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Original Article

The influence of alternative pathways of respiration that utilize branched-chain amino acids following water shortage in *Arabidopsis*

Marcel V. Pires[1,2], Adilson A. Pereira Júnior[1], David B. Medeiros[2], Danilo M. Daloso[1,2], Phuong Anh Pham[1], Kallyne A. Barros[2], Martin K. M. Engqvist[3,4], Alexandra Florian[1], Ina Krahnert[1], Veronica G. Maurino[3], Wagner L. Araújo[2] & Alisdair R. Fernie[1]

[1]*Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany,* [2]*Max-Planck Partner Group, Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil,* [3]*Plant Molecular Physiology and Biotechnology, Institute of Plant Developmental and Molecular Biology, Cluster of Excellence on Plant Sciences (CEPLAS), Heinrich-Heine-University, Universitätsstr 1, D-40225 Düsseldorf, Germany and* [4]*Department of Chemical and Biological Engineering, Chalmers University of Technology, Göterborg, Sweden*

ABSTRACT

During dark-induced senescence isovaleryl-CoA dehydrogenase (IVDH) and D-2-hydroxyglutarate dehydrogenase (*D-2HGDH*) act as alternate electron donors to the ubiquinol pool via the electron-transfer flavoprotein/electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETFQO) pathway. However, the role of this pathway in response to other stresses still remains unclear. Here, we demonstrated that this alternative pathway is associated with tolerance to drought in *Arabidopsis*. In comparison with wild type (WT) and lines overexpressing *D-2GHDH*, loss-of-function *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutants displayed compromised respiration rates and were more sensitive to drought. Our results demonstrated that an operational ETF/ETFQO pathway is associated with plants' ability to withstand drought and to recover growth once water becomes replete. Drought-induced metabolic reprogramming resulted in an increase in tricarboxylic acid (TCA) cycle intermediates and total amino acid levels, as well as decreases in protein, starch and nitrate contents. The enhanced levels of the branched-chain amino acids in loss-of-function mutants appear to be related to their increased utilization as substrates for the TCA cycle under water stress. Our results thus show that mitochondrial metabolism is highly active during drought stress responses and provide support for a role of alternative respiratory pathways within this response.

Key-words: branched-chain amino acids; drought; ETF/ETFQO pathway; metabolomics; respiration; tricarboxylic acid cycle.

INTRODUCTION

It is widely recognized that plant productivity largely depends on the balance between photosynthesis and respiration (Krömer *et al.* 1993; Nunes-Nesi *et al.* 2011; Araújo *et al.* 2014). 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 (Ribas-Carbo *et al.* 2005; Atkin & Macherel 2009). This fact apart, the effects of water limitation on mitochondrial respiration have also strong impacts on agricultural yield (Ribas-Carbo *et al.* 2005; Atkin & Macherel 2009). This is most likely because the respiratory process involves a variety of physiological functions including ATP synthesis, supply of carbon (C) skeletons for biosynthetic processes and regulation of the cellular redox potential (Bauwe *et al.* 2010; Foyer & Noctor 2011; van Dongen *et al.* 2011; Araújo *et al.* 2012). Moreover, respiration plays an important role in the adaptive responses of plants to several abiotic stresses (Pastore *et al.* 2007; Atkin & Macherel 2009; Dinakar *et al.* 2010). Despite being such a fundamental and robust process, the flexibility of respiration renders it highly sensitive to environmental changes. Unlike photosynthesis that is limited by a range of environmental conditions and to certain cell types, respiration occurs continuously in each cell of every plant organ. Given this, it seems reasonable to assume that the process of respiration plays an important role as a mechanism for controlling yield. This is especially true when photosynthesis is affected, for example, under limited water availability conditions (Atkin & Macherel 2009).

Compelling evidence has recently demonstrated that, although plant respiration is mainly dependent on carbohydrate oxidation under stress conditions (Plaxton & Podesta 2006; Araújo *et al.* 2012), metabolism is altered such that other pathways are induced in order to provide alternative respiratory substrates to the respiratory processes (Ishizaki *et al.* 2005; Ishizaki *et al.* 2006; Engqvist *et al.* 2009; Araújo *et al.* 2010). In mammals, the electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETFQO), a nuclear-encoded protein located in the inner mitochondrial membrane, accepts electrons from the ETF that is localized in the mitochondrial matrix and then reduces ubiquinone (Ruzicka & Beinert 1977; Beckmann & Frerman 1985; Zhang *et al.* 2006; Watmough & Frerman 2010). In these species, the ETF/ETFQO complex is essential for the catabolism of fatty acid, diverse amino acids and choline,

Correspondence: W. L. Araújo. Fax: +55 31 3899 2580; e-mail: wlaraujo@ufv.br

supplying the mitochondrion with alternative respiratory substrates to glucose (Frerman 1988; Watmough & Frerman 2010). Similarly to the situation observed in mammal cells, the ETF/ETFQO complex was identified in plants as being located in the mitochondrial membrane (Heazlewood *et al*. 2004). This complex has been shown to be highly induced at a transcriptional level during dark-induced senescence (Buchanan-Wollaston *et al*. 2005) oxidative stress (Lehmann *et al*. 2009), and under conditions in which free amino acids are plentiful (Weigelt *et al*. 2008). Using a combination of enzymatic, metabolic and isotopic labelling approaches in *Arabidopsis thaliana* mutants, it has been recently demonstrated that catabolic products of both aromatic and branched-chain amino acids (BCAAs) (e.g. isoleucine, leucine and valine) metabolism can act as substrates of ETF/ETFQO pathway and are alternative electron donors to the mitochondrial electron transport chain (mETC) (Araújo *et al*. 2010). This donation occurs either directly, through the transfer of electrons to the mETC via ETF complex, or indirectly, by the direct feeding of catabolic products into the tricarboxylic acid (TCA) cycle (Araújo *et al*. 2010). We have previously demonstrated that both 2-hydroxyglutarate dehydrogenase (*D-2HGDH*) and isovaleryl-CoA dehydrogenase (IVDH) are able to donate electrons to the ubiquinone pool via ETF/ETFQO complex (Engqvist *et al*. 2009; 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 *D-2HGDH*, because it seems to be capable of using a wider range of substrates (Araújo *et al.* 2010). This finding is additionally consistent with recent suggestions that like in animals, *D-2HGDH* largely functions as an enzyme of metabolite repair in plants (Hüdig *et al*. 2015).

Although the role of alternative substrates under dark-induced senescence is now well accepted (Fahnenstich *et al.* 2007; Araújo *et al*. 2010; Engqvist *et al*. 2011), their participation during other stressful conditions such as, for example, water shortage has received little attention to date. This is somewhat surprising given the fact that the role of mitochondrial metabolism in water stress has been documented in several studies (Giraud *et al*. 2008; Skirycz *et al*. 2010a, 2010b; Geisler *et al*. 2012). Bearing this in mind, studies aiming at fully understanding the role of the BCAA as alternative substrate supplying electrons to the mETC under water deficit conditions will likely be highly revealing. For this reason, in an attempt to elucidate the physiological role of enzymes involved in the alternative supply of electrons to the mETC in *A. thaliana* under limited water conditions, a metabolic and physiological approach was undertaken using a range of loss-of-function mutants as well as overexpression lines of enzymes known to donate electrons to the mETC. The results presented here reveal that both the ETF/ETFQO pathway and BCAA catabolism, but not the accumulation of BCAAs *per se*, appear to play an important role in the tolerance mechanisms to short-term episodes of drought, most likely by delaying the onset of stress. Our findings also demonstrated that although knockout (KO) and overexpression lines lose water faster and slower than wild type (WT) plants, respectively, this is not associated with changes in stomatal behaviour but most likely linked with metabolic changes following episodes of water limitation. The combined data are discussed in the context of current models of metabolic regulation of classical and alternative pathways of plant respiration in the illuminated leaf during episodes of water shortage.

MATERIAL AND METHODS

Plant material

All *A. 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), SAIL844G06 (*d2hgdh-2*) and SALK_007870 (*etfqo-1*, Ishizaki *et al*. 2005) were handled exactly as previously described. Three different *D-2HGDH* overexpressor (OX) lines (*D-2HGDH* OX 6.3, 8.4 and 9.3; Engqvist *et al*. 2011) were also used. The *Arabidopsis* genome initiative locus numbers for the major genes discussed in this study can be found in the GenBank/EMBL databases under the following accession numbers: *IVDH*, At3g45300; *D-2HGDH*, At4g36400 and *ETFQO*, At2g43400 (The Arabidopsis Genome Initiative; http://www.arabidopsis.org/).

Growth conditions and water stress treatment

Seeds were surface-sterilized and imbibed for 2 d at 4 °C in the dark on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog (MS) media (Sigma-Aldrich; pH 5.7). Seeds were subsequently germinated and grown at 22 °C under short-day conditions (8 h light/16 h dark) with 150 μmol m^{-2} s^{-1}. For examination of phenotype, seedlings were transferred to soil 7 to 10 d after germination and placed in a growth chamber at 22 °C under long-day conditions (16 h light/8 h dark). Four-week-old plants were subjected to a progressive water deficit by suspension of irrigation and then given recovery irrigation aiming analyses, the capacity of recovery vegetative growth of those plants. 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. At days 0, 5 and 10 of drought stress and following 1, 3 and 5 d of recovery irrigation samples were harvested at around 12 h (middle of the photoperiod) for further analysis. We additionally grew plants side by side in a similar manner in large pots (6 L) to allow comparison of the responses in similar soil conditions and to be confident that the water restriction was identical on all genotypes. To this end, 4-week-old plants were subjected to water limitation exactly as described previously, and after 0, 8 and 16 d of drought stress, the relative water content (RWC) was determined, and the samples were harvested at about 12 h (middle of the photoperiod) for further metabolic analysis. It should be mentioned that both experiments were repeated at least three times (and even in different growth facilities) with similar phenotypes observed each time. For phenotype characterization, plants were also grown under a short-day photoperiod (8 h of light/16 h of dark), and similar responses were generally observed. Whole rosettes of six independent samples by genotype were harvested, at each harvest

point, and were immediately immersed in liquid nitrogen and stored at −80 °C until further use.

For metabolic analysis, frozen leaf tissues were ground in a mixer mill (Retsch MM301, Retsch, Haan, Germany), in appropriate containers with steel balls for 45 s at 25 Hz. After grinding, samples were aliquoted and stored at −80 °C until further use.

Water loss measurements and relative water content

For water loss measurements, the weight of detached leaves left in air, abaxial side up, under greenhouse conditions was determined between 8 and 12 h, at 20 min intervals. Water loss was calculated as a percentage of the initial fresh weight (FW) (Araújo *et al.* 2011b).

Leaf RWC was assessed to monitor the status of leaf hydration at 0, 5 and 10 d without watering as well as at 1, 3 and 5 d after the recovery of irrigation. In our large pot experiments, leaf RWC was assessed to monitor the status of leaf hydration at 0, 8 and 16 d without watering. One leaf from each replicate was excised and weighed in order to obtain the FW. Afterwards, leaves were hydrated for 2 h 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 °C for 72 h and weighed in order to obtain the dry weight (DW). For the calculation of RWC, the following equation was used:

$$RWC(\%) = \frac{FW - DW}{TW - DW} \quad (1)$$

Respiration measurements

Dark respiration was measured in an oxygen electrode following the protocol detailed previously (Geigenberger *et al.* 2000). Each measurement took ~5 min during which oxygen depletion in the buffer was less than 20% of the initial value.

Measurement of senescence parameters

Protein and chlorophyll content were determined photometrically as described in the literature (Bradford 1976; Porra *et al.* 1989), respectively. The ratio of F_v to F_m, which corresponds to the potential quantum yield of the photochemical reactions of PSII and represents a measure of the photochemical efficiency, was measured after dark adaptation of leaves for 30 min, as previously described (Oh *et al.* 1996).

Photosynthetic gas exchange parameters

Gas exchange parameters were determined using an open-flow infrared gas exchange analyser system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chambers (LI-6400-40; Li-Cor Inc.). Dark respiration (R_d) was measured after at least 1 h during the dark period, and it was divided by two (R_d/2) to estimate the mitochondrial respiration rate in the light (R_L) (Niinemets *et al.* 2005, 2006; Niinemets *et al.* 2009). The photorespiration rate (P_R) was estimated by using the values of A_N, R_d and C_i, and the compensation point in the absence of respiration (Γ^*), following the model proposed by Sharkey (1988), where $P_R = 0.5\,((A_N + R_d)/[(C_i/2\Gamma^*) - 0.5])$.

Photosynthetic light-response curves [A/photosynthetic photon flux density (PPFD)] were initiated at ambient CO_2 concentration (C_a) of 400 μmol mol^{-1} and PPFD of 600 μmol m^{-2} s^{-1}. Then, the PPFD was increased to 1000 μmol m^{-2} s^{-1} and after decreased until 0 μmol m^{-2} s^{-1} (11 different PPFD steps). Simultaneously, Chl *a* fluorescence parameters were obtained. The responses of A_N to C_i (A/C_i curves) were performed at 700 μmol m^{-2} s^{-1} at 25 °C under ambient O_2. Briefly, the measurements started at ambient CO_2 concentration (C_a) of 400 μmol mol^{-1}, and once the steady state was reached, C_a was decreased stepwise to 50 μmol mol^{-1}. Upon completion of the measurements at low C_a, C_a was returned to 400 μmol mol^{-1} to restore the original A_N. Next, C_a was increased stepwise to 1600 μmol mol^{-1} in a total of 13 different C_a values (Long and Bernacchi, 2003). Corrections for the leakage of CO_2 into and water vapour out of the leaf chamber of the LI-6400 were applied to all gas exchange data as described by Rodeghiero *et al.* (2007). A/C_i and A_N/PPFD curves were obtained using the ninth leaf totally expanded from 10 different plants per genotype in two independent assays (five plants in each assay). The estimation of mesophyll conductance (g_m), maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}) and photosynthetic limitations was carried out as detailed in Medeiros *et al.* (2016).

Determination of metabolite levels

Leaf material was sampled at the indicated time points, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. Extraction was performed by grinding the frozen materials in a ball–mill pre-cooled with liquid nitrogen and immediate addition of the appropriate extraction buffers. The levels of starch, nitrate and total amino acids were determined as described previously in Sienkiewicz-Porzucek *et al.* (2010).

Metabolite profiling was performed using approximately 75 mg of fully expanded rosette leaves. The extraction, derivation, standard addition and sample injection were performed exactly as described previously (Lisec *et al.* 2006). The gas chromatography–mass spectrometry (GC-MS) system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a Leco Pegasus III (Leco Corporation, St. Joseph, MI, USA) time-of-flight–mass spectrometry running in EI+ mode. Metabolites were identified in comparison with database entries of authentic standards (Kopka *et al.* 2005).

Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco, http://www.leco.com/) and TAGFINDER 4.0 software (Luedemann *et al.* 2008). Data presentation and experimental details follow recent recommendations (Fernie *et al.* 2011) and are recorded in Supporting Information Dataset S1, and the full data are additionally available in Supporting Information Tables S1 and S2.

Quantitative real-time PCR

Quantitative real-time PCR was carried out exactly as described previously (Zanor *et al.* 2009). The primers that were used here are described in the Supporting Information Table S3. RNA was extracted from at least six biological replicates and at least two technical replicates. Analyses were performed on fully expanded leaves of 4-week-old plants following harvesting and immediate snap freezing of the samples in liquid nitrogen. Extraction of total RNA was made by phenol separation and LiCl precipitation as previously described (Bugos *et al.* 1995). Digestion with DNase I (Ambion, http://www.ambion.com/) was performed according to the manufacturer's instructions. To confirm the absence of genomic DNA contamination, a quantitative PCR analysis using three primer pairs was performed. 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 (http://www.nanodrop.com/). cDNA was synthesized from 2 μg total RNA using superscript III reverse transcriptase (Invitrogen, http://www.invitrogen.com/) according to the manufacturer's instructions. PCR reactions were performed using an ABI prism 7900 HT sequence detection system (Applied Biosystems, http://www.appliedbiosystems.com/). Data analysis was performed using SDS software version 2.3 (Applied Biosystems). 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.

Statistical analysis

The experiments were conducted in a completely randomized design with 3–6 replicates of each genotype. 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.

RESULTS

Phenotype of *Arabidopsis* mutants under water deficit conditions

To investigate the involvement of BCAAs in alternative pathways of respiration during water shortage, we analysed three loss-of-function mutants of (i) the ETF/ETFQO complex (*etfqo-1*; Ishizaki *et al.* 2005); (ii) IVDH (*ivdh-1*; Araújo *et al.* 2010) and (iii) *D-2HGDH* (*d2hgdh-2*; Engqvist *et al.* 2009; Araújo *et al.* 2010), and OX lines of *D-2HGDH* (*D-2HGDH* OX; Engqvist *et al.* 2011). *Arabidopsis* KO and OX lines developed like the WT under normal watering conditions (Supporting Information Fig. S1). After suspension of irrigation, *etfqo-1*, *d2hgdh-2* and *ivdh-1* showed early symptoms of chlorosis and leaf wilting 4 to 5 d after the onset of water deficit. By contrast, dehydration symptoms in both WT and the OX lines become visible 6 to 7 d after the onset of water deficit. Interestingly, 10 d after the onset of the water deficit period, almost all KO mutants were completely dehydrated and showed severe leaf necrosis, while both WT and OX lines displayed higher survival rates, despite showing intense signs of dehydration. Notably, while soil-drying techniques are generally regarded as the most practical means of approximating field drought conditions for laboratory-based research, we are aware that their use leads to complicating factors such as variation in leaf or soil water loss rates due to differences in plant size and soil composition (Verslues *et al.* 2006). However, this seems to not be of great consequence in our work given that we are using plants of similar initial size (Supporting Information Fig. S1), grown on the same substrate.

The water loss observed in detached leaves from KO mutants was higher (30–32%) than in WT and OX lines (22–25%) after 180 min (Fig. 1A). Interestingly, detached leaves of the OX lines showed significantly lower FW losses than the WT during the first 2 h of treatment indicating a higher water conservation capacity in these lines. In addition, all genotypes studied showed

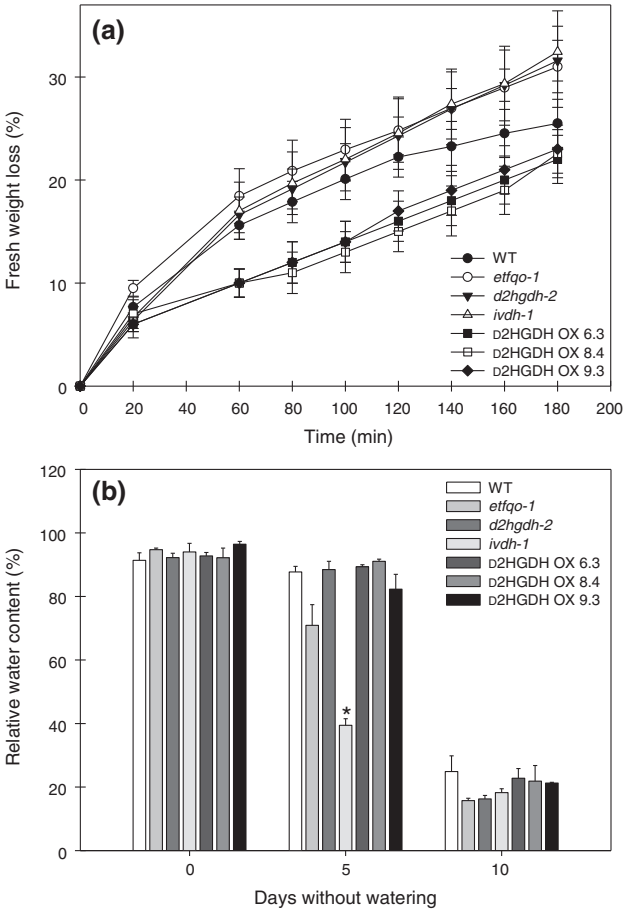

Figure 1. Fresh weight loss from detached leaves (a) and relative water content (b) of leaves of 4-week-old, long-day-grown *Arabidopsis* plants after further treatment for 0, 5 and 10 d without watering. Values are means ± SE of six independent samplings; an asterisk indicates values that were determined by the Student's *t*-test to be significantly different ($P < 0.05$) from the wild type (WT).

similar values of leaf RWC (approximately 93%) before the imposition of water stress (Fig. 1B). After 5 d without watering, a significant reduction of the RWC was observed in *ivdh-1* (39% of the control levels). At the end of the imposed treatment, all genotypes showed similar RWC values (15–24%); however, despite this fact, the visible phenotypes of the WT and OX lines more closely reflected the water content at earlier time points.

To evaluate the development of drought stress symptoms, we next determined chlorophyll content and maximum photochemical efficiency of PSII (F_v/F_m). These parameters are related to the function of chloroplasts and are commonly associated with the diagnosis of leaf senescence (Oh *et al.* 1996) as well as water stress status (Woo *et al.* 2008). Following the cessation of irrigation, total chlorophyll content ($a+b$) decreased and was lower in the KO mutants (*etfqo-1* and *ivdh-1*) in comparison with both WT and OX lines following 10 d of drought stress (Supporting Information Fig. S2). Reductions in the F_0 value were observed in all genotypes over the experimental period (Supporting Information Fig. S2) probably as a consequence of the earlier reductions in chlorophyll content. In agreement with the phenotypic responses of the plants under water deficit, the KO mutants displayed significant decreased F_0 value relative to WT and OX lines after 10 d of treatment (Supporting Information Fig. S2). Moreover, a decrease in F_v/F_m values was observed after 5 d without watering in *ivdh-1* and after 10 d in all other KO mutants relative to WT and OX values (Supporting Information Fig. S2). Taken together, these results indicate that *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutants are likely more sensitive to water deficit than WT and OX lines.

Phenotype of *Arabidopsis* mutants following restoration of irrigation

In order to assess the capacity of the plants to recover following water shortage, irrigation was restored 10 d after the onset of the water deficit. Previous observations of Woo *et al.* (2008) demonstrated that different ecotypes and genotypes of *Arabidopsis* are generally able to recover from severe drought stress as long as the F_v/F_m value is not reduced to lower than approximately 30% of the well-watered value (usually 0.8). In our study, although the RWC was around 20% for all genotypes after 10 d without irrigation, the F_v/F_m was around 0.40 (corresponding to 50% of the well-watered value) for the most stressed lines. Thus, on the basis of the work of Woo *et al.* (2008), all genotypes would be anticipated to be able to rescue growth after re-irrigation. However, following re-irrigation, only WT and OX lines were able to fully recover vegetative and reproductive growth, while all KO mutants displayed intense dehydration and necrosis and did not even fully recover after an additional 5 d of irrigation (Fig. 2).

Increases of RWC were only observed at 3 (68–72%) and 5 (88–92%) d after the re-establishment of irrigation in WT

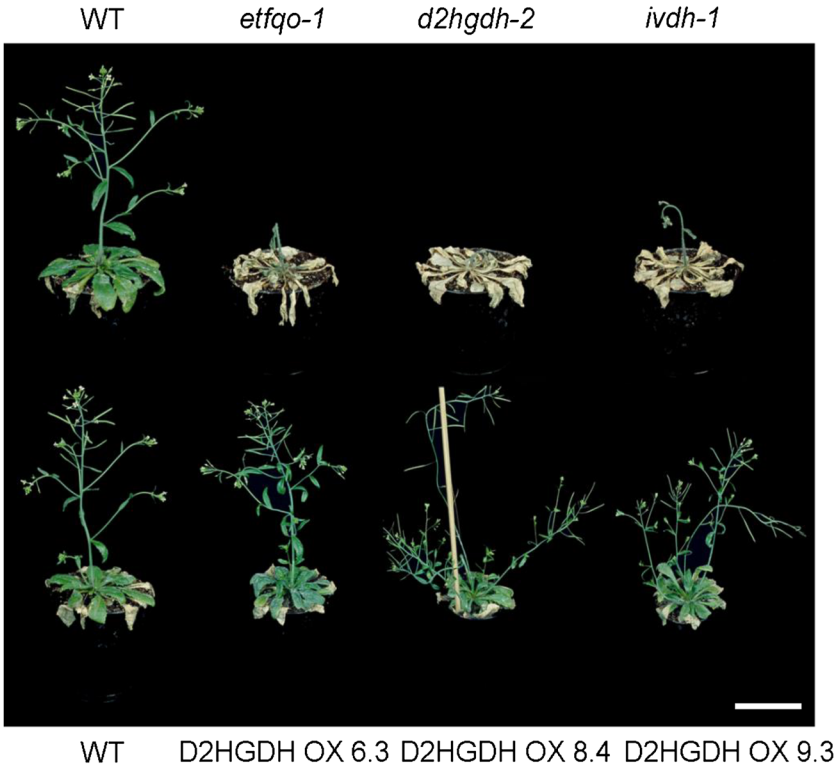

Figure 2. Recovery phenotype observed in 6-week-old, long-day-grown *Arabidopsis* mutants and wild type plants (WT). Images are representative of 4-week-old plants that were subjected to a progressive water deficit by suspension of irrigation for 10 d and then given recovery irrigation for further 5 d. This experiment was repeated at least three times (and even in different growth facilities) with similar phenotypes observed each time. Scale bar represents 5 cm.

plants and OX lines. All KO genotypes displayed significantly lower RWC values (25–29%) than the WT and OX lines even after 5 d of re-establishment of irrigation (Fig. 3A). WT and OX lines were additionally able to recover to the F_v/F_m values (Fig. 3B) observed at the beginning of the experiment (day 0, non-stress condition, Supporting Information Fig. S2), indicating that the water availability was enough to fully recover the photosynthetic capacity of these plants. Following the recovery of irrigation, the KO mutants showed F_v/F_m values higher than those observed at the end of the water deficit period (Supporting Information Fig. S2 and Fig. 3B); they were, however, not able to recover to their initial values (day 0, non-stress condition, Supporting Information Fig. S2).

The effects of water stress on leaf respiration

In order to estimate the impact of the water stress on the respiration of the genotypes analysed, we next assayed the rate of respiration. To this end, we incubated leaf discs in an oxygen electrode following water restriction. As expected, no differences in respiration were observed between WT and KO lines prior to the imposition of water deficit (Fig. 4A) indicating that the ETF/ETFQO pathways might be important mostly under stress situations. Under stress conditions, these measurements revealed a reduction in the rate of oxygen consumption in all genotypes with that of the KO lines being less than 70% of that observed in the WT and OX lines after 10 d without watering (Fig. 4A). As observed for F_v/F_m values, oxygen consumption was fully recovered following the re-irrigation in WT and OX lines, but not in the KO lines (Fig. 4B).

Metabolic characterization of the electron-transfer flavoprotein:ubiquinone oxidoreductase system under water deficit conditions

To investigate the functional link between the ETF/ETFQO system and the altered drought tolerance, we next performed an extensive metabolic characterization in the mutants and WT plants during water deficit treatment. It is important to mention that all genotypes used in this study exhibited similar levels of total soluble proteins, total amino acids, nitrate and starch (Supporting Information Fig. S3) in samples harvested immediately prior to the start of the drought treatment (day 0). This indicates that silencing of the ETF/ETFQO pathway has only a negligible impact on leaf primary metabolism under normal growth conditions. However, as early as 5 d following the onset of drought, all genotypes showed a decrease of over 55% of the total protein content with a greater impact observed in the KO lines (Supporting Information Fig. S3). Conversely, total amino acids increased during the course of the experiment in all genotypes (Supporting Information Fig. S3). In contrast, the content of nitrate and starch (Supporting Information Fig. S3) decreased steadily over the experimental period, with these changes being more pronounced in the KO mutants. After 10 d without watering, the starch content in the *d2hgdh-2* and *ivdh-1* mutants was below the detection limit (Supporting Information Fig. S3).

Metabolic regulation is one of the main mechanisms involved in the maintenance of osmotic potential under water deficit (Bowne *et al.* 2012; Obata & Fernie 2012). Thus, an established GC-MS protocol for metabolite profiling (Lisec *et al.* 2006) was used for the simultaneous identification and quantification of a total of 50 metabolites in KO, OX and WT plants. In response to the imposed treatments, changes in the levels of a wide range of organic acids, amino acids and sugar were evident (Figs 5, 6 and Supporting Information Fig. S4).

The levels of most amino acids increased significantly, particularly after 10 d without watering (Fig. 5). The main differences were observed in the levels of aromatic amino acids (phenylalanine and tryptophan) and BCAA (isoleucine, leucine and valine). Compared with WT plants, all KO mutants additionally displayed increases in the levels of 4-aminobutyric acid (GABA), isoleucine, lysine, proline and valine. Moreover, increases in the levels of alanine, ornithine, phenylalanine and tryptophan were observed in *d2hgdh-2* and *ivdh-1* and of glutamine in *d2hgdh-2* compared with the WT. By contrast, while mild increases in the levels of aspartate and decreases of glutamate in all KO lines were observed (Fig. 5), significant reductions in both metabolite levels were observed for *etfqo-1* and *ivdh-1* plants (Fig. 5). Moreover, a clear pattern of down-regulation of the levels of some metabolites in those lines in

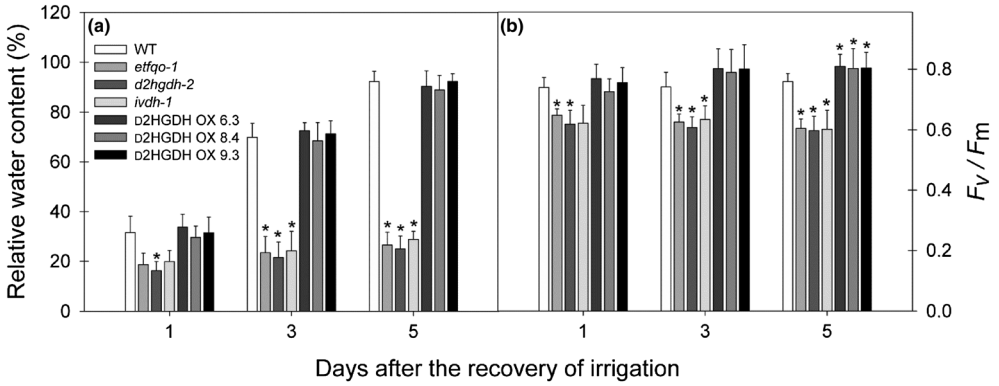

Figure 3. Relative water content (a) and the maximum quantum yield of PSII electron transport and F_v/F_m (b) of leaves of 6-week-old, long-day-grown *Arabidopsis* plants after further treatment for 1, 3 and 5 d after re-watering. Values are means ± SE of six independent samplings; an asterisk indicates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

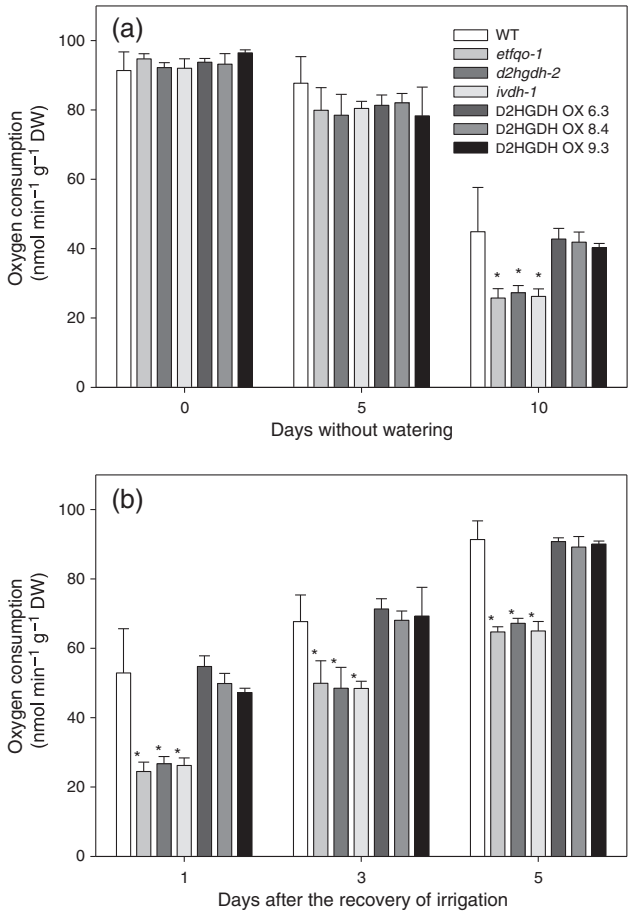

Figure 4. The effects of water stress on leaf respiration in *Arabidopsis* plants after further treatment for 0, 5 and 10 d without watering (a) and 1, 3 and 5 d after re-watering (b). Freshly prepared leaf discs were transferred into the temperature-controlled measuring chamber of an oxygen electrode containing 1 mL of 10 mM MES-KOH, pH 6.5. Each determination was performed in five biological replicates, and data presented are means ± SE of these replicates. 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.

comparison with the pattern of change documented in the KO mutants (Fig. 5) was observed. After 10 d without watering, at least two OX lines showed significant increments in the content of most amino acids with respect to the WT (Fig. 5). Exceptions were glutamate, glutamine and tryptophan, which showed significantly reduced contents with respect to the WT and lysine, which showed WT levels.

Drought conditions also promoted increases in the levels of sugars in all genotypes analysed (Fig. 6). After 5 d without irrigation, all KO mutants displayed increased levels of fructose, galactose, glucose, mannose and ribose. Moreover, after 10 d without watering, more pronounced increments in the levels of sugars were observed in the KO mutants with respect to the WT: (i) all KO mutants showed increased levels of glucose; (ii) *etfqo-1* and *ivdh-1* showed increased contents of fructose, maltose and ribose; (iii) *etfqo-1* and *d2hgdh-2* showed increased levels of mannose and (iv) *etfqo-1* showed increased levels of galactose. The levels of sucrose and raffinose in the KO mutants were unaltered with respect to WT (Fig. 6).

After 10 d without watering, the OX lines displayed increased contents of galactose, glucose, maltose, mannose and sucrose with respect to the WT (Fig. 6). On the other hand, all OX lines showed reduced contents of raffinose, ribose, trehalose and dehydroascorbate (Fig. 6).

The levels of organic acids increased with the progress of drought stress treatment in all genotypes (Fig. 6). Regarding TCA cycle intermediates, the levels of *cis*-aconitate and isocitrate were increased in all KO mutants after 5 d without watering, while citrate was decreased in all KO mutants when compared with WT plants. In addition, at this time point, fumarate was increased in *etfqo-1* and *ivdh-1* with regard to the WT. After 10 d without watering, the KO mutants showed increased contents of most TCA cycle intermediates with respect to the WT: (i) all KO mutants showed increased contents of 2-oxoglutarate, citrate and succinate; (ii) *etfqo-1* and *ivdh-1* showed increased contents of *cis*-aconitate and isocitrate and (iii) *ivdh-1* and *d2hgdh-2* showed increased contents of fumarate and malate, respectively (Fig. 6). Glycolate levels were increased in all KO mutants being significant in *ivdh-1* and *d2hgdh-2*. After 10 d without watering, the OX lines were characterized by decreased levels of 2-oxoglutarate and *cis*-aconitate and increased levels of citrate, isocitrate, fumarate, malate and succinate with respect to the WT (Fig. 6).

Following 10 d without watering, increased levels of some sugar alcohols (e.g. erythritol, glycerol and mannitol), as well as polyamines such as putrescine and spermidine, were observed (Supporting Information Fig. S4). Furthermore, similarly to the situation observed in KO mutants, water restriction promoted increases in sugar alcohols in OX lines, such as glycerol and myo-inositol, as well as polyamines, such as putrescine and spermidine (Supporting Information Fig. S4).

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 d, the expression of selected genes was also determined in KO mutants and WT plants (Fig. 7 and Supporting Information Fig. S5). Firstly, we demonstrated that the genes associated with the ETF/ETFQO pathway are clearly induced in WT plants following 5 d of water stress and return to the levels found prior water stress after 10 d without watering (Fig. 7). Secondly, although the only KO line where no expression of the related gene was observed was the *etfqo-1*, our results demonstrated that in both *ivdh-1* and *d2hgdh-2*, a strong reduction in the expression of its respective gene compared with WT levels was observed (Fig. 7). We did not observe an up-regulation of genes related to alternative respiration in KO plants (Fig. 7). Altogether, these results indicate that the genes involved in the ETF/ETFQO pathway seem to be highly regulated and that the down-regulation of one will most likely culminate with no up-regulation of others. Our result also

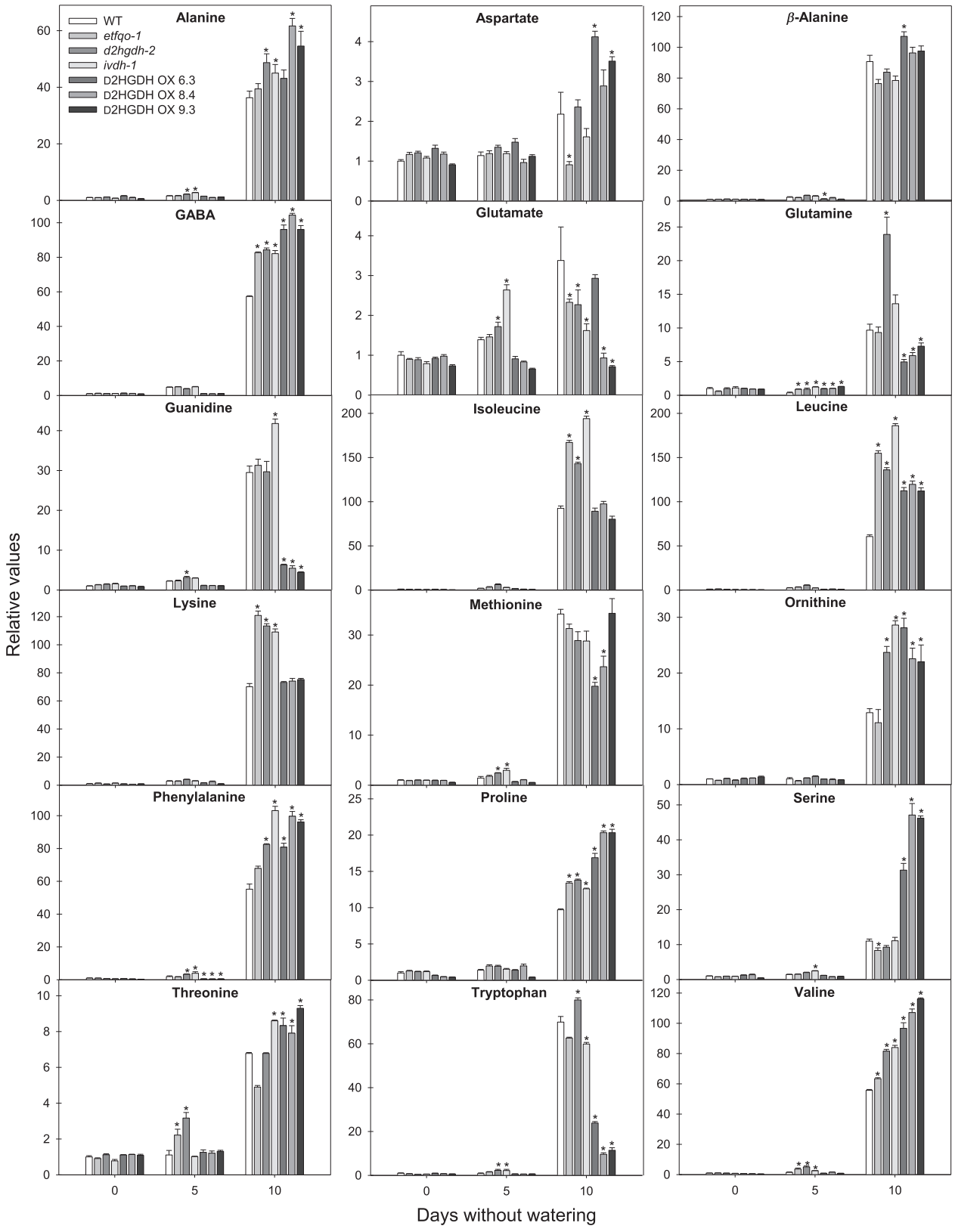

Figure 5. Relative levels of amino acids in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, overexpression lines *D-2HGDH* OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 d without watering as measured by gas chromatography–mass spectrometry. The *y*-axis values represent the metabolite level relative to the WT. Data were normalized to the mean response calculated for the 0 d 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. GABA, 4-aminobutyric acid.

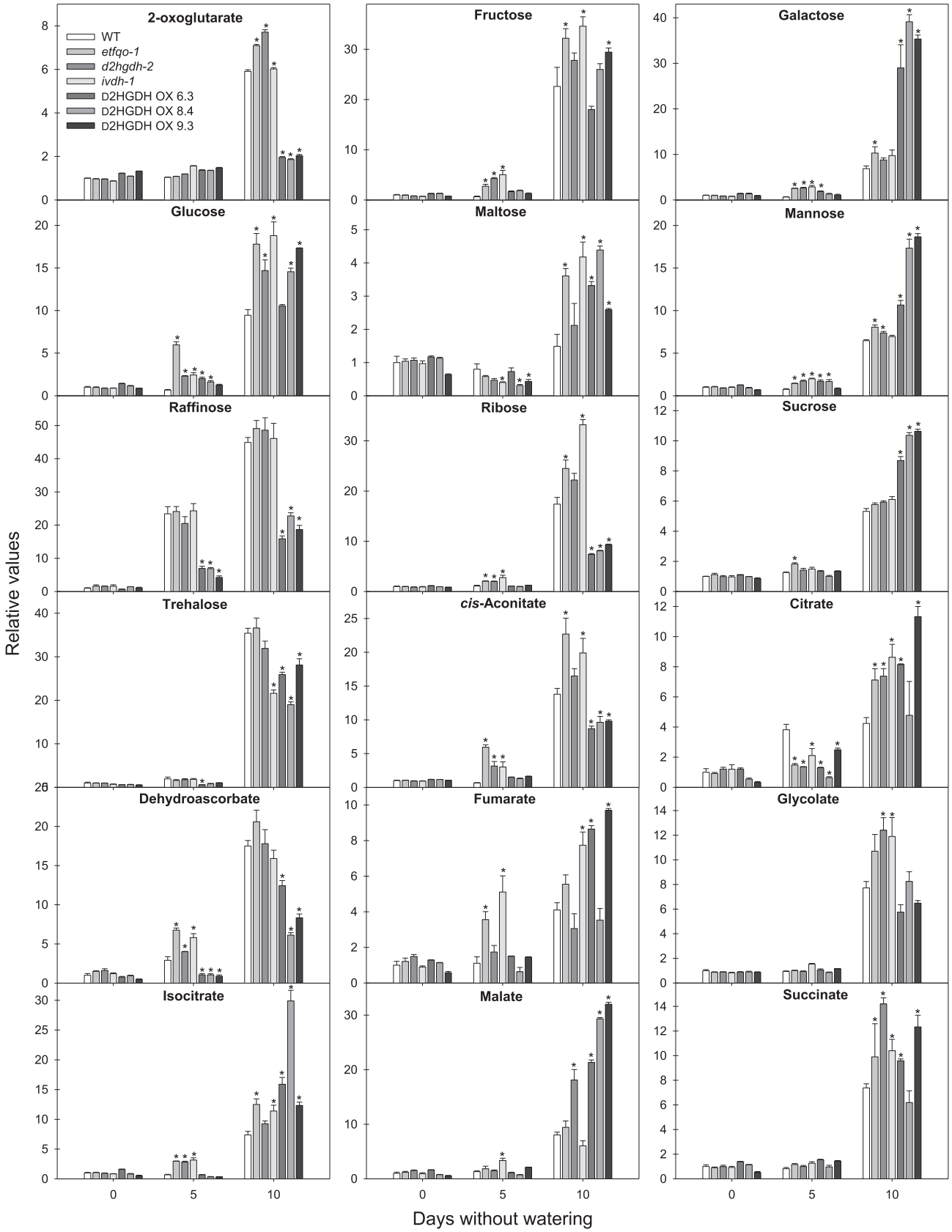

Figure 6. Relative levels of sugars and organic acids in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1*, overexpression lines *D-2HGDH* OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 5 and 10 d without watering as measured by gas chromatography–mass spectrometry. Levels of the indicated sugars and organic acids are presented as in Figure 5.

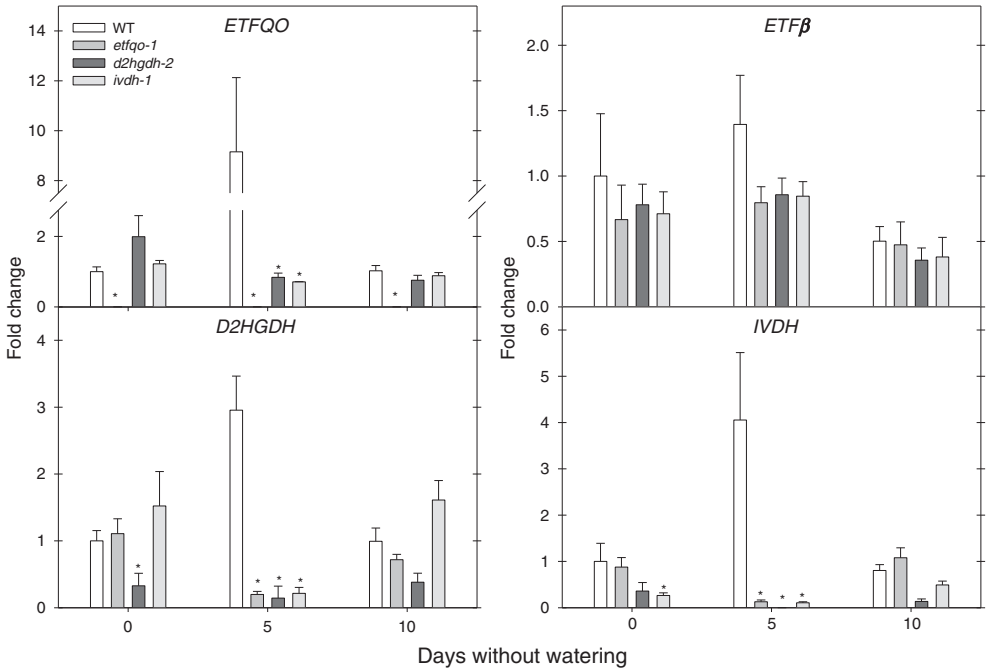

Figure 7. Transcript levels of genes related to alternative pathways of respiration in *Arabidopsis* knockout mutants *etfqo-1*, *d2hgdh-2* and *ivdh-1* and wild type plants (WT) after further treatment for 0, 5 and 10 d without watering as quantified by qRT-PCR. The *y*-axis values represent the transcript level relative to the WT. Data were normalized to the mean response calculated for the 0 d 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 the WT. ETFQO, electron-transfer flavoprotein:ubiquinone oxidoreductase; IVDH, isovaleryl-CoA dehydrogenase.

indicates a good co-expression amongst the genes of this pathway, as previously observed, particularly under stress conditions (Araújo *et al.* 2010).

Intriguingly, a down-regulation of the transcription of photorespiration-related genes, such as 2-phosphoglycolate phosphatase 1 (*PGLP1*), peroxisomal catalase 2 (*CAT2*), serine hydroxymethyl transferase 1 (*SHMT1*) and 2 (*SHMT2*), ferredoxin-dependent glutamate synthase (*Fd-GOGAT*) and glutamine synthetase (*GS*), was observed in all genotypes (Supporting Information Fig. S5). More specifically, the transcripts levels of *PGLP1*, *Fd-GOGAT* and *GS* were extremely low, and those of *SHMT1* and *SHMT2* were not detected after 10 d without watering in all genotypes. Moreover, a decrease of the expression of genes related to photosynthesis (rubisco activase, *RCA*), alternative respiration pathways (uncoupling protein, *UCP*) and metabolism of nitrogen and amino acids (glutamate dehydrogenase 1, *GDH1* and the aforementioned *Fd-GOGAT*; and *GS*) was observed in all genotypes (Supporting Information Fig. S5). Taken together, these results are in good agreement with the unexpectedly low levels of both photorespiratory metabolites and transcripts described previously. Additionally, minor changes in the expression levels of TCA cycle-related genes, such as malate dehydrogenase (*MDH*) and fumarase 1 (*FUM1*), were observed during the course of drought in all genotypes. In addition, other genes evaluated [e.g. ADP-glucose pyrophosphorylase 1 (*AGPase1*), NADP-dependent isocitrate dehydrogenase (*NADP-ICDH*), aspartate aminotransferase 1 (*AspAT1*) and aminomethyltransferase glycine decarboxylase (*GDCT*)] behaved similarly in all genotypes during the drought treatment.

The absence of the electron-transfer flavoprotein: ubiquinone oxidoreductase pathways does not alter stomatal behaviour

Given the changes in water loss from detached leaves (Fig. 1A) and RWC (Fig. 1B), we further asked whether stomatal function might be affected in KO mutants. To this end, we fully characterized the photosynthetic performance by analysing diffusional, photochemical and biochemical constraints to photosynthesis in well-watered plants. Net photosynthetic rates (A_N), *g*s, transpiration rates (E), dark respiration (R_d) and photorespiratory rates (P_R) were invariant between WT and mutant lines. Indeed, the absence of changes in photosynthetic parameters cannot be linked with photochemical constraints given that both the maximum quantum efficiency of PSII (F_v/F_m) and capture efficiency of excitation energy (F_v'/F_m') remained invariant. Additionally, the electron transport rate (J_{flu}) was similar between the genotypes analysed (Supporting Information Table S4).

In order to access the general effects of alternative pathways of respiration on stomatal function, we further analysed the response of A_N to the internal CO_2 concentration – A_N/C_i curves (Supporting Information Fig. S6A), which were further converted into responses of A_N to chloroplastidic CO_2 concentration – A_N/C_c curves (Supporting Information Fig. S6B). Estimations of C_i and C_c were similar between mutant lines and WT plants. Mesophyll conductance (g_m), estimated using a combination of gas exchange and chlorophyll *a* fluorescence parameters via two independent methods, was also invariant in the genotypes analysed here (Supporting Information

Table S5). In addition, the maximum carboxylation velocity (V_{cmax}) and maximum capacity for electron transport rate (J_{max}) were not altered in mutant lines whether estimated on a C_i basis or on a C_c basis. The overall photosynthetic limitations were partitioned into their functional components: stomatal (l_s), mesophyll (l_m) and biochemical (l_b) (Supporting Information Table S5) with no significant changes being observed between WT plants and mutant lines. Altogether, the results obtained within this experiment indicated that the phenotype observed in *etfqo-1*, *d2hgdh-2* and *ivdh-1* mutants under water restriction is not directly associated with an altered stomatal behaviour.

Metabolic responses following water limitation are similar in plants with identical soil water status

Our GC-MS-based metabolite profile (Figs 5, 6 and Supporting Information Fig. S4) strongly suggests that the ETF/ETFQO pathway is involved in the metabolic acclimation of *Arabidopsis* plants under water shortage conditions. However, given the changes in water loss and RWC in mutant plants, it is reasonable to assume that plants were facing different soil water status following water restriction. Thus, we next performed a drought stress experiment in which plants grew side by side in large pots in order to exclude the possibility that the metabolic changes observed could be related to different stress intensities that might be reached by growing plants separately. This experiment was carried out at the same growth conditions as the first experiment (see Material and Methods section for further details). The RWC reduced over time with no differences between the genotypes (Supporting Information Fig. S7). Given that plants growing under bigger pots have access to higher water availability, which leads to a slower water limitation, no differences were observed in RWC between WT and KO mutant lines in this experiment (Supporting Information Fig. S7), which is in keeping with the lack of difference in stomatal behaviour in these genotypes (Supporting Information Tables S4 and S5). However, the metabolic responses and the differences between KO mutants, WT and OX lines under drought conditions were mostly conserved (Supporting Information Fig. S7). Briefly, we observed increases in the levels of proline and lysine in all KO mutants, valine in *ivdh-1* and *d2hgdh-2* mutants and isoleucine and phenylalanine in the *ivdh-1* KO mutant compared with WT plants. Increased levels of proline and valine were also observed in all OX lines and in the line OX 6.3, respectively. Furthermore, we observed increased maltose levels in all KO mutants and fructose in both *etfqo-1* and *ivdh-1* under drought conditions (Supporting Information Fig. S7). The results from this experiment are in good agreement with those obtained in plants growing separately and thus exclude the possibility that the metabolic effects observed are due to an artefact of our experimental system where plants grown separately and as such support our contention of the significance of the ETF/ETFQO system as one metabolic pathway involved in water deficit acclimation in *Arabidopsis*.

DISCUSSION

Plant growth and development are both negatively affected by environmental stresses, such as drought (Dinneny *et al.* 2008; Skirycz *et al.* 2010a; Skirycz *et al.* 2011). However, plants respond and adapt to continuous environmental fluctuations via suitable physiological, developmental and biochemical responses in order to accommodate stressful conditions. Interestingly, one such adaptive mechanism observed here was the reduction in respiration rate coupled with growth arrest of all genotypes during water deficit – a response that was exacerbated in the KO mutants. Growth arrest can be viewed as a mechanism to preserve carbohydrates for sustained metabolism, prolonged energy supply and for more efficient recovery after the cessation of the imposed stress (Zhu 2002; Bartels & Sunkar 2005; Witt *et al.* 2012; Gechev *et al.* 2013). Interestingly, growth recovery after water supply was only observed in WT and OX lines (Fig. 2). Although the functional mechanism remains to be fully investigated, this result supports our previous suggestion that the catabolism of BCAA might lead to them acting as an important alternative respiratory substrate under conditions of stress (Araújo *et al.* 2011c) and adds further supports for the contention that delayed onset of stress (Lawlor 2013) might explain, at least partially, the results obtained. However, caution must be taken with the interpretation of these results because the higher water loss observed in the KO lines (Fig. 1) might be associated with alterations in cuticle thickness, leaf hydraulic conductance or impairments in stomata closure in the KO lines during situations of stress. Thus, it should be mentioned that drought-sensitivity phenotype observed in mutants of the ETF/ETFQO pathway may also reflect differences in water uptake from soil (lower water use efficiency) and altered transpiration rates under water limitation leading to growth delayed. Although similar responses have been already observed in other mitochondrial-related mutants (Meyer *et al.* 2009; Zhu *et al.* 2014), this hypothesis represents an exciting topic for future research.

Several enzymes involved in the biosynthesis (Singh & Shaner 1995; Dumas *et al.* 2001; Binder *et al.* 2007) and degradation of BCAA (Däschner *et al.* 2001; Schuster & Binder 2005; Engqvist *et al.* 2009; Araújo *et al.* 2010; Binder 2010; Kochevenko *et al.* 2012) have been already identified and characterized in *Arabidopsis* and other plants. However, thus, far relatively little is known concerning their regulation at the transcriptional, protein or enzyme activity level. The transcriptional behaviour of ETF/ETFQO-related genes in WT and KO lines observed here (Fig. 7) further suggests that a better understanding of the physiological role of the enzymes involved in this pathway will require a more exhaustive definition of their expression profile and whether these genes are affected at the post-translational level in response to water deprivation. Because *Arabidopsis* and other plant species are capable of both BCAA biosynthesis and degradation, these counteracting pathways have to be carefully balanced in order to maintain the homeostasis of this important group of amino acids. This is particularly true under stress situations and critically under conditions of energy deprivation (Binder 2010; Ding *et al.* 2012). In addition, it was recently demonstrated that BCCA catabolism

provides TCA cycle substrates under energy-limited conditions, interacting with energy metabolism at multiple points of primary metabolism (Peng *et al.* 2015). Furthermore, recent studies have shown that the degradation of BCAA plays an important role not only in plant responses to both dark-induced and developmental senescence (Engqvist *et al.* 2009; Araújo *et al.* 2010; Angelovici *et al.* 2013; Peng *et al.* 2015) but also acts as an important respiratory substrate during the process of fruit ripening in tomato (Kochevenko *et al.* 2012) as well as being required for seed viability (Ding *et al.* 2012). These unique features of BCAA metabolism clearly indicate its pivotal role in the regulation of amino acid metabolism under different growth conditions and that our understanding of the metabolic network of those amino acids is still revealing new aspects and connections of such important metabolites. Taken together, the results obtained here and those discussed previously further suggest that the manipulation of amino acid catabolism might have substantial importance under stress situations and that the orthologues from crop plants should be considered as candidates for biotechnological purposes.

Following from the observation that BCAAs accumulate on the imposition of drought stress in a wide range of species including *Arabidopsis* (Skirycz *et al.* 2010a), tomato (Semel *et al.* 2007), barley (Bowne *et al.* 2012), maize (Witt *et al.* 2012), the resurrection glacial relic *Haberlea rhodopensis* (Gechev *et al.* 2013) and grasses of the *Sporobolus* family (Oliver *et al.* 2011), we set out here to address the reason for their accumulation under these conditions. The mutant lines used were highly effective in addressing this question. Our findings are in agreement with our early studies that demonstrated that chlorophyll degradation is also able to support respiratory metabolism during dark-induced senescence (Ishizaki *et al.* 2005; Ishizaki *et al.* 2006; Araújo *et al.* 2010), however, not via an ETF/ETFQO-mediated route (Araújo *et al.* 2011a). Despite this fact, chlorophyll degradation alongside the use of other non-osmotic carbon reserves such as those in starch represents important alternative strategies to maintain respiration in times of water stress (Gechev *et al.* 2013). Interestingly, starch reserves were entirely depleted in *d2hgdh-2* and *ivdh-1* mutants but were higher in the OXs than in the WT by the end of the experiment (Supporting Information Fig. S3). These combined results, alongside the lack of accumulation of total amino acids, indicate that while the mutants are also reliant on chlorophyll and starch degradation to support respiration, the overexpression of *D-2HGDH* reduces this dependency. This is mostly likely because the degradation of such compounds would not imbalance osmotic potential and allows mutants to keep using carbohydrates for this end. Although strong correlations between the accumulation of several sugars (sucrose, galactinol and trehalose) and sugar alcohols (such as mannitol and sorbitol) and drought tolerance in different plant species have been reported (Taji *et al.* 2002; Bartels & Sunkar 2005; Obata & Fernie 2012), it has been recently recognized that this tolerance is achieved also by reducing growth and stomatal conductance, therefore slowing the water loss and the onset of the drought (Lawlor 2013). This is most likely one of the reasons allowing the maintenance or even the increase of carbohydrate levels during drought episodes, as observed here for several sugar and sugar-related compounds (Fig. 6). Collectively, our results demonstrate therefore that not only the usage of BCAA but also carbohydrate availability may explain the differential drought tolerance observed here.

Notably, while all mutants displayed a reduced ability to recover their photosynthetic rates following re-watering, the *D-2GHDH* overexpression lines exhibited a mild, yet significant increase in maximum quantum yield of PSII 5 d following re-irrigation (Fig. 3B). Intriguingly, this opposite behaviour in photosynthetic recovery cannot be linked to the BCAA levels *per se*. Our results suggest that the proper function of the ETF/ETFQO pathway is likely important in supporting drought episodes in *A. thaliana*. Nevertheless, the changes in several other amino acids indicate a complex metabolic reprogramming following water limitation and that proteolysis has commenced, similarly to other stress situations (Obata & Fernie 2012). It also suggests that more integrative analysis of the role of all aspects of protein degradation and consequent amino acid remobilization during its catabolism should be performed within the context of understanding metabolic responses to stress at the whole plant level.

The metabolic results obtained here revealed many interesting changes in response to the water stress with the response of WT being highly similar to those previously described for *Arabidopsis* (Urano *et al.* 2009; Skirycz *et al.* 2010a; Obata & Fernie 2012). Furthermore, the response of the mutants partially resembled those seen following dark-induced (Araújo *et al.* 2010) and developmental (Engqvist *et al.* 2011) senescence, however, additionally displayed water stress-specific features. Examples of common responses in the mutants include the exaggerated increases in GABA, BCAAs and aromatic amino acids and the albeit less pronounced increase in proline and serine. Similarly, a decrease in glutamate was ultimately observed following both water and senescence stresses. By sharp contrast, the increases in alanine and glutamate observed here (Fig. 5) were opposite to their pattern of change following dark-induced senescence. Comparison of responses of organic acids revealed that their patterns of change were largely conserved between these two stresses; however, as would be anticipated, the levels of sugars were opposite (Fig. 6). In parallel to the increase in sugars was an exaggerated increase in the mutants of some sugar alcohols and polyamines such as glycerol, *myo*-inositol, putrescine and spermidine, however, levels of mannitol were considerably lower than those seen in the WT (Supporting Information Fig. S4). Thus, although our results are in agreement with recent suggestion that with reductions in photosynthesis, protein degradation occurs rapidly, and subsequent amino acid catabolism serves as the main cellular energy supply (Caldana *et al.* 2011; Hildebrandt *et al.* 2015); the metabolic response observed here also indicates that extensive protein degradation coupled with osmotic adjustment to cope with water limitation might be the most likely explanation for the phenotypes observed. Intriguingly, similar alterations in the levels of several metabolites including BCAA were observed in other crop species (Bowne *et al.* 2012; Witt *et al.* 2012).

The complexity of the response described here is further underlined by the expression levels of select transcripts that reflect a clear prioritization of certain pathways above others. Particularly prominent here are the conserved levels of NADP-dependent isocitrate dehydrogenase, fumarase and malate dehydrogenase at time points at which photorespiration-associated transcripts as well as AGPase and the uncoupling protein are dramatically reduced (Supporting Information Fig. S5). The majority of these changes are consistent either with the changes in metabolism observed in the mutants or the need to reduce production of reactive oxygen species (ROS) during drought stress (Dinakar *et al*. 2010; Møller & Sweetlove 2010; Jacoby *et al*. 2012; Obata & Fernie 2012). In light of this observation, the down-regulation of the expression of the uncoupling protein is perhaps surprising. That said, despite strong evidence for a role in maintaining optimal photosynthesis in *Arabidopsis*, detailed studies failed to reveal evidence for an important role in the mediation of ROS levels (Carrari *et al*. 2006). Returning to the metabolite levels, the partially commonality of response between water and carbon stress is not surprising particularly given the overlap between mitochondrial and oxidative stress responses that underlie most abiotic stresses (Obata & Fernie 2012). While the precise nature of the interaction between both mitochondrial and energy metabolism associated to drought tolerance mechanisms could not be fully resolved in the present study, the link between the two is rather clear.

In summary, we provide circumstantial evidence that the ETF/ETFQO pathway is of critical importance in drought-tolerance mechanisms in *Arabidopsis*. As previously described (Ishizaki *et al*. 2005; Ishizaki *et al*. 2006; Araújo *et al*. 2010; Peng *et al*. 2015), carbon starvation associated to dark-induced senescence induces the ETF/ETFQO alternative pathway of respiration. These studies were initiated by the widespread observation that BCAA levels increased on water stress and given our relatively poor understanding of the function of these metabolites, we set out to test if this accumulation was due to properties of the metabolites themselves or was rather linked to their availability to fuel alternative pathways of mitochondrial respiration. The information from our previous studies coupled with the data presented here demonstrates that the enzymes IVDH and *D-2HGDH* integrate electron donation to this complex also during water deficit episodes and thus reveal that functional electron transfer to the mETC via the ETF/ETFQO pathway is important for plants to withstand drought. This pathway seems to be also equally important for plants to recover growth after re-watering rather than properties of the metabolites *per se* as evidenced by similar trends within several metabolites analysed here. The dehydration tolerance associated with the proper function of the ETF/ETFQO pathway involves plant survival after severe stress, maintenance of respiratory rates and reduced loss of water and chlorophyll content and increments in the usage of amino acids, allowing a better metabolic performance under episodes of water restriction. It is also worth noting that the data presented here exclude the possibility of altered stomatal function because of lack of a functional ETF/ETFQO under well-watered conditions but does not allow us to rule out that changes in sugars metabolism (Daloso *et al*. 2015) or organic acid transport (Medeiros *et al*. 2016) could take place following drought episodes impacting stomatal function. Further experimentation with specific metabolite flux experiments using ^{13}C-labelled substrates will be required to fully ascertain this issue. As such, a novel aspect of this intriguingly ETF/ETFQO pathway was revealed in the present work. Although compelling evidence showing that BCAA metabolic network is of pivotal importance in regulation energy metabolism at different steps has been recently provided (Ding *et al*. 2012; Kirma *et al*. 2012; Peng *et al*. 2015), future studies will be required to investigate the role of the supply of other amino acids such as lysine and BCAA to the ETF/ETFQO pathway under water stress. That said, the enhanced recovery of photosynthesis in the OX lines, alongside their lesser dependence on stored carbon reserves, suggests that manipulation of this pathway may represent an important novel strategy for tailoring drought-resistant crops.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Phenotype of *Arabidopsis* mutants and wild type plants (WT) under normal growth conditions. Images of 4-week-old, long-day-grown *Arabidopsis* plants before imposition of water stress.

Figure S2. Total chlorophyll content (A), initial fluorescence, F_0 (B) and F_v/F_m, the maximum quantum yield of PSII electron transport (C) of leaves of 4-week-old, long-day-grown, *Arabidopsis* plants after further treatment for 0, 5 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.

Figure S3. 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 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.

Figure S4. Relative levels of sugar alcohols and polyamines in *Arabidopsis* knockout mutants *etfqo-1, d2hgdh-2* and *ivdh-1*, overexpression lines D-2HGDH 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 sugar alcohols and polyamines are presented as in Fig. 5.

Figure S5. 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. 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 the WT.

Figure S6. Net photosynthesis (A_N) curves in response to substomatal (Ci) or chloroplastic (C_c) CO_2 concentrations in Arabidopsis knockout mutant plants *etfqo-1, d2hdgh-2, ivdh-1*, and overexpression lines D2HGDH OX 6.3, 8.4, 9.3, and wild type (WT) plants. **(A)** A_N/C_i curves and **(B)** A_N/C_c curves. Values are presented as means ± SE (n = 10); values in mutant lines were determined by the Student's *t* test as not significantly different ($P < 0.05$) from WT.

Figure S7. Relative water content and relative levels of metabolites in *Arabidopsis* knockout mutants *etfqo-1, d2hgdh-2* and *ivdh-1*, overexpression lines D-2HGDH OX 6.3, 8.4 and 9.3, and wild type plants (WT) after further treatment for 0, 8 and 16 days without watering as measured by GC-MS. Values are means ± SE of five independent samplings. Asterisks indicates values that were determined by the Student's *t* test to be significantly different ($P < 0.05$) from the WT.

Table S1. Relative metabolite content of the fully expanded leaves of *Arabidopsis* knockout mutants. The metabolites were measured in *etfqo-1, d2hgdh-2* and *ivdh-1*, and wild type (WT) plants after further treatment for 0, 5 and 10 days without watering 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).

Table S2, Relative metabolite content of the fully expanded leaves of *Arabidopsis* overexpressor lines. Relative metabolite levels were determined in D2HGDH OX 6.3, 8.4 and 9.3, and wild type (WT) plants after further treatment for 0, 5 and 10 days without watering 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).

Table S3. Primers used in the RT-PCR analyses performed in this study.

Table S4. Gas exchange and chlorophyll a fluorescence parameters in Arabidopsis knockout mutant plants *etfqo-1, d2hdgh-2, ivdh-1*, and overexpression lines D2HGDH OX 6.3, 8.4, 9.3, and wild type (WT) plants. Values are presented as means ± SE (n = 10); values in mutant lines were determined by the Student's *t* test as not significantly different ($P < 0.05$) from WT.

Table S5. Photosynthetic characterization of Arabidopsis knockout mutant plants *etfqo-1, d2hdgh-2, ivdh-1*, and overexpression lines D2HGDH OX 6.3, 8.4, 9.3, and wild type (WT) plants. Values are presented as means ± SE (n = 10); values in mutant lines were determined by the Student's *t* test as not significantly different ($P < 0.05$) from WT.