Seasonal variation in transcript abundance in cork tissue analyzed by real time RT-PCR

MARÇAL SOLER,[1] OLGA SERRA,[1] MARISA MOLINAS,[1] EMILI GARCÍA-BERTHOU,[2] ANTÒNIA CARITAT[2] and MERCÈ FIGUERAS[1,3]

[1] *Laboratori del Suro, Department of Biology, Universitat de Girona, Campus Montilivi s/n, 17071 Girona, Spain*

[2] *Department of Environmental Sciences, Universitat de Girona, Campus Montilivi s/n, 17071 Girona, Spain*

[3] *Corresponding author (merce.figueras@udg.edu)*

Summary The molecular processes underlying cork biosynthesis and differentiation are mostly unknown. Recently, a list of candidate genes for cork biosynthesis and regulation was made available opening new possibilities for molecular studies in cork oak (*Quercus suber* L.). Based on this list, we analyzed the seasonal variation in mRNA abundance in cork tissue of selected genes by real time reverse-transcriptase polymerase chain reaction (RT-PCR). Relative transcript abundance was evaluated by principal component analysis and genes were clustered in several functional subgroups. Structural genes of suberin pathways such as *CYP86A1*, *GPAT* and *HCBT*, and regulatory genes of the *NAM* and *WRKY* families showed highest transcript accumulation in June, a crucial month for cork development. Other cork structural genes, such as *FAT* and *F5H*, were significantly correlated with temperature and relative humidity. The stress genes *HSP17.4* and *ANN* were strongly positively correlated to temperature, in accord with their protective role.

Keywords: cork cambium, cork oak, periderm, phellem, phenology, Quercus suber, suberin biosynthesis.

Introduction

Cork (referred to in the botanical literature as phellem) is a water-resistant tissue commonly found in the outer bark of trees, but which also occurs in roots and wounded tissues. Cork tissue protects woody plant organs and healing tissues from dehydration, solar irradiation and pathogens. Cork is formed on the external side of the cork cambium (phellogen) as part of the periderm, which replaces the epidermis in plant secondary growth. It consists of multiple layers of dead cells whose walls are made impervious by the deposition of suberin and waxes. Suberin, the main component of cork and the substance responsible for the imperviousness of the tissue, is a complex polymer comprising both aliphatic and aromatic (phenolic) domains (Bernards 2002).

Cork used to seal bottles is a well-known raw material obtained from the outer bark of cork oak (*Quercus suber* L.), whose cork cambium is a permanent layer that produces cork indefinitely. Cork thickness is achieved by the accumulation of annual cork rings about 2–3 mm thick that adhere to one another (Caritat et al. 2000). Cork cellular structure, chemical composition, and physical and mechanical properties have been well described (Silva et al. 2005). Its average chemical composition is 41% aliphatic suberin, 22% aromatic suberin (also referred to as cork lignin), 7.5% waxes (mostly terpenes and sterols) and 7.5% tannins. However, the molecular processes underlying cork biosynthesis and differentiation are mostly unknown.

Cork oak is an evergreen species native to the Mediterranean region that has hot, dry and sunny summers, mild winters, rainy autumns (Lavorel et al. 1998). Features such as sclerophylly, presence of a lignotuber (Molinas and Verdaguer 1993*a*, 1993*b*), litterfall pattern (Caritat et al. 2006) and stomatal response to water deficit (Tenhunen et al. 1984, Oliveira et al. 1994) confirm the xerophytic character of this oak species. The thick corky bark of the tree is stripped off for commercial use when it reaches a thickness of at least 25 mm (about every 10–12 years, depending on the geographical area). Radial growth of cork starts at the beginning of the spring and continues until autumn, with the growth rate peaking in late spring (June) (Natividade 1950, Costa et al. 2001, Fialho et al. 2001). Annual rings have both light and dark cork (Figure 1). The light-colored cork is produced early in the season when cell production is more active and the cells are relatively large. Later, cork turns darker as cell production decelerates and cells become smaller. The winter period when no cork growth occurs is marked by a dark zone, that separates distinct annual rings. Both wood and cork rings are sensitive to climate, showing inter-annual fluctuations reflecting accumulated precipitation and temperature (Caritat et al. 1996, Nunes 1998, Ferreira et al. 2000). Precipitation during fall and winter and moderately low temperatures during the summer favor cork ring growth (Caritat et al. 2000). Little is known at the molecular level about the seasonal activity of cork cambium and its response to climatic factors, mainly drought and temperature.

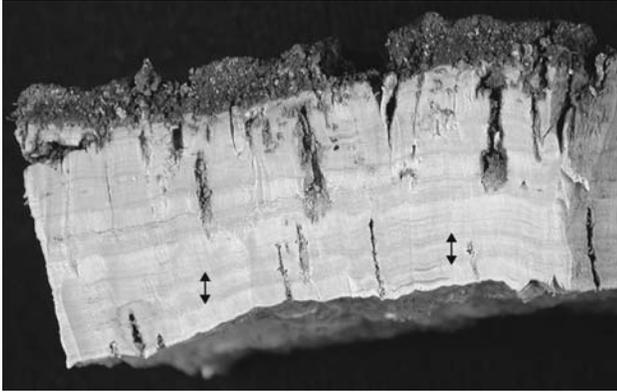The biochemical and molecular analyses of cork are espe-

Figure 1. Transverse section of a cork sample peeled from a cork oak tree in Romanyà de la Selva (Girona, NE Spain) in early summer 2007. Annual cork-rings are seen as light and dark zones. Arrows indicate the cork ring corresponding to the 2005 growing season.

cially difficult because cork cells are dead at maturity (Franke and Schreiber 2007). Therefore, most of what is known about the enzymatic reactions of the cork biosynthetic pathway has been deduced from cork chemical composition (Kolattukudy 1981, Graça and Santos 2007). Recently, we identified genes important for cork biosynthesis and differentiation in cork oak based on a two-step strategy (Soler et al. 2007). First, a set of expressed sequence tags (ESTs) preferentially induced in cork tissue was obtained by means of a suppression subtractive hybridization (SSH) library. Second, those genes likely associated with suberin and cork biosynthesis were confirmed by a cork-versus-wood microarray comparison. This approach yielded a substantial list of genes likely related to cork biosynthesis and regulation, consisting for the most part of enzymes involved in the biosynthesis of suberin and other cork chemical components, stress-related proteins and regulatory transcription factors identified in cork for the first time.

In the present study, we investigated the seasonal behavior of cork by real time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. A set of cork genes (Table 1) was analyzed during the growing season in mature cork oak trees growing in a typical cork oak forest in northeastern Spain. The genes were chosen according to their putative roles in cork tissue and the relative abundance of their transcripts in cork-forming tissues (Soler et al. 2007). The set of genes included representatives of the major biosynthetic pathways involved in cork biosynthesis: the acyl-lipid pathway, required for the synthesis of the aliphatic suberin domain (Kolattukudy 1981, Nawrath 2002); the phenylpropanoid pathway, needed for the synthesis of the aromatic suberin domain (Bernards 2002); the isoprenoid pathway, necessary for wax terpenes and sterols (Laule et al. 2003, Benveniste 2004); and the flavonoid pathway, necessary for tannins (Koes et al. 2005). The set of genes also included representatives of some stress genes and transcription factors. Correlations between transcript abundance and the main climatic variables (precipitation, temperature, relative humidity and solar radiation) were statistically analyzed.

Materials and methods

Samples and data collection

Cork samples were obtained from trees growing in a typical cork oak (*Quercus suber*) forest in Romanyà de la Selva, Girona, Spain (41°51′42.5″ N, 3°2′7.9″ E; UTM X = 502951; Y = 4634516.2) at a density of 1178 trees ha^{-1}. The cork oak trees had a maximum diameter of 38 cm and a mean diameter of 17.3 ± 5.4 cm. Mean tree height was 8.2 m. At this site, cork trees are periodically stripped at a mean height of 91 ± 17 cm.

Cork was harvested from the trunks of 15–20-year-old trees at breast height. Harvesting took place six times during the growing season of 2005 (April 26, May 14, June 17, July 19, August 11 and September 16). Four trees were used as replicates at each harvest. Different trees were used as replicates for cork harvesting at each sampling to avoid effects of wounding. The external bark (cork bark) of the tree was removed, and the exposed phellem tissue was harvested with sterile scalpels to obtain fractions rich in differentiating cork. The cork samples were immediately frozen in liquid nitrogen and stored at –80 °C.

Validated climatic data for the 2005 growing season were obtained from the nearby weather station of Castell d'Aro (UTM X = 502731; Y = 4628777), belonging to the Servei Meteorològic de Catalunya (SMC). Measurements included daily precipitation, temperature, relative humidity and solar radiation.

RNA extraction and cDNA synthesis

Total RNA was extracted from cork tissue by a modification of the method described by Chang et al. (1993). Five g of tissue was ground in liquid nitrogen and then mixed with extraction buffer (2% CTAB, 2% PVP, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl and 2.67% 2-mercaptoethanol). After a 10-min incubation at 65 °C, two chloroform:isoamyl alcohol 24:1 (v/v) extractions at room temperature were performed, followed by an overnight precipitation with 2 M LiCl at 4 °C. The precipitate was collected by centrifugation, and the dry pellet was resuspended in buffer containing 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA and incubated at 60 °C for 5 min. Then, one acid phenol:chloroform:isoamyl alcohol (125:24:1 (v/v)) and two 24:1 (v/v) chloroform:isoamyl alcohol extractions were performed at room temperature. The RNA in the upper phase was precipitated with 2 volumes of 100% ethanol and the precipitate collected by centrifugation. The pellet was washed twice with 70% (v/v) ethanol and resuspended in 50 μl of RNase-free water. RNeasy MinElute Cleanup (Qiagen) was used to remove any remaining protein fragments. To verify the absence of degradation, RNA was separated by electrophoresis on a formamide-formaldehyde denaturing agarose gel. The purity and quantity of RNA were checked with a Nanodrop spectrophotometer. For each sample, single-stranded cDNA was synthesized from 1 μg of total RNA with the QuantiTect Reverse Transcription kit (Qiagen), which includes one step to eliminate any contaminating genomic DNA in the total RNA sample. First-strand cDNA synthesis was carried out in a T-Gradient thermocycler (Biometra).

Table 1. List of the genes selected for study. Seven structural genes are representatives of the main metabolic pathways involved in cork biosynthesis (acyl-lipids, isoprenoids, phenylpropanoids and flavonoids), two genes encode regulatory transcription factors and three genes encode stress-related proteins.

Gene name and functional group	Description	EST GenBank Accession no.	Best TAIR TBLASTX	Similarity (%)	Primers used
Acyl-lipids					
FAT	Palmitoyl-acyl carrier protein thioesterase	EE743843	At1g08510	81	F: GCACCCCTTCATGCAATCTT R: GTTTTGTCTGGCCCAATCTCA
CYP86A1	Fatty acid omega-hydroxylase	EE743846	At5g58860	84	F: CCACGTGGACTTACAGGATTTG R: ACAGTGTTTCCGGGTCTTTACC
GPAT	Glycerol-3-phosphate acyltransferase	EE743865	At3g11430	96	F: TTGACTCGGAGAAAATCAAGCA R: GCTACCGGCACAATTCGATC
Isoprenoids					
bAS	Beta-amyrin synthase	EE743683	At1g78955	81	F: TGGCCCGATGTTTTTCATTC R: CCCAGCCACCATCTTCATTT
Phenylpropanoids					
F5H	Ferulate-5-hydroxylase	EE743847	At4g36220	72	F: CAAGTGTTTGGACTGAGCCAGAT R: CGGGATAAGCTCAAAGTCATGTC
HCBT	N-Hydroxycinnamoyl/ benzoyltransferase	EE743861	At5g41040	78	F: TGGCATTGGTGCTATGGAGTT R: GGGTTGCGGGCCTTAAGTA
Flavonoids					
ANR	Anthocyanidin reductase	EE743804	At1g61720	85	F: CCCAAAACCTCGGAAAGAAGA R: CGGCATAGCCTTTCTGAAGC
Regulatory proteins					
WRKY	WRKY transcription factor	EE743809	At2g46130	90	F: CGGCCTAGGTTTGCATTTCA R: TGCCCATACTTTCTCCATCGA
NAM	NAM transcription factor	EE743827	At3g18400	82	F: AACGCTGCCATCTCTTGAGTCT R: CCCACTTGATGAATTTGCAATG
Stress-related proteins					
HSP17.4	Heat shock protein 17.4	AJ000691	At5g59720	81	F: GCTACAGCACTGAAGCCCTCA R: AAATCCAATGGCGCTCAGTC
ANN	Annexin	EE743878	At1g35720	89	F: ATCCCCTCTGTGTCCGAAGACT R: GCATTTCTATGACCCAAGATGGAG
APX	Ascorbate peroxidase	EE743659	At3g09640	80	F: GGTCCAAAGCACACAAAAATCC R: GGCTGCTGTCTGGTGTCTAGTCT

Validation of cork housekeeping genes

To select a cork constitutive gene as a reference for the relative mRNA quantification, transcript abundances of four genes were measured. Their constitutive expression was validated with the BestKeeper excel-based tool (Pfaffl et al. 2004) and the following primers: actin (*ACT*), forward: GCCCCACGA-GCTGTGTTC, reverse: TCTGGCCCATTCCAACCA; tubulin (*TUB*), forward: 5′-AAGAACATGATGTGCGCTGCT-3′, reverse: 5′-TCCACCTCCTTGGTGCTCA-3′; polymerase elongation factor (*EF*), forward: 5′-TTGTGCCGTCCTCAT-TATTGACT-3′, reverse: 5′-TCACGGGTCTGACCATCCT-T-3′; and phytosulfokine receptor (*PSKR*), forward: 5′-GGG-CCTTGGAGATTGTTGTAAC-3′, reverse: 5′-TCGACAAG-GTGGACCAATCTTA-3′. All of these genes showed standard deviations of the threshold cycle (Ct) lower than one cycle and therefore could serve as valid references. The *ACT* and *TUB* genes showed the highest stability (lower standard deviations), but *TUB* showed the highest abundance (lower Ct; Figure 2) and therefore was selected as the reference gene for normalizing data.

Real time RT-PCR

Gene-specific primers were designed with Primer Express 2.0 (Applied Biosystems). Real time polymerase chain reactions were performed in an optical 96-well plate with an ABI PRISM 7300 Sequence Detector System (Applied Biosystems), using SYBR Green to monitor dscDNA synthesis. Reactions contained 1× Power SYBR Green Master Mix reagent (Applied Biosystems), 300 nM of gene-specific primer (except for the *WRKY* oligonucleotides, which were amplified with 50 nM of gene-specific primers) and 1 μl of a 100-fold dilution of the previously synthesized cDNA in a final volume of 20 μl. The following standard thermal profile was used for all PCRs: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation step was performed after amplification to confirm the presence of a single amplicon. To estimate variation in the technique, three technical replicates were carried out for each biological replicate. Data were analyzed with 7300 SDS 1.3.1 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were
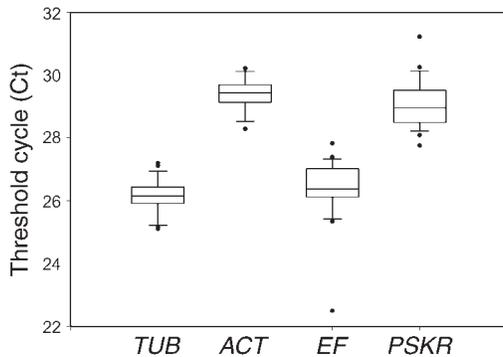
Figure 2. Box and whisker plot of the variation in validated house-keeping genes. Threshold cycle (Ct) variation in four housekeeping gene transcripts (tubulin, *TUB*; actin, *ACT*; elongation factor, *EF*; phytosulfokine receptor, *PSKR*) among samples is plotted. Boxes indicate the 25/75 percentiles, and the line inside each square indicates the median. Whisker caps indicate 1.5-fold the interquartile range (IQR), and outliers are indicated by dots. Among the housekeeping transcripts, *TUB* showed a low standard deviation and the highest transcript abundance (lowest Ct values) and therefore was chosen as the reference for real time RT-PCR.

collected between cycles 3 and 15. All amplification plots were analyzed with an R_n threshold of 0.2 to obtain Ct (threshold cycle) values. The amplification efficiency for each gene was calculated based on five dilutions of template ranging from 1 to 10^{-5} and the equation $E = 10^{-1/\text{slope}} - 1$. The mRNA abundances for each candidate gene were calculated as: Relative Transcript Abundance = $E^{\Delta\text{Ct(control)}}$(Target)/ $E^{\Delta\text{Ct(control)}}$(Reference) (Pfaffl 2001). A mix with equal amounts of each sample was used as the control to standardize data, and tubulin (*TUB*) was used as the reference to normalize data. The absence of genomic DNA contamination was checked using Non-Retrotranscriptase Controls (RT–) and the absence of environmental contamination using Non-Template Controls (NTC). Specific primer sequences are in Table 1.

Statistical analysis

Differences in transcript abundance among months were tested by one-way analysis of variance (ANOVA) (Sokal and Rohlf 1995). When significant results were found, a repeated contrast was performed to determine in which month there were changes; a repeated contrast compares only adjacent categories and is more powerful and less ambiguous than other conventional post-hoc tests, when the factor (such as month) is ordinal not categorical (Gaines and Rice 1990).

Principal components analysis (PCA) was applied to summarize the variation in the table of 12 variables (mRNA levels) and 24 samples (6 months × 4 trees). Principal components analysis is a multivariate technique of data reduction that analyzes the correlation matrix to compute a few independent factors that are linear combinations of the variables and that explain most of the variation in the data (Sokal and Rohlf 1995). The results are shown in bivariate diagrams corresponding to the main factors extracted. The usefulness of the PCA was assessed by Kaiser-Meyer-Olkin's measure of sampling ade-

quacy (KMO). The KMO ranges from 0 to 1 and should be well above 0.5 if variables are interdependent and a PCA is useful. The computed PCA sample scores were then correlated with meteorological data (maximum, minimum and mean temperature, relative humidity, solar radiation and precipitation) for further interpretation. We employed the values of meteorological variables just seven days before sampling, including the sampling day, because they showed higher correlations with mRNA levels.

Transcript abundance data were log transformed to improve the normality, homogeneity of variances, and linearity of the data in conformity with assumptions of conventional statistical methods.

Results

To determine cork seasonal activity, the relative abundances of mRNAs of a set of 12 cork candidate genes (Table 1) were analyzed by real time RT-PCR. Figure 3 shows the relative mRNA abundances of these genes during the growing season. All the genes except *GPAT* (one-way ANOVA, $n = 24$, $P = 0.368$) and *NAM* ($P = 0.057$) showed significant seasonal variation in transcript abundance ($P < 0.05$), which was strongest for *HSP17.4* and *F5H*. For the genes with significant seasonal variation, the repeated contrasts identified significant shifts ($P < 0.05$) in the following cases: *FAT*, April > May; *CYP86A1*, May < June; *F5H*, May > June > July = Aug < Sept; *HCBT*, May < June = July > Aug; *bAS*, April < May; *WRKY*, April < May = June > July; for *ANR*, April > May; *HSP17.4*, May < June = July > Aug; for *ANN*, April = May < June = July > Aug = Sept; for *APX*, April > May < June (none of the other month pairs was significantly different, $P > 0.05$). Therefore, all the variables with significant seasonal variations had significant changes in May or June, and a few also had significant changes in other months.

Many of the transcript abundance variables were interdependent (KMO = 0.67; Table 2) and the two first axes of the PCA explained 35.1 and 28.5%, respectively, of the variation. Figure 4A shows the PCA factor loadings for the 12 gene transcript profiles, and Figure 4B shows the scores of the six sampling days for the two first PCA components. The first PCA axis plots a group of genes (*HCBT*, *NAM*, *CYP86A1*, *WRKY*, *GPAT* and, to a lower degree, *bAS*) that were strongly and positively correlated (Figure 4A, Table 2). The factor scores of this axis only marginally varied with season (ANOVA, $F_{5,18} = 2.5$, $P = 0.069$) because, although they mostly separated the June samples from the rest, these factor scores also reflected inter-individual variation. Therefore, the transcript abundance of *HCBT*, *NAM*, *CYP86A1*, *WRKY*, *GPAT* and, to a lesser degree, *bAS* tended to be positively correlated and particularly high in June (Figure 4B). The second PCA axis highlighted two groups of two genes (*HSP17.4* and *ANN* on the one hand, and *F5H* and *FAT* on the other). The *HSP17.4* and *ANN* genes were positively correlated with each other and negatively correlated with *F5H* and *FAT* (Figure 4A, Table 2). The *HSP17.4* and *ANN* transcripts were most abundant in July, high in June and August and lower in April and May, whereas *F5H* and *FAT*
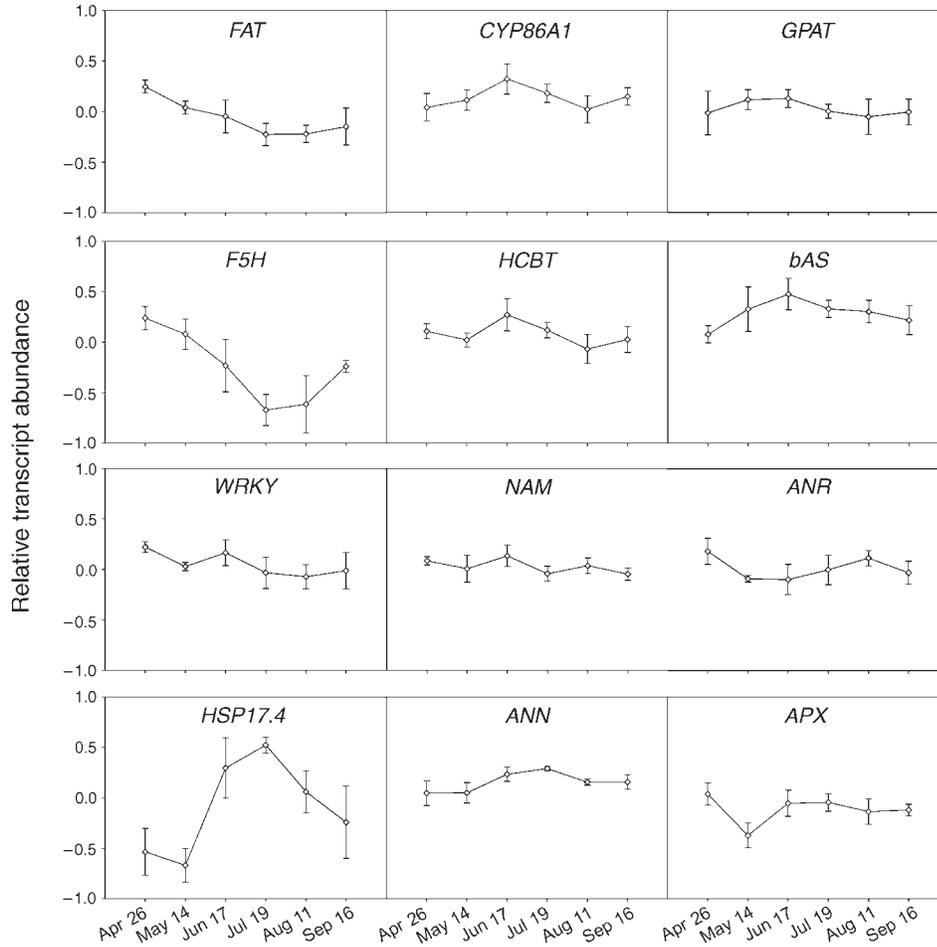
Figure 3. Transcript profiles of cork genes. Relative transcript accumulation (log-transformed) in mature trees growing in a representative cork oak forest (Romanyà de la Selva, Girona, NE Spain) sampled six times during the 2005 growing season. Values were calculated by the Pfaffl method (2001) with tubulin (*TUB*) as the reference gene.

showed the opposite pattern (Figure 4B). The second axis scores were significantly related to season ($F_{5,18} = 42.1$, $P < 0.0005$), significantly correlated to maximum, minimum and mean temperature variables ($r > 0.92$, $P < 0.0005$) and negatively correlated with relative humidity ($r > -0.672$, $P < 0.0005$), but unrelated to other meteorological descriptors (precipitation and solar radiation; $P > 0.1$). Conversely, *APX*

and especially *ANR* were placed in the center of the factorial plot (Figure 4A) because they were the least correlated variables, being higher in April, lower in May and quite constant in the other months.

In summary, most genes (*HCBT*, *NAM*, *CYP86A1*, *WRKY*, *GPAT* and, to a lesser degree, *bAS*) were positively correlated and displayed similar seasonal variation, with maxima in June.

Table 2. Correlation matrix among the relative transcript abundance values (log-transformed) of the genes studied ($n = 24$. *, $P < 0.05$; and **, $P < 0.01$).

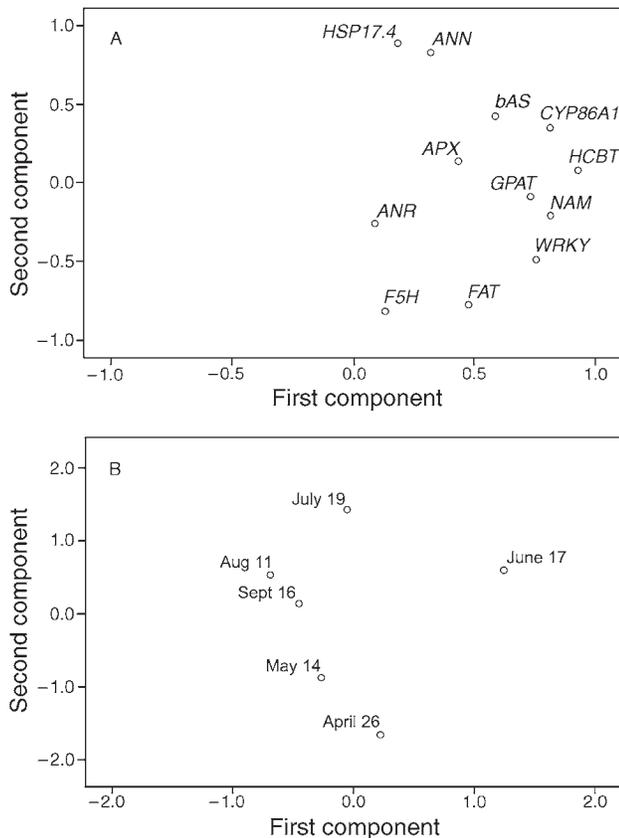|	ANN	ANR	APX	bAS	F5H	NAM	FAT	HCBT	CYP86A1	GPAT	HSP17.4
ANR	−0.01										
APX	0.44*	0.39									
bAS	0.44*	−0.32	−0.05								
F5H	−0.57**	−0.05	−0.04	−0.27							
NAM	0.06	0.24	0.35	0.49*	0.26						
FAT	−0.39	0.38	0.17	−0.09	0.62**	0.49*					
HCBT	0.34	0.02	0.41*	0.45*	0.04	0.61**	0.39				
CYP86A1	0.47*	−0.29	0.24	0.62**	−0.04	0.53**	0.03	0.81**			
GPAT	0.04	−0.13	−0.01	0.49*	0.18	0.49*	0.32	0.68**	0.70**		
HSP17.4	0.85**	0.02	0.35	0.32	−0.74**	−0.02	−0.52**	0.28	0.38	−0.10	
WRKY	−0.13	0.33	0.34	0.18	0.38	0.69**	0.79**	0.70**	0.34	0.43*	−0.21

Figure 4. Principal component analysis of the transcript abundances of the 12 genes analyzed on six dates during the 2005 growing season. (A) Factor loadings of the 12 genes for the two PCA axes. (B) Mean PCA scores of the sampling dates for the two axes extracted.

However, the seasonal variation was stronger for *HSP17.4* and *F5H*. Moreover, *HSP17.4* together with *ANN* on the one hand, and *F5H* together with *FAT* on the other hand, displayed opposite patterns, with minima in April and maxima in July for *HSP17.4* and *ANN* and vice versa for *F5H* and *FAT*.

Discussion

Real time RT-PCR proved a useful technique to investigate the molecular processes underlying cork ring formation. Data from the real time RT-PCR analysis of the set of genes putatively involved in cork biosynthesis and differentiation were consistent with sustained general activity of the cork cambium during the growing season (Costa et al. 2001). Small but significant variations were observed that accord with the maximum activity of cork cambium in late spring (June) (Natividade 1950) and with decreased cork growth during periods of high temperature or drought (Caritat et al. 2000).

Principal component analysis (PCA) detected common trends among the transcripts from the cork genes investigated. One group of genes was positively related to the first principal component. This group comprised most of the cork secondary metabolism genes (acyl-lipid pathway: *CYP86A1* and *GPAT*;

phenylpropanoid pathway: *HCBT*; isoprenoid pathway: *bAS*) together with two genes encoding two putative cork regulators, the *WRKY* and the *NAM* transcription factors. The first component was unrelated to climatic variables. All genes in this group show their greatest transcript abundance in June which is the month when cork oak trees are full of sap, leaves are renewed (Caritat et al. 2006), cork growth rate is maximal and cork harvesting starts in northeastern Spain.

The *CYP86A1* gene encodes a fatty acid omega-hydroxylase, an enzyme thought to provide fatty acid precursors for the suberin polyester (Franke and Schreiber 2007). The *Arabidopsis thaliana* L. ortholog to cork *CYP86A1* catalyzes the hydroxylation of fatty acids that are compatible with aliphatic suberin monomers (Benveniste et al. 1998) and shows specific expression in root (Duan and Schuler 2005), a tissue in which suberin is deposited. The *GPAT* gene encodes an acyl-CoA: glycerol-3-phosphate acyltransferase. The *Arabidopsis* ortholog of cork *GPAT* binds fatty acids to a glycerol-containing acceptor to generate the suberin lipid polyester network (Beisson et al. 2007). The *HCBT* gene encodes a hydroxycinnamoyl transferase, a key enzyme in the synthesis of the suberin aromatic (phenolic) monomers (Bernards 2002). *HCBT* was also the second highest up-regulated gene in cork tissue in the cork versus wood comparison (Soler et al. 2007). Moreover, for *HCBT Arabidopsis* ortholog, in silico expression analysis (Zimmermann et al. 2004, https://www.genevestigator.ethz.ch) showed a preferential expression in suberized tissues. The *bAS* enzyme catalyzes the cyclization of oxidosqualene into β-amyrin, the most common type of triterpene found in plants (Kushiro et al. 1998). Terpenes are major components of suberin-associated waxes in cork that make cork impervious to water (Conde et al. 1999). We studied two regulatory genes, *NAM* and *WRKY* transcription factors, which belong to large families with multiple functions in different tissues (Ooka et al. 2003, Ülker and Somssich 2004). The *Arabidopsis* ATTED-II database (Obayashi et al. 2007, http://www.atted.bio.titech.ac.jp) analysis showed high co-expression correlation between *NAM* and *WRKY* orthologs and between *NAM*, *HCBT* and *CYP86A1* orthologs. The co-regulation of these genes suggests that they are involved in related pathways.

The second PCA extracted component was related to the genes with strong seasonal variation in transcript abundance. Both *FAT* and *F5H* were strongly negatively correlated with this component, whereas *HSP17.4* and *ANN* were strongly positively correlated. The second component was influenced positively by temperature and negatively by relative humidity. Therefore, we conclude *F5H* and *FAT* are preferentially expressed in spring, when temperature is lower and humidity higher, whereas *HSP17.4* and *ANN* are preferentially expressed in summer, when temperature is higher and humidity lower.

The *FAT* and *F5H* genes encode enzymes with key functions in the pathways for acyl-lipids and phenylpropanoids, respectively. The *FAT* enzyme is likely involved in providing the basic saturated fatty acid monomers for the synthesis of aliphatic suberin. The *Arabidopsis* orthologous gene to cork *FAT* en-

codes an enzyme that regulates the amount of saturated fatty acids exported from plastids, acting as a major control point for saturated fatty acid fluxes (Bonaventure et al. 2003). The *F5H* enzyme catalyzes the hydroxylation of ferulic acid, coniferaldehyde and coniferyl alcohol leading to sinapic acid and syringyl lignin biosynthesis (Franke et al. 2000). It has been demonstrated that this enzyme regulates lignin monomeric composition, because *Arabidopsis* plants bearing a mutation in the *F5H* gene accumulate guaiacyl (G) lignin, whereas plants over-expressing *F5H* accumulate syringyl (S) lignin (Meyer et al. 1998). However, it is unknown whether the relative down-regulation of the *FAT* and *F5H* genes in cork, when temperature is high and humidity is low, induces a change in suberin monomeric composition.

Both *HSP17.4* and *ANN* are stress-related proteins that may play pivotal roles in abiotic stress tolerance. *HSP17.4* is a Class I small heat shock protein (sHsp) that accumulates in cork oak tissues in response to heat, water or oxidative stress (Puigderrajols et al. 2002). Moreover, cork *HSP17.4* (Jofre et al. 2003) and chestnut (*Castanea sativa* L.) *HSP17.5* (Soto et al. 1999) confer tolerance against heat stress. Seasonal studies in stems of chestnut (Lopez-Matas et al. 2004) and holm oak (Verdaguer et al. 2003) showed that Class I sHsps are induced in summer. The *ANN* gene encodes a plant annexin, a member of a multi-gene family of Ca^{2+}-dependent membrane binding proteins implicated in cellular responses to a variety of abiotic stresses (Gorecka et al. 2007) and to abscisic acid (Lee et al. 2004). The ortholog of the cork *ANN* gene showed a 39-fold induction in response to dehydration but increased only marginally under high temperature stress in *Arabidopsis* (Cantero et al. 2006). The seasonal patterns shown by *HSP17.4* and *ANN* are in accord with their protective roles against heat and drought. Their high seasonal fluctuations in response to climatic factors make these genes good candidates as markers of tree stress.

The transcript profiles of *ANR* and *APX* were less related to those of the other genes studied and showed a significant seasonal variation that did not match the two patterns just described. The *ANR* gene codes for an enzyme involved in the biosynthesis of condensed tannins (proanthocyanidins) because a mutation in an *ANR* orthologous gene is associated with a decrease of condensed tannins in *Arabidopsis* (Devic et al. 1999, Xie et al. 2003). Tannins are secondary compounds with antioxidant activity (Barbehenn et al. 2006, Kim et al. 2007) playing a role in defense against herbivores (Peters and Constabel 2002). They are abundant in cork (Silva et al. 2005), but the exact role of condensed tannins in cork tissue is unclear. The *APX* gene codes for a cytosolic ascorbate peroxidase, which is a key enzyme for scavenging hydrogen peroxide in plant cells (Smirnoff 2000, Davletova et al. 2005). Cork cells suffer high oxidative stress as a result of a peroxidase-catalyzed H_2O_2-mediated free radical coupling process during the assembly of suberin aromatic compounds (Razem and Bernards 2002, 2003).

References

Barbehenn, R.V., C.P. Jones, M. Karonen and J.P. Salminen. 2006. Tannin composition affects the oxidative activities of tree leaves. J. Chem. Ecol. 32:2235–2251.

Beisson, F., Y. Li, G. Bonaventure, M. Pollard and J.B. Ohlrogge. 2007. The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of *Arabidopsis*. Plant Cell 19: 351–368.

Benveniste, P. 2004. Biosynthesis and accumulation of sterols. Annu. Rev. Plant Biol. 55:429–457.

Benveniste, I., N. Tijet, F. Adas, G. Philipps, J.P. Salaun and F. Durst. 1998. CYP86A1 from *Arabidopsis thaliana* encodes a cytochrome P450-dependent fatty acid omega-hydroxylase. Biochem. Biophys. Res. Commun. 243:688–693.

Bernards, M.A. 2002. Demystifying suberin. Can. J. Bot. 80: 227–240.

Bonaventure, G., J.J. Salas, M.R. Pollard and J.B. Ohlrogge. 2003. Disruption of the FATB gene in *Arabidopsis* demonstrates an essential role of saturated fatty acids in plant growth. Plant Cell 15:1020–1033.

Cantero, A., S. Barthakur, T.J. Bushart, S. Chou, R.O. Morgan, M.P. Fernandez, G.B. Clark and S.J. Roux. 2006. Expression profiling of the *Arabidopsis* annexin gene family during germination, de-etiolation and abiotic stress. Plant Physiol. Biochem. 44:13–24.

Caritat, A., M. Molinas and E. Gutierrez. 1996. Annual cork-ring width variability of *Quercus suber* L. in relation to temperature and precipitation (Extremadura, southwestern Spain). For. Ecol. Manage. 86:113–120.

Caritat, A., E. Gutierrez and M. Molinas. 2000. Influence of weather on cork-ring width. Tree Physiol. 20:893–900.

Caritat, A., E. García-Berthou, R. Lapeña and L. Vilar. 2006. Litter production in a *Quercus suber* forest of Montseny (NE Spain) and its relationship to meteorological conditions. Ann. For. Sci. 63: 791–800.

Chang, S., J. Puryear and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11:113–116.

Conde, E., M.C. Garcia-Vallejo and E. Cadahia. 1999. Waxes composition of *Quercus suber* reproduction cork from different Spanish provenances. Wood Sci. Technol. 33:271–283.

Costa, A., H. Pereira and A. Oliveira. 2001. A dendroclimatological approach to diameter growth in adult cork-oak trees under production. Trees 15:438–443.

Davletova, S., L. Rizhsky, H. Liang, Z. Shengqiang, D.J. Oliver, J. Coutu, V. Shulaev, K. Schlauch and R. Mittler. 2005. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell 17:268–281.

Devic, M., J. Guilleminot, I. Debeaujon, N. Bechtold, E. Bensaude, M. Koornneef, G. Pelletier and M. Delseny. 1999. The BANYULS gene encodes a DFR-like protein and is a marker of early seed coat development. Plant J. 19:387–398.

Duan, H. and M.A. Schuler. 2005. Differential expression and evolution of the *Arabidopsis* CYP86A subfamily. Plant Physiol. 137: 1067–1081.

Ferreira, A., F. Lopes and H. Pereira. 2000. Characterization of cork growth and quality in one region of production. Ann. For. Sci. 57:187–193.

Fialho, C., F. Lopes and H. Pereira. 2001. The effect of cork removal on the radial growth and phenology of young cork oak trees. Forest Ecol. Manage. 141:251–258.

Franke, R. and L. Schreiber. 2007. Suberin—a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. 10:252–259.

Franke, R., C.M. McMichael, K. Meyer, A.M. Shirley, J.C. Cusumano and C. Chapple. 2000. Modified lignin in tobacco and poplar plants over-expressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. Plant J. 22:223–234.

Gaines, S.D. and W.R. Rice. 1990. Analysis of biological data with ordered expectations. Am. Nat. 135:310–317.

Gorecka, K.M., C. Thouverey, R. Buchet and S. Pikula. 2007. Potential role of annexin AnnAt1 from *Arabidopsis thaliana* in pH-mediated cellular response to environmental stimuli. Plant Cell Physiol. 48:792–803.

Graça, J. and S. Santos. 2007. Suberin: a biopolyester of plants' skin. Macromol. Biosci. 7:128–135.

Jofre, A., M. Molinas and M. Pla. 2003. A 10-kDa class-CI sHsp protects *E. coli* from oxidative and high-temperature stress. Planta 217:813–819.

Kim, M.-J., J.-N. Hyun, J.-A. Kim, J.-C. Park, M.-Y. Kim, J.-G. Kim, S.-J. Lee, S.-C. Chun and I.-M. Chung. 2007. Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. J. Agric. Food Chem. 55: 4802–4809.

Koes, R., W. Verweij and F. Quattrocchio. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10:236–242.

Kolattukudy, P. 1981. Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. 32:539–567.

Kushiro, T., M. Shibuya and Y. Ebizuka. 1998. Beta-amyrin synthase—cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. Eur. J. Biochem. 256:238–244.

Laule, O., A. Furholz, H.S. Chang, T. Zhu, X. Wang, P.B. Heifetz, W. Gruissem and M. Lange. 2003. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 100:6866–6871.

Lavorel, S., J. Canadell, S. Rambal and J. Terradas. 1998. Mediterranean terrestrial ecosystems: research priorities on global change effects. Glob. Ecol. Biogeogr. Lett. 7:157–166.

Lee, S., E.J. Lee, E.J. Yang, J.E. Lee, A.R. Park, W.H. Song and O.K. Park. 2004. Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in *Arabidopsis*. Plant Cell 16:1378–1391.

Lopez-Matas, M.A., P. Nunez, A. Soto, I. Allona, R. Casado, C. Collada, M.A. Guevara, C. Aragoncillo and L. Gomez. 2004. Protein cryoprotective activity of a cytosolic small heat shock protein that accumulates constitutively in chestnut stems and is up-regulated by low and high temperatures. Plant Physiol. 134:1708–1717.

Meyer, K., A.M. Shirley, J.C. Cusumano, D.A. Bell-Lelong and C. Chapple. 1998. Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 95:6619–6623.

Molinas, M. and D. Verdaguer. 1993*a*. Lignotuber ontogeny in the cork-oak (*Quercus suber*; Fagaceae). I. Late embryo. Am. J. Bot. 80:172–181.

Molinas, M. and D. Verdaguer. 1993*b*. Lignotuber ontogeny in the cork-oak (*Quercus suber*; Fagaceae). II. Germination and young seedling. Am. J. Bot. 80:182–191.

Natividade, J.V. 1950. Subericultura. Ministério da Economia. Direcção Geral dos Serviços Florestais e Aquícolas, Lisboa, 387 p.

Nawrath, C. 2002. The biopolymers cutin and suberin. *In* The *Arabidopsis* Book. Eds. C.R. Somerville and E.M. Meyerowitz. Am. Soc. Plant Biol., Rockville, MD, pp 1–14.

Nunes, E. 1998. Estudo da influência da precipitação e temperatura no crescimento juvenil de *Quercus suber* L. através dos anéis anuais de crescimento. Ms.D. Thesis, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisboa, 109 p.

Obayashi, T., K. Kinoshita, K. Nakai, M. Shibaoka, S. Hayashi, M. Saeki, D. Shibata, K. Saito and H. Ohta. 2007. ATTED-II: a database of co-expressed genes and cis elements for identifying co-regulated gene groups in *Arabidopsis*. Nucleic Acids Res. 35: D863–D869.

Oliveira, G., O. Correia, M.A. Martins-Loução and F.M. Catarino. 1994. Phenological and growth patterns of the Mediterranean oak *Quercus suber* L. Trees 9:41–46.

Ooka, H., K. Satoh, K. Doi et al. 2003. Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Res. 10:239–247.

Peters, D.J. and C.P. Constabel. 2002. Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J. 32:701–712.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:2001–2007.

Pfaffl, M.W., A. Tichopad, C. Prgomet and T.P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26:509–515.

Puigderrajols, P., A. Jofre, G. Mir, M. Pla, D. Verdaguer, G. Huguet and M. Molinas. 2002. Developmentally and stress-induced small heat shock proteins in cork oak somatic embryos. J. Exp. Bot. 53:1445–1452.

Razem, F.A. and M.A. Bernards. 2002. Hydrogen peroxide is required for poly(phenolic) domain formation during wound-induced suberization. J. Agric. Food. Chem. 50:1009–1015.

Razem, F.A. and M.A. Bernards. 2003. Reactive oxygen species production in association with suberization: evidence for an NADPH-dependent oxidase. J. Exp. Bot. 54:935–941.

Silva, S.P., M.A. Sabino, E.M. Fernandes, V.M. Correlo, L.F. Boesel and R.L. Reis. 2005. Cork: properties, capabilities and applications. Int. Mater. Rev. 50:345–365.

Smirnoff, N. 2000. Ascorbate biosynthesis and function in photoprotection. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355: 1455–1464.

Sokal, R.R. and F.J. Rohlf. 1995. Biometry: the principles and practice of statistics in biological research. W.H. Freeman and Co., New York, 887 p.

Soler, M., O. Serra, M. Molinas, G. Huguet, S. Fluch and M. Figueras. 2007. A genomic approach to suberin biosynthesis and cork differentiation. Plant Physiol. 144:419–431.

Soto, A., I. Allona, C. Collada, M.A. Guevara, R. Casado, E. Rodriguez-Cerezo, C. Aragoncillo and L. Gomez. 1999. Heterologous expression of a plant small heat-shock protein enhances *Escherichia coli* viability under heat and cold stress. Plant Physiol. 120:521–528.

Tenhunen, J.D., O.L. Lange, J. Gebel, W. Beyschlag and J.A. Weber. 1984. Changes in photosynthetic capacity, carboxylation efficiency, and CO_2 compensation point associated with midday stomatal closure and midday depression of net CO_2 exchange of leaves of *Quercus suber*. Planta 162:193–203.

Ülker, B. and I.E. Somssich. 2004. WRKY transcription factors: from DNA binding towards biological function. Curr. Opin. Plant Biol. 7:491–498.

Verdaguer, D., X. Aranda, A. Jofré, M. El Omari, M. Molinas and I. Fleck. 2003. Expression of low molecular weight heat-shock proteins and total antioxidant activity in the Mediterranean tree *Quercus ilex* L. in relation to seasonal and diurnal changes in physiological parameters. Plant Cell Environ. 26:1407–1417.

Xie, D.Y., S.B. Sharma, N.L. Paiva, D. Ferreira and R.A. Dixon. 2003. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. Science 299:396–399.

Zimmermann, P., M. Hirsch-Hoffmann, L. Hennig and W. Gruissem. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. Plant Physiol. 136:2621–2632.