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Temperature stress effects in *Quercus suber* leaf metabolism

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ABSTRACT

Based on projections that climate changes are will intensify in the near future, it is important to understand how plants respond to climate. Consequently, we have been studying the effect of contrasting temperatures on leaf metabolism of *Quercus suber*, an important Mediterranean oak.

Potted plants were grown under controlled conditions for 53 days at 28 °C or 10 °C. The accumulation of major soluble metabolites was analyzed by NMR. The relative levels of transcripts of genes encoding key enzymes of the shikimate and phenylpropanoid pathway (CS, PAL, CAD and ChS) were examined by means of quantitative, real-time RT-PCR. At 10 °C, in the pre-existing leaves, the concentrations of sucrose, quercitol and catechin were higher, as were PAL and ChS transcripts. At 28 °C, however, it was the concentration of quinic acid that was higher, as were the concentrations of CS and CAD transcripts.

We conclude that contrasting temperatures greatly influence *Q. suber* metabolism and that a deeper analysis of the effects of more extreme temperatures is needed to understand the possible effects of temperature changes on *Q. suber* metabolism and physiology.

Introduction

Temperate oaks (*Quercus* spp.) represent one of the major components of the European broadleaved forest resources with high economic and ecological importance, contributing greatly to the social and economic well-being of the populations (Varela, 1995; Euforgen, 2003). Oak forests are important for the conservation of soil and water, biodiversity, natural landscape and climate, and give rise to wood products that have a variety of important applications. In the Mediterranean area, cork oak has great ecological importance and a high economic impact due to the cork's value. It is predicted that the future climate scenarios of global warming will be particularly severe in the Mediterranean region (Pereira and Chaves, 1995; Pereira et al., 2002), with negative impacts on productivity and the quality of the plant products. It is therefore important to understand how temperature affects cork oak metabolism, and in particular the compounds with a protective role against stress (Buchana et al., 2000). *Q. suber* has xerophytic characteristics and we showed, in a previous study (Passarinho et al., 2006), that leaf metabolite concentrations, namely sugars and quinic acid (a compound of the shikimate pathway), changed throughout the year in field-grown *Q. suber*. This suggests that *Q. suber* leaf metabolism can adapt to changes in the environment. In view of the importance of phenolic compounds both for protection against environmental stress and for the synthesis of cell wall constituents, we studied the effects of long-term exposure of young cork oak trees to contrasting temperatures (10 °C and 28 °C) on leaf metabolites and on gene expression of key enzymes of phenolic metabolism. Indeed, there is a scarcity of information about the molecular responses of *Q. suber* to temperature, and this fact is reflected in GenBank databases, where there is little information on genes encoding key enzymes of secondary metabolism. This study has the potential to provide insight to support an early genetic selection of trees well adapted to thermal stress. Using nuclear magnetic resonance (NMR), we identified and quantified the major water-soluble organic metabolites. We also analyzed the accumulation of the transcripts of four key enzymes involved in phenolic synthesis: chorismate synthase (CS; from the shikimate/arogenate pathway), phenylalanine ammonia-lyase (PAL; first enzyme from phenylpropanoid pathway), NADPH-dependent cinnamyl alcohol dehydrogenase (CAD; enzyme from phenylpropanoid pathway) and chalcone synthase (ChS; first enzyme of the flavonoids biosynthesis branch). CS was partially sequenced (Soler et al., 2007), CAD and PAL have been fully sequenced for *Q. suber* (Coelho et al., 2006; Coelho, 2009), but for ChS, heterologous primers had to be designed in order to obtain the gene sequence.

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Materials and methods

Experimental design

Half-sibling plants of *Quercus suber* were grown on a soil:peat:sand (1:1:1) mixture in a greenhouse under semi-controlled conditions (November–January, mean air temperature of about 15 °C). When they were 3 months old, the plants were transferred to growth chambers (AraLab) at the constant temperatures of 10 °C and 28 °C, 50% humidity, 12 h photoperiod and 500 $\mu Em^{-2} S^{-1}$ of light intensity. After 53 days of treatment, pre-existing (PE) leaves and the leaves formed during the temperature treatment (newly formed leaves – NF leaves) were harvested from 20 plants for biomass determination. Leaves were collected in triplicate samples from each treatment, frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. The water soluble metabolites were quantified by NMR. We used quantitative RT-PCR to determine the relative expression of genes encoding key enzymes of the shikimate and phenylpropanoid pathways.

Phenylpropanoid pathway enzymes

To study chorismate synthase (CS – EC 4.2.3.5), phenylalanine ammonia-lyase (PAL – EC 4.3.1.5), and cinnamyl alcohol dehydrogenase [QsCAD1, with motives of cinnamyl-Co reductase (Coelho et al., 2006) – EC 1.1.1.1 or EC 1.1.1.219], mRNA sequences deposited in the GenBank data base (accessions no. EE743660.1, AY443341.1 and AY362455.1, respectively) were used to design specific primers with the Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). For chalcone synthase (ChS – EC 2.3.1.74), degenerated primers were designed after the alignment of chalcone synthase ESTs sequences of *Betula nigra*, *Casuarina glauca*, *Fagus sylvatica*, *Juglans nigra*, *Populus alba*, *Populus trichocarpa*, *Vitis rotundifolia*, (forward GTYGTSTGCTCKGARATCAC and reverse GACATGTTKCCR-TAMTCACT) to produce a fragment of 432 bp after PCR. The PCR product was sequenced and the partial cds for *Quercus suber* putative chalcone synthase gene deposited in the GenBank (accession no EU016228.1) and specific primers were designed (Table 1 and Fig. 1).

Total RNA extraction and quantitative real-time PCR

RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN) using the RLC buffer with 2% 2-mercaptoethanol. DNA contaminants were removed using RNase-Free DNase set (QIAGEN) for on-column DNase digestion. Total RNA was quantified using a NanoDrop® ND-1000 spectrometer (Thermo Scientific) and the quality analyzed by agarose gel electrophoresis.

Quantitative real-time PCR was performed using the iScript cDNA Synthesis Kit (BIO-RAD) and iQ SYBR Green Supermix (BIO-RAD) according to the manufacturers' protocols, with specific primers (Table 1). Each PCR was performed with three technical replicates of three independent cDNA for each treatment, using an iQ5 apparatus (5 min at 95 °C for enzyme activation, 40 cycles of 30 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 30 s at 72 °C for extension). The absence of non-specific PCR products and primer dimmer artefacts was checked by melting curves, which showed a single peak, and agarose gel electrophoresis revealed a band of the expected size for all specific primer pairs (data not shown). We tested 4 genes to be used as endogenous controls: actin (Soler et al., 2007), elongation factor (accession no EE743684.1), 18S rRNA (accession no EE743696.1) and HMG-CoA synthase (accession no EE743693.1). The evaluation of the expression using the Genex software resulted in the selection of EF and HMG-CoA synthase to be used as reference genes. Before using the comparative C_T method (Livak and Schmittgen, 2001), the efficiency of reference and target genes were ca. 100% calculated by a standard curve method, and the samples were diluted from 1 to 10,000, using the iQ5 Optical System software (BIO-RAD).

NMR analysis of extracts

At each sampling time, leaves were collected from plants in the morning (10.00 h) and stored at −80 °C until analyzed. For metabolite determination, frozen leaf samples (0.2–0.3 g) were ground to a fine powder on liquid nitrogen in a mortar and dropped into boiling water for 5 min. The slush was centrifuged, the recovered supernatant lyophilized and the residue resuspended in 2 mL of an aqueous solution containing D_2O (5.8 M), Na_2EDTA (2.5 mM) and NaN_3 (2.5 mM). The qualitative and quantitative characterization of osmotic solutes in extracts was carried out by ^1H and ^{13}C nuclear magnetic resonance (^1H NMR, ^{13}C NMR), using dioxan as an internal concentration standard. All experiments were performed on a Bruker Avance II$^+$ 400 spectrometer (Germany) operating at ^1H frequency of 400.13 MHz and ^{13}C frequency of 100.61 MHz, using a 5 mm diameter broadband probe head. ^1H NMR spectra were acquired using a zg pr30 pulse sequence with the following parameters: number of scans 48, a recycle time of 5.48 s, acquisition of 65 k data points over a spectral width of 8224 Hz and pulse width of 10.6 μs, corresponding to a flip angle of 30°. ^{13}C NMR spectra were acquired using a zg ig30 pulse program with a total of 2500 scans collected into 65 k points over a spectral width of 24 kHz, a recycle time of 2.9 s, and a pulse width of 8 μs corresponding to a flip angle of 30°. Proton decoupling was applied during the acquisition only. The temperature of the probe head was kept at 298 K. Data were processed with 4 Hz exponential line broadening. Chemical shifts are expressed in ppm relative to dioxan at 67.0 ppm. Resonances due to fructose, glucose, sucrose, catechin and quinic acids were identified from their chemical shifts of pure substances. Resonances due to quercitol were assigned by comparison with chemical shifts

Table 1
List of primers used for quantitative real-time PCR.

Gene name	GenBank accession no	Forward primer (5′ 3′)	Reverse primer (5′ 3′)	Position of the primers	Product length (bp)
ACT	–	TGACAATGGAACTGGAATGG[a]	CATCACCAACATAGGCATCC[a]	–	–
EF	EE743684.1	ACAGCCTTATAGCCAGCAG	GGTTGAAGAGGAGGACATATTG	152–293	141
18S rRNA	EE743696.1	TGATGTATTCAACGAGTTTATAGC	CGGGCAGGTACAAAGGG	43–210	168
HMG-CoA	EE743693.1	GCAAGCGAGTGATACTGTTC	TCCATACCTGTGCTCCATTAG	64–263	200
PAL	AY443341.1	ATTAGCAGGGATTGATGG	CAAGTGGTCTGTAAATTCG	927–1122	196
CAD	AY362455.1	CAGATGATAAGCCATTTGCG	AGGAACTTCAGGGTGCTAC	896–1060	165
ChS	EU016228.1	TGAGATCACAGCAGTTAC	CAAGTTGAAACAGTGGAC	136–281	146
CS	EE743660.1	TGGATTGATTGGAAACAGATTAC	CAAGGAAGCAGCACACAG	114–292	179

ACT, actin; EF, elongation factor; HMG-CoA, HMG CoA synthase; PAL, phenyalaline ammonia-lyase; CAD, cinnamyl alcohol dehydrogenase; ChS, chalcone synthase; CS, chorismate synthase.

[a] Primers used in Soler (2007) or Soler et al. (2007).