as observed in the present work. Clearly the importance of this work is demonstrated by the fact that alteration of alternative mitochondrial metabolism can be highly effective for drought tolerance in *Arabidopsis* plants. Further studies under a range of other environmental stress conditions might reveal the full importance not only of this pathway but also of the mitochondrial metabolism under stress situations. Specific examples in which there is good reason to believe in an important role of not only classical but also alternative mitochondrial metabolism function include tolerance to heavy metals as well as nutrient limitations. It should be mention, however, that the evidence for the involvement of mitochondrial process is rather fragmentary and thus desperately in need of further experimental study. Understanding the control and regulation of the alternative pathways is therefore of vital importance to further engineer plant biomass production and to explain plant growth as well as its variability under different environmental conditions.

6. CONCLUSIONS

The results presented in this study demonstrated that ETFQO, IVDH and D2HGDH seem to play a significant role in drought-tolerance mechanisms in *Arabidopsis*. As previously described (Ishizaki et al., 2005, 2006; Araújo et al., 2010), carbon starvation associated to dark-induced senescence induces the ETF/ETFQO alternative pathway of respiration. This information coupled with the data presented here demonstrate the enzymes IVDH and D2HGDH integrate electron donation to this complex also during water deficit episodes and that impairment in electron transfer to mETC mediated by the ETF/ETFQO pathway is important for plants to withstand drought as well as to recover growth after re-watering. Thus, a novel aspect of this intriguingly ETF/ETFQO pathway was revealed in the present work.

Furthermore, the metabolic pattern of all genotypes showed a large number of metabolites that were significantly altered due to the water deficit. Firstly, protein degradation and the consequent decreased level of nitrate are likely to be associated to the increased amino acids levels. The high levels of BCAA (isoleucine, leucine and valine) seem to indicate a fundamental role of those amino acids in drought tolerance. However, whether this is mediated by an osmoprotectant effect or by increased usage of those amino acids as alternative source of electrons to mETC remains to fully elucidate. Whilst the precise nature of the interaction between energy metabolism, BCAA metabolism and drought tolerance could not be fully resolved in the present study, it remains an exciting topic for future research. Gene expression analyses revealed that photorespiration seems to be operating at

negligible rates under water deficit and does not constitute an effective loss on C assimilation. This coupled with the minor changes in the expression of TCA cycle related genes seem to indicate a possible maintenance of respiratory pathway under drought most likely through the operation of non-cyclic modes of the TCA cycle.

Finally, germination assays indicated that ETFQO, IVDH and D2HGDH seem to play a relevant role in salt- and osmotic-tolerance mechanisms in *Arabidopsis* germination events, as well as that the BCAA catabolism plays an important role in regulating levels of those amino acid in seeds. Although the last is not completely surprisingly additional studies are clearly needed to fully elucidate the role of the BCAA deficiency which most likely leads to major adjustments of the seeds *Arabidopsis* transcriptome and primary metabolome in a manner considerably similar to the adjustment of these two networks associated with exposure to drought stress, known to exert significant energy deprivation. It is, however, clear that future experiments are required to furnish direct evidence in support of this hypothesis.

REFERENCES

Abebe T, Guenzi AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol **131**: 1748–1755

Achouri Y, Noel G, Vertommen D, Rider MH, Veiga-Da-Cunha M, van Schaftingen E (2004) Identification of a dehydrogenase acting on D-2 hydroxyglutarate. Biochem J **381**: 35–42

Alcázar R, Marco F, Cuevas JC, Patron M, Ferrando A, Carrasco P, Tiburcio AF, Altabella T (2006a) Involvement of polyamines in plant response to abiotic stress. Biotechnol Lett **28**: 1867‑1876

Alcázar R, Cuevas JC, Patrón M, Altabella T, Tiburcio AF (2006b) Abscisic acid modulates polyamine metabolism under water stress in *Arabidopsis thaliana*. Physiol Plant **128**: 448‑455

Alcázar R, Bitrián M, Bartels D, Koncz C, Altabella T, Tiburcio AF (2011) Polyamine metabolic canalization in response to drought stress in *Arabidopsis* and the resurrection plant *Craterostigma plantagineum*. Plant Signal Behav **6**: 243-250

Alvarez S, Marsh EL, Schroeder SG, Schachtman DP (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant Cell Environ **31**: 325–340

Angelovici R, Fait A, Zhu X, Szymanski J, Feldmesser E, Fernie AR, Galili G (2009) Deciphering transcriptional and metabolic networks associated with lysine metabolism during *Arabidopsis* seed development. Plant Physiol **151**: 2058–2072

Angelovici R, Fait A, Fernie AR, Galili G (2011) A seed high-lysine trait is negatively associated with the TCA cycle and slows down *Arabidopsis* seed germination. New Phytol **189**: 148–159

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373–399

Apse MP, Blumwald E (2002) Engineering salt tolerance in plants. Curr Opin Biotechnol **13**: 146–150

Araújo WL, Ishizaki K, Nunes-Nesi A, Larson TR, Tohge T, Krahnert I, Witt S, Obata T, Schauer N, Graham IA, Leaver CJ, Fernie AR (2010) Identification of the 2-Hydroxyglutarate and Isovaleryl-CoA dehydrogenases as alternative electron donors linking Lysine catabolism to the electron transport chain of *Arabidopsis* mitochondria. Plant Cell **22**: 1549–1563

Araújo WL, Ishizaki K, Nunes-Nesi A, Tohge T, Larson TR, Krahnert I, Balbo I, Witt S, Dörmann P, Graham IA, Leaver CJ, Fernie AR (2011a) Analysis of a range of catabolic mutants provides evidence that phytanoyl-CoA does not act as a substrate of the ETF/ETFQO complex in *Arabidopsis thaliana* during dark induced senescence. Plant Physiol **157**: 55–69

Araújo WL, Nunes-Nesi A, Fernie AR (2011b) Fumarate: Multiple functions of a simple metabolite. Phytochemistry **72**: 838–843

Araújo WL, Nunes-Nesi A, Osorio S, Usadel B, Fuentes D, Nagy R, Balbo I, Lehmann M, Studart-Witkowski C, Tohge T, Martinoia E, Jordana X, Damatta FM, Fernie AR (2011c) Antisense inhibition of the iron-sulphur subunit of succinate dehydrogenase enhances photosynthesis and growth in tomato via an organic acid-mediated effect on stomatal aperture. Plant Cell **23**: 600–627

Araújo WL, Tohge T, Ishizaki K, Leaver CJ, Fernie AR (2011d) Protein degradation – an alternative respiratory substrate for stressed plants. Trends Plant Sci **16**: 489–498

Araújo WL, Nunes-Nesi A, Nikoloski Z, Sweetlove LJ, Fernie AR (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant Cell Environ **35**: 1–21

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24**: 1–15

Atkin OK, Bruhn D, Hurry VM, Tjoelker MG (2005) The hot and the cold: unraveling the variable response of plant respiration to temperature. Func Plant Biol **32**: 87–105

Atkin OK, Macherel D (2009) The crucial role of plant mitochondria in orchestrating drought tolerance. Ann Bot **103**: 581–597

Aubert S, Bligny R, Day DA, Whelan J, Douce R (1997) Induction of alternative oxidase synthesis by herbicides inhibiting branched-chain amino acid synthesis. Plant J **11**: 649–657

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol **59**: 89–113

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci **24**: 23–58

Bartoli CG, Gomez F, Martinez DE, Guiamet JJ (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). J Exp Bot **55**: 1663–1669

Bartoli CG, Gomez F, Gergoff G, Guiamet JJ, Puntarulo S (2005) Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. J Exp Bot **56**: 1269–1276

Basu PS, Ali M, Chaturvedi SK (2007) Osmotic adjustment increases water uptake, remobilization of assimilates and maintains photosynthesis in chickpea under drought. Indian J Exp Biol **45**: 261–267

Bauwe H, Hagemann M, Fernie AR (2010) Photorespiration: players, partners and origin. Trends Plant Sci **15**: 330–336

Beckmann JD, Frerman FE (1985) Electron-transfer flavoprotein:ubiquinone oxidoreductase from pig liver: Purification and molecular, redox, and catalytic properties. Biochemistry **24**: 3913–3921

Begcy K, Mariano ED, Mattiello L, Nunes AV, Mazzafera P, Maia IG, Menossi M (2011) An *Arabidopsis* mitochondrial uncoupling protein confers tolerance to drought and salt stress in transgenic tobacco plants. PLoS ONE **6**: e23776

Binder S, Knill T, Schuster J (2007) Branched-chain amino acid metabolism in higher plants. Physiol Plant **129**: 68–78

Bogdan J, Zagdanska B (2006) Changes in the pool of soluble sugars induced by dehydration at the heterotrophic phase of growth of wheat seedlings. Plant Physiol Biochem **44**: 787–794

Bouaziz A, Hicks DR (1990) Consumption of wheat seed reserves during germination and early growth as affected by soil water potential. Plant Soil **128**: 161–165

Bouche N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci **9**: 110–115

Bouma TJ, Devisser R, Janssen JHJA, Dekock MJ, Vanleeuwen PH, Lambers H (1994) Respiratory energy requirements and rate of protein turnover in vivo determined by the use of an inhibitor of protein synthesis and a probe to assess its effect. Physiol Plant **92**: 585–594

Bown AW, Shelp BJ (1997) The metabolism and functions of gamma-aminobutyric acid. Plant Physiol **115**: 1–5

Bowne JB, Erwin TA, Juttner J, Schnurbusch T, Langridge P, Bacic A, Roessner U (2012) Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at the metabolite level. Mol Plant **5**: 418–429

Boyer JS (1982) Plant productivity and the environment. Science **218**: 443–448

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlach J (2001) Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. Plant Cell **13**: 1499–1510

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248–254

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci **2**: 48–54

Bray EA (2002) Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. Ann Bot **89**: 803–811

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot **55**: 2331–2341

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin J-F, Wu S-H, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J **42**: 567–585

Burg MB, Kwon ED, Kultz D (1996) Osmotic regulation of gene expression. FASEB J **10**: 1598–1606

Bykova NV, Keerberg O, Parnik T, Bauwe H, Gardestrom P (2005) Interaction between photorespiration and respiration in transgenic potato plants with antisense reduction in glycine decarboxylase. Planta **222**: 130–140

Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D, Fernie AR, Willmitzer L, Hannah MA (2011) High-density kinetic analysis of the metabolomics and transcriptomic response of *Arabidopsis* to eight environmental conditions. Plant J **67**: 869–884

Capell T, Escobar C, Liu H, Burtin D, Lepri O, Christou P (1998) Overexpression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns in vitro and results in putrescine accumulation in transgenic plants. Theor Appl Gen **97**: 246–254

Capell T, Bassie L, Christou P (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc Natl Acad Sci USA **101**: 9909–9914

Carmo-Silva AE, Powers SJ, Keys AJ, Arrabacxa MC, Parry MAJ (2008) Photorespiration in C4 grasses remains slow under drought conditions. Plant Cell Environ **31**: 925–940

Charlton AJ, Donarski JA, Harrison M, Jones SA, Godward J, Oehlschlager S, Arques JL, Ambrose M, Chinoy C, Mullineaux PM, Domoney C (2008) Responses of the pea (*Pisum sativum* L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy. Metabolomics **4**: 312–327

Chaves MM, Pereira JS, Maroco JP, Rodrigues ML, Ricardo CPP, Osorio ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field? Photosynthesis and growth. Ann Bot **89**: 907–916

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought – from genes to the whole plant. Funct Plant Biol **30**: 239–264

Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot **55**: 2365–2384

Chen TH, Murata N (2011) Glycine betaine protects plants against abiotic stress: mechanisms and biotechnological applications. Plant Cell Environ **34**: 1–20

Chia DW, Yoder TJ, Reiter WD, Gibson SI (2000) Fumaric acid: an overlooked form of fixed carbon in *Arabidopsis* and other plant species. Planta **211**: 743–751

Considine MJ, Goodman M, Echtay KS, Laloi M, Whelan J, Brand MD, Sweetlove LJ (2003) Superoxide stimulates a proton leak in potato mitochondria that is related to the activity of uncoupling protein. J Biol Chem **278**: 22298–22302

Cornic G, le Gouallec JL, Briantais JM, Hodges M (1989) Effect of a high light treatment during a drought stress on photosynthetic capacities of two C3 plants: *Phaseolus vulgaris* and *Elatostema repens*. Planta **177**: 84–90

Cortina C, Culianez-Macia F (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Sci **169**: 75–82

Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc Natl Acad Sci USA **97**: 13430–13435

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. Plant Physiol **110**: 1–2

Dekankova K, Luxova M, Gasparikova O, Kolarovic L (2004) Response of maize plants to water stress. Biologia **59**: 151–155

Demirevska K, Simova-Stoilova L, Vassileva V, Vaseva I, Grigorova B, Feller U (2008) Drought-induced leaf protein alterations in sensitive and tolerant wheat varieties. Gen Appl Plant Physiol **34**: 79–102

Dennis DT, Blakeley SD (2000) Carbohydrate metabolism. *In* Buchanan B, Gruissem W, Jones RL, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, USA, pp 630–675

Deuschle K, Funck D, Hellmann H, Daeschner K, Binder S, Frommer WB (2001) A nuclear gene encoding mitochondrial Δ^1-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant J **27**: 345–356

Diebold R, Schuster J, Daschner K, Binder S (2002) The branched-chain amino acid transaminase gene family in *Arabidopsis* encodes plastid and mitochondrial proteins. Plant Physiol **129**: 540–550

Dinakar C, Abhaypratap V, Yearla SR, Raghavendra AS, Padmasree K (2010) Importance of ROS and antioxidant system during the beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. Planta **231**: 461–474

Dodd GL, Donovan LA (1999) Water potential and ionic effects on germination and seedling growth of two cold desert shrubs. Am J Bot **86**: 1146–1153

Dodd AN, Kudla J, Sanders D (2010) The language of calcium signaling. Annu Rev Plant Biol **61**: 593–620

van Dongen JT, Gupta KJ, Ramírez-Aguilar SJ, Araújo WL, Nunes-Nesi A, Fernie AR (2011) Regulation of respiration in plants: A role for alternative metabolic pathways. J Plant Physiol **168**: 1434–1443

Engqvist M, Drincovich MF, Flügge UI, Maurino VG (2009) Two D-2-hydroxy-acid dehydrogenases in *Arabidopsis thaliana* with catalytic capacities to participate in the last reactions of the methylglyoxal and beta-oxidation pathways. J Biol Chem **284**: 25026–25037

Engqvist MK, Kuhn A, Wienstroer J, Weber K, Jansen EEW, Jakobs C, Weber APM, Maurino VG (2011) Plant D-2-hydroxyglutarate dehydrogenase participates in the catabolism of lysine especially during senescence. J Biol Chem **286**: 11382–11390

Escobar MA, Franklin KA, Svensson AS, Salter MG, Whitelam GC, Rasmusson AG (2004) Light regulation of the *Arabidopsis* respiratory chain: multiple discrete photoreceptor responses contribute to induction of type II NAD(P)H dehydrogenase genes. Plant Physiol **136**: 2710–2721

Fahnenstich H, Saigo M, Niessen M, Drincovich MF, Flügge UI, Maurino VG (2008) Malate and fumarate emerge as key players in primary metabolism: *Arabidopsis thaliana* overexpressing C4-NADP-ME offer a way to manipulate the levels of malate and to analyse the physiological consequences. *In* Allen JF, Gantt E, Golbeck JH, Osmond B, eds, Photosynthesis Energy From the Sun. Springer-Verlag, Heidelberg, Germany, pp 971–975

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2008) Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci **13**: 14–19

Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ (2001) Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta **212**: 250–263

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Curr Opin Plant Biol **7**: 254–261

Fernie AR, Aharoni A, Willmitzer L, Stitt M, Tohge T, Kopka J, Carroll AJ, Saito K, Fraser PD, DeLuca V (2011) Recommendations for reporting metabolite data. Plant Cell **23**: 2477–2482

Fernie AR, Stitt M (2012) On the discordance of metabolomics with proteomics and transcriptomics: coping with increasing complexity in logic, chemistry, and network interactions scientific correspondence. Plant Physiol **158**: 1139–1145

Fernie AF, Bauwe H, Eisenhut M, Florian A, Hanson D, Hagemann M, Keech O, Mielewczik M, Nikoloski Z, Peterhänsel C, Roje S, Sage R, Timm S, von Caemmerer S, Weber APM, Westhoff P (2012) Perspectives on plant photorespiratory metabolism. Plant Biol (doi: 10.1111/j.1438-8677.2012.00693.x)

Finnegan PM, Soole KL, Umbach AL (2004) Alternative mitochondrial electron transport proteins in higher plants. *In* Day DA, Millar AH, Whelan J, eds, Plant Mitochondria: From Genome to Function. Advances in Photosynthesis and Respiration Series. Kluwer Academics Publishers, Dordrecht, The Netherlands, pp 163–230

Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of *Arabidopsis* AOX1a transgenic plants. Plant Physiol **139**: 1795–1805

Fleury D, Jefferies S, Kuchel H, Langridge P (2010) Genetic and genomic tools to improve drought tolerance in wheat. J Exp Bot **61**: 3211–3222

Flexas J, Galmes J, Ribas-Carbo M, Medrano H (2005) The effects of water stress on plant respiration. *In* Lambers H, Ribas-Carbo M, eds, Plant Respiration: From Cell to Ecosystem. Springer, Dordrecht, The Netherlands, pp 85–94

Flexas J, Bota J, Galmes J, Medrano H, Ribas-Carbo M (2006) Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol Plant **127**: 343–352

Foyer CH, Valadier MH, Migge A, Becker TW (1998) Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. Plant Physiol **117**: 283–292

Fratianni A, Pastore D, Pallotta ML, Chiatante D, Passarella S (2001) Increase of membrane permeability of mitochondria isolated from water stress adapted potato cells. Biosci Rep **21**: 81–91

Frerman FE, Goodman SI (2001) Defects of electron transfer flavoprotein and electron transfer flavoprotein-ubiquinone oxidoreductase: Glutaric acidemia type II. *In* Scriver CR, Sly WS, Childs B, Beaudet AL, Valle D, eds, The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, USA, pp 2357–2365

Frerman FE (1987) Reaction of electron-transfer flavoprotein ubiquinone oxidoreductase with the mitochondrial respiratory chain. Biochim Biophys Acta **893**: 161–169

Frerman FE (1988) Acyl-CoA dehydrogenases, electron transfer flavoprotein and electron transfer flavoprotein dehydrogenase. Biochem Soc Trans **16**: 416–418

Fresneau C, Ghashghaie J, Cornic G (2007) Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal CO_2. J Exp Bot **58**: 2983–2992

Fujiki Y, Sato T, Ito M, Watanabe A (2000) Isolation and characterization of cDNA, clones for the E1 beta and E2 subunits of the branched-chain alpha-ketoacid dehydrogenase complex in *Arabidopsis*. J Biol Chem **275**: 6007–6013

Fujiki Y, Ito M, Nishida I, Watanabe A (2001) Leucine and its keto acid enhance the coordinated expression of genes for branched-chain amino acid catabolism in *Arabidopsis* under sugar starvation. FEBS Lett **499**: 161–165

Galili G (1995) Regulation of lysine and threonine synthesis. Plant Cell **7**: 899–906

Galili G, Tang G, Zhu X, Gakiere B (2001) Lysine catabolism: a stress and development super-regulated metabolic pathway. Curr Opin Plant Biol **4**: 261–266

Galili G (2002) New insights into the regulation and functional significance of lysine metabolism in plants. Annu Rev Plant Biol **53**: 27–43

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99**: 15898–15903

Gastón S, Ribas-Carbo M, Busquets S, Berry JA, Zabalza A, Royuela M (2003) Changes in mitochondrial electron partitioning in response to herbicides inhibiting branched-chain amino acid biosynthesis in soybean. Plant Physiol **133**: 1351–1359

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline M-T, Deleens E (2001) $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. Plant Cell Environ **24**: 505–515

Gibon Y, Pyl E, Sulpice R, Lunn JE, Höhne M, Günther M, Stitt M (2009) Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when *Arabidopsis* is grown in very short photoperiods. Plant Cell Environ **32**: 859–874

González-Meler MA, Matamala R, Peñuelas J (1997) Effects of prolonged drought stress and nitrogen deficiency on the respiratory O_2 uptake of bean and pepper leaves. Photosynthetica **34**: 505–512

Gornall J, Betts R, Burke E, Clark R, Camp J, Willett K, Wiltshire A (2010) Implications of climate change for agricultural productivity in the early twenty-first century. Phil Trans R Soc B **365**: 2973–2989

Gu L, Jones AD, Last RL (2010) Broad connections in the *Arabidopsis* seed metabolic network revealed by metabolite profiling of an amino acid catabolism mutant. Plant J **61**: 579–590

Guo P, Baum M, Grando S, Ceccarelli S, Bai G, Li R, von Korff M, Varshney RK, Graner A, Valkoun J (2009) Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Bot **60**: 3531–3544

Hannah MA, Zuther E, Buchel K, Heyer AG (2006) Transport and metabolism of raffinose family oligosaccharides in transgenic potato. J Exp Bot **57**: 3801–3811

Harb A, Krishnan A, Ambavaram MM, Pereira A (2010) Molecular and physiological analysis of drought stress in *Arabidopsis* reveals early responses leading to acclimation in plant growth. Plant Physiol **154**: 1254-1271

Hare PD, Cress WA, Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ **21**: 535–553

Heazlewood JL, Millar AH (2005) AMPDB: the *Arabidopsis* mitochondrial protein database. Nucleic Acids Res **33**: 605–610

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH (2004) Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell **16**: 241–256

Hellmann H, Funck D, Rentsch D, Frommer WB (2000) Hypersensitivity of an *Arabidopsis* sugar signaling mutant toward exogenous proline application. Plant Physiol **123**: 779–789

Herppich WB, Peckmann K (2000) Influence of drought on mitochondrial activity, photosynthesis, nocturnal acid accumulation and water relations in the CAM plants *Prenia sladeniana* (ME-type) and *Crassula lycopodioides* (PEPCK-type). Ann Bot **86**: 611–620

Hoefnagel MHN, Atkin OK, Wiskich JT (1998) Interdependence between chloroplasts and mitochondria in the light and the dark. BBA-Bioenergetics 1366: 235–255

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci **6**: 431–438

Ishizaki K, Larson TR, Schauer N, Fernie AR, Graham IA, Leaver CJ (2005) The critical role of *Arabidopsis* electrontransfer flavoprotein:ubiquinone oxidoreductase during dark-induced starvation. Plant Cell **17**: 2587–2600

Ishizaki K, Schauer N, Larson TR, Graham IA, Fernie AR, Leaver CJ (2006) The mitochondrial electron transfer flavoprotein complex is essential for survival of *Arabidopsis* in extended darkness. Plant J **47**: 751–760

Jacoby RP, Taylor NL, Millar AH (2011) The role of mitochondrial respiration in salinity tolerance. Trends Plant Sci **16**: 614-623

Jacoby RP, Li L, Huang S, Pong Lee C, Millar AH, Taylor NL (2012) Mitochondrial composition, function and stress response in plants. J Integr Plant Biol **54**: 887–906

Jander G, Norris SR, Joshi V, Fraga M, Rugg A, Yu SX, Li LL, Last RL (2004) Application of a high-throughput HPLC-MS/MS assay to *Arabidopsis* mutant screening: evidence that threonine aldolase plays a role in seed nutritional quality. Plant J **39**: 465–475

Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Choi YD, Nahm BH, Kim JK (2003). Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131**: 516–524

Joshi V, Laubengayer KM, Schauer N, Fernie AR, Jander G (2006) Two *Arabidopsis* threonine aldolases are nonredundant and compete with threonine deaminase for a common substrate pool. Plant Cell **18**: 3564–3575

Juszczuk IM, Malusa E, Rychter AM (2001) Oxidative stress during phosphate deficiency in roots of bean plants (*Phaseolus vulgaris*). J Plant Physiol **158**: 1299–1305

Kakumanu A, Ambavaram MM, Klumas C, Krishnan A, Batlang U, Myers E, Grene R, Pereira A (2012) Effects of drought on gene expression in maize reproductive and leaf meristem tissue revealed by RNA-Seq. Plant Physiol **160**: 846-867

Kaplan F, Guy CL (2004) beta-Amylase induction and the protective role of maltose during temperature shock. Plant Physiol **135**: 1674–1684

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004) Exploring the temperature-stress metabolome of *Arabidopsis*. Plant Physiol **136**: 4159–4168

Kempa S, Krasensky J, Dal Santo S, Kopka J, Jonak C (2008) A central role of abscisic acid in stress-regulated carbohydrate metabolism. PLoS ONE **3**: e3935

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Crit Rev Plant Sci **19**: 479–509

Kishor PBK, Hong Z, Miao G-H, Hu C-AA, Verma DPS (1995) Over-expression of Δ-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol **108**: 1387–1394

Kochevenko A, Klee HJ, Fernie AR, Araújo WL (2012) Molecular identification of a further branched-chain aminotransferase 7 (BCAT7) in tomato plants. J Plant Physiol **169**: 437–443

Korkina LG (2007) Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. Cell Mol Biol **53**: 15–25

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot **63**: 1593–1608

Krauss S, Zhang CY, Lowell BB (2005) The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol **6**: 248–261

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol **130**: 2129–2141

Krömer S, Malmberg G, Gärdestrom P (1993) Mitochondrial contribution to photosynthetic metabolism. Plant Physiol **102**: 947–955

Krömer S (1995) Respiration during photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **46**: 45–70

Lambers H, Robinson SA, Ribas-Carbo M (2005) Regulation of respiration in vivo. *In* Lambers H, Ribas-Carbo M, eds, Plant Respiration: From Cell to Ecosystem. Advances in Photosynthesis and Respiration Series. Springer, Dordrecht, The Netherlands, pp 1–15

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ **25**: 275–294

Lawlor DW, Fock H (1977) Water stress induced changes in the amounts of some photosynthetic assimilation products and respiratory metabolites of sunflower leaves. J Exp Bot **28**: 329–337

Lawlor DW (2002) Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Ann Bot **89**: 871–885

Lawlor DW, Tezara W (2009) Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. Ann Bot **103**: 561–579

Lehmann M, Schwarzlander M, Obata T, Sirikantaramas A, Burow M, Olsen C, Tohge T, Fricker M, Moller B, Fernie A, Sweetlove LJ, Laxa M (2009) The metabolic response of *Arabidopsis* roots to oxidative stress is distinct from that of heterotrophic cells in culture and highlights a complex relationship between the levels of transcripts, metabolites, and flux. Mol Plant **2**: 390–406

Less H, Galili G (2008) Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses. Plant Physiol **147**: 316–330

Lin CC, Kao CH (1995) NaCl stress in rice seedlings: starch mobilization and the influence of gibberellic acid on seedling growth. Bot Bull Acad Sin **36**: 169–173

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat Protoc **1**: 387–396

Luedemann A, Strassburg K, Erban A, Kopka J (2008) TagFinder for the quantitative analysis of gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling experiments. Bioinformatics **24**: 732–737

Lugan R, Niogret MF, Leport L, Guegan JP, Larher FR, Savoure A, Kopka J, Bouchereau A (2010) Metabolome and water homeostasis analysis of *Thellungiella salsuginea* suggests that dehydration tolerance is a key response to osmotic stress in this halophyte. Plant J **64**: 215–229

Mani S, van de Cotte B, Van Montagu M, Verbruggen N (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in *Arabidopsis*. Plant Physiol **128**: 73–83

Martinelli T, Whittaker A, Bochicchio A, Vazzana C, Suzuki A, Masclaux-Daubresse C (2007) Amino acid pattern and glutamate metabolism during dehydration stress in the 'resurrection' plant *Sporobolus stapfianus*: a comparison between desiccation-sensitive and desiccation-tolerant leaves. J Exp Bot **58**: 3037–3046

Maxwell DP, Nickels R, McIntosh L (2002) Evidence of mitochondrial involvement in the transduction of signals required for the induction of genes associated with pathogen attack and senescence. Plant J **29**: 269–279

Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA (2003) Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. Plant Physiol **131**: 298–308

Miyashita Y, Good AG (2008) NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidopsis thaliana* during dark-induced carbon starvation. J Exp Bot **59**: 667–680

Møller IM (2001) Plant mitochondria and oxidative stress: electron transport. NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Phys **52**: 561–591

Møller IM, Sweetlove LJ (2010) ROS signaling – specificity is required. Trends Plant Sci **15**: 370–374

Morgan J (1992) Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. Aust J Plant Physiol **19**: 67–76

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15**: 473–497

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett **461**: 205–210

Nishizawa A, Yabuta Y, Shigeoka S (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol **147**: 1251–1263

Noctor G, De Paepe R, Foyer CH (2007) Mitochondrial redox biology and homeostasis in plants. Trends Plant Sci **12**: 125–134

Noguchi K, Terashima I (2006) Responses of spinach leaf mitochondria to low nitrogen availability. Plant Cell Environm **29**: 710–719

Nonami H, Boyer JS (1990) Primary events regulating stem growth at low water potentials. Plant Physiol **94**: 1601–1609

Nuccio ML, Rhodes D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol **2**: 128–134

Nunes-Nesi A, Carrari F, Lytovchenko A, Smith AMO, Loureiro ME, Ratcliffe RG, Sweetlove LJ, Fernie AR (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol **137**: 611–622

Nunes-Nesi A, Carrari F, Gibon Y, Sulpice R, Lytovchenko A, Fisahn J, Graham J, Ratcliffe RG, Sweetlove LJ, Fernie AR (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J **50**: 1093–1106

Nunes-Nesi A, Sulpice R, Gibon Y, Fernie AR (2008) The enigmatic contribution of mitochondrial function in photosynthesis. J Exp Bot **59**: 1675–1684

Nunes-Nesi A, Araújo WL, Fernie AR (2011) Targeting mitochondrial metabolism and machinery as a means to enhance photosynthesis. Plant Physiol **155**: 101–117

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol **30**: 739–754

Osorio J, Osorio ML, Chaves MM, Pereira JS (1998) Water deficits are more important in delaying growth than in changing patterns of carbon allocation in *Eucalyptus globulus*. Tree Physiol **18**: 363–373

Parry MAJ, Andralojc PJ, Khan S, Lea PJ, Keys AJ (2002) Rubisco activity: effects of drought stress. Ann Bot **89**: 833–839

Parsons HL, Yip JYH, Vanlerberghe GC (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. Plant Physiol **121**: 1309–1320

Pastore D, Stoppelli MC, Di FN, Passarella S (1999) The existence of the K^+ channel in plant mitochondria. J Biol Chem **274**: 26683–26690

Pastore D, Trono D, Laus MN, Di Fonzo N, Flagella Z (2007) Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: durum wheat mitochondria. J Exp Bot **58**: 195–210

Petti AA, Crutchfield CA, Rabinowitz JD, Botstein D (2011) Survival of starving yeast is correlated with oxidative stress response and nonrespiratory mitochondrial function. Proc Natl Acad Sci USA **108**: 1089–1098

Pichersky E, Noel JP, Dudareva N (2006) Biosynthesis of plant volatiles: nature's diversity and ingenuity. Science **311**: 808–811

Plaxton WC, Podesta FE (2006) The functional organization and control of plant respiration. Crit Rev Plant Sci **25**: 159–198

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta **975**: 384–394

Portis AR (2003) Rubisco activase: Rubisco's catalytic chaperone. Photosynth Res **75**: 11–27

Prakash L, Prathapasenan G (1988) Putrescine reduces NaCl induced inhibition of germination and early seedling growth of rice (*Oryza sativa* L.). Aust J Plant Physiol **15**: 761–767

Radwanski ER, Last RL (1995) Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. Plant Cell **7**: 921–934

Raghavendra AS, Padmasree K (2003) Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. Trends Plant Sci **8**: 546–553

Ramagopal S (1990) Inhibition of seed germination by salt and its subsequent effect on embryonic protein synthesis in barley. J Plant Physiol **136**: 621–625

Rasmusson AG, Soole KL, Elthon TE (2004) Alternative NAD(P)H dehydrogenases of plant mitochondria. Annu Rev Plant Biol **55**: 23–39

Rasmusson AG, Escobar MA (2007) Light and diurnal regulation of plant respiratory gene expression. Physiol Plant **129**: 57–67

Rasmusson AG, Geisler DA, Møller IM (2008) The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. Mitochondrion **8**: 47–60

Rasmusson AG, Fernie AR, van Dongen JT (2009) Alternative oxidase: a defence against metabolic fluctuations? Physiol Plant **137**: 371–382

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol **161**: 1189–1202

Reinhold T, Alawady A, Grimm B, Beran KC, Jahns P, Conrath U, Bauer J, Reiser J, Melzer M, Jeblick W, Neuhaus HE (2007) Limitation of nocturnal import of ATP into *Arabidopsis* chloroplasts leads to photooxidative damage. Plant J **50**: 293–304

Ribas-Carbo M, Taylor NL, Giles L, Busquets S, Finnegan PM, Day DA, Lambers H, Medrano H, Berry JA, Flexas J (2005) Effects of water stress on respiration in soybean leaves. Plant Physiol **139**: 466–473

Ricquier D, Bouillaud F (2000) Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. J Physiol **529**: 3–10

Rizhsky L, Liang HJ, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. Plant Physiol **134**: 1683–1696

Rocha M, Licausi F, Araujo WL, Nunes-Nesi A, Sodek L, Fernie AR, van Dongen JT (2010) Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. Plant Physiol **152**: 1501–1513

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell **13**: 11–29

Rontein D, Basset G, Hanson AD (2002) Metabolic engineering of osmoprotectant accumulation in plants. Metab Eng **4**: 49–56

Ruzicka FJ, Beinert H (1977) A new iron-sulfur flavoprotein of the respiratory chain. A component of the fatty acid beta oxidation pathway. J Biol Chem **252**: 8440–8445

Sanchez DH, Schwabe F, Erban A, Udvardi MK, Kopka J (2011) Comparative metabolomics of drought acclimation in model and forage legumes. Plant Cell Environ **35**: 136–149

Santos C, Pereira A, Pereira S, Teixeira J (2004) Regulation of glutamine synthetase expression in sunflower cells exposed to salt and osmotic stress. Sci Hort **103**: 101–111

Scheibe R, Backhausen JE, Emmerlich V, Holtgrefe S (2005) Strategies to maintain redox homeostasis during photosynthesis under changing conditions. J Exp Bot **56**: 1481–1489

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31**: 279–292

Sharp RE, Davies WJ (1979) Solute regulation and growth by roots and shoots of water-stressed maize plants. Planta **147**: 43–49

Shen W, Wei Y, Dauk M, Zheng Z, Zou J (2003) Identification of a mitochondrial glycerol-3-phosphate dehydrogenase from *Arabidopsis thaliana*: evidence for a mitochondrial glycerol-3-phosphate shuttle in plants. FEBS Lett **11**: 92–96

Shen WY, Wei YD, Dauk M, Tan YF, Taylor DC, Selvaraj G, Zou JT (2006) Involvement of a glycerol-3-phosphate dehydrogenase in modulating the NADH/NAD^+ ratio provides evidence of a mitochondrial glycerol-3-phosphate shuttle in *Arabidopsis*. Plant Cell **18**: 422–441

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol **6**: 410–417

Siedow JN, Day DA (2000) Respiration and Photorespiration. *In* Buchanan B, Gruissem W, Jones RL, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, USA, pp 676–728

Sienkiewicz-Porzucek A, Nunes-Nesi A, Sulpice R, Lisec J, Centeno DC, Carillo P, Leisse A, Urbanczyk-Wochniak E, Fernie AR (2008) Mild reductions in mitochondrial citrate synthase activity result in a compromised nitrate assimilation and reduced leaf pigmentation but have no effect on photosynthetic performance or growth. Plant Physiol **147**: 115–127

Silva P, Thompson E, Bailey S, Kruse O, Mullineaux CW, Robinson C, Mann NH, Nixon PJ (2003) FtsH is involved in the early stages of repair of Photosystem II in *Synechocystis* sp PCC 6803. Plant Cell **15**: 2152–2164

Skirycz A, Inzé D (2010) More from less: plant growth under limited water. Curr Opin Biotech **21**: 197–203

Sluse FE, Jarmuszkiewicz W, Navet R, Douette P, Mathy G, Sluse-Goffart CM (2006) Mitochondrial UCPs: new insights into regulation and impact. BBA-Bioenergetics **1757**: 480–485

Smith AMO, Ratcliffe RG, Sweetlove LJ (2004) Activation and function of mitochondrial uncoupling protein in plants. J Biol Chem **279**: 51944–51952

Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell **11**: 431–444

Spollen WG, Sharp RE, Saab IN, Wu Y (1993) Regulation of cell expansion in roots and shoots at low water potentials. *In* Smith JAC, Griffiths H, eds, Water Deficits: Plant Responses from Cell to Community. BIOS Scientific Publishers, Oxford, pp 37–52

Stepansky A, Galili G (2003) Synthesis of the *Arabidopsis* bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme of lysine catabolism is concertedly regulated by metabolic and stress associated signals. Plant Physiol **133**: 1407–1415

Stewart CR, Martin BA, Reding L, Cerwick S (1990) Respiration and alternative oxidase in corn seedlings tissues during germination at different temperatures. Plant Physiol **92**: 755–760

Studart-Guimarães C, Fait A, Nunes-Nesi A, Carrari F, Usadel B, Fernie AR (2007) Reduced expression of succinyl-coenzyme A ligase can be compensated for by up-regulation of the gamma-aminobutyrate shunt in illuminated tomato leaves. Plant Physiol **145**: 626–639

Sweetlove LJ, Lytovchenko A, Morgan M, Nunes-Nesi A, Taylor NL, Baxter CJ, Eickmeier I, Fernie AR (2006) Mitochondrial uncoupling protein is required for efficient photosynthesis. Proc Natl Acad Sci USA **103**: 19587–19592

Sweetlove LJ, Beard KF, Nunes-Nesi A, Fernie AR, Ratcliffe RG (2010) Not just a circle: flux modes in the plant TCA cycle. Trends Plant Sci **15**: 462–470

Tardieu F, Reymond M, Hamard P, Granier C, Muller B (2000) Spatial distributions of expansion rate, cell division rate and cell size in maize leaves: a synthesis of the effects of soil water status, evaporative demand and temperature. J Exp Bot **51**: 1505–1514

Taylor NL, Rudhe C, Hulett JM, Lithgow T, Glaser E, Day DA, Millar AH, Whelan J (2003) Environmental stresses inhibit and stimulate different protein import pathways in plant mitochondria. FEBS Lett **547**: 125–130

Taylor NL, Heazlewood JL, Day DA, Millar AH (2004) Lipoic acid-dependent oxidative catabolism of α-keto acids in mitochondria provides evidence for branched-chain amino acid catabolism in *Arabidopsis*. Plant Physiol **134**: 838–848

Tempone AG, Sartorelli P, Mady C, Fernandes F (2007) Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. Cell Mol Biol **15**: 15–25

Tezara W, Mitchell VJ, Driscoll SD, Lawlor DW (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature **401**: 914–917

Timm S, Nunes-Nesi A, Pärnik T, Morgenthal K, Wienkoop S, Keerberg O, Weckwerth W, Kleczkowski LA, Fernie AR, Bauwe H (2008) A cytosolic pathway for the conversion of hydroxypyruvate to glycerate during photorespiration in *Arabidopsis*. Plant Cell **20**: 2848–2859

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The botany array resource: e-Northerns, expression angling, and promoter analyses. Plant J **43**: 153–163

Urano K, Yoshiba Y, Nanjo T, Igarashi Y, Seki M, Sekiguchi K, Yamaguchi-Shinozaki K, Shinozaki K (2003) Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. Plant Cell Environ **26**: 1917–1926

Urano K, Maruyama K, Ogata Y, Morishita Y, Takeda M, Sakurai N, Suzuki H, Saito K, Shibata D, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2009) Characterization of the ABA-regulated global responses to dehydration in *Arabidopsis* by metabolomics. Plant J **57**: 1065–1078

Usadel B, Blasing OE, Gibon Y, Poree F, Hohne M, Gunter M, Trethewey R, Kamlage B, Poorter H, Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. Plant Cell Environ **31**: 518–547

Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci **9**: 573–577

Vanková R, Dobrá J, Storchová H (2012) Recovery from drought stress in tobacco: an active process associated with the reversal of senescence in some plant parts and the sacrifice of others. Plant Signal Behav **7**: 19–21

Vanlerberghe GC, McIntosh L (1992) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. Plant Physiol **100**: 115–119

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: from gene to function. Annu Rev Plant Physiol Plant Mol Biol **48**: 703–734

Venekamp JH, Lampe JEM, Koot JT (1989) Organic acids as sources for drought-induced proline synthesis in field bean plants, *Vicia faba* L. J Plant Physiol **133**: 654–659

Vercesi AE, Borecky J, de Godoy Maia I, Arruda P, Cuccovia IM, Chaimovich H (2006) Plant uncoupling mitochondrial proteins. Annu Rev Plant Biol **57**: 383–404

Vertucci CW, Farrant JM (1995) Acquisition and loss of desiccation tolerance. *In* Kigel J, Galili G, eds, Seed development and germination. Marcel Dekker, New York, USA, pp 237–271

Virlouvet L, Jacquemot MP, Gerentes D, Corti H, Bouton S, Gilard F, Valot B, Trouverie J, Tcherkez G, Falque M, Damerval C, Rogowsky P, Perez P, Noctor G, Zivy M, Coursol S (2011) The ZmASR1 protein influences branched-chain amino acid biosynthesis and maintains kernel yield in maize under water-limited conditions. Plant Physiol **157**: 917–936

Wagner AM, Krab K (1995) The alternative respiration pathway in plants: role and regulation. Physiol Plant **95**: 318–325

Weigelt K, Kuster H, Rutten T, Fait A, Fernie AR, Miersch O, Wasternack C, Emery RJ, Desel C, Hosein F, Muller M, Saalbach I, Weber H (2009) ADP-glucose pyrophosphorylase-deficient pea embryos reveal specific transcriptional and metabolic changes of carbon-nitrogen metabolism and stress responses. Plant Physiol **149**: 395–411

Widodo, Patterson JH, Newbigin E, Tester M, Bacic A, Roessner U (2009) Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. J Exp Bot **60**: 4089–4103

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An 'electronic fluorescent pictograph' browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2**: e718.

Witt S, Galicia L, Lisec J, Cairns J, Tiessen A, Araus JL, Palacios-Rojas N, Fernie AR (2012) Metabolic and phenotypic responses of greenhouse grown maize hybrids to experimentally controlled drought stress. Mol Plant **5**: 401–417

Wolfe J, Bryant G (1999) Freezing, drying, and/or vitrification of membrane–solute–water systems. Cryobiology **39**: 103–129

Xue GP, McIntyre CL, Glassop D, Shorter R (2008) Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. Plant Mol Biol **67**: 197–214

Yan SP, Tang ZC, Su W, Sun WN (2005) Proteomic analysis of salt stress responsive proteins in rice root. Proteomics **5**: 235–244

Yan D (2007) Protection of the glutamate pool concentration in enteric bacteria. Proc Natl Acad Sci USA **104**: 9475–9480

Yang S, Vanderbeld B, Wan J, Huang Y (2010) Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops. Mol Plant **3**: 469–490

Yeaman SJ (1989) The 2-oxo acid dehydrogenase complexes: recent advances. Biochem J **257**: 625–632

Zagdanska B (1995) Respiratory energy demand for protein turnover and ion transport in wheat leaves upon water deficit. Physiol Plant **95**: 428–436

Zeeman SC, Rees T (1999) Changes in carbohydrate metabolism and assimilate export in starch-excess mutants of *Arabidopsis*. Plant Cell Environ **22**: 1445–1453

Zhang J, Frerman FE, Kim JJ (2006) Structure of electron transfer flavoprotein-ubiquinone oxidoreductase and electron transfer to the mitochondrial ubiquinone pool. Proc Natl Acad Sci USA **103**: 16212–16217

Zhang L, Xi D, Li S, Gao Z, Zhao S, Shi J, Wu C, Guo X (2011) A cotton group C MAP kinase gene, GhMPK2, positively regulates salt and drought tolerance in tobacco. Plant Mol Biol **77**: 17–31

Zhu X, Galili G (2003) Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in *Arabidopsis* seeds. Plant Cell **15**: 845–853

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53**: 247–273

Zhu J, Gong Z, Zhang C, Song C-P, Damsz B, Inan G, Koiwa H, Zhu J-K, Hasegawa PM, Bressan RA (2002) OSM1/SYP61: A syntaxin protein in *Arabidopsis* controls abscisic acid-mediated and non-abscisic acid mediated responses to abiotic stress. Plant Cell **14**: 3009–3028

SUPPLEMENTAL DATA

Supplemental Table S1. Fatty acid methyl esters (FAMES) used as standard retention times

Compound	Retention Time (min)	Retention Time Index (s)
Octanoic acid methyl ester	7.81	262320
Nonanoic acid methyl ester	9.25	323120
Decanoic acid methyl ester	10.65	381020
Dodecanoic acid methyl ester	13.25	487220
Tetradecanoic acid methyl ester	15.60	582620
Hexadecanoic acid methyl ester	17.72	668720
Octadecanoic acid methyl ester	19.66	747420
Eicosanoic acid methyl ester	21.44	819620
Docosanoic acid methyl ester	23.08	886620
Tetracosanoic acid methyl ester	24.60	948820
Hexacosanoic acid methyl ester	26.02	1006900
Octacosanoic acid methyl ester	27.35	1061700
Triacontanoic acid methyl ester	28.72	1113100

Supplemental Table S2. Relative metabolite content of the fully expanded leaves of *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Values are means ± SE of six independent samplings. Bold numbers indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT)

	WT			***etfqo-1***			***d2hgdh-2***			***ivdh-1***		
	0 d	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**
2-oxoglutarate	1 ± 0.01	1.04 ± 0.01	**5.91 ± 0.07**	0.97 ± 0.01	1.08 ± 0.01	**7.09 ± 0.06**	0.96 ± 0.01	1.19 ± 0.01	**7.71 ± 0.11**	0.87 ± 0.01	**1.56 ± 0.02**	**6.02 ± 0.06**
Alanine	1 ± 0.06	1.59 ± 0.09	**36.3 ± 2.33**	0.98 ± 0.04	1.63 ± 0.12	39.4 ± 1.88	1.17 ± 0.05	**2.19 ± 0.07**	**48.7 ± 3.11**	0.77 ± 0.04	**2.74 ± 0.09**	**45 ± 3.03**
Asparagine	1 ± 0.23	1.76 ± 0.54	**57.4 ± 3.01**	1.07 ± 0.11	1.90 ± 0.41	**45.3 ± 1.56**	1.30 ± 0.03	**5.07 ± 0.44**	**33 ± 1.89**	1.19 ± 0.12	**7.45 ± 1.22**	51.2 ± 2.63
Aspartate	1 ± 0.04	1.14 ± 0.09	2.18 ± 0.55	1.17 ± 0.05	1.19 ± 0.07	0.91 ± 0.08	1.21 ± 0.04	1.35 ± 0.05	2.36 ± 0.18	1.08 ± 0.04	1.19 ± 0.05	1.61 ± 0.21
β-Alanine	1 ± 0.06	**2.41 ± 0.39**	**90.7 ± 4.11**	1.09 ± 0.08	2.22 ± 0.16	76.4 ± 2.71	1.26 ± 0.05	**3.59 ± 0.21**	83.8 ± 2.09	1.07 ± 0.15	3.14 ± 0.36	78.4 ± 2.89
cis-Aconitate	1 ± 0.08	**0.66 ± 0.04**	**13.8 ± 0.85**	1.04 ± 0.04	**5.94 ± 0.37**	**22.7 ± 2.36**	0.94 ± 0.05	**3.16 ± 0.67**	16.5 ± 1.08	0.92 ± 0.06	**3.02 ± 0.75**	**19.9 ± 2.16**
Citrate	1 ± 0.24	**3.82 ± 0.36**	**4.24 ± 0.39**	0.92 ± 0.07	**1.48 ± 0.10**	**7.12 ± 0.75**	1.21 ± 0.12	**1.36 ± 0.01**	**7.38 ± 0.50**	1.20 ± 0.30	**2.11 ± 0.46**	**8.63 ± 0.86**
Dehydroascorbate	1 ± 0.22	**2.91 ± 0.46**	**17.5 ± 0.71**	1.51 ± 0.05	**6.76 ± 0.27**	20.6 ± 1.48	1.61 ± 0.22	**4.01 ± 0.04**	17.8 ± 1.79	1.20 ± 0.11	**5.81 ± 0.50**	15.9 ± 1.07
Erythritol	1 ± 0.12	**1.76 ± 0.04**	**53.6 ± 2.76**	1.43 ± 0.07	**2.70 ± 0.19**	76 ± 3.71	1.38 ± 0.05	**2.34 ± 0.09**	65.6 ± 2.40	1.45 ± 0.08	**2.79 ± 0.09**	45 ± 1.10
Fructose	1 ± 0.09	0.63 ± 0.13	**22.6 ± 3.80**	0.96 ± 0.03	**2.74 ± 0.38**	**32.2 ± 1.91**	0.79 ± 0.03	**4.27 ± 0.19**	27.8 ± 1.48	0.71 ± 0.04	**5.04 ± 0.85**	**34.6 ± 1.87**
Fumarate	1 ± 0.22	1.11 ± 0.36	**4.10 ± 0.41**	1.21 ± 0.19	**3.56 ± 0.45**	5.55 ± 0.52	1.49 ± 0.11	1.74 ± 0.37	3.06 ± 0.83	0.90 ± 0.06	**5.11 ± 0.91**	**7.74 ± 0.74**
GABA	1 ± 0.09	**4.76 ± 0.21**	**57.3 ± 0.44**	1.21 ± 0.13	4.99 ± 0.17	**82.7 ± 0.44**	1.15 ± 0.06	3.88 ± 0.17	**84.3 ± 1.11**	1.18 ± 0.03	5.03 ± 0.17	**82.1 ± 1.79**
Galactinol	1 ± 0.14	**15.5 ± 1.91**	**26.5 ± 0.53**	1.65 ± 0.22	**28.8 ± 3.37**	29.5 ± 1.45	1.99 ± 0.14	18.9 ± 2.13	**18.1 ± 1.09**	1.43 ± 0.25	**7.39 ± 1.42**	**37.4 ± 2.86**
Galactose	1 ± 0.08	**0.64 ± 0.08**	**6.88 ± 0.59**	0.99 ± 0.06	**2.55 ± 0.02**	**10.3 ± 1.38**	0.86 ± 0.03	**2.62 ± 0.15**	8.79 ± 0.45	0.79 ± 0.03	**2.87 ± 0.31**	9.75 ± 1.21
Glycerate	1 ± 0.08	**0.48 ± 0.04**	**25.9 ± 2.57**	0.88 ± 0.08	0.85 ± 0.15	**43.9 ± 1.40**	0.77 ± 0.05	0.68 ± 0.08	**42.7 ± 1.12**	0.71 ± 0.03	**1.24 ± 0.08**	**38.9 ± 1.41**
Glycolate	1 ± 0.09	0.94 ± 0.04	**7.72 ± 0.52**	0.87 ± 0.04	1.01 ± 0.03	10.7 ± 1.36	0.90 ± 0.01	0.94 ± 0.04	**12.4 ± 1.02**	0.83 ± 0.04	1.54 ± 0.05	**11.9 ± 1.53**
Gluconate	1 ± 0.14	2.01 ± 0.19	**29.7 ± 1.06**	1.17 ± 0.13	**4.33 ± 0.51**	**34.8 ± 1.19**	1.17 ± 0.05	**3.94 ± 0.19**	30 ± 0.74	1.10 ± 0.14	**5.01 ± 0.34**	27.3 ± 1.79
Glucose	1 ± 0.09	**0.66 ± 0.08**	**9.45 ± 0.66**	0.97 ± 0.07	**5.97 ± 0.36**	**17.8 ± 1.25**	0.87 ± 0.05	**2.3 ± 0.06**	**14.7 ± 1.25**	0.88 ± 0.04	**2.43 ± 0.27**	**18.8 ± 1.61**
Glutamate	1 ± 0.09	**1.39 ± 0.06**	**3.38 ± 0.84**	0.90 ± 0.02	1.46 ± 0.06	**2.33 ± 0.08**	0.89 ± 0.05	**1.72 ± 0.11**	**2.27 ± 0.37**	0.79 ± 0.05	**2.64 ± 0.13**	**1.62 ± 0.17**
Glutamine	1 ± 0.15	**0.36 ± 0.11**	**9.67 ± 0.89**	0.58 ± 0.08	**0.89 ± 0.06**	9.33 ± 0.81	0.99 ± 0.13	**0.89 ± 0.18**	**23.9 ± 2.57**	1.09 ± 0.18	**1.22 ± 0.08**	13.6 ± 1.32
Glycerol	1 ± 0.01	0.97 ± 0.11	**14.6 ± 0.96**	1.01 ± 0.03	1.01 ± 0.05	**28.5 ± 1.79**	1.10 ± 0.09	1.16 ± 0.07	18.4 ± 1.21	0.88 ± 0.01	1.39 ± 0.09	**26.8 ± 1.69**
Glycine	1 ± 0.06	0.75 ± 0.12	**48.4 ± 0.17**	0.69 ± 0.03	0.72 ± 0.09	47.1 ± 0.93	0.77 ± 0.09	**1.17 ± 0.08**	**59.7 ± 0.63**	0.63 ± 0.04	**1.38 ± 0.07**	53.7 ± 1.13
Guanidine	1 ± 0.09	2.24 ± 0.11	**29.5 ± 1.62**	1.31 ± 0.08	2.30 ± 0.19	31.3 ± 1.55	1.46 ± 0.13	**3.18 ± 0.26**	29.7 ± 2.60	1.56 ± 0.13	2.99 ± 0.10	**41.8 ± 1.14**

Supplemental Table S2. continued

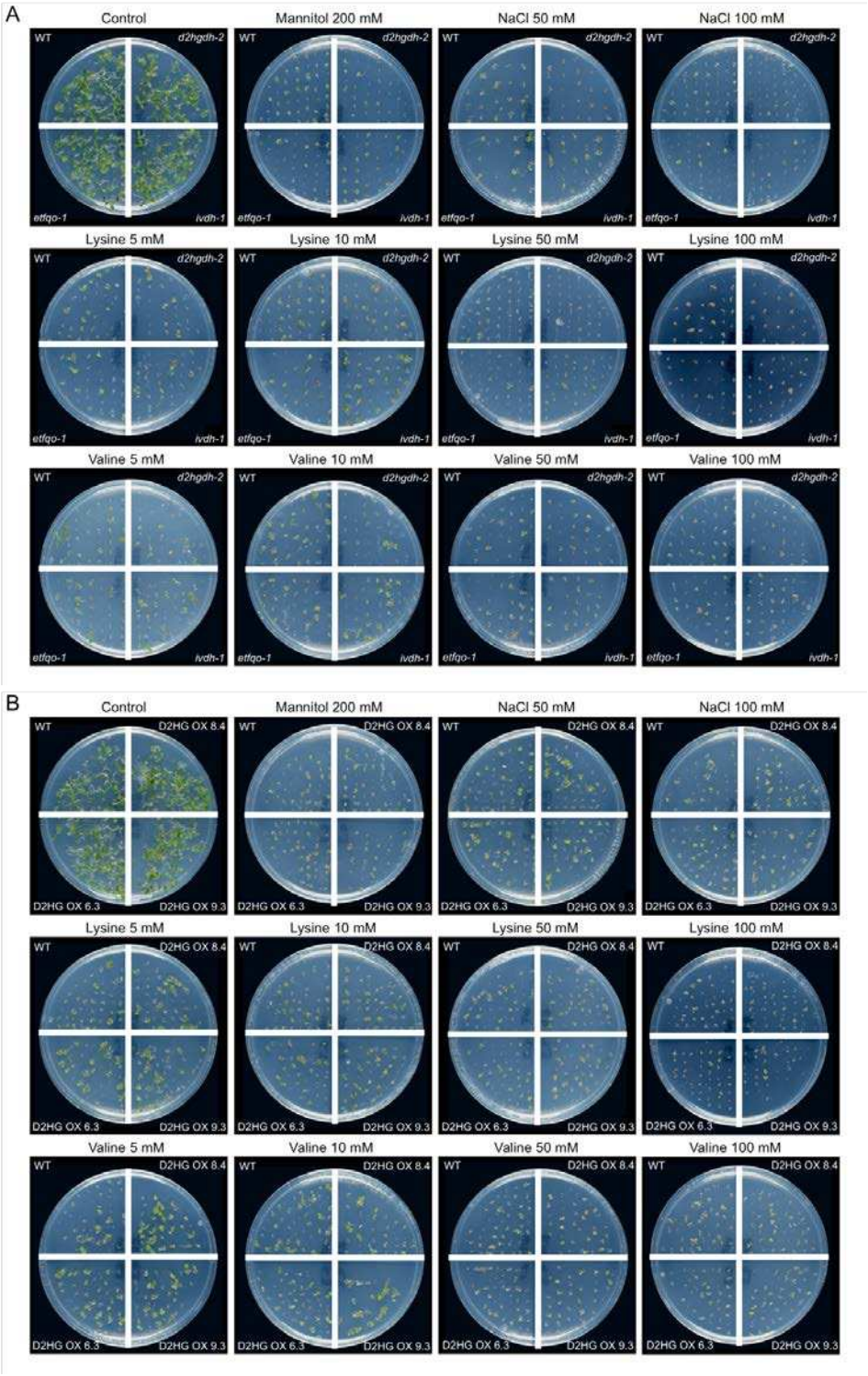
Isocitrate	1 ± 0.08	**0.67 ± 0.10**	**7.38 ± 0.60**	1.05 ± 0.07	**2.97 ± 0.03**	**12.5 ± 0.93**	0.94 ± 0.06	**2.82 ± 0.15**	9.26 ± 0.48	0.84 ± 0.04	**3.16 ± 0.37**	**11.4 ± 0.97**
Isoleucine	1 ± 0.12	1.79 ± 0.39	**92.5 ± 2.68**	0.87 ± 0.05	**3.29 ± 0.61**	**167.1 ± 2.3**	0.78 ± 0.02	**6.13 ± 0.87**	**143.1 ± 1.7**	0.63 ± 0.02	**2.94 ± 0.07**	**194.1 ± 2.7**
Leucine	1 ± 0.11	**2.35 ± 0.43**	**60.6 ± 1.90**	1.20 ± 0.09	**3.26 ± 0.47**	**154.9 ± 2.8**	0.83 ± 0.02	**5.47 ± 0.42**	**136.1 ± 2.5**	0.65 ± 0.04	2.39 ± 0.07	**186.1 ± 2.3**
Lysine	1 ± 0.22	**2.86 ± 0.39**	**70.1 ± 2.32**	1.44 ± 0.15	2.84 ± 0.31	**120.9 ± 3.1**	0.83 ± 0.09	**4.14 ± 0.11**	**113.4 ± 1.6**	1.47 ± 0.11	**3.12 ± 0.09**	**109.1 ± 2.2**
Malate	1 ± 0.22	1.31 ± 0.14	**8.04 ± 0.52**	1.18 ± 0.18	1.84 ± 0.48	9.44 ± 1.16	1.51 ± 0.12	1.47 ± 0.17	**18.1 ± 1.94**	0.94 ± 0.18	**3.35 ± 0.43**	6.05 ± 0.91
Maltose	1 ± 0.19	0.80 ± 0.16	**1.49 ± 0.36**	1.04 ± 0.07	0.58 ± 0.03	**3.61 ± 0.22**	1.07 ± 0.07	0.47 ± 0.05	2.12 ± 0.66	0.97 ± 0.08	**0.40 ± 0.02**	**4.18 ± 0.45**
Mannitol	1 ± 0.16	**5.97 ± 0.88**	**74.4 ± 2.47**	1.66 ± 0.13	**11.3 ± 1.65**	**47.8 ± 1.11**	0.87 ± 0.03	**14.9 ± 0.60**	**42 ± 0.06**	0.60 ± 0.05	6.13 ± 0.08	**37.4 ± 0.99**
Mannose	1 ± 0.06	0.75 ± 0.09	**6.47 ± 0.11**	1.05 ± 0.05	**1.45 ± 0.03**	**8.06 ± 0.26**	0.91 ± 0.03	**1.73 ± 0.10**	**7.38 ± 0.18**	0.99 ± 0.06	**1.99 ± 0.08**	6.95 ± 0.15
Methionine	1 ± 0.10	1.43 ± 0.32	**34.2 ± 0.93**	0.91 ± 0.06	1.79 ± 0.19	31.3 ± 0.88	1.03 ± 0.02	**2.43 ± 0.09**	28.9 ± 1.76	0.98 ± 0.05	**2.98 ± 0.40**	28.8 ± 1.98
myo-Inositol	1 ± 0.14	**2.01 ± 0.19**	**29.7 ± 1.06**	1.17 ± 0.13	**3.46 ± 0.36**	**34.8 ± 1.19**	1.21 ± 0.04	**4.12 ± 0.22**	30 ± 0.95	1.10 ± 0.14	**5.01 ± 0.34**	27.3 ± 0.79
Octadecanoic ac.	1 ± 0.09	1.80 ± 0.47	**5.98 ± 0.64**	0.92 ± 0.03	0.95 ± 0.02	6.92 ± 0.69	1.22 ± 0.02	1.50 ± 0.04	**8.86 ± 0.60**	0.93 ± 0.04	1.69 ± 0.11	6.12 ± 0.47
Ornithine	1 ± 0.04	1.03 ± 0.22	**12.9 ± 0.71**	0.74 ± 0.06	0.67 ± 0.08	11.1 ± 2.36	1.09 ± 0.05	1.19 ± 0.02	**23.7 ± 1.07**	0.79 ± 0.06	1.48 ± 0.11	**28.6 ± 0.77**
Phenylalanine	1 ± 0.12	1.91 ± 0.46	**55.2 ± 3.11**	0.99 ± 0.07	1.72 ± 0.21	67.9 ± 1.35	0.75 ± 0.03	**3.35 ± 0.21**	**82.5 ± 0.46**	0.59 ± 0.06	**3.95 ± 0.92**	**103.1 ± 2.6**
Proline	1 ± 0.17	1.38 ± 0.11	**9.71 ± 0.12**	1.27 ± 0.09	1.96 ± 0.17	**13.4 ± 0.19**	1.18 ± 0.06	1.93 ± 0.13	**13.8 ± 0.14**	1.18 ± 0.09	1.52 ± 0.11	**12.6 ± 0.09**
Putrescine	1 ± 0.11	**0.54 ± 0.14**	**18.6 ± 0.39**	0.74 ± 0.04	1.01 ± 0.16	20.5 ± 0.34	0.86 ± 0.05	**1.75 ± 0.20**	**37.5 ± 1.94**	1.58 ± 0.14	**5.47 ± 0.09**	**33.5 ± 0.53**
Raffinose	1 ± 0.14	**23.4 ± 2.15**	**44.9 ± 1.51**	1.62 ± 0.39	24.1 ± 1.48	49.1 ± 2.42	1.60 ± 0.09	20.5 ± 2.06	48.6 ± 3.75	1.60 ± 0.42	24.3 ± 2.19	46.1 ± 4.57
Ribose	1 ± 0.08	1.09 ± 0.16	**17.4 ± 1.34**	0.99 ± 0.03	**2.05 ± 0.08**	**24.5 ± 1.67**	0.89 ± 0.03	**2.01 ± 0.07**	22.2 ± 1.33	0.91 ± 0.10	**2.77 ± 0.47**	**33.2 ± 1.03**
Serine	1 ± 0.06	**1.43 ± 0.10**	**11 ± 0.59**	0.77 ± 0.04	1.50 ± 0.09	**8.34 ± 0.72**	0.96 ± 0.03	1.99 ± 0.11	9.26 ± 0.49	0.91 ± 0.06	**2.45 ± 0.10**	11.1 ± 0.97
Shikimate	1 ± 0.07	**1.48 ± 0.14**	**28.6 ± 1.04**	1.45 ± 0.10	1.83 ± 0.12	**46.4 ± 1.78**	0.81 ± 0.06	**2.13 ± 0.15**	40.7 ± 1.06	0.86 ± 0.04	**3.15 ± 0.21**	30.9 ± 1.77
Spermidine	1 ± 0.13	0.96 ± 0.08	**32 ± 2.62**	0.93 ± 0.07	**1.53 ± 0.17**	**54.7 ± 1.61**	1.12 ± 0.03	**2.09 ± 0.18**	**51.9 ± 2.68**	1.16 ± 0.08	**2.82 ± 0.24**	**43.4 ± 1.54**
Succinate	1 ± 0.13	0.82 ± 0.10	**7.38 ± 0.33**	0.90 ± 0.06	1.16 ± 0.08	**9.90 ± 2.69**	0.99 ± 0.11	1 ± 0.09	**14.2 ± 0.49**	0.92 ± 0.09	1.27 ± 0.12	**10.4 ± 0.92**
Sucrose	1 ± 0.02	1.26 ± 0.04	**5.32 ± 0.19**	1.12 ± 0.09	**1.83 ± 0.08**	5.77 ± 0.11	0.99 ± 0.07	1.42 ± 0.12	5.93 ± 0.09	0.97 ± 0.08	1.49 ± 0.12	6.11 ± 0.19
Threonine	1 ± 0.07	1.11 ± 0.25	**6.78 ± 0.05**	0.91 ± 0.04	**2.22 ± 0.33**	4.90 ± 0.09	1.12 ± 0.06	**3.17 ± 0.31**	6.78 ± 0.04	0.78 ± 0.08	1.02 ± 0.02	**8.6 ± 0.04**
Trehalose	1 ± 0.19	**1.94 ± 0.41**	**35.4 ± 1.14**	0.98 ± 0.04	1.58 ± 0.19	36.6 ± 2.30	0.97 ± 0.03	1.76 ± 0.23	31.9 ± 1.68	0.71 ± 0.06	1.84 ± 0.18	**21.6 ± 0.79**
Tryptophan	1 ± 0.14	0.81 ± 0.32	**69.9 ± 2.56**	0.75 ± 0.05	1.54 ± 0.19	62.6 ± 0.42	0.51 ± 0.01	**2.27 ± 0.47**	**80 ± 0.95**	0.58 ± 0.02	**2.21 ± 0.54**	**59.9 ± 0.72**
Tyrosine	1 ± 0.15	**2.14 ± 0.25**	**46.3 ± 0.79**	1.19 ± 0.14	**3.75 ± 0.42**	**57.3 ± 1.86**	1.24 ± 0.04	**4.61 ± 0.27**	48.4 ± 1.27	1.11 ± 0.15	**5.98 ± 0.39**	42.7 ± 1.26
Valine	1 ± 0.08	1.49 ± 0.28	**55.7 ± 0.57**	1.06 ± 0.07	**3.66 ± 0.69**	**63.4 ± 0.65**	0.97 ± 0.03	**5.09 ± 0.62**	**81.6 ± 1.17**	0.81 ± 0.04	**2.59 ± 0.08**	**84 ± 1.45**

Supplemental Table S3. Relative metabolite content of the fully expanded leaves of *Arabidopsis* overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 days without watering as measured by GC-MS. Values are means ± SE of six independent samplings. Bold numbers indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the wild type (WT)

	WT			**D2HGDH OX 6.3**			**D2HGDH OX 8.4**			**D2HGDH OX 9.3**		
	0 d	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**	**0 d**	**5 d**	**10 d**
2-oxoglutarate	1 ± 0.02	1.12 ± 0.02	**1.97 ± 0.06**	1.10 ± 0.02	1.23 ± 0.04	1.76 ± 0.07	0.98 ± 0.03	1.22 ± 0.03	1.67 ± 0.03	1.19 ± 0.03	1.33 ± 0.04	1.83 ± 0.04
Alanine	1 ± 0.03	0.65 ± 0.04	**46.4 ± 7.53**	1.44 ± 0.11	**1.32 ± 0.05**	38.8 ± 2.97	0.88 ± 0.14	**0.92 ± 0.02**	55.5 ± 2.64	0.54 ± 0.05	**1.04 ± 0.05**	49.1 ± 5.22
Aspartate	1 ± 0.08	**0.63 ± 0.03**	1.09 ± 0.08	1.19 ± 0.08	**1.33 ± 0.09**	**3.71 ± 0.14**	1.06 ± 0.05	0.87 ± 0.08	**2.6 ± 0.40**	0.82 ± 0.02	**1.01 ± 0.04**	**3.16 ± 0.11**
β-Alanine	1 ± 0.04	**1.34 ± 0.05**	**93.9 ± 2.51**	1.06 ± 0.04	1.23 ± 0.11	96.4 ± 2.99	0.97 ± 0.02	1.84 ± 0.32	86.7 ± 3.71	0.88 ± 0.04	1.07 ± 0.07	87.8 ± 3.32
cis-Aconitate	1 ± 0.04	**1.22 ± 0.07**	**3.51 ± 0.24**	1.05 ± 0.03	1.34 ± 0.05	**7.83 ± 0.37**	1.05 ± 0.01	1.17 ± 0.08	**8.67 ± 0.87**	0.95 ± 0.01	1.45 ± 0.06	**8.82 ± 0.21**
Citrate	1 ± 0.19	1.24 ± 0.09	**11.96 ± 3.0**	1.09 ± 0.08	1.17 ± 0.03	7.33 ± 0.52	0.49 ± 0.07	**0.58 ± 0.06**	**4.3 ± 2.25**	0.29 ± 0.06	**2.24 ± 0.11**	10.2 ± 0.67
Dehydroascorbate	1 ± 0.12	0.86 ± 0.01	**2.83 ± 0.25**	0.70 ± 0.08	0.95 ± 0.17	**11.2 ± 0.65**	0.85 ± 0.06	0.95 ± 0.13	**5.51 ± 0.31**	0.44 ± 0.04	0.79 ± 0.16	**7.51 ± 0.48**
Erythritol	1 ± 0.06	**2.78 ± 0.14**	**12.1 ± 2.25**	0.99 ± 0.09	**1.44 ± 0.06**	**18.3 ± 0.22**	0.99 ± 0.03	**1.37 ± 0.18**	**18.5 ± 2.66**	0.81 ± 0.04	1.8 ± 0.16	16.8 ± 1.84
Fructose	1 ± 0.18	1.15 ± 0.15	**14.42 ± 0.7**	1.14 ± 0.05	1.49 ± 0.14	16.2 ± 0.66	1.16 ± 0.06	**1.68 ± 0.1**	**23.4 ± 1.14**	0.69 ± 0.02	1.14 ± 0.14	**26.5 ± 0.81**
Fumarate	1 ± 0.06	1.19 ± 0.03	**4.71 ± 0.05**	1.16 ± 0.01	**1.35 ± 0.02**	**7.78 ± 0.2**	1.02 ± 0.02	0.57 ± 0.24	**3.18 ± 0.65**	0.52 ± 0.07	1.31 ± 0.02	**8.73 ± 0.10**
GABA	1 ± 0.14	1.04 ± 0.03	**56.6 ± 2.04**	1.18 ± 0.11	0.97 ± 0.06	**86.5 ± 2.59**	1.06 ± 0.05	**0.87 ± 0.03**	**94 ± 1.01**	0.83 ± 0.03	0.99 ± 0.07	**86.5 ± 2.25**
Galactinol	1 ± 0.13	**6.61 ± 1.05**	**3.33 ± 0.35**	1.27 ± 0.19	**12.2 ± 0.25**	**17.8 ± 1.31**	0.59 ± 0.12	6.37 ± 0.89	**11.3 ± 1.05**	1.06 ± 0.09	4.9 ± 0.02	**10.9 ± 0.54**
Galactose	1 ± 0.08	**1.77 ± 0.21**	**26.9 ± 2.06**	1.25 ± 0.09	1.68 ± 0.14	26.1 ± 5.07	1.21 ± 0.15	1.15 ± 0.19	**35.2 ± 1.56**	0.84 ± 0.05	0.98 ± 0.17	31.8 ± 0.88
Gluconate	1 ± 0.09	0.83 ± 0.07	**61.6 ± 0.99**	0.99 ± 0.03	1.15 ± 0.14	**44.8 ± 4.89**	1.02 ± 0.08	1.02 ± 0.14	57.3 ± 4.62	0.63 ± 0.04	0.75 ± 0.05	60.2 ± 3.42
Glucose	1 ± 0.13	0.97 ± 0.09	**8.08 ± 0.66**	1.28 ± 0.04	**1.81 ± 0.17**	9.49 ± 0.16	1.03 ± 0.06	1.45 ± 0.15	**13.1 ± 0.42**	0.79 ± 0.02	1.13 ± 0.09	**15.6 ± 0.03**
Glutamate	1 ± 0.06	**0.59 ± 0.04**	**0.50 ± 0.09**	0.83 ± 0.03	**0.82 ± 0.06**	**2.64 ± 0.09**	0.88 ± 0.04	**0.75 ± 0.02**	**0.84 ± 0.12**	0.66 ± 0.03	0.59 ± 0.02	0.64 ± 0.03
Glutamine	1 ± 0.02	0.98 ± 0.04	**4.80 ± 0.38**	0.92 ± 0.04	0.87 ± 0.05	4.46 ± 0.37	0.82 ± 0.04	0.92 ± 0.03	5.3 ± 0.45	0.82 ± 0.01	1.17 ± 0.01	6.56 ± 0.48
Glycerate	1 ± 0.11	0.92 ± 0.11	**14.2 ± 2.22**	0.78 ± 0.02	**1.49 ± 0.14**	20.2 ± 3.28	0.61 ± 0.02	1.29 ± 0.12	**26.7 ± 4.08**	0.54 ± 0.02	1.43 ± 0.05	20.9 ± 0.8
Glycerol	1 ± 0.09	0.78 ± 0.05	**26.1 ± 0.98**	0.89 ± 0.04	0.77 ± 0.06	33.5 ± 2.72	0.91 ± 0.04	0.91 ± 0.03	**41.8 ± 1.34**	0.82 ± 0.03	0.94 ± 0.02	**50.8 ± 0.17**
Glycolate	1 ± 0.03	**0.76 ± 0.03**	**3.08 ± 0.32**	0.80 ± 0.03	0.96 ± 0.08	**5.18 ± 0.60**	0.81 ± 0.06	0.77 ± 0.04	**7.42 ± 0.79**	0.79 ± 0.03	1.04 ± 0.01	**5.84 ± 0.21**
Guanidine	1 ± 0.04	0.91 ± 0.01	**2.20 ± 0.26**	0.89 ± 0.03	1.02 ± 0.07	**5.71 ± 0.15**	0.96 ± 0.03	1.01 ± 0.04	**4.94 ± 0.61**	0.78 ± 0.07	0.99 ± 0.01	**4.03 ± 0.09**
Isocitrate	1 ± 0.06	**0.35 ± 0.03**	**6.75 ± 0.18**	1.43 ± 0.07	**0.62 ± 0.04**	**14.3 ± 1.15**	0.74 ± 0.08	0.29 ± 0.07	**26.9 ± 1.78**	0.46 ± 0.11	0.31 ± 0.02	**11.1 ± 0.55**
Isoleucine	1 ± 0.03	1.11 ± 0.07	**92.2 ± 3.36**	0.87 ± 0.04	**1.52 ± 0.04**	80.3 ± 3.58	0.67 ± 0.03	0.90 ± 0.08	87.8 ± 2.86	0.32 ± 0.01	0.72 ± 0.02	**72.2 ± 3.39**

Supplemental Table S3. continued

Leucine	1 ± 0.03	1.18 ± 0.11	**98.1 ± 6.69**	0.57 ± 0.01	0.89 ± 0.06	101.2 ± 3.6	0.50 ± 0.02	1.03 ± 0.09	**107.7 ± 3.8**	0.37 ± 0.01	0.83 ± 0.03	**100.9 ± 3.7**
Lysine	1 ± 0.02	**1.67 ± 0.19**	**69.0 ± 4.06**	0.85 ± 0.04	1.51 ± 0.11	65.9 ± 0.58	0.64 ± 0.01	2.33 ± 0.40	66.7 ± 1.92	0.87 ± 0.04	0.91 ± 0.1	67.7 ± 0.81
Malate	1 ± 0.04	0.99 ± 0.07	**11.6 ± 1.51**	1.47 ± 0.02	1.01 ± 0.09	**19.2 ± 0.46**	0.68 ± 0.05	**0.66 ± 0.04**	**26.4 ± 0.21**	0.49 ± 0.08	**1.89 ± 0.05**	**28.8 ± 0.36**
Maltose	1 ± 0.06	**0.25 ± 0.01**	**1.64 ± 0.11**	1.17 ± 0.03	**0.73 ± 0.11**	**3.32 ± 0.12**	1.13 ± 0.03	0.31 ± 0.03	**4.39 ± 0.12**	0.64 ± 0.02	0.43 ± 0.06	**2.59 ± 0.04**
Mannitol	1 ± 0.17	**2.71 ± 0.12**	**73.3 ± 5.15**	0.52 ± 0.01	**0.68 ± 0.04**	**13.9 ± 2.25**	0.77 ± 0.02	**0.83 ± 0.10**	**13.6 ± 0.78**	0.61 ± 0.01	2.06 ± 0.16	**15.3 ± 0.35**
Mannose	1 ± 0.14	0.74 ± 0.09	**10.1 ± 0.59**	1.14 ± 0.01	**1.53 ± 0.16**	9.59 ± 0.54	0.83 ± 0.07	**1.51 ± 0.22**	**15.6 ± 1.06**	0.62 ± 0.02	0.75 ± 0.09	**16.8 ± 0.38**
Methionine	1 ± 0.10	0.71 ± 0.07	**7.85 ± 0.08**	0.85 ± 0.09	0.63 ± 0.04	**17.8 ± 0.77**	0.86 ± 0.03	**0.97 ± 0.05**	**21.3 ± 2.12**	0.49 ± 0.05	0.47 ± 0.03	**30.9 ± 2.94**
myo-Inositol	1 ± 0.08	1.05 ± 0.09	**12.5 ± 0.34**	0.88 ± 0.04	**1.93 ± 0.04**	**22.5 ± 0.23**	0.76 ± 0.04	**1.83 ± 0.05**	**20.5 ± 1.63**	0.89 ± 0.08	1.16 ± 0.02	**26.6 ± 0.33**
Octadecanoic ac.	1 ± 0.04	1.25 ± 0.06	**4.28 ± 0.54**	1.24 ± 0.06	**2.12 ± 0.09**	**8.49 ± 0.44**	1.08 ± 0.06	1.13 ± 0.14	**9.28 ± 0.02**	0.89 ± 0.03	1.52 ± 0.09	**7.49 ± 0.29**
Ornithine	1 ± 0.09	**0.59 ± 0.04**	**8.43 ± 0.33**	0.97 ± 0.10	**0.89 ± 0.01**	**25.3 ± 1.71**	1.05 ± 0.03	0.80 ± 0.16	**20.3 ± 1.91**	1.25 ± 0.13	0.77 ± 0.04	**19.8 ± 3.03**
Phenylalanine	1 ± 0.12	0.74 ± 0.06	**68.4 ± 4.02**	0.67 ± 0.01	**0.51 ± 0.03**	72.7 ± 2.42	0.47 ± 0.03	**0.43 ± 0.08**	**89.7 ± 2.91**	0.32 ± 0.01	**0.48 ± 0.04**	**86.6 ± 1.3**
Proline	1 ± 0.07	1.45 ± 0.52	**10.0 ± 0.31**	0.59 ± 0.06	1.25 ± 0.08	**15.2 ± 0.59**	0.43 ± 0.03	1.78 ± 0.24	**18.3 ± 0.24**	0.39 ± 0.02	**0.38 ± 0.03**	**18.3 ± 0.47**
Putrescine	1 ± 0.05	**0.63 ± 0.09**	**19.7 ± 1.18**	1.13 ± 0.17	0.63 ± 0.08	**27.9 ± 1.92**	0.62 ± 0.03	0.38 ± 0.04	**33.2 ± 1.68**	0.91 ± 0.09	0.41 ± 0.01	**33.9 ± 0.83**
Raffinose	1 ± 0.10	**4.12 ± 0.38**	**9.77 ± 0.48**	0.64 ± 0.03	**6.26 ± 0.64**	**14.3 ± 0.85**	1.29 ± 0.03	**6.20 ± 0.33**	**20.5 ± 0.94**	1.03 ± 0.15	3.82 ± 0.46	**16.8 ± 1.24**
Rhamnose	1 ± 0.07	**1.57 ± 0.07**	**95.9 ± 3.68**	0.85 ± 0.02	**1.23 ± 0.06**	**42.2 ± 2.37**	0.98 ± 0.04	**1 ± 0.06**	**49.9 ± 2.09**	0.83 ± 0.03	1.44 ± 0.1	**53.4 ± 2.21**
Ribose	1 ± 0.03	1 ± 0.02	**6.99 ± 0.49**	1.04 ± 0.02	0.98 ± 0.01	6.64 ± 0.19	0.86 ± 0.01	**0.90 ± 0.02**	7.29 ± 0.11	0.77 ± 0.01	1.13 ± 0.01	**8.4 ± 0.12**
Serine	1 ± 0.12	**0.53 ± 0.04**	**49.3 ± 7.65**	1.13 ± 0.08	**1.06 ± 0.04**	**28.2 ± 1.92**	1.23 ± 0.18	**0.73 ± 0.01**	42.4 ± 3.29	0.43 ± 0.04	0.83 ± 0.04	41.6 ± 0.61
Spermidine	1 ± 0.07	0.68 ± 0.07	**30.2 ± 1.49**	1 ± 0.03	0.69 ± 0.05	30.7 ± 1.44	0.65 ± 0.05	0.61 ± 0.09	34.6 ± 1.71	0.39 ± 0.05	**0.23 ± 0.01**	35.7 ± 2.62
Succinate	1 ± 0.06	1.21 ± 0.04	**4.83 ± 0.31**	1.25 ± 0.03	1.39 ± 0.04	**8.62 ± 0.16**	1.03 ± 0.02	0.87 ± 0.13	5.57 ± 0.96	0.46 ± 0.08	1.30 ± 0.04	**11.1 ± 0.95**
Sucrose	1 ± 0.03	1.10 ± 0.01	**5.09 ± 0.13**	1 ± 0.01	1.22 ± 0.04	**7.82 ± 0.27**	0.89 ± 0.01	**0.91 ± 0.06**	**9.33 ± 0.18**	0.78 ± 0.05	1.21 ± 0.03	**9.57 ± 0.14**
Threonine	1 ± 0.05	1.12 ± 0.03	**4.70 ± 1.27**	1 ± 0.02	1.13 ± 0.14	**7.5 ± 0.42**	1.02 ± 0.02	1.09 ± 0.12	**7.12 ± 0.42**	0.98 ± 0.06	1.18 ± 0.06	**8.37 ± 0.15**
Trehalose	1 ± 0.10	**1.59 ± 0.10**	**65 ± 6.43**	0.55 ± 0.02	**0.52 ± 0.02**	**23.3 ± 0.55**	0.57 ± 0.04	**0.75 ± 0.06**	**17.1 ± 0.63**	0.47 ± 0.05	0.92 ± 0.02	**25.3 ± 1.43**
Tryptophan	1 ± 0.13	0.64 ± 0.07	**4.67 ± 0.26**	0.87 ± 0.05	0.59 ± 0.04	**21.5 ± 0.63**	0.68 ± 0.03	0.53 ± 0.03	**8.64 ± 0.72**	0.57 ± 0.06	0.57 ± 0.05	**10.3 ± 1.16**
Valine	1 ± 0.07	1.43 ± 0.20	**51.3 ± 2.94**	0.7 ± 0.01	**0.79 ± 0.07**	**87 ± 3.70**	0.58 ± 0.01	1.42 ± 0.21	**96.4 ± 2.36**	0.40 ± 0.02	**0.78 ± 0.03**	**104.5 ± 0.7**



Supplemental Figure S1. Germination of *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1* (A) and overexpressor lines D2HGDH OX 6.3, 8.4 and 9.3 (B). Morphology of the germinating seedlings seven days after stratification