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Analysis of the expression of putative heat-stress related genes in relation to thermotolerance of cork oak

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SUMMARY

Cork oak (*Quercus suber* L.) is a research priority in the Mediterranean area and because of cork oaks' distribution these stands are experiencing daily stress. Based on projections of intensifying climate change and considering the key role of exploring the recovery abilities, cork oak seedlings were subjected to a cumulative temperature increase from 25 °C to 55 °C and subsequent recovery. CO_2 assimilation rate, chlorophyll fluorescence, anthocyanins, proline and lipid peroxidation were used to evaluate plant performance, while the relative abundance of seven genes encoding for proteins of cork oak with a putative role in thermal/stress regulation (*POX1*, *POX2*, *HSP10.4*, *HSP17a.22*, *CHS*, *MTL* and *RBC*) was analyzed by qPCR (quantitative Polymerase Chain Reaction). A temperature change to 35 °C showed abundance alterations in the tested genes; at 45 °C, the molecular changes were associated with an antioxidant response, possibly modulated by anthocyanins. At 55 °C, *HSP17a.22*, *MTL* and proline accumulation were evident. After recovery, physiological balance was restored, whereas *POX1*, *HSP10.4* and *MTL* abundances were suggested to be involved in increased thermotolerance. The data presented here are expected to pinpoint some pathways changes occurring during such stress and further recovery in this particular Mediterranean species.

Introduction

Oak forest is a very particular and sensitive ecosystem where cork oak (*Quercus suber* L.) plays a central role. Cork oak represents the mainstay of multiple-use agro forestry systems with great socio-economic value mainly because of cork exploitation and acorns production (Hidalgo et al., 2008; Vaz et al., 2012). These trees are also valued for their ecological role to restrain desertification and soil erosion and for their contribution to biodiversity maintenance. Cork oak is thus a major research priority in the Mediterranean area and problems affecting oak and holm stands deserve a particular attention (Faria et al., 1996; Scarascia-Mugnozza et al., 2000; Ghouil et al., 2003; Ramírez-Valiente et al., 2010; Vaz et al., 2012).

As consequence of the climate change, temperatures are expected to increase about 2–4 °C during this century within the cork oak distribution range (IPCC, 2007) and selective drivers exerted by climate are expected to increase (Lindner et al., 2010; Ramírez-Valiente et al., 2010). Several authors (Ramírez-Valiente et al., 2010; Vaz et al., 2012) have highlighted the need to understand this species' capacity of coping with the expected climate change, which will be crucial in coming sustainable management strategies. In fact, improvement of traditional forest management strategies based in classical genetics using the information provided by physiological and molecular biology studies will be essential to tackle this matter (Wahid et al., 2012).

Cork oak is widely distributed in the Mediterranean area, where it withstands a variety of climates with contrasting temperatures and rainfall and can be exposed to temperature near 40–45 °C (in shade) (Ghouil et al., 2003). Elevated temperatures are known to

Abbreviations: *A*, net CO_2 assimilation rate; ER, endoplasmic reticulum; F_v/F_m, maximum quantum yield of photosystem II; FW, fresh weight; HSF, heat shock factor; HSP, heat shock protein; MDA, malondialdehyde; PSII, photosystem II; qPCR, quantitative Reverse-Transcriptase Polymerase Chain Reaction; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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induce an array of physiological and biochemical changes in plants, which greatly affect plant performance and further survival (Wahid et al., 2007; Ahuja et al., 2010; Hüve et al., 2011). Among cellular functions, photosynthesis is frequently considered as one of the most sensitive to high temperatures (Berry and Björkman, 1980). Photosynthetic performance is thus largely used as an indicator of plant thermotolerance (Wahid et al., 2007). To deal with heat stress, plants resort to several mechanisms, including: production of antioxidants, e.g. type III peroxidases, a multigenic family formed by several isoforms with different roles in plant development and defense differentially regulated, that deplete H_2O_2 (hydrogen peroxide) levels in different cellular compartments (Cosio and Dunand, 2009); scavenging reactive oxygen species (ROS) and oxidative stress by different mechanisms, like metallothioneins, which have been recently reported important roles on abiotic stress as a ROS scavenger (Brosché et al., 2005; Hassinen et al., 2011; Du et al., 2012) or chalcone synthase, regulating the synthesis of flavonoids and anthocyanins with a photoprotective and antioxidant role (Dao et al., 2011; Zhang et al., 2012); adjustment of compatible solutes and chaperone signal activation, with a very important role of heat shock factors (HSFs) that activate expression of heat shock proteins (HSPs) with particular importance in thermotolerance response, acting as molecular chaperones to prevent denaturation or aggregation of target proteins, as well as facilitating protein refolding in what is called ER (endoplasmic reticulum) stress (Wang et al., 2003; Ahuja et al., 2010; Howell, 2013). All of these mechanisms are regulated at the molecular level and can lead to two different responses: acquisition of thermotolerance or programmed cell death (Wahid et al., 2007; Ahuja et al., 2010).

As far as we know, few studies are available reporting the physiological effect of high temperatures in *Quercus* (Peñuelas and Llusià, 2002; Ghouil et al., 2003; Haldimann and Feller, 2004), while at the molecular level the information is even scarcer for such high temperatures. We recently reported an epigenetic regulation study that investigated heat tolerance in cork oak (Correia et al., 2013) and the results indicated that the studied epigenetic mechanisms, DNA methylation and histone H3 acetylation, had opposite and particular dynamics, probably crucial for the stepwise establishment of this species into such high stress (55 °C). Besides, other authors (Chaves et al., 2011) have highlighted the need for a deeper analysis on the effects of more extreme temperatures to understand the possible effects of temperature changes on cork oak metabolism and physiology, a knowledge gap that we expect to reduce with this research. Moreover and taking into account that the potential of recovery after stress dictates survival and growth (Chaves et al., 2009), plants' recovery capacity emerges as a crucial topic for managing productive forests, considering both the economic pressure and the increase in frequency and severity of extreme environmental stress (Allen et al., 2010). As already reported for water limitation (Vaz et al., 2012), the capacity to cope with stress and the ability to recover after the stress relief may represent adaptive traits of these trees. However, this has never been reported to these elevated temperatures.

In the present study, we subjected *Q. suber* seedlings to a cumulative temperature increase from 25 °C to 55 °C (in 10 °C steps) with subsequent one-month recovery. Net CO_2 assimilation rate (A), the maximum quantum yield of photosystem II (PSII; F_v/F_m), anthocyanins, proline content and lipid peroxidation were assessed as physiological markers to evaluate plant performance under stress and after recovery.

The relative abundance of stress-related transcripts was evaluated by quantitative Reverse-Transcriptase Polymerase Chain Reaction (qPCR). A set of seven genes encoding for proteins of *Q. suber* with a putative role in thermal/stress regulation was analyzed: two class III peroxidase precursor (*POX1* and *POX2*), two small HSPs (*HSP10.4* and *HSP17a.22*), a chalcone synthase (*CHS*), a metallothionein-like protein (*MTL*) and Rubisco large subunit (*RBC*). We aim to provide new insights to understand heat response and to reduce the gap knowledge related to changes occurring during such stress and further recovery in this particular Mediterranean species.

Materials and methods

Plant material and experimental design

Eight-month-old cork oak plants were acquired from a forest plant producer ANADIPLANTA (located in Central Portugal) and transferred from semi controlled greenhouse conditions to a climate chamber for a 2-week acclimation period. The climate chamber environment was kept constant (air temperature = 25 °C; relative humidity = 60–70%; photosynthetic photon flux density = 250 μmol m^{-2} s^{-1}; watering = field capacity; long photoperiod = 16 h light). During the experimental treatment, relative humidity, irradiance, watering and photoperiod were held constant, while air temperature was gradually increased by 10 °C every 3 days from 25 °C to 55 °C; peak temperature was maintained for 3 h. Minimum daily temperature was 20 °C during the 8 night hours. After this period, plants recovered at 25 °C during one month. Twenty plants were maintained at 25 °C during the whole experiment as control.

Sampling occurred on the third day during peak heat hours (around 12 a.m.) for each temperature (25 °C, 35 °C, 45 °C and 55 °C). Fully expanded leaves were collected at each treatment, frozen in groups of five individuals (pools) in liquid nitrogen and stored at −80 °C for subsequent analyses. After one month recovering, leaf samples were collected (≥10) from newly formed leaves (fully expanded).

Percentage of survival and recovery capacity

The percentage of plant survival was recorded at each temperature of the experimental ramp and one month after recovery.

Net CO_2 assimilation rate and chlorophyll fluorescence

At each temperature of the experimental ramp, *in vivo* determination of net CO_2 assimilation rate (A) was performed with a portable IRGA (LCpro, ADC, Hoddesdon, United Kingdom), operating in the open mode under growth chamber conditions at 200 mmol photon m^{-2} s^{-1}. The relative humidity of air entering the cuvette was set at 60% and the cuvette temperature was 23 °C. Flow rate of air through the cuvette was set at 300 mmol s^{-1}. Measurements were performed on fully expanded leaves of cork oak plants under ambient CO_2 concentration. Chlorophyll fluorescence was determined on the same leaves using a portable modulated fluorometer Mini-PAM, including the leaf clip holder part 2030-B (Heinz Walz GmbH, Effeltrich, Germany). As described in Peña-Rojas et al. (2004), maximal photochemical efficiency of PSII (given by F_v/F_m) was estimated after dark adaptation for, at least 30 min, to obtain F_o (minimum fluorescence), F_m (maximum fluorescence) and F_v (variable fluorescence, equivalent to $F_m - F_o$).

Anthocyanins quantification

Anthocyanins were extracted from 100 mg of leaf samples in cold methanol:HCl:H_2O (90:1:1) (v/v/v) solution, following the procedure described by Sims and Gamon (2002). Absorbance was measured at 529 nm and 650 nm using a Thermo Fisher Scientific (USA) spectrophotometer (Genesys 10-uv S). Anthocyanin concentration was calculated using the equation of Sims and