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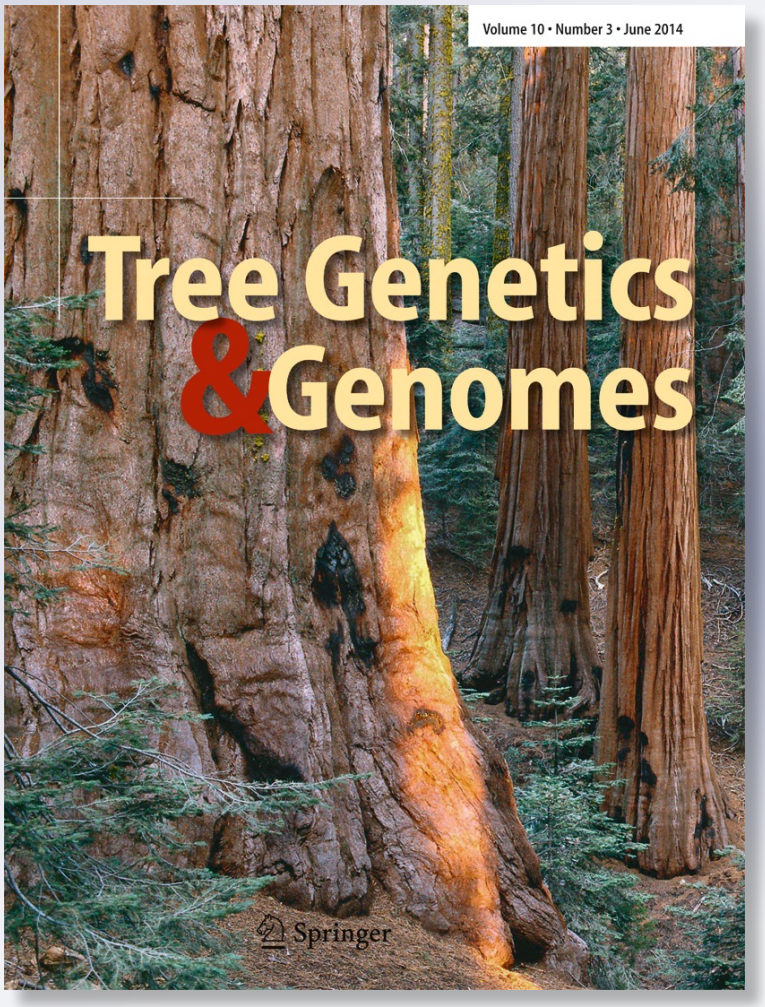

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ORIGINAL PAPER

miRNA profiling in leaf and cork tissues of *Quercus suber* reveals novel miRNAs and tissue-specific expression patterns

Inês Chaves · Yao-Cheng Lin · C. Pinto-Ricardo · Yves Van de Peer · Célia Miguel

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Abstract The differentiation of cork (phellem) cells from the phellogen (cork cambium) is a secondary growth process observed in the cork oak tree conferring a unique ability to produce a thick layer of cork. At present, the molecular regulators of phellem differentiation are unknown. The previously documented involvement of microRNAs (miRNAs) in the regulation of developmental processes, including secondary growth, motivated the search for these regulators in cork oak tissues. We performed deep sequencing of the small RNA fraction obtained from cork oak leaves and differentiating phellem. RNA sequences with lengths of 19–25 nt derived from the two libraries were analysed, leading to the identification of 41 families of conserved miRNAs, of which the most abundant were miR167, miR165/166, miR396 and miR159. Thirty novel miRNA candidates were also unveiled, 11 of which were unique to leaves and 13 to phellem. Northern blot detection of a set of conserved and novel miRNAs confirmed their differential expression profile. Prediction and analysis of putative miRNA target genes provided clues regarding processes taking place in leaf and phellem tissues, but further experimental work will be needed for functional characterization. In conclusion, we here provide a first characterization of the miRNA population in a Fagacea species, and the comparative analysis of miRNA expression in leaf and phellem libraries represents an important step to uncovering specific regulatory networks controlling phellem differentiation.

Keywords Small RNA · miRNA · *Quercus suber* · Phellem · Tree · Secondary growth

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I. Chaves · C. Miguel (✉)
Instituto de Biologia Experimental e Tecnológica (iBET), Apartado 12, 2780-901 Oeiras, Lisbon, Portugal
e-mail: cmiguel@itqb.unl.pt

I. Chaves · C. Pinto-Ricardo · C. Miguel
Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Lisbon, Portugal

Y.-C. Lin · Y. Van de Peer
Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Ghent, Belgium

Y.-C. Lin · Y. Van de Peer
Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

Y. Van de Peer
Genomics Research Institute (GRI), University of Pretoria, Private bag X20, Pretoria 0028, South Africa

Introduction

Cork oak is a long-lived evergreen tree endemic to the western Mediterranean, where it is found both in native forests and in the human-shaped ecosystem of high socio-economic and ecological value, known as 'montado' (Bugalho et al. 2011; Varela 1995). The economic value of the cork oak tree comes from cork, the out-bark of cork oak, which the tree continuously produces and is removed from the tree every 9 years. Due to properties such as elasticity, thermal insulation and impermeability, conferred by its characteristic chemical composition (Pereira 2007), cork has several commercial applications of which cork stoppers are probably best known. Most importantly, the cork layer allows the tree to withstand adverse environmental conditions and confers protection against the periodic fires that are common throughout the Mediterranean (Pausas 1997). This rare feature among plant species results from the secondary growth sustained by the activity of cork cambium or phellogen, the lateral meristem that envelopes the

tree trunk, giving rise each year to a new cork ring that is not shed naturally. Through periclinal divisions, the pluripotent cork cambium gives rise to daughter cells that differentiate either as phelloderm (inwards) or phellem (outwards). Differentiation of phellem or cork cells involves the deposition of a high amount of suberin in the cell walls (Pereira 1988) culminating in cell death.

The first genomic approach to suberin biosynthesis and cork differentiation used a suppression subtractive hybridization (SSH) strategy (Soler et al. 2007). In that work, a list of candidate genes isolated from cork tissues was identified including genes for the synthesis, transport and polymerization of suberin monomers as well as a number of regulatory genes including members of the NO APICAL MERISTEM (NAM), MYB and WRKY transcription factor families with putative functions in meristem identity and cork differentiation. Since then, a number of studies have been published characterizing the role of a few selected candidate orthologues in potato tuber suberin formation by reverse genetics approaches (Serra et al. 2009a, b, 2010a, b) and identifying additional candidates in potato tuber periderm (Barel and Ginzberg 2008; Chaves et al. 2009; Ginzberg et al. 2009; Soler et al. 2011). Despite the remarkable ability of cork oak to produce cork, potato has been the preferred model to study these processes since it is more amenable to in vitro manipulation and transformation having, in addition, its genome fully sequenced (Xu et al. 2011). However, it is becoming increasingly evident that species-specific regulators are crucial in the regulation of a wide range of processes including developmental processes (Osorio et al. 2012; Sunkar et al. 2007).

MicroRNAs (miRNAs) represent an important class of plant small RNAs regulating gene expression at the post-transcriptional level during growth and development (Bartel 2004) and in response to abiotic stress and pathogens (Liu and Vance 2010; Sunkar 2010). These non-coding RNAs usually range in size from 20 to 24 nucleotides and negatively regulate gene expression by degrading target mRNAs or inhibiting their translation. There are a number of plant miRNAs that are highly conserved between species and play crucial roles in the regulation of large transcriptional networks, but the majority are family or species specific (Cuperus et al. 2011). In the latest version of the miRBase (release 19, http://www.mirbase.org) with data from 67 plant species (15 eudicotyledoneous families), no miRNAs are present for *Quercus* species or other members in the Fagaceae family. Because cork oak is probably the species where the most extensive process of phellem differentiation is observed, it represents an attractive system to address the miRNA-mediated regulatory processes controlling phellem differentiation.

Although the similarities between the processes that occur as a result of the activity of vascular cambium and cork cambium suggest that conserved regulatory mechanisms may exist, there is much less knowledge about the molecular regulation of phellem differentiation than xylem differentiation, which has been the subject of intensive research due to its role in wood formation. During xylem differentiation, a few miRNAs are implicated in the process. By comparing miRNAs from developing xylem in *Populus trichocarpa* stems and in *Arabidopsis*, Lu et al. (2005) found that conserved miRNAs exhibited species-specific developmental expression patterns. In that work, Lu et al. further suggest that even conserved miRNAs may have different regulatory roles in different species and possible roles in tree-specific processes (Lu et al. 2005) such as secondary cell wall synthesis and deposition in developing xylem cells. A microarray analysis comparing primary and secondary xylem growth in poplar revealed a dominant expression of miR164, miR162a and miR168 in the secondary development zone (Dharmawardhana et al. 2010), reinforcing the role of miRNAs during secondary growth. In a recent study using *Acacia mangium* as lignification model system, the conserved miR166 was shown to be strongly down-regulated in xylem when compared to phloem, suggesting its potential role in xylem development by indirect regulation of the genes involved in lignin biosynthesis (Ong and Wickneswari 2012). Transgenic *Populus* expressing a miR166-resistant target gene have developed phenotypic abnormalities affecting the primary and secondary growth, including the abnormal formation of cambia within cortical parenchyma that can produce secondary vascular tissues in reverse polarity (Robischon et al. 2011). More recently, Puzey et al. (2012) identified several xylem-enriched miRNAs predicted to target genes known to be important in secondary growth, including xyloglucan endotransglycosylase/hydrolase and vascular-related transcription factors (Puzey et al. 2012).

In this work, we aimed to identify novel and conserved miRNAs in cork oak and look for leaf and cork tissue-specific patterns of miRNA expression. Since no whole-genome sequence data is available for cork oak yet, we have conducted deep sequencing of small RNA libraries and used available EST data for miRNA profiling and prediction of miRNA target genes in the sampled tissues. To our knowledge, this represents the first report on small RNA analysis in *Quercus* and Fagaceae species and provides novel resources to address the molecular regulation of phellem differentiation.

Material and methods

Small RNA isolation and sequencing

The first fully expanded leaves were collected from 2-year-old plants. The phellem tissues were harvested from adult cork oak trees by scraping the inner surface of the removed cork plank with a scalpel. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

A protocol allowing the isolation of good quality low molecular weight RNA from the leaves and phellem was established based on the CTAB method described by Chang et al. (1993), with the following modifications: 0.5 ml of pre-warmed (65 °C) extraction buffer (300 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 2 % CTAB, 2 % PVP10, 0.05 % spermidine trihydrochloride, 2 % β-mercaptoethanol) was added to 100 mg ground tissue in 1.5 ml tube, followed by incubation at 65 °C for 10 min. Samples were extracted twice with 1 ml of chloroform/isoamyl alcohol (24:1) and centrifuged at 15,000×*g* for 10 min at 4 °C; 0.1 vol 3 M NaOAc (pH 5.2) and 1 vol isopropanol were added to the supernatant and the tubes were kept at −80 °C for 3 h. Nucleic acids were recovered by centrifugation at 15,000×*g* (30 min at 4 °C); the nucleic acid pellet was resuspended in 375 μl TE (pH 7.5) and the high molecular weight (HMW) RNA was precipitated overnight at 4 °C with 140 μl of 8 M LiCl. After 30 min of centrifugation at 15,000×*g* at 4 °C, the pellet for HMW RNA recovery can be kept if needed and the low molecular weight (LMW) RNA fraction is precipitated from the supernatant with 1 vol isopropanol and 0.1 % SDS at −80 °C for 3 h. After centrifugation at 15,000×*g*, for 30 min at 4 °C, the pellet was washed with ice-cold ethanol (1 ml), resuspended in 10 μl RNAse-free water and treated with DNAse Turbo (Ambion® TURBO™ DNase, Invitrogen-Life Technologies).

RNA quality and yield was checked in all samples by measuring the UV absorbance (*A*) at 230, 260 and 280 nm using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA). The RNA integrity of both the HMW and the LMW RNA fractions was also evaluated on the Agilent 2100 Bioanalyzer. The purified LMW RNA fraction was prepared for sequencing by Illumina HiSeq 2000 (Fasteris, Switzerland). The small RNA sequences obtained for leaf and cork are deposited in the NCBI GenBank database under the accession numbers SRR988108 and SRR988109, respectively.

Small RNA sequence processing

After removal of the sequencing adapter, reads were further processed using the UEA sRNA toolkit (Moxon et al. 2008b). 'Filter' function and only read length ranging from 15 to 25 bp with no match with the plant rRNA/tRNA were kept for further analysis. We searched for the conserved mature miRNAs in the miRBase database release 18 by miRProf allowing two mismatches. The identified conserved miRNA were further grouped by match signature and combined by organism name and the miRNA family members. The number of reads of the conserved miRNAs was normalised with the total number of sequenced reads in each library.

Due to the lack of a reference cork oak genome, we collected available genomic and transcriptome sequence information to identify the miRNA precursors. To identify novel miRNA, we combined the Oak Gene Index EST (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=oak), Oak EST from NCBI (http://www.ncbi.nlm.nih.gov/dbEST/index.htm), Oak Genome Survey Sequence (GSS, http://www.ncbi.nlm.nih.gov/projects/dbGSS/), an in-house cork oak transcript database including three EST leaf libraries (http://www.corkoakdb.org/) (Pereira-Leal et al., submitted) and two phellem libraries from SRA (NCBI accessions SRX022024 and SRX022025) subsequently assembled by Newbler, as a customised enlarged Oak reference database (CEOR DB), and *P. trichocarpa* genome. We applied the miRCat tool in the UEA package to identify the novel miRNAs with the following settings: minimum free energy (MFE), −10; maximum number of genome hits, −5; maximum distance between consecutive hits on the genome, −100; minimum length of the miRNA, 19; and maximum length of the miRNA, 24. Overview of the analysis workflow is summarized in Fig. 1.

Northern blot hybridization

For experimental validation of conserved and non-conserved miRNAs, northern blot hybridization was performed using LMW RNA from cork oak phellem and leaves. LMW RNA-enriched samples (1 μg) were separated in a 17 % acrylamide gel containing 8 M urea in TBE buffer. Gels were run at 180 V in TBE buffer and RNA was subsequently transferred to Hybond N^+ membrane (Amersham Biosciences) in TBE buffer at 300 mA for 1 h (mini-protean® 3 Bio-Rad). After UV crosslinking, the membrane was pre-hybridized in 10 ml of PerfectHyb™ (Sigma) hybridization solution for 1 h at 42 °C. Afterwards, each specifically designed miRNA probe was generated using DIG labeling (DIG oligonucleotide 3-end labeling kit, second generation, Roche) following the instructions of the manufacturer. Five picomoles of the probe was added and the membrane was hybridized overnight at 42 °C. The membrane was rinsed twice in 4×SSC for 20 min followed by 2×SSC/0.1 % SDS for 15 min and incubated for 30 min in blocking solution (1 g blocking reagent (Roche) in 0.1 M maleic acid buffer pH 7.5), followed by 1 h in antibody solution (anti-digoxigenin-AP, Roche). After four washes in washing buffer (0.1 M maleic acid buffer, 0.3 % Tween 20, pH 7.5) for 15 min, the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), then incubated in CDP-Star (Ambion) working solution (1:100 dilution in detection buffer) and finally exposed to X-ray film. U6 DNA probe was used as sample loading control. The X-ray film images were acquired using the ChemiDoc™ XRS+ System, and the intensity of the bands detected in the northern blot was measured using the Image Lab™ Software.

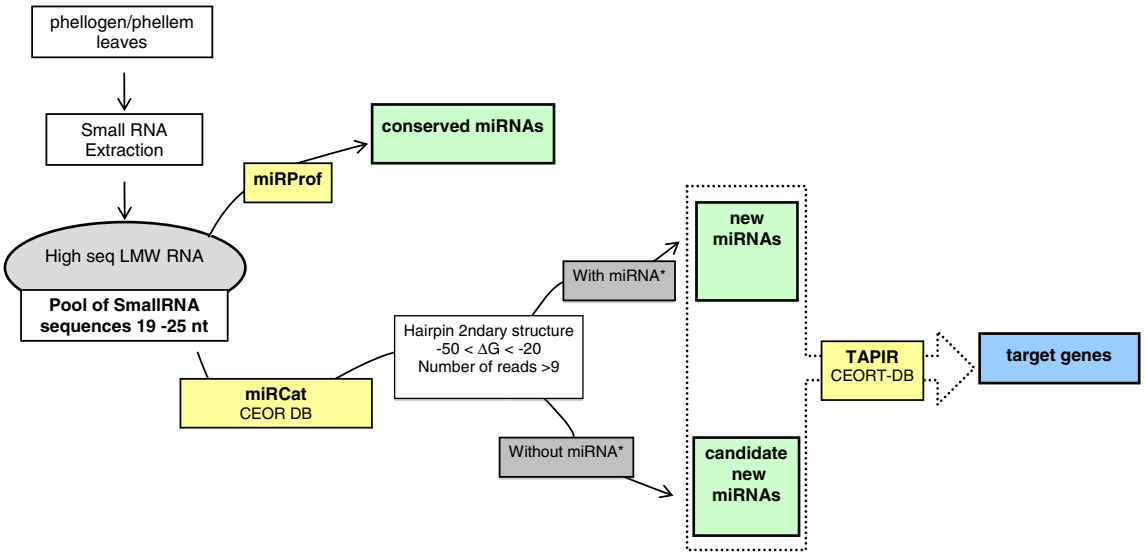


Fig. 1 Workflow of the bioinformatics analysis of small RNAs from cork oak leaves and phellem tissues

Prediction of miRNA target genes

Target prediction was performed for all predicted non-conserved miRNAs. An in-house cork oak transcript database containing information from cork oak (CEORT DB) was used. Due to the lack of directional information in the Cork Oak EST database and since the prediction algorithm only searches the target site on the forward strand, the transcript database was constructed to contain both the forward and the reverse complement sequences. The target gene search was performed using the TAPIR software (Bonnet et al. 2010) with the following settings: TAPIR hybrid (combining FASTA and RNAhybrid); maximum internal loop size, 5; and number of hits per target, 10.

Results

Small RNA isolation and deep sequencing

To uncover regulatory mechanisms involving small RNA pathways that are specifically functioning in phellem differentiation, we have sequenced the small RNA transcriptome of cork oak developing phellem and leaves and performed expression analysis of the miRNAs present in both tissues.

To this end, we have used a modified CTAB extraction method which is effective for tissues with high polyphenol content such as cork oak phellem. The buffer described in this method stabilizes the polyphenols allowing the efficient isolation of total RNA. To increase the effectiveness of the extraction and recovery of high quality low molecular weight (LMW) RNA, we performed additional precipitation steps adapted from Zhu et al. (2008). The $A(260)$ / $A(230)$ and $A(260)$ / $A(280)$ RNA absorbance ratios of the samples varied from 1.7 to 2.2 and 1.9 to 2.1, respectively, which reveals the absence of polysaccharide and protein contaminants. Additionally, the obtained yield was in the range of 10–15 μg high molecular weight (HMW) RNA and 1–1.5 μg LMW RNA per 100 mg of tissue. The electropherograms obtained for the HMW RNA fraction from phellem tissues showed a profile with a RNA integrity number (RIN) above 8 which is indicative of high quality RNA with no degradation. The electropherograms of the LMW RNA fraction obtained from the same tissue samples showed peaks corresponding to expected LMW RNA species (small ribosomal RNA (rRNA), transfer RNA (tRNA) and miRNAs) (Supplementary material—SM1_Electropherogram of RNA fractions).

A total of 19,620,275 and 12,731,020 sequence reads were obtained from the leaves and phellem of cork oak, respectively (Table 1). After removing reads shorter than 19 nt and longer than 25 nt and with an abundance of 3 or lower, 85.1 % (16,692,453) and 68.9 % (8,775,468) of the reads from the leaves and phellem, respectively, remained for subsequent analysis.

The distribution by sequence length of the reads between 19 and 25 nt showed that the 24-nt length sequences were

Table 1 General statistics for cork oak leaf and phellem small RNA sequence reads processing

Sample type	Leaves	Phellem
Raw reads	19,620,275	12,731,020
Reads with 19<sequence length<25 nt	16,684,977	8,771,865
Reads with abundance>= 3	8,988,329	4,352,306
After filtering rRNA/tRNA	8,820,356	3,426,924
Non-redundant reads	869,583 (4.4 %)	298,116 (2.3 %)

expressed at higher levels followed by the 21-nt length (Fig. 2). When considering the total number of small RNA reads, the 21-nt class was as abundant in the leaves (12.8 %; 2,519,032 reads) as in the phellem (12.7 %; 1,616,326 reads), but interestingly, the 24-nt class was approximately twice more expressed in the leaves (60 %; 11,766,735 reads) than in the phellem (36.8 %; 4,687,046 reads) (Fig. 2a).

The other classes of small RNAs such as those derived from rRNA and tRNA identified in the libraries were afterwards discarded leading to a decrease of 2 % of the reads in the leaves and 21 % in the phellem (Table 1). The filtering of the rRNA/tRNA had a significant effect on the size class profile (Fig. 2b). While the 21-nt class remained similar in both tissues, decreasing approximately 1 % in the leaves (from 12.8 to 11.3 %) and 4 % in the phellem (from 12.7 to 8.7 %), the number of reads in the 24-nt class decreased more than twofold in the leaves (from 60.0 % to 28.0 %) and over threefold in the phellem (from 36.8 to 10.6 %). The removal of rRNA/tRNA resulted in almost identical levels of the 21-nt and the 24-nt size classes in the phellem, but in the leaves, the 24-nt size class was about twice more expressed. However, when comparing the levels of the non-redundant sequences according to size (Fig. 2c), the 24-nt size class was much more diverse in both tissues, with the maximum number of non-redundant reads being detected in the leaves. The level of non-redundant reads was very low in the other size classes evidencing the fact that the 21-nt class is much less diverse that the 24-nt class in the analysed cork oak tissues.

Conserved miRNAs and expression profile

Although a diverse set of miRNAs has been already identified in several plant species, miRNAs from *Quercus* species have been up to now absent from the miRBase repository. Many miRNA families are greatly conserved among plant species allowing their identification through BLAST searches using the annotated miRNA deposited on miRBase (Griffiths-Jones 2006). After filtering the reads according to size, abundance and rRNA/tRNA, as described above, the conserved miRNAs in the leaves and phellem of cork oak were identified by BLAST searches of the small RNA reads against miRBase release 18, which includes miRNAs from 52 plant species. The search for the conserved mature miRNAs led to the identification of members of 34 and 35 conserved miRNAs in the cork oak leaves and phellem, respectively, out of a total of 41 miRNA families (Table 2). It was also possible to identify precursor sequences for 22 conserved miRNA from the leaves and 21 from the phellem (Supplementary material—SM2_Leaf_miRCat_results.xls and SM3_cork-tissues_miRCat_results.xls).

The most highly expressed families in both tissues were miR167, miR165/166, miR396 and miR159. Although high similarity was observed regarding the conserved families of miRNA present in the leaves and phellem, their expression level was, in some cases, quite different. The main difference was found for miR164 which was approximately 108 times more expressed in the leaves than in the phellem, followed by miR395 (85 times), mir156/157 (80 times) and miR399 (59 times). The miRNAs whose expression was more different between tissues had a maximum expression level below 500 normalised weighted counts. The differences between tissues were smaller for the miRNA families that presented higher expression in the phellem, which was the case of miR390 and miR168, and were 49 and 15 times more expressed in the phellem than in the leaves, respectively.

The analysis of our data revealed that six miRNA families were leaf specific (miR163, miR2089, miR2950, miR477, miR529 and miR394), while seven miRNA families were phellem specific (miR1140, miR158, miR1863, miR2916, miR479, miR5083 and miR530). It should be noted that the miRNAs with a tissue-specific pattern generally showed low expression levels.

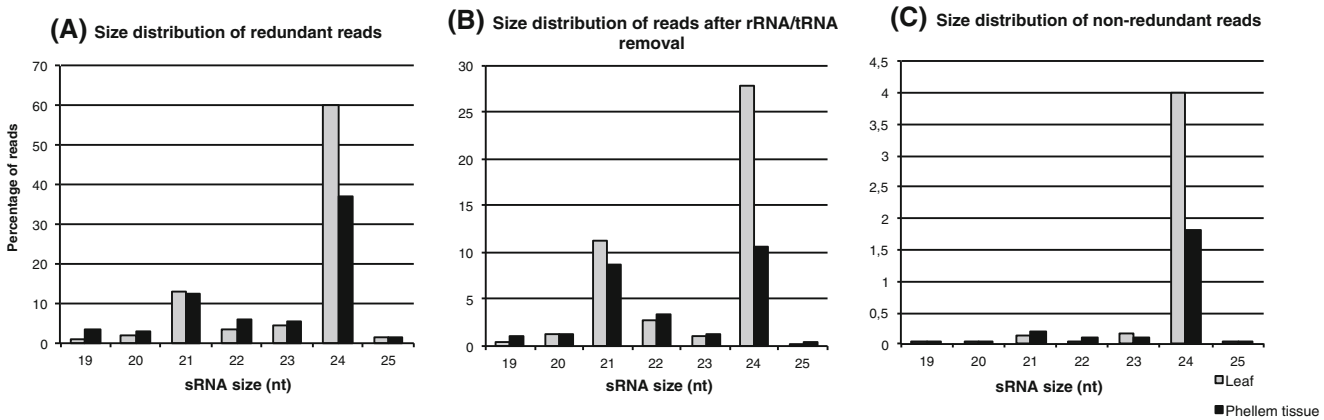


Fig. 2 Size distribution of small RNA sequences obtained from cork oak leaves and phellem: **a** redundant reads; **b** after removal of rRNA/tRNA exact matches; **c** non-redundant reads. *Bars* representing numbers of reads are in *grey* (leaves) and *black* (phellem)

Table 2 Conserved miRNA in cork oak

miR family	Normalised weighted count				Fold change	
	Leaf		Phellem		Leaf/phellem	
	miRNA	miRNA*	miRNA	miRNA*	miRNA	miRNA*
miR1140	0	0	2.24	0	–	–
miR156/157	2,382.54	238.06	29.85	9.21	79.8	25.8
miR158	0	0	3.55	0	–	–
miR159	8,637.86	11.76	7,200.74	48.53	1.2	−4.1
miR160	30.84	1.28	3.55	2.89	8.7	–
miR162	389.11	0.97	394.83	3.03	−1.0	–
miR163	0.55	0	0	0	–	–
miR164	353.71	4.14	3.29	0.26	107.5	–
miR165/166	10,749.60	683.34	6,664.95	14,900.00	1.6	−21.8
miR167	15,936.72	4.63	20,940.64	9.73	−1.3	−2.1
miR168	553.75	10.48	8,068.14	196.36	−14.6	−18.7
miR169	73.85	6.64	30.78	2.63	2.4	2.5
miR171	51.00	15.47	379.70	29.41	−7.4	–
miR172	14.44	74.40	18.41	152.57	−1.3	−2.0
miR1863	0	0	0.66	0	–	–
miR2089	0.43	0	0	0	–	–
miR2911	0.85	0	3.81	0	−4.5	–
miR2916	0	0	1.71	0	–	–
miR2950	1.71	0	0	0	–	–
miR319	168.11	1.16	302.63	15.13	−1.8	−13.0
miR3627	0.24	0	0.53	0	−2.2	–
miR390	10.78	0.73	531.48	26.96	−49.3	
miR393	24.01	24.68	4.73	38.01	5.1	−1.5
miR394	6.7	0	0	0	–	–
miR395	488.36	2.86	5.79	0	84.3	–
miR396	7,720.49	12,600.00	5,470.56	4,502.55	1.4	2.8
miR397	121.01	0	419.43	0	−3.5	–
miR398	767.19	0.61	463.75	1.18	1.6	–
miR399	434.56	0	7.37	0	59.0	–
miR403	93.71	0	30.12	2.24	3.1	–
miR408	26.02	21.51	42.61	48.14	−1.6	–
miR472	1.46	0	6.84	0	−4.7	–
miR477	0.67	0	0	0	–	–
miR479	0	0	2.76	0	–	–
miR482	1,067.52	0.91	2,425.67	7.23	−2.3	–
miR894	1.65	0	24.85	0	−15.1	–
miR5072	14.01	0	156.78	0	−11.2	–
miR5083	0	0	1.58	0	–	–
miR5139	1.04	0	6.58	0	−6.3	–
miR529	0.43	0	0	0	–	–
miR530	0	0	0.79	0	–	–

Normalised count: count of matching sequence reads normalised to the total number of reads after last filtering step search miRBase database

Based on recent reports (Devers et al. 2011; Todesco et al. 2012; Zhou et al. 2010), referring the putative importance of the miRNA* form, we have also performed the analysis of these forms in our data. There was a general agreement

between the relative abundance of the mature miRNA and its star form but exceptions were found for four miRNA* which showed different profiles when compared to the mature miRNA: miR165*/166*, miR172*, miR393* and miR396* (Table 2). In the leaves, the miR165/166 mature form was expressed at higher levels than the star form, but in the phellem, the miR165*/166* was twice the expression level of the mature form; the miR172* was expressed at higher levels than miR172 in both tissues; the miR393 and miR393* had the same expression level in the leaves, but miR393* was expressed at higher levels in the phellem; and the miR396 and miR396* were expressed at similar levels in the phellem, but the miR396* was expressed at higher levels in the leaves.

Novel miRNAs in *Quercus suber*

In order to perform the prediction of the stem-loop secondary structure characteristic of the pre-miRNA, we have taken into account the previously described structure and minimum free energy criteria (Jones-Rhoades et al. 2006). Since a reference cork oak genome is not yet available, we customised an enlarged oak reference database: CEOR DB (see "Material and methods" for details) for the prediction of the stem-loop secondary structures using miRCat from UEA package (Moxon et al. 2008b) (Supplementary material—SM2_Leaf_miRCat_results.xls, SM3_cork-tissues_miRCat_results.xls).

Using this approach, a total of 30 novel miRNA candidates were identified (Table 3) of which 11 were unique to the leaves and 13 were unique to the phellem. The average expression level of the novel miRNAs was approximately 362 reads. However, the tissue-specific novel miRNAs showed lower average expression levels that ranged from 71 to 162 in the leaves and phellem, respectively.

An additional criteria proposed by Meyers et al. (2008) to confirm the annotation of a novel miRNA is the isolation in the same library of the miRNA* form with an extensive base-pairing with the miRNA. Of the 30 novel miRNAs, eight had the corresponding star form present in the same library. The expression level of the new miRNAs was lower in comparison with the levels observed for the conserved miRNA, and the new miRNAs were more abundant in the phellem than in the leaves. The secondary structure for phellem-specific novel miRNA is shown in Table 4.

Validation of identified miRNAs by northern blot analysis

In order to confirm the deep sequencing data and the differential expression of the miRNAs, 19 miRNAs were selected for further analysis by northern blot (Fig. 3), including 13 conserved miRNAs (miR156/157, miR159, miR164, miR165/166, miR166*, miR168, miR171, miR319, miR390, miR395, miR399, miR482 and miR894) and four novel miRNAs (miR-A, miR-B, miR-3P and miR-13P). We designed DNA probes complementary to the miRNA sequence (Supplementary material—SM4_northernblot_probes.pdf) and detected signal for the miRNAs under validation, even for those with low expression levels. Remarkably, we validated four non-conserved miRNAs despite their low expression levels, two in both leaf and phellem (miR-A and miR-B) and the other two specifically in phellem (miR-3P and miR-13P).

Estimation of the relative expression levels between the leaves and phellem by comparing the intensity of hybridization signal obtained by northern blot was consistent with the deep sequencing data. For instance, the identified novel miRNAs miR-A and the miR-B were less expressed in the leaves, while the miR-3P and the miR-13P were only expressed in the phellem. Considering the expression level observed both by deep sequencing and northern blot analysis, miR-13P is the best miRNA phellem-specific candidate (Table 3, Fig. 3). However, it is likely that the conserved miRNAs miR166*, miR168 and miR390 play important roles in the differentiating phellem as judged by analysis of their expression patterns by northern blot hybridization (Table 2, Fig. 3).

Target prediction for the tissue-specific novel miRNAs

To better understand the putative functions of the conserved and novel miRNAs in the analysed tissues, their corresponding target genes were predicted using TAPIR, a bioinformatic tool developed specifically for plants (Bonnet et al. 2010). Since complete genome sequence information is not available for oaks, we have constructed a database called CEORT DB (see "Material and methods" for details) that we have used to predict the miRNA target genes. Considering that the miRNA and its target gene should coexist in the same tissue for interaction to take place, only the predicted target genes found in the same tissue as the corresponding miRNAs were considered as putative targets (Supplementary material—SM5_non-conserved miRNAs_targets.xls, Supplementary material—SM6_target_contigs_leaf.fasta, Supplementary material—SM7_target_contigs_cork.fasta). Using these criteria, we have found that several miRNAs have more than one predicted target gene but some of them had the same annotation (Table 3).

To obtain further insights into the regulatory mechanisms involving miRNA pathways and their relevance concerning tissue specificity, all the specific targets for each tissue (72 for leaves and 219 for phellem) were annotated using Blast2GO (Conesa and Götz 2008; Conesa et al. 2005; Götz et al. 2008, 2011). The genes with functional annotation (54 for the leaves and 156 for the phellem) were grouped by gene ontology using Blast2GO. We further assigned the GO terms based on the plant GOslim classification with the combined graph category (Fig. 4a, b).

Table 3 Novel miRNA in cork oak and the predicted target genes

miRNA sequence	Length	miRNA*	Normalised weight count		Predicted target proteins
			Leaf	Phellem	
Qsu-miR-A AGTGGAAGGATTGGAAAGACA	21	Yes	1,951.26	4,634.11	– rcc1 domain-containing protein – (TF) tubby-like f-box protein 5-like – Subunit of NADH dehydrogenase
Qsu-miR-B CAGCCCTGTGTCGCTTCGATTCGT	24	Yes	328.32	3,123.12	– Binding protein/chloroplast thylakoid membrane/signal peptide – na
Qsu-miR-D GTCGTTGTAGTATAGTGGTG	20	No	14.58	122.33	Leaves – Protein disulfide-isomerase – Squalene monooxygenase – Phytol kinase chloroplast Phellem – Sieve element-occluding protein – Protein disulfide-isomerase – gpn-loop gtpase
Qsu-miR-E TCGGAAGAACAGCAGCCACCG	21	No	95.22	70.85	– bzip protein – na
Qsu-miR-F ATGATGGCCTAGGAATTTGAA	21	Yes	14.82	148.07	– Dihydrolipoyl dehydrogenase 1. mitochondrial – Beta-amylase 1. chloroplastic – Probable protein phosphatase 2C 52
Qsu-miR-G GTTTTGTCAAAGGATTAGCCAATT	24	No	6.42	13.91	– Ubiquitin-like-specific protease ESD4 – Sentrin-specific protease 2
Leaf specific					
Qsu-miR-1 L GTTGGTTAGGATACTCGGCTCT	22	No	182.34		– 60S ribosomal protein L4
Qsu-miR-2 L TCGACAAAATGCTTGTGGCGA	21	Yes	171.84		– Cytochrome P450 – Trafficking protein complex – fbd-associated f-box protein
Qsu-miR-3 L TGAAGCTGCCAGCATGATCTTA	22	No	165.12		– Auxin response factor – Quinone oxidoreductase-like protein
Qsu-miR-4 L TGCCGGAGTGCGGGACGATGCGGG	24	No	78.30		– Actin
Qsu-miR-5 L CTTGATGATGCTGCATCGGCA	21	No	42.60		– Amino acid binding – 4-Coumarate—CoA ligase 2 – Tropinone reductase
Qsu-miR-6 L TGCCAAAGGAGAATTGCCCT	20	No	39.96		– Leucoanthocyanidin reductase – Glycolate oxidase – Peptidase family m48
Qsu-miR-7 L AGGGGAGTAGAGTAGAGTTGGCT	23	No	37.26		– Related lipid transfer protein
Qsu-miR-8 L CGGGGCGTGGACCGATGCG	19	No	24.96		– gata transcription factor 9-like
Qsu-miR-9 L TTTGAGCCAAGGATGACTTGC	21	No	20.58		– f-Box protein – RCD1-like – 12-Oxophytodienoate reductase
Qsu-miR-10 L TTGGTGGAACAAAAAGTGGTA	21	No	12.30		– Cinnamoyl reductase – Dehydratation-induced protein – Elongation factor
Qsu-miR-11 L ATTTTAGGAAACTTTTTATGGAA	23	No	6.48		– NADHP hc-toxin reductase
Phellem/phellogen specific					
Qsu-miR-13P AAATGGGTGCGTTGGCAAGAA	21	No		1,053.52	– Fumarylacetoacetase – Early nodulin-like protein
Qsu-miR-1P GGTGTCGTGGTGTAGTTGG	19	No		273.26	– Oligopeptide transporter – Probable 26S proteasome non-ATPase regulatory subunit 3 – Ferredoxin-dependent glutamate synthase

Table 3 (continued)

miRNA sequence	Length	miRNA*	Normalised weight count		Predicted target proteins
			Leaf	Phellem	
Qsu-miR-2P GTCTGGGTGGTGTAGTTGGTTA	22	No		226.59	– Receptor serine-threonine protein – Histone deacetylase
Qsu-miR-3P CCTGGACGGGGTCAATGGGTGA	22	No		189.67	– gdsl esterase lipase activity
Qsu-miR-4P GTTGAGATGGCCGAGTTGG	19	No		102.70	– Putative copper-transporting ATPase – Heat shock protein 90 – Probable rhamnose biosynthetic enzyme
Qsu-miR-12P CCTGGACGGGGTCAATGGG	19	No		85.80	– Integrin-linked protein kinase family – Phosphatise protein – na
Qsu-miR-5P TTCCCCAGCGGAGTCGCCA	19	No		35.23	– Calcium-transporting ATPase – 4-Coumarate—CoA ligase – Enolase (chloroplastic-like)
Qsu-miR-6P ATCCCCAGCGGAGTCGCCA	19	No		34.45	– Calcium-transporting ATPase – gdp-mannose-dehydratase – Enolase
Qsu-miR-7P CATAGGTTTGGGTCCGAGGA	20	No		27.56	– Histone acetyl transferase
Qsu-miR-8P GATCACCCATGACTTCTGTGTA	22	Yes		23.79	– ATP-synthase alpha-subunit – Glycerol-3-phosphate dehydrogenase – Ubiquitin-conjugating enzyme E2
Qsu-miR-9P CATTGAGTGCAGCGTTGATGA	21	No		23.53	– Calcium-transporting ATPase – Laccase – Transposase protein
Qsu-miR-10P ATCCCCAGTGGAGTCGCCA	19	No		18.85	– Enolase – gdsl esterase lipase 5-like – Serine palmitoyltransferase
Qsu-miR-11P GCGGGAGGTCTTGAGTTCG	19	No		13.26	– Lupeol synthase – ATPase-related DNA repair protein – Syntaxin-like protein

Normalised count: count of matching sequence reads normalised to the total number of reads after last filtering step (see table). Only the top 3 best blastx hits (*e*-value < 1e -10) with oak transcripts were selected (all homologous proteins were collapsed)

na not available

Discussion

miRNAs regulate development, metabolism, adaptation and evolution of plants, but despite their importance, relatively few studies were hitherto performed in woody plants (Sun et al. 2012). The control of secondary growth in trees and the formation of highly specialized types of secondary cell walls in wood and cork cells likely involve several small RNA-mediated networks which are still largely unknown. In this work, we report for the first time the sequencing and analysis of the small RNA transcriptome of a *Quercus* species and, to our knowledge, the first in the Fagaceae. Moreover, a comparative analysis between the small RNA transcriptome of the leaves and phellem of cork oak was conducted aiming to identify and characterize the putative involvement of miRNAs in phellem differentiation. Over 25 million small RNA sequences with 19–25 nt length derived from two libraries representing leaf and phellem tissues were analysed.

A higher number of total small RNA sequences were obtained from the leaves when compared to the phellem. This was not surprising considering that more cell types are present in whole leaves than in the isolated phellem tissues which are expected to contain mostly differentiating phellem cells. Therefore the higher number of small RNAs in the leaves probably reflects a wider range of regulatory functions being performed in the leaves. The 24-nt length class of small RNAs was the most abundant followed by the 21-nt class in both analysed tissues but the 24-nt/21-nt proportion was higher in the leaves than in the phellem. It is well established that the relative abundance of the 24-nt and the 21-nt length

Table 4 Predicted pre-miRNA secondary structure for phellem-specific novel miRNA in cork oak. The hairpin structures were plotted based on miRCat prediction using the RNA fold software. The miRNA is highlighted in blue and the miRNA* in red. The adjusted minimum free energy (MFE) and the number of mismatches are included

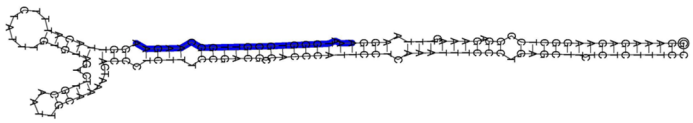
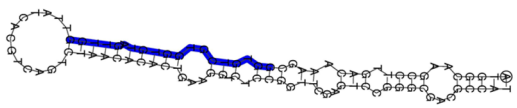
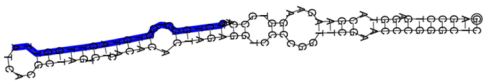
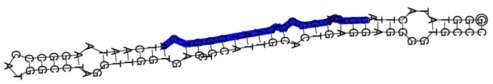
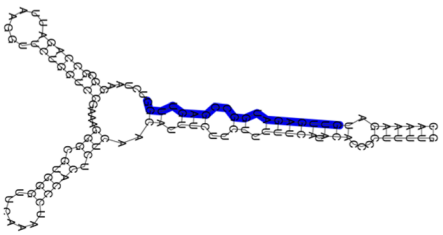
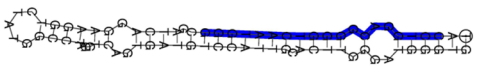
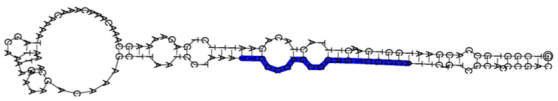
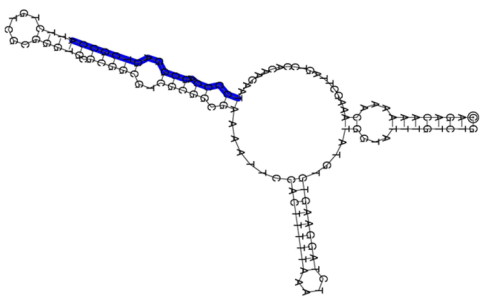
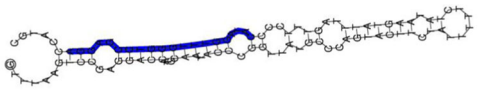
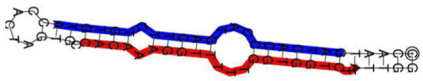
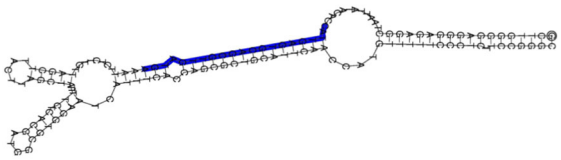
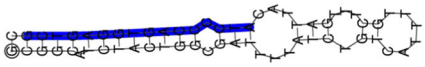
Novel miRNA	Hairpin structure Hairpin structure in Vienna format
Qsu-miR- 13P AAATGGGTGCGTTGGCAAGAA **MFE** -45.75 **mismatches** 3	 (((((((((((((((.((.((((.(((.(((<<-<<<<<<<<<<<<<-<<<<-(((((((((.....)))))...((((....))))....))).)))).)))))).)))))))))).)))))))).))))))))))
Qsu-miR-1P GGTGTCGTGGTGTAGTTGG **MFE** -32.76 **mismatches** 4	 ((((((..((((.(((.((.(<<-<<<<--<<<<<-<<<<<.....)))))))))...)).).)))..))...))).))))...))))))
Qsu-miR-2P GTCTGGGTGGTGTAGTTGGTTA **MFE** -40.83 **mismatches** 6	 (((((((.(((.((((...((..((..-<<<<<---<<<<<<<<<<<--....))))))..)).)))))))))..))...))))).))))))))))
Qsu-miR-3P CCTGGACGGGGTCAATGGGTGA **MFE** -40.67 **mismatches** 6	 ((((((..(((.<<<---<<<-<-<<<<<<<<<-<((((((.((((((...))))))..)))))))..))..)))))))).)))))))..)))))
Qsu-miR-4P GTTGAGATGGCCGAGTTGG **MFE** -29.48 **mismatches** 5	 ((((((.(<<<<<<<-<<--<<<-<<<-.....((.((((((.......)))))))).....(((.(((((.......)))))..)))..))..)))))..)))...))))))
Qsu-miR-12P CCTGGACGGGGTCAATGGG **MFE** -40.65 **mismatches** 3	 ((<<<<--<-<<<<<<<<<<<<(.((((.((((((.....))))...)).)).)))))))))))))).))))..)..))))))
Qsu-miR-5P TTCCCCAGCGGAGTCGCCA **MFE** -26.41 **mismatches** 5	 (((((((((.((((((((((((.(((..((...((((((..((...((((.....................((....)).....(....).....))))..))..)))>>>---->>--->>>>>>>>>>))))).)).)))).))))))
Qsu-miR-6P ATCCCCAGCGGAGTCGCCA **MFE** -30.89 **mismatches** 5	 (((((((((...((.....................-<<-<<-<<<<--<<<<<<<(((.....)))))..)))))..)))))..)).....(((((((((....)))))))))....))...))))))))

Table 4 (continued)

Qsu-miR-7P CATAGGTTTGGGTCCGAGGA **MFE** -25.35 **mismatches** 5	 ((....((((..((((((...(((.(((..(.((..(((((((.((.....)).)))))))..)).)..)>---->>>>>>>>>>>>>-->>>)...))
Qsu-miR-8P GATCACCCATGACTTCTGTGTA **MFE** -36.25 **mismatches** 4	 (((((<<<<<<<---<<<<<-<<<<<<(.....)))).}}}}=}}}}}===}}}}}}}==})))))
Qsu-miR-9P CATTGAGTGCAGCGTTGATGA **MFE** -43.77 **mismatches** 3	 ((((((((((((((((((.....--<<<<<<<<<<<<<<<<-<<<(((.....((((.....))))).((((.((((....)))))))..)))))).))))))))))))....)))))))))).)))))))
Qsu-miR-10P ATCCCCAGTGGAGTCGCCA **MFE** -37.54 **mismatches** 1	 (((((.(((((((((.(((...(((.((.....)))...)))))...>>>->>>>>>>>>>>>>>
Qsu-miR-11P GCGGGAGGTCTTGAGTTCG **MFE** -38.64 **mismatches** 4	 (((((((((.(...((((((((((((.(.((((((.....)>>>>>>->->>>>>>-->>>)))..).))))))))

classes may vary depending on the species and the tissue type, among other factors. In fact, previous reports refer that the 24-nt small RNAs (sRNAs) are the predominant size class, for instance in *Arabidopsis thaliana*, *Nicothiana benthamiana*, *Solanum lycopersicum*, *Oryza sativa* and *Olea europaea* (Morin et al. 2008; Pantaleo et al. 2010; Donaire et al. 2011; Kasschau et al. 2007), while the 21-nt class is more abundant in species such as *Eschscholzia californica*, *Hordeum vulgare*, *Vitis vinifera*, *Pinus contorta* and *Panax ginseng* (Pantaleo et al. 2010; Morin et al. 2008; Schreiber et al. 2011; Wu et al. 2012; Barakat et al. 2007). The tissue-dependent variation in the pools of 24 and 21 nt classes has also been observed in other plant species (Pantaleo et al. 2010), and in a few cases, striking variation has been detected (Slotkin et al. 2009), reflecting the sRNA biogenesis pathways operating in such tissues. The determined level of non-redundancy further showed that in both cork oak tissue types, the 21-nt class is less diverse than the 24-nt class, which is in agreement with previous reports in other species (Kasschau et al. 2007; Martínez et al. 2011; Pantaleo et al. 2010; Rajagopalan et al. 2006).

The rRNA/tRNA-derived small RNAs were mainly present in the 24-nt length class and their number was higher in the phellem. Interestingly, recent studies report that these elements may contribute to gene regulation, being cleaved by DICER RNAse to produce stable RNA products rather than being just randomly generated degradation products (Haussecker et al. 2010; Cole et al. 2009; Sobala and Hutvagner 2011). Their functions are still largely unknown; however, a recent report from Li et al. (2012) based on human and mouse constitutively expressed non-coding RNAs suggests a role for these products in the regulation of unwarranted expression of endogenous viruses through the RNA interference pathway (Li et al. 2012). The presence of tRNA-derived RNA fragments (Lee et al. 2009) has been detected also in plants (Chen et al. 2011; Hackenberg et al. 2012; Hsieh et al. 2009) as well as their putative target genes (Loss-Morais et al. 2013).

Following the commonly used guidelines for miRNA annotation (Meyers et al. 2008), we have found 41 families of conserved miRNA and 30 novel miRNA families, some of which were tissue specific. The prediction of precursor sequences for about 60 % of the conserved miRNAs in our analysis was high when compared to previous reports in other species without an available genome sequence such as *Arachis hypogaea* L., for which precursors of nine conserved miRNA families out of 75 were identified (Zhao et al. 2010). These results suggest that the database used in this analysis adequately covers the transcriptome of the studied tissues.

The most abundant miRNAs were those of the families miR167, followed by miR165/166, miR396 and miR159 which are described as the most widespread miRNA families in plants (Barakat et al. 2007; Sun 2012). MiR167 is one of the miRNA families involved in the regulation of auxin signalling

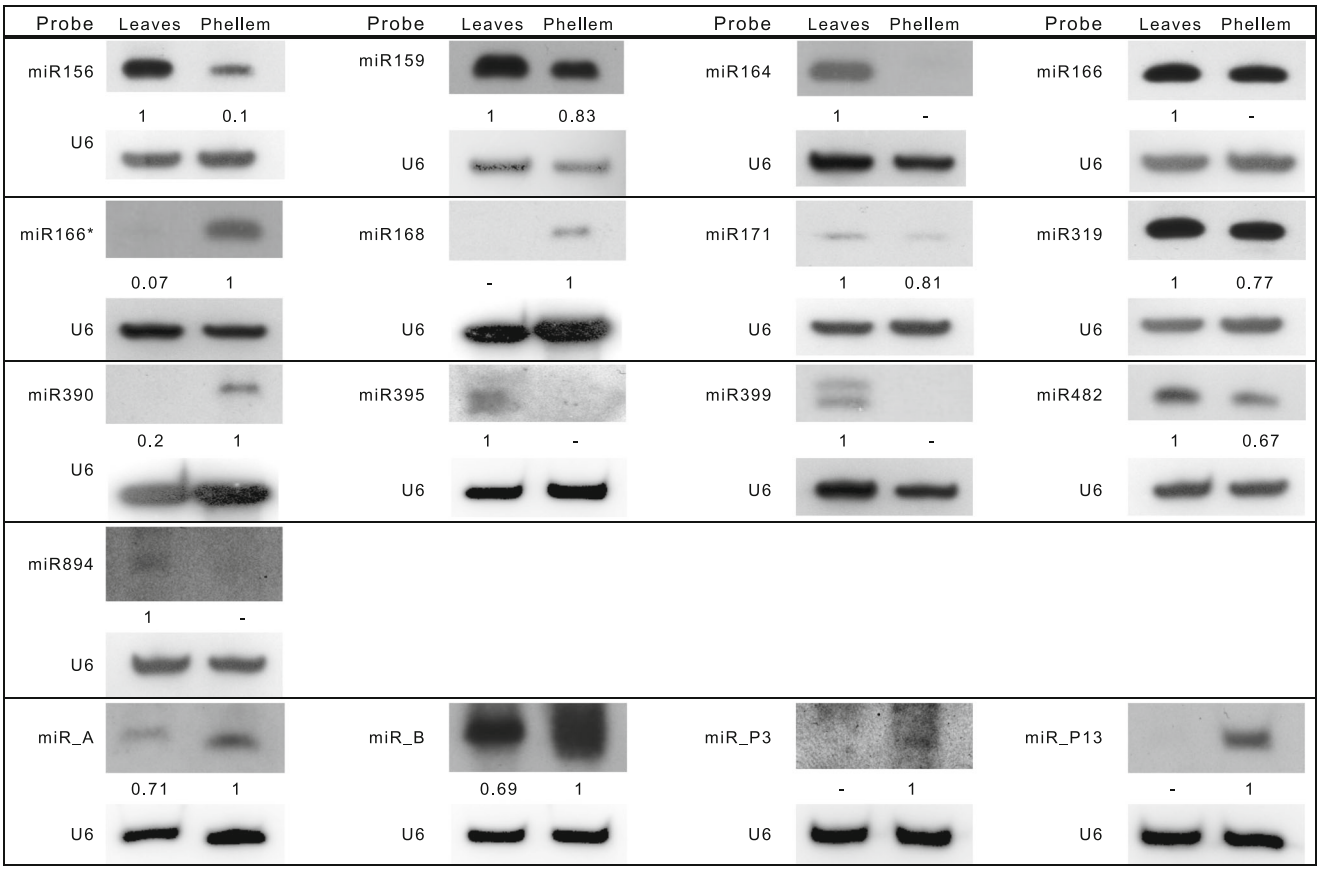


Fig. 3 Expression analysis by northern blot hybridization of selected conserved miRNAs in phellem and leaves of cork oak. The *numbers below blots* represent relative abundance of each miRNA normalised to U6 expression

pathways. It targets members of the AUXIN RESPONSE FACTOR (ARF) family of transcription factors, namely ARF6/8, involved in early auxin response through activation of auxin-responsive genes (Wu et al. 2006), as well as IAA-Ala Resistant3 (IAR3) which hydrolyzes an inactive form of auxin (indole-3-acetic acid [IAA]-alanine) and releases bioactive auxin (IAA) (Kinoshitaa et al. 2012). The regulation of auxin signalling pathways by miR167 and its targets has been related to male and female flower development (Wu et al. 2006) and osmotic stress-induced root architecture changes (Kinoshitaa et al. 2012). In contrast, miR160 which also targets members of the ARF family showed a very low expression level, especially in the phellem. The targets of miR160 are the ARF10/16/17 involved in auxin-mediated developmental processes including root growth, blade outgrowth and fruit development (Hendelman et al. 2012; Khan et al. 2011; Xing et al. 2011). We also have found another miRNA mainly present in the phellem, miR390, which directs cleavage of ta-siRNA locus 3 (TAS3) leading to the production of ta-siRNAs that target ARF2/3/4 (Allen et al. 2005; Marin et al. 2010) involved in lateral root initiation (Marin et al. 2010). Although the regulatory function of these miRNAs in cork oak phellem needs to be studied in detail, these results suggest that auxin signalling-mediated events play a relevant role in phellem differentiation.

When comparing the level of expression of conserved miRNAs in the leaves and phellem, we observed that the families of miRNAs expressed at higher levels in the leaves are miR156, miR160, miR164, miR395 and miR399, while miR166*, miR168, miR171 and miR390 are expressed at higher levels in the phellem. These miRNAs had already been described in other woody plants including *P. trichocarpa* (Tuskan et al. 2006) and *V. vinifera* (Mica et al. 2009) and their targets are known (Khraiwesh et al. 2012). Based on the description of the role of conserved plant miRNAs by Khraiwesh et al. (2012), the miR156, miR160, miR395 and miR399 are controlling *adaptive responses to stress* in the leaves, whereas *adaptive responses to stress* (miR168, miR171 and miR319) and *leaf polarity* (miR166, miR168 and miR390) are the two dominant roles identified in the phellem. We have also identified some miRNAs partially conserved across vascular plants such as miR403 only present in the Eudicots clade and miR482 from the Core rosids clade (Cuperus et al. 2011). From an evolutionary point of view, the ancient miRNAs have roles in growth, development and differentiation, while young miRNAs have a more specific set of functions and are usually less abundant (Sun et al. 2012). Interestingly, we have found one 'young' miRNA, miR482, more expressed in the phellem which was found to be also involved in poplar xylem differentiation (Lu et al. 2005).

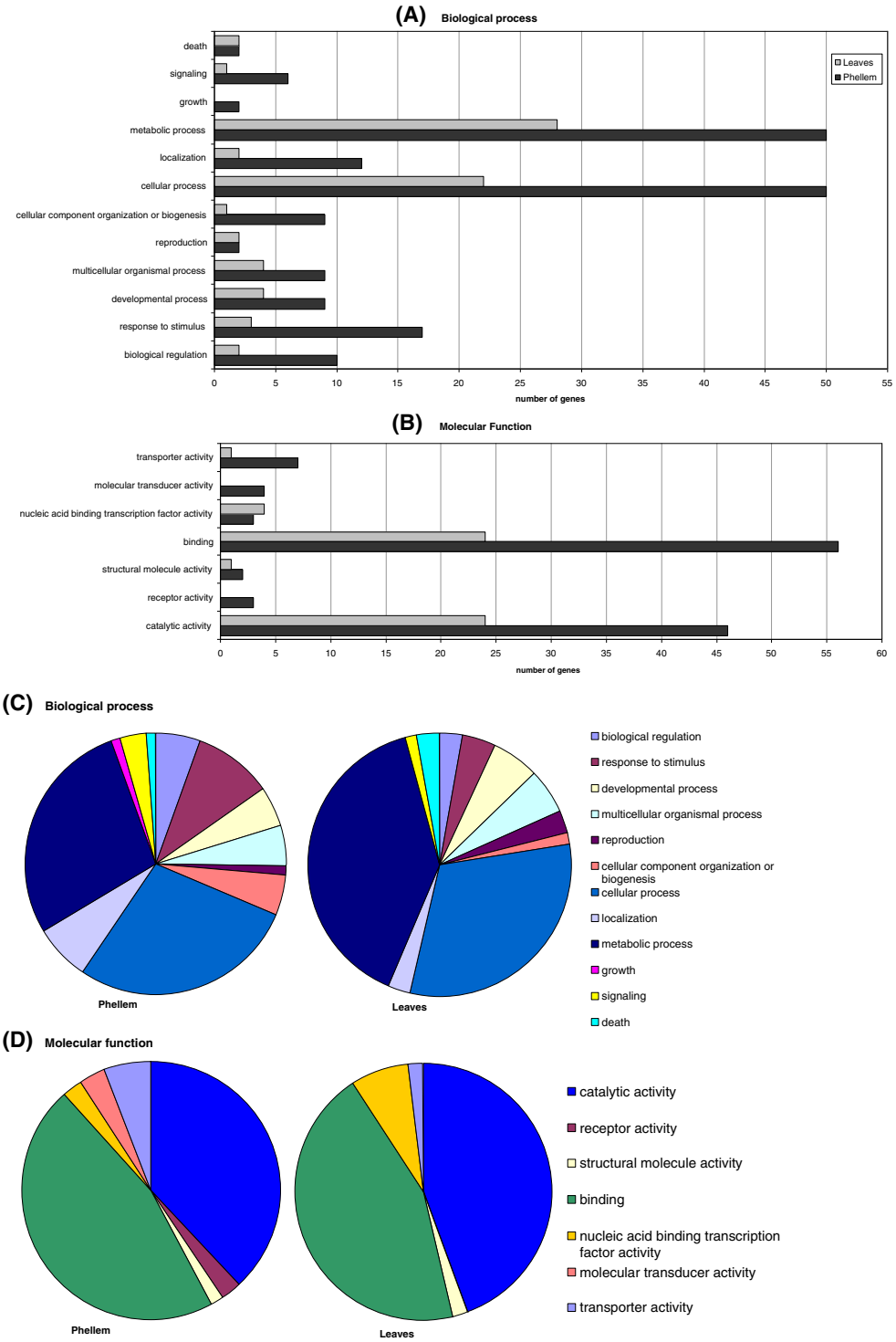


Fig. 4 Gene ontology of the predicted target genes for the novel miRNAs considering the number of genes obtained for biological process (**a**) and molecular function (**b**) and their relative value for the biological process (**c**) and molecular function (**d**)

It has been generally considered that the miRNA* is expressed in lower abundance (<10 %) than the mature form of a miRNA (Rajagopalan et al. 2006), but the idea that the miRNA* is an inactive form with no functional relevance has been questioned (Devers et al. 2011). For instance, in human plasmacytoid dendritic cells, there is evidence for a cooperative action of the star form and the mature form of a miRNA (Zhou et al. 2010). Interestingly, in this study, we have found that the miR166 was expressed at higher levels in the leaves compared to its star form, but inversely, miR166* was expressed at higher levels in the phellem. The predicted target for the miR166* was a putative beta-1,6-*N*-acetylglucosaminyltransferase coding transcript. These enzymes are known to catalyze attachment of oligosaccharide side chains to glycoproteins (Siddiqui

et al. 2005). Interestingly, a recent report by Zalepa-King and Citovsky (2013) describes the location of one of these enzymes in the plasmodesmata, suggesting that it plays a role in these structures. Also, it has been shown that the number of plasmodesmata might significantly increase in the cambial zone of tomato stems (Ehlers and van Bel 2010). Establishing a parallel between the differentiation process of xylem cells from vascular cambium and phellem cell differentiation from cork cambium derivatives, it is expected that rapid cell expansion occurs before active cell wall deposition in the phellem cells. It is therefore tempting to speculate that the number of plasmodesmata might also significantly increase in differentiating phellem cells and that the regulation of this process may require the function of specific miRNA molecules.

A difficult challenge in this type of studies is the identification of species-specific miRNAs. The approach usually consists in the identification of the secondary stem-loop structure characteristic of the precursor of miRNAs (pre-miRNA) by computational prediction. This prediction is best performed using the full genome information, but when this information is lacking, the ESTs can also be used to perform this analysis. We have been able to predict 30 novel miRNAs, some of them tissue specific. The level of expression of these new miRNAs was lower in comparison with the levels observed for the conserved miRNAs which is consistent with previous studies reporting that non-conserved miRNAs were expressed at lower levels and were usually characteristic of a tissue or developmental stage (Fahlgren et al. 2007; Moxon et al. 2008a; Rajagopalan et al. 2006; Yao et al. 2007). Despite their lower expression, four new miRNAs were validated by northern blotting, two of them phellem specific (miR-3P and miR-13P). The difficulties in finding the miRNA* for many of the novel miRNA candidates can be explained by the low expression levels making it less likely to identify the star form, usually less expressed than the corresponding miRNA. Since the miRNA and its true target should coexist in the cell, we excluded the predicted targets that were not present in the same tissue as the corresponding novel miRNA in order to increase the confidence in the prediction. In the phellem, miRNA target genes related to *response to stimulus*, *biological regulation* and *signalling* were predominant when compared to the leaves, while in the biological process, *growth* was only present in the phellem. Moreover, in the molecular function categories, *receptor activity* and *molecular transducer activity* were only found for target genes present in the phellem. We have predicted two targets related to chromatin modifications, a histone deacetylase and a histone acetyltransferase, for the newly identified phellem-specific miRNA Qsu-miR-2P and Qsu-miR-7P, respectively. These two enzymes have opposite roles in the acetylation of the histones, where acetylation is usually associated with activation of transcription (Shahbazian and Grunstein 2007). According to the expression levels of the miRNAs targeting these genes, it could be hypothesized that histone deacetylases are being repressed resulting in transcription activation. However, complex interactions exist between different chromatin modifications to regulate gene transcription and further studies would be necessary to clarify the roles of these miRNAs. It was shown by Conde et al. (2013) that winter dormancy in poplar stems is epigenetically controlled depending on the level of histone acetylation (Conde et al. 2013). Moreover, genes related to histone acetylation such as histone acetyltransferases and histone deacetylases are differentially expressed during regeneration of *Populus tomentosa* secondary vascular tissue (Zhang et al. 2011). Since phellem formation was highly increased at the time of sample collection, the regulation of the gene transcriptional activity should be crucial to control tissue differentiation, and histone acetylation may play an important role during this process. When comparing the putative proteins encoded by the predicted target genes of phellem-specific miRNAs with the proteins from the same tissues identified in a previous study (Ricardo et al. 2011), we found some overlapping, notably in the case of enolases and laccases. When considering the biological process, overlap was found for heat shock proteins, ATPases and proteasome complex proteins.

Overall, these results suggest that tightly controlled regulatory mechanisms are operating in the phellem and may be relevant for the differentiation process. The identification of conserved and novel miRNAs of cork oak as well as of their target genes performed in this work complements the recent effort to sequence the coding transcriptome (Pereira-Leal et al., submitted) and provides new tools to dissect relevant regulatory mechanisms operating in cork oak phellem.

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Data Archiving Statement The small RNA sequences obtained for leaf and cork are deposited in the NCBI GenBank database under the accession numbers SRR988108 and SRR988109, respectively.

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