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A molecular approach to drought-induced reduction in leaf CO_2 exchange in drought-resistant *Quercus ilex*

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Drought-induced reduction of leaf gas exchange entails a complex regulation of the plant leaf metabolism. We used a combined molecular and physiological approach to understand leaf photosynthetic and respiratory responses of two-year-old *Quercus ilex* seedlings to drought. Mild drought stress resulted in glucose accumulation while net photosynthetic CO_2 uptake (P_n) remained unchanged, suggesting a role of glucose in stress signaling and/or osmoregulation. Simple sugars and sugar alcohols increased throughout moderate to very severe drought stress conditions, in parallel to a progressive decline in P_n and the quantum efficiency of photosystem II; by contrast, minor changes occurred in respiration rates until drought stress was very severe. At very severe drought stress, 2-oxoglutarate dehydrogenase complex gene expression significantly decreased, and the abundance of most amino acids dramatically increased, especially that of proline and ³-aminobutyric acid (GABA) suggesting enhanced protection against oxidative damage and a re-organization of the tricarboxylic cycle acid (TCA) cycle via the GABA shunt. All together, our results point to *Q. ilex* drought tolerance being linked to signaling and osmoregulation by hexoses during early stages of drought stress, and enhanced protection against oxidative damage by polyols and amino acids under severe drought stress.

Abbreviations – Ala, alanine; AOX, alternative oxidase; CS, citrate synthase; ESTs, Expressed Sequence Tags; GABA, ³-aminobutyric acid; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; GGAT, glutamate-glyoxylate aminotransferase; g_s, stomatal conductance to water vapor; LS, mild drought stress; MD, malate dehydrogenase; MS, moderate drought stress; OGDH, 2-oxoglutarate dehydrogenase complex; P_n, leaf net photosynthetic CO_2 uptake; PR, Leaf photorespiration; PPFD, photosynthetic photon flux density; R_d, leaf dark respiration; RuBP, ribulose-1,5-bisphosphate; SDH, succinate dehydrogenase; SS, severe drought stress; SGAT, serine-glyoxylate aminotransferase; TCA cycle, tricarboxylic cycle acid cycle; VS, very severe drought stress; Ψ_{pd}, predawn leaf water potential; Ψ_{md}, midday leaf water potential; ΦPSII, quantum efficiency of photosystem II.

Introduction

Plants respond to fluctuations in soil water availability by regulating leaf gas exchange rates. In response to mild drought, early stomata closure reduces transpiration but limits carbon dioxide (CO_2) diffusion and photosynthesis. Changes in the mesophyll in response to drought further limit CO_2 diffusion to the chloroplasts (Flexas et al. 2008, Chaves et al. 2009). As the drought severity increases, low chloroplastic CO_2 concentration triggers metabolic changes (e.g. restricted abundance or activity of chloroplastic enzymes and proteins) that further impair photosynthesis (Flexas and Medrano 2002, Cano et al. 2013). The respiratory metabolism is also affected by drought. Leaf photorespiration (PR) increases in moderate drought stress but decreases with increasing drought severity (Biehler and Fock 1996, Wingler et al. 1999, Lawlor and Cornic 2002, Guan et al. 2004, Peguero-Pina et al. 2009). On the contrary, leaf dark respiration (R_d) usually decreases during the onset of moderate to severe drought, but can increase with increasing drought severity (Atkin and Macherel 2009). These changes in the rates of leaf net photosynthetic CO_2 uptake (P_n), PR and R_d are controlled by precise biochemical and molecular adjustments. However, more studies on molecular ecophysiology are needed to link molecular changes to organ function and plant resistance to drought.

Plant leaf primary metabolic adjustments are well-known to occur in response to drought. In general, under mild to moderate drought, increased abundance of polyols, amino acids, hexose sugars, and other low-molecular weight compounds contribute to a decrease of the cell osmotic potential that moderate the decrease in turgor pressure and P_n (Morgan 1984, Clifford et al. 1998, Evers et al. 2010, Warren et al. 2011, Bowne et al. 2012, Barchet et al. 2014, Barnaby et al. 2015). Under limited chloroplastic CO_2 and ribulose-1,5-bisphosphate (RuBP) carboxylation conditions, RuBP oxygenase activity and PR can help to maintain a non-assimilatory electron flow that protects against photoinhibition (Wingler et al. 1999, Peñuelas and Llusià 2002, Voss et al. 2013). However, under severe drought stress, PR can decrease as a consequence of an imbalance between the synthesis and use of chloroplastic NADPH, which might lead to the accumulation of reactive oxygen species (ROS) and oxidative stress (Peñuelas and Llusià 2002, Guan et al. 2004, Gallé et al. 2007, Feller 2016). To mitigate oxidative stress under increasing drought severity, both the expression of genes coding for enzymes involved in the synthesis of metabolites with antioxidant functions (e.g. polyols and amino acids) as well as the levels of those metabolites increase (Taji et al. 2002, Verdaguer et al. 2003, Nishizawa et al. 2008, Jansen et al. 2014).

The mechanisms governing the response of R_d to drought are not clear. Upon drought, the increase in soluble sugars (Wingler et al. 1999, Rodríguez-Calcerrada et al. 2011, Sala et al. 2012), the

overexpression of genes coding for glycolytic enzymes (Rizhsky et al. 2002, Chaves et al. 2003, Jorge et al. 2006), and the role of lipids and proteins in feeding the tricarboxylic cycle acid (TCA) cycle besides carbohydrates (Tcherkez et al. 2003; but see Fischer et al. 2015) suggests that R_d does not decrease during drought due to a limitation in carbon substrates. Low recycling of ADP could be a key factor in controlling R_d during drought (Atkin and Macherel 2009). Flexas et al. (2005) suggested that ATP demand could initially increase in response to the onset of mechanisms of acclimation to mild drought and then subside in response to an attenuation of energy-demanding processes (e.g. reduced phloem upload, protein synthesis). Studies in which ADP and ATP levels in cells and organelles have been measured under drought stress are consistent with the hypothesis that ATP demand exerts a strong control on both R_d and the flow of electrons between the cytochrome and alternative respiratory pathways in the mitochondrial electron transport chain (Bartoli et al. 2005, Ribas-Carbó et al. 2005, Galle et al. 2010).

The high plasticity of the respiratory machinery in response to water availability is also reflected in the TCA cycle. Fluxomic and genomic analyses have revealed that most TCA-cycle steps can be bypassed through alternative pathways with just minor consequences for plant function. Only citrate synthase (CS) and succinate dehydrogenase (SDH) appear to have a role in the cycle that cannot be bypassed, without a negative impact to the plant biological functions (e.g. sterility, leaf curling, or altered organ functions) (Sweetlove et al. 2010, Millar et al. 2011, Araújo et al. 2012, Tcherkez et al. 2012). Sweetlove et al. (2010) suggested that the TCA cycle operates in a non-cyclic flux mode when the demand of ATP is low, favoring the synthesis of amino acids in lieu of ATP. António et al. (2016) demonstrated the existence of an alternative carbon flux that explained the accumulation of alanine (Ala), ³-aminobutyric acid (GABA), and succinate upon hypoxia via pathways mediated by an Ala and GABA shunts. The study provided evidence for a non-cyclic flux mode that explained the down-regulation of the respiration rate measured during hypoxic stress (António et al. 2016). A similar re-organization of the TCA cycle can occur under drought stress (Bouché and Fromm 2004, Warren et al. 2011, Rabara et al. 2015).

Few studies have addressed leaf responses to soil drought stress in tree species by combining molecular (e.g. metabolomics and transcriptomics) and physiological approaches (Jansen et al. 2014, Du et al. 2015), and even fewer addressing long drought periods in non-commercial tree species of slow growth and high drought resistance (Valero-Galván et al. 2013, Rivas-Ubach et al. 2014). To shed light on the mechanisms underpinning reduced rates of photosynthesis and respiration under drought, in our study, leaf gas exchange rates were analyzed together with gene expression and gas

chromatography time-of-flight mass spectrometry (GC-TOF-MS) primary metabolite profiling in *Quercus ilex* L. seedlings exposed to increasing levels of drought severity. *Q. ilex* is one of the dominant tree species in natural forest ecosystems of the Western Mediterranean Basin and was chosen for its well-known resistance to summer droughts (Peguero-Pina et al. 2009, Rodríguez-Calcerrada et al. 2011, Gratani et al. 2013). We hypothesized that leaf metabolism would be progressively altered by drought; specifically, that with increasing levels of drought stress: (1) P_n would decline and amino acids and soluble sugars (involved in stress signaling, osmoregulation and antioxidation) would increase; (2) PR and related metabolites (e.g. glycine) would initially increase and later decrease; (3) while R_d, organic acids and expression of some genes coding for enzymes of the TCA cycle would decrease.

Material and methods

Plant material

Q. ilex seedlings from pre-germinated acorns collected from several mother trees were cultivated in 16-l pots under optimal nutrient, irradiance, and water conditions for one year. All pots were filled with a mixture of sand and sieved peat TKS2 (Floragard Vertriebs GmbH, Oldenburg, Germany) in 1:3 (v/v) enriched with 1 g l^3 of slow-release fertilizer (Osmocote Pro 5-6 months, 17N+11P+10K+2MgO+trace elements; Everris International BV, Waanderburg, the Netherlands). Seedlings were cultivated outdoors under a neutral shade cloth to reduce 40% of sunlight, and a rainout shelter.

Experimental design

Drought stress was imposed in the summer of the second year of plant growth by withholding watering. The large pot size and shading ensured a slow drought stress imposition. Conditions of very severe drought stress were reached after approximately 3 months of withholding watering (from July to October). Control plants were maintained with regular watering. Leaf sampling for metabolite profiling and gene expression was carried out at mild (LS), moderate (MS), severe (SS) and very severe (VS) drought stress conditions, established as a function of predawn (Ψ_{pd}) and midday (Ψ_{md}) leaf water potential as follows: LS, $-0.5 < \Psi_{pd} < -1$ MPa and $-2 < \Psi_{md} < -2.5$ MPa; MS, $-1 < \Psi_{pd} < -4$

MPa and -2.5 < Ψ_{md} < -5 MPa; SS, -4 < Ψ_{pd} < -7 MPa and -5 < Ψ_{md} < -7.5 MPa; VS, Ψ_{pd} < -7 MPa and Ψ_{md} < -7.5 MPa. Only one exception was made with one plant with Ψ_{pd} = -2.2MPa and Ψ_{md} = -2.5 MPa, which was included in the SS group for exhibiting rates of stomatal conductance to water vapor (g_s) as low as the other plants in this group (Fig. 1) (Flexas and Medrano 2002).

Four replicate plants and the respective controls were harvested at each drought stress time-point (LS, MS, SS, VS) at approximately 10:00 to 15:00 local time. Gene expression analysis was carried out using three replicates. Leaf material was harvested, immediately frozen in liquid nitrogen, and stored at -80°C until processing. A cryogenic ball mill supplied with liquid nitrogen (CryoMill, Retsch GmbH, Germany) was used to grind the leaves to a fine powder.

Leaf water potential and gas exchange

As a surrogate of plant water status, leaf water potential was measured at dawn and midday in one leaf per plant using a pressure chamber (PMS Instrument Co., Albany, OR). Leaf gas exchange was measured with a portable photosynthesis system (Li-6400, Li-Cor INC., Lincoln, NE) at 400 ppm CO_2 concentration (using the Li 6400-01 CO_2 mixer), 25°C (using dual Peltier devices on the sensor head), ambient air relative humidity (35-65%), and either 1200 or 0 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), depending on whether net photosynthetic CO_2 uptake (P_n) or dark respiration (R_d) was measured, respectively. Measurements of P_n were made early in the morning, in the 2-cm^2 6400-40 leaf chamber fluorometer to concurrently measure chlorophyll *a* fluorescence. The quantum efficiency of photosystem II (ΦPSII) was calculated from maximal (F_m') and steady-state (F_s) fluorescence as: (F_m' - F_s)/ F_m' according to Genty et al. (1989). Measurements of R_d were made at dawn, in the 6 cm^2 standard leaf chamber to sample a larger leaf area and have a better CO_2 flux signal (6400-02B LED Light Source, Li-Cor INC., Lincoln, NE).

Photorespiration (PR) was estimated according to Valentini et al. (1995):

$$PR = \frac{1}{12}\left[(\theta PSII \times 0.5 \times 0.932 \times PPFD) - 4(P_n + R_d)\right];$$

where ΦPSII is used to calculate the rate of electron transport through PSII, assuming 93.2% of irradiance is absorbed by the leaf (Sperlich et al. 2014) and equally distributed between PSII and PSI.

Extraction of primary metabolites and GC-TOF-MS metabolite profiling analysis

Primary metabolites were extracted using a well-established methanol/chloroform extraction protocol as previously described in the literature (Lisec et al. 2006). A total of 50 mg fresh weight (FW) of finely homogenized leaf material were weighed into 2.0 ml safe-lock Eppendorf tubes and 700 µL ice-cold 100% (v/v) methanol with 30 µL of ribitol (0.2 mg mL^{-1} ribitol in water) as an internal standard were added to each tube. The mixture was vortex-mixed, incubated for 15 min on a shaker (Thermomixer C, Eppendorf, Hamburg, Germany) for 15 min at 70°C and at 950 rpm. Subsequently, each tube was centrifuged at room temperature at 12 000 *g* for 10 min. The supernatant was transferred to a new 2.0 mL safe-lock Eppendorf tube, mixed with 300 µL chloroform and 700 µL water, and vortex-mixed. Tubes were centrifuged at room temperature, 12 000 *g* for 15 min. A total of 150 µL of the polar (upper) aqueous/methanol phase were evaporated to dryness using a centrifugal concentrator for a minimum of 3 h at 30 ºC (Vacufuge Plus, Eppendorf, Hamburg, Germany), and stored at -80 ºC. Primary metabolites were derivatized and 1 µl was analyzed using an established GC-TOF-MS protocol (Lisec et al. 2006) at the LabMet metabolomics facility (CTBE, Campinas, Brazil). Biological variations were controlled by analyzing quality control (QC) standards by fatty acid methyl esters (FAMES) internal standard markers and a QC standard solution of 41 pure reference compounds (i.e. the most detected and abundant metabolites) throughout the analysis. GC-TOF-MS chromatograms were subsequently evaluated at the Plant Metabolomics Lab (ITQB NOVA, Oeiras, Portugal) using TagFinder (Luedemann et al., 2008). Analytes were manually identified using the TargetFinder plug-in of the TagFinder software and a reference library of ambient mass spectra and retention indices from the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/) (Kopka et al. 2005, Schauer et al. 2005). GC-TOF-MS relative primary metabolite levels were normalized to the internal standard (ribitol) and the FW of the samples.

Gene expression

The expression of 12 genes involved in the respiratory metabolism was analyzed using RT-PCR. Total RNA was extracted from 500 mg FW of frozen leaf powder per plant following the CTAB–LiCl precipitation method (Chang et al. 1993), including on-column DNAse treatment and RNA purification using the Qiagen RNAeasy kit (Qiagen, CA). First-strand cDNA was synthesised from 1 µg total RNA from each sample using PowerScriptIII reverse transcriptase (Invitrogen) according to the supplier's manual. 18S rRNA was used as a control, after verifying that the signal intensity remained unchanged during the drought experiment.

Genes were selected based on the importance of the encoded enzyme for carbon metabolism, glycolysis, photorespiratory pathway, TCA cycle and mitochondrial electron transport (Table 1). The process for identifying appropriate genes was as follows: selected enzymes in different pathways were identified in the 'Kyoto Encyclopedia of Genes and Genomes' (KEGG) from *Arabidopsis thaliana*. When different proteins were identified for the same enzyme, we selected the most suitable according to the variation observed in previous drought or CO_2-enrichment studies in this model species. Expressed Sequence Tags (ESTs) for selected enzymes were identified using Blast software throughout searches in CorkOakDB (Pereira-Leal et al. 2014) such as in 'The oak gene expression atlas' (Lesur et al. 2015).

The primers (Supplementary Table S1**)** were located in a conserved region of DNA and identified by comparing the ESTs from both CorkOakDB and 'The oak gene expression atlas' databases, to optimize transferability to *Q. ilex*. The primer pairs were designed using Primer 3 software (Rozen and Skaletsky 1999). Polymerase chain reactions were performed in an optical 96-well plate with a CFX 96 Detection system (BIO-RAD, CA), using EvaGreen to monitor dsDNA synthesis. Reactions containing 2x SsoFast EvaGreen Supermix reagent (BIO-RAD, CA), 12.5 ng cDNA and 500 nM of primers in a final volume of 10 µl were subjected to the following standard thermal profile: 95°C for 3 min, 40 cycles of 95°C for 10 s and 60°C for 10 s. Three technical replicates were performed for each PCR run. To compare the data from different PCR runs or cDNA samples, the mean of the threshold cycle (CT) values of the three technical replicates was normalized to the mean CT value of Ri18S. The expression ratios were then obtained using the " " CT method corrected for the PCR efficiency for each gene (Pfaffl 2001).

Statistical analysis

Means of gas exchange variables, GC-TOF-MS relative metabolite levels and gene expression levels described in the text as significantly different from the respective controls were determined with the Student's *t* test ($P < 0.05$) using the algorithm incorporated into Microsoft Excel 2016 (Microsoft Corporation, Seattle, WA). Comparisons were made within each level of drought (vs control) separately. Correlation coefficients were calculated by applying the Pearson algorithm using the statistical software R (R Core Team, 2016). Heatmap plots and clustering were also performed in the R software (R Core Team 2016) using the "heatmap.2" function from the "gplots" package (Warnes et al. 2016).

Results

Leaf carbon exchange and quantum efficiency of photosystem II (ΦPSII)

As drought stress severity increased, leaf water potential and g_s decreased in *Q. ilex* 2 year-old seedlings (Fig. 1). Similarly, the rate of P_n per unit leaf area and ΦPSII also decreased (Fig 2). P_n was significantly lower than that exhibited by control plants in moderate, severe, and very severe drought stress conditions, reaching negative rates of P_n (Fig. 2). PR and R_d rates were less sensitive to drought. Although both PR and R_d decreased with increasing drought severity, they were only significantly lower than control plants in very severe drought stress conditions (Fig. 2).

GC-TOF-MS metabolite profiling

Gas chromatography coupled to mass-spectrometry detected a set of 31 primary metabolites in leaves subjected to different drought stress conditions (Fig. 3). Among these, amino acids and derivatives were the most abundant metabolites with a total of 15 chemical species, followed by sugars and sugar-alcohols (7 and 3 chemical species, respectively) and 6 organic acids. At mild and moderate drought stress conditions simple sugars increased relative to controls (significantly for glucose, fucose and xylose). At severe and very severe stress conditions, all the detected simple sugars and sugar alcohols showed a significant increase, whereas compound sugars decreased (sucrose) or did not change (raffinose and trehalose). Amino acids were little responsive to mild and moderate stress, but increased dramatically at severe and especially very severe drought stress conditions (Fig. 3). The highest increases were observed for asparagine, ²-alanine, GABA and proline, which accumulated 9, 10, 63 and 92 fold, respectively (Fig. 3; Table S2). The only exception was glutamate, which showed a significant decrease relative to control plants, and cysteine and ornithine, which were little responsive to any degree of drought stress. A similar response was found for the detected organic acids; threonate and malate increased at moderate drought stress, and most organic acids peaked at very severe drought stress, with the exception of succinate which did not change relative to controls at any stress level (Fig. 3; Table S2).

Although individual sugars, organic and amino acids exhibited both positive and negative correlations with P_n, PR and R_d, two general patterns emerged from the correlation analysis of

metabolite concentrations with gas exchange variables (Fig. 4). First, the levels of most organic and amino acids and their derivatives were negatively correlated with leaf gas exchange rates, with only glutamate exhibiting clear and consistent positive correlations. Second, the levels of simple sugars and sugar alcohols were negatively correlated with gas exchange rates, while this was not true for compound sugars. Because most metabolites exhibited significant correlations among them, multivariate models with metabolites could not be applied to explain gas exchange rates (supplementary Fig. S1).

Gene expression

Q ilex samples from control and drought stress showed minor differences in mean expression values of genes involved in glycolysis, the TCA cycle and the mitochondrial electron transport at all stress levels (Fig. 5). There was a trend for CS and SDH transcript levels to be higher in drought stressed plants, with SDH transcript levels being 2.5-fold higher in severely stressed than control plants at $P < 0.05$. However, most gene expression levels tended to decrease with increasing drought severity, particularly in very severe drought stress conditions. For example, the two analyzed genes involved in electron transport, cytochrome *c* oxidase in phosphorylating pathways, and alternative oxidase 1a (AOX 1a) in non-phosphorylating pathways, showed decreased levels of transcripts, particularly AOX in very severe drought stress conditions (Fig. 5). However, differences between mean values were generally not significant due to high variability between biological replicates. Only mean levels of transcripts of the 2-oxoglutarate dehydrogenase complex (OGDH) exhibited a 4-fold significant decrease at very severe drought stress, relative to control plants ($P < 0.05$).

The two genes involved in the photorespiratory pathway showed that serine-glyoxylate aminotransferase (SGAT) and glutamate-glyoxylate aminotransferase (GGAT) transcript levels decreased at severe and particularly very severe drought stress conditions. The PR rates estimated from gas exchange and chlorophyll *a* fluorescence were positively related with the abundance of transcripts of SGAT (r^2=0.51, p=0.046) and GGAT (r^2=0.65, p=0.016) across the 8 treatments (LS, MS, SS, VS and respective controls).

Discussion

Changes in leaf photosynthetic and respiratory metabolism occurred in *Q. ilex* seedlings in response to a long period of drought stress. Our data showed primary metabolic adjustments to (1) maintain photosynthetic carbon uptake at mild drought stress, and (2) to protect leaves from oxidative damage at more severe levels of drought stress. Additional adjustments involving the production of small heat shock proteins (Verdaguer et al. 2003), peroxidases (Echevarría-Zomeño et al. 2009) and terpenes (Peñuelas and Llusià 2002, Blanch et al. 2009) have been observed in *Q. ilex* leaves under high temperatures and low water availability. These adjustments also act against oxidative stress by increasing thermotolerance, ROS scavenging, or cell repairing. Leaf tissue protection from drought-induced photodamage is crucial to rapidly recover carbon after periods of drought in evergreen and deciduous oaks growing in nutrient- and water-poor sites (Gallé et al. 2007, Rodríguez-Calcerrada et al. 2011, Feller 2016). The fact that many metabolites serving as substrates for respiration and playing a role in osmoprotection and photoprotection (e.g. simple sugars and amino acids) increase with drought stress severity (Fig. 3), suggests that a decrease in R_d reflects a down-regulation of energy-demanding processes. This allows mitigating the loss of carbon compounds crucial to withstand and recover from drought stress events. All together, these results provide evidence of the strong ability of this species to survive in dry to semi-arid sites.

Photosynthetic response to drought

Mild reductions in water potential (i.e. $-0.5 < \Psi_{pd} < -1$ MPa) had no effect on gas exchange; however, it resulted in the significant increase of glucose (Fig. 3). The increase of glucose during mild drought stress suggests a role in cellular stress signaling (Chaves et al. 2003, Rosa et al. 2009). Moreover, this increase in glucose levels could be a metabolic response to the need of osmolytes to sustain turgor maintenance and P_n, as suggested in previous studies (Clifford et al. 1998, Chen and Jiang 2010). The importance of simple sugars in signal transduction pathways and osmoregulation is supported by the increase in fructose, fucose, xylose, and glucose at moderate drought stress (i.e. $-1 < \Psi_{pd} < -4$ MPa), when P_n was only exhibiting moderate reductions (Fig. 2). A higher reduction in leaf growth (and carbon sink strength) than P_n can result in the accumulation of sugars in response to drought (Sala et al. 2012). However, because leaves were already fully expanded when sampled, the accumulation of simple sugars induced by drought was probably the result of reduced phloem transport (Quick et al. 1992, Wingler et al. 1999, Lemoine et al. 2013) and enhanced hydrolysis of reserve sugars,

particularly starch (Rosa et al. 2009, Rodríguez-Calcerrada et al. 2011, Sala et al. 2012) and sucrose, which decreased with drought stress.

A general response to drought stress was the increase in most amino acids. These metabolites play an important role in reducing osmotic potential and maintaining cell turgor under drought (Morgan 1984, Clifford et al. 1998). However, in this study, amino acids tended to decrease or remain unchanged at mild and moderate stress, but increase with increasing drought severity, suggesting that they might play a minor role in osmoregulation, but a major role in the protection against drought-induced oxidative stress (Xiao et al. 2009; Szabados and Savoure 2010, Bowne et al. 2012, Barchet et al. 2014). Of all amino acids, proline exhibited the highest increase during drought stress (>90-fold; Table S2). This is consistent with the multiple roles described for proline in stress tolerance, which include ROS scavenging, and enhancement of cellular homeostasis and protein protection (Szabados and Savoure 2010). In general, the accumulation of amino acids might be due to an increased allocation of carbon and nitrogen for their biosynthesis, but also to an increased rate of proteolysis (Good and Zaplachinski 1994, Reguera et al. 2013) or a lowered use of amino acids in protein synthesis (Reguera et al. 2013, Barchet et al. 2014).

The increase of antioxidant polyols, such as *myo*-inositol, glycerol and erythritol in response to severe drought stress in *Q. ilex* leaves, is also consistent with the reduced rates of photosynthetic and photorespiratory processes at these conditions (Shen et al. 1997, Rodríguez-Calcerrada et al. 2013, Jansen et al. 2014). The increase in all antioxidant metabolites can also help preventing chlorophyll loss during drought stress in *Q. ilex*; in fact, leaf chlorophyll a and b were shown to exhibit constant concentrations over a range of 7 MPa in water potential in a study by Peguero-Pina et al. (2009).

Photorespiratory response to drought

The rate of PR is difficult to quantify (Busch 2013). Here, using an indirect PR estimation based on gas exchange and chlorophyll fluorescence data (Valentini et al. 1995), it was observed that PR remained unchanged until drought stress conditions were very severe (Ψ_{pd} < -8 MPa), when it decreased relative to control plants. The same result has been reported in previous studies (Biehler and Fock 1996, Guan et al. 2004, Peguero-Pina et al. 2009), suggesting that in severe drought stress, the electron transport through the PSII and regeneration of RuBP are limiting factors for photosynthetic and photorespiratory fluxes. This conclusion is partly supported by the expression of genes coding for peroxisomal enzymes SGAT and GGAT, as the upregulation of SGAT transcripts in conditions of

drought stress has been related to increased PR rates (Abogadallah 2011, Cramer et al. 2013). However, results of gene expression and PR rates appeared to be at odds with metabolite analyses. The synthesis and accumulation of glycine in the peroxisomes are considered a proxy of the PR rate that results from the cooperative role of GGAT and SGAT (Igarashi et al. 2006). In our study, glycine levels increased up to 6-fold under severe stress conditions, despite decreased PR rates and transcript abundance of these genes. Consistently with the increase in glycine, glutamate – which acts as an amino donor for photorespiratory glycine formation in the peroxisome – exhibited a clear decrease in response to drought (Fig. 3). This result suggests that drought stress might have triggered an increased conversion of glutamate to glycine and glycerate, an organic acid that also increased significantly in response to drought stress. On the other hand, the accumulation of proline could also explain the decrease in glutamate at very severe stress levels, given the inter-conversion between both metabolites (Szabados and Savoure 2010).

Further studies using independent methods such as ^{13}C-stable isotope labelling techniques to measure CO_2 release from glycine decarboxylation (Busch 2013) are needed to confirm the impact of drought stress on fluxes via the PR pathway.

Response of mitochondrial respiration to drought

This study also aimed at better understanding the drought-induced decrease in R_d that generally occurs with increasing drought severity (Atkin and Macherel 2009). Compared to previous studies in *Q. ilex* (e.g. Rodríguez-Calcerrada et al. 2011), in this current study, R_d only significantly decreased at very severe drought conditions. At this stage, simple sugars and amino acids increased, suggesting that the observed drought-induced decrease of R_d was not related to a photosynthetic limitation of carbon substrates. The reduced transcript levels of OGDH in drought stressed plants are consistent with the reduction in R_d (Araújo et al. 2012). However, metabolites and genes coding for other enzymes of the glycolytic pathway or the TCA cycle did not exhibit responses to drought stress that matched that of R_d. Transcript profiling studies on drought stressed plants reveal high variability in the expression of genes encoding enzymes related with respiration or carbohydrate metabolism. For instance, rice plants subjected to 2-3 days of no watering showed increased levels of transcripts in all key enzymes along the glyoxylate cycle, with significant inductions in expression of isocitrate lyase and malate synthase (Maruyama et al. 2015). In contrast, no significant changes were identified in the genes of homologous enzymes from *Arabidopsis thaliana* plants subjected to 6 days of no watering and similar soil relative

water content than above; just a weak induction was identified in a transcript encoding malate dehydrogenase (MD), and a weak repression in a transcript encoding CS (Maruyama et al. 2015). High variability in the expression of TCA-cycle genes were also observed within *Populus balsamífera* genotypes subjected to drought. The leaves from drought stressed plants showed induction or repression in CS and MD genes depending on the genotype analyzed (Hamanishi et al. 2015).

While changes in the TCA-cycle flux modes are only unequivocally revealed by flux profiling approaches (Tcherkez et al. 2012, António et al. 2016), our results suggest that a change in the TCA-cycle operation mode occurred in response to very severe levels of drought stress. In these conditions, glutamate levels decreased concomitantly with a significant increase in the levels of GABA, while the expression of the gene coding for OGDH was reduced. Taken together, these results suggest that the carbon flux from 2-oxoglutarate to succinate via succinyl-CoA was bypassed via the GABA shunt. The activation of the GABA shunt in conditions of drought stress has been reported to control for cytoplasmic pH and protect against oxidative stress (Bouché and Fromm 2004, Warren et al. 2011, Rabara et al. 2015). Changes in the TCA cycle at very severe drought conditions could be related to leaf senescence (Ansari et al. 2014). In fact, some *Q. ilex* plants experienced early symptoms of leaf dieback at such low levels of predawn water potential (< -8 MPa). However, the drought-tolerant nature of this species allows that, upon rewatering, severely stressed *Q. ilex* plants recover leaf functionality and survive (unpublished data; Peguero-Pina et al. 2009). Thus, the re-organization of the TCA cycle under very severe drought conditions might play a role in drought acclimation by diverging TCA-cycle intermediates toward synthesis of antioxidant amino acids and other carbon compounds, instead of production of ATP (Bouché and Fromm 2004, Sweetlove et al. 2010, Araújo et al. 2012, Jansen et al. 2014, António et al. 2016).

Drought can also alter the efficiency of R_d in synthesizing ATP by modifying the activity of the non-phosphorylating AOX pathway. The induction of an AOX gene was associated to the prevention of accumulation of ROS and enhancement of drought tolerance in durum wheat (Pastore et al. 2007). Here, the fact that transcripts of the AOX pathway did not increase in *Q. ilex* leaves under drought stress (Fig. 4) suggests that increased rates of *in vivo* AOX activity, as previously reported in drought stress studies, are likely to be a consequence of biochemical regulation of AOX (Bartoli et al. 2005, Ribas-Carbó et al. 2005). The activity of AOX is known to be strongly regulated via allosteric activation by organic acids (Millar et al. 1993, Vanlerberghe et al. 2013) and/or reduction of protein disulfide bonds (Umbach et al. 1994). Thus, presumably, AOX activity varies independently of AOX

transcripts in response to drought and other abiotic factors such as temperature (Armstrong et al. 2008).

Conclusions

This study shows that leaf metabolism of *Q. ilex* seedlings was affected by drought. The accumulation of glucose presumably acted in stress signaling and osmoregulation, which might contribute to the maintenance of P_n under mild drought stress. Subsequent increases in drought severity caused a progressive decrease in P_n and photosynthetic electron transport rates in parallel with a general increase in some organic acids. The accumulation of soluble sugars, polyols and amino acids (particularly GABA and proline) under severe to very severe drought stress suggests that these metabolites were mostly involved in protection against oxidative stress. An upregulation of the respiratory metabolism was apparently not needed to conduct these acclimation changes, as rates of R_d generally decreased, especially under very severe stress conditions. There was no evidence of R_d rates being limited by the availability of carbon substrates, suggesting that other factors such as adenylate limitation associated to reduced consumption of ATP might be involved. The reduction in the R_d rates and the activation of the GABA shunt reflect a significant shift in the carbohydrate and respiratory metabolism that favor the accumulation of osmoprotectant and antioxidant compounds crucial for drought-tolerant *Q. ilex* seedlings to survive long periods of low water potentials and little CO_2 uptake.

Author contributions

J. R-C and L.G designed the experiments. J. R-C, A. M. R, P. P, C. A, M. L and C. C performed the experiments. J. R-C, A. M. R, P. P, C. A, O. K. A, and C.C analyzed the data. J. R-C, L. G, A. M. R, P. P, C. A, O. K. A and C.C wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer pairs used in RT-PCR.

Table S2. Relative values of selected leaf primary metabolites.

Fig. S1. Metabolite-metabolite pairwise correlation heat map.

Figure legends

Fig. 1. Means (±SE; n=3-4) of (A) leaf predawn and midday water potential (Ψ_w) and (B) stomatal conductance to water vapor (g_s), considered to establish different groups of drought stress (mild (LS), moderate (MS), severe (SS) and very severe (VS) drought stress) in seedlings of *Quercus ilex* subjected to either cessation of watering or frequent irrigation (C). Asterisks denote significant differences between each drought level and the respective controls using Student's *t*-test as: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Asterisks in the upper side of panel A refer to differences in leaf predawn water potential.

Fig. 2. Means (±SE; n=3-4) of leaf physiological variables in seedlings of *Quercus ilex* subjected to either drought (mild (LS), moderate (MS), severe (SS) and very severe (VS) drought stress) or well-watered conditions (C). (A) Net photosynthetic CO_2 uptake (P_n); (B) quantum efficiency of photosystem II electron transport (ΦPSII); (C) photorespiration (PR); and (D) dark respiration (R_d). Asterisks denote significant differences between each drought level and the respective controls using Student's *t*-test as: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Fig. 3. Heat map of fold changes in leaf primary metabolite relative values in *Quercus ilex* seedlings subjected to various levels of drought stress (mild (LS), moderate (MS), severe (SS) and very severe (VS)) in relation to their well-watered controls. Relative values are normalized to the internal standard (ribitol) and fresh weight (FW) of the samples; false color imaging was performed on log10-transformed GC-TOF-MS metabolite data. Values are means (± SE; n=3-4). Significant changes using Student's *t*-test are indicated as Ë $P < 0.05$; Ê $P < 0.01$; Δ $P < 0.001$, with respect to controls. Metabolites grouped in sugars & sugar alcohols (S), amino acids & derivatives (AA) and organic acids (OA).

Fig. 4. Metabolite pairwise correlation with leaf dark respiration (R_d), net photosynthetic CO_2 uptake (P_n) and photorespiration (PR) heat map. Significance of correlations indicated as: Ë $P < 0.05$; Ê $P < 0.01$; Δ $P < 0.001$. Metabolites grouped in sugars & sugar alcohols (S), amino acids & derivatives (AA) and organic acids (OA).

Fig. 5. Schematic view of main respiratory pathways showing mean (±SE; n=3) expression of 10 genes coding for selected enzymes in seedlings of *Quercus ilex* subjected to either drought (mild (LS), moderate (MS), severe (SS) and very severe (VS) drought stress) or well-watered conditions (C).

Fig. 6. Mean (±SE; n=3) expression levels of photorespiratory enzymes (A) SGAT (serine-glyoxylate aminotransferase) and (B) GGAT (glutamate-glyoxylate aminotransferase) in leaves of *Quercus ilex* subjected to either drought (mild (LS), moderate (MS), severe (SS) and very severe (VS) drought stress) or well-watered conditions (C). Asterisks denote significant differences between each drought level and the respective controls using Student's *t*-test as: *, $P < 0.05$ and ***, $P < 0.001$.

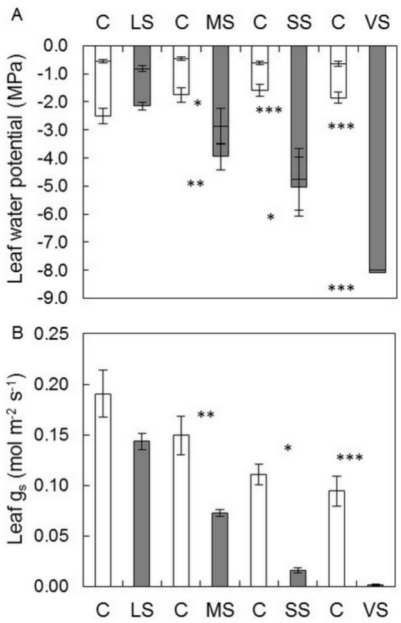
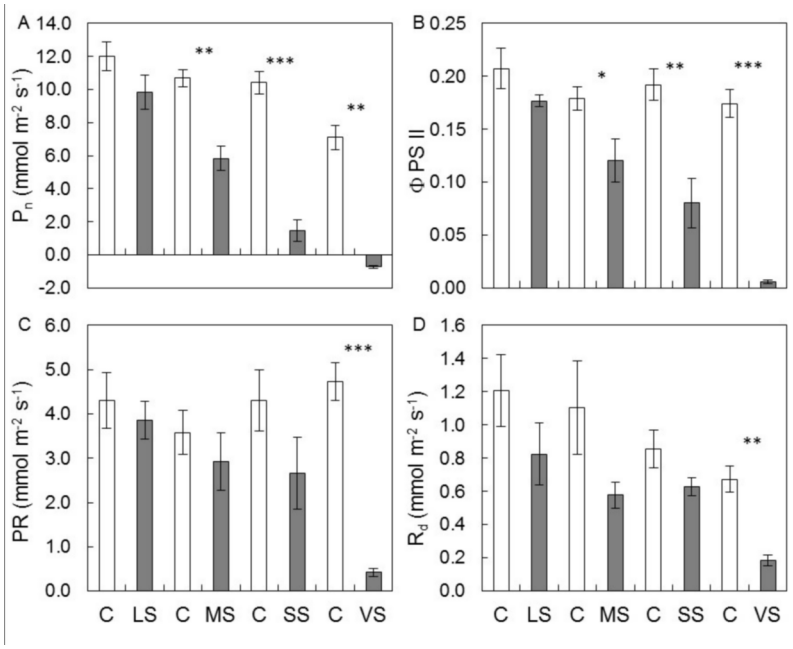


FIG1.tif



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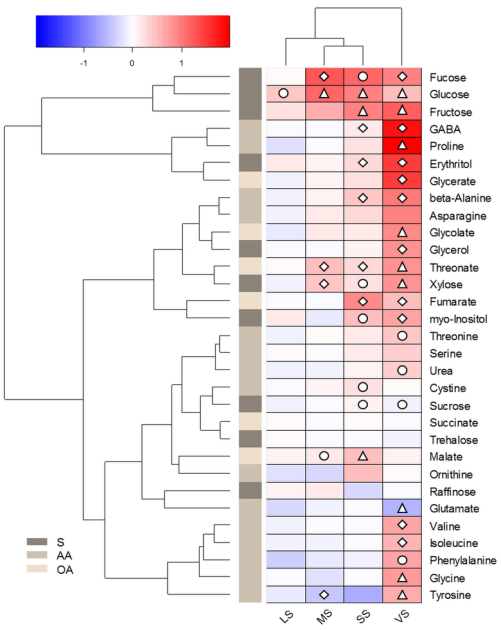

Figure 3.tif

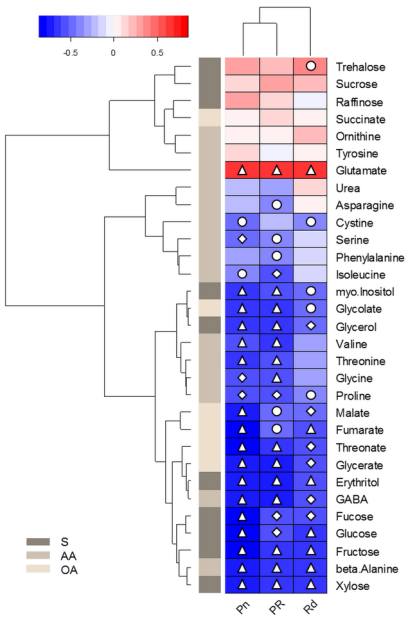

Figure 4.tif

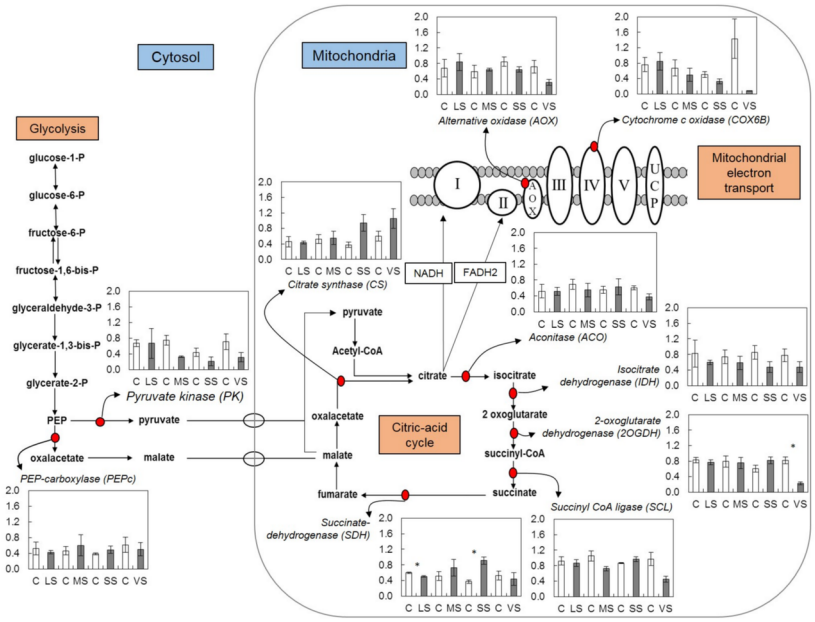

Figure 5.tif

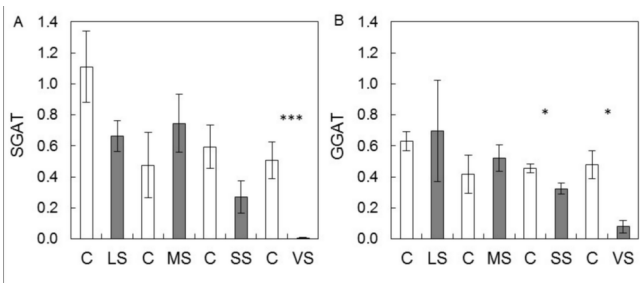

Figure 6.tif