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Proceeding Paper

Transcriptome Analysis in Cork Oak Using Laser Microdissection and RNA-Seq †

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Abstract: In order to identify specific candidate genes linked to secondary growth, a transcriptomic analysis of single-cells, isolated by laser microdissection technology, of cork oak was performed. Thus, an optimized protocol for single-cell isolation laser microdissection of phellogen, lenticels and xylem was successfully achieved, followed by RNA isolation, cDNA libraries preparation and RNA sequencing. Differential expression and GO terms analysis clearly reveal enzyme active players on lignin and suberin critical point pathways. The scRNA-Seq analysis contributed to increasing the knowledge of potential genes associated with the development processes of secondary growth in cork oak.

Keywords: *Quercus suber*; periderm; xylem; laser microdissection; single-cell RNA sequencing; transcriptomics

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1. Introduction

Laser microdissection microscopy (LM), a technology for isolate cells or tissue of interest, combined with RNA-sequencing are powerful techniques for investigating the transcriptome profile at a cellular level of specific tissues or cells type [1]. This type of analysis, more specific and precise, enhance the detection of low-abundance transcripts that are expressed in only few and specialized cells.

The periderm (outer bark in trees) is a protective tissue against the environment, differentiated by a secondary meristem, the phellogen or cork cambium. The outward layers of periderm are constituted by cork or phellem tissue, which is composed of several compacted layers of suberized cells [2,3]. At particular regions of phellogen, a specific meristem called lenticular phellogen is responsible for the lenticular channels (or lenticels) differentiation. These channels, filled with non-suberized tissue, cross the cork layers, allowing gas exchange between the interior and the exterior of the tree [3,4]. The phellogen is also responsible for the production of phelloderm, an inner layer of the bark [3]. Moreover, xylem, responsible for the transportation of water and nutrients, from the roots upwards, is originated from another secondary meristem, the vascular cambium.[5].

Cork from *Quercus suber* L. is a non-wood forest product with high economic and commercial value, as well social and ecological role. In Portugal, cork represents 33% of all national forestry products placing Portugal as the world leader in cork production, industrial processing, and trade of cork. The impermeability, fire retardancy, and sound insulation properties of cork make it the optimum material for a variety of applications,

such as wine bottles stoppers, insulation corkboard, shoe soles, and others fashion purposes [2,6].

The aim of our research is to compare the gene expression of several tissues from cork oak, namely phellogen, xylem and lenticular phellogen. The isolation of single-cells using LM technique and RNA isolation method for RNA-seq analysis was optimized in *Q.suber* tissues. The single-cell RNA-sequencing allowed gene expression analysis in individual tissues of oaks, contributing to understand the molecular mechanisms associated with the development processes of secondary growth.

2. Materials and Methods

Cork oak trees were selected in the Parque Biológico da Cabeça Gorda, Beja (37°54′43.6" N 7°47′22.7" W), Alentejo region, Portugal. Small twigs with 5–8 mm length were cut from three-years-old branches, immediately frozen in liquid nitrogen and stored at −80 °C until use for cryosectionning/LM.

The samples were embedded in Optimal Cutting Temperature medium (OCT) (VWR Chemicals) and transversal sections were sliced using a CryoStar NX50 Cryostat (Thermo Scientific, USA). The sections were mounted on PET-membrane slides with some drops of 70% and 100% ethanol, to remove the OCT and facilitating the adhesion of sections respectively.

The cells of interest selected from xylem, phellogen and lenticular phellogen tissues were microdissected using a PALM Zeiss Microbeam Microscope. Total RNA isolation, from pooled harvested cells from several LM sessions, was carried out with PicoPure RNA Isolation kit (Thermo Fisher Scientific) according to manufacturer's instructions, followed by DNase treatment with RNase-Free DNase I Set (Qiagen) to eliminate genomic DNA. At the end of each extraction, a quality control was assessed by LabChip technology (Fragment Analyser System) to check quality and quantity of the RNA extracted.

At the final stage, RNA from xylemic and phellogen tissues was converted to cDNA using the SMART-seq protocol [7] and small fragments of cDNA were created by Nextera fragmentation [8]. Quality of cDNA synthesis and fragmentation were analysed by LabChip technology (Fragment Analyser System). For sequencing, Ultra-low Sequencing method was performed through Illumina HiSeq 2500 platform.

For the paired-end (PE) RNA sequencing data, the raw reads were trimmed, to remove Illumina adaptors sequences and duplicates reads, with Trimmomatic software (v0.36; [9]). Clean pairs of reads from each library were independently mapped to *Quercus suber* genome (v1.0; [10]), using STAR (Spliced Transcripts Alignment to a Reference) software (v2.3.0; [11]). Blast2GO will be employed to annotate the GO terms, enzymatic protein codes and KEGG pathways. The number of unique match reads to each reference unigene was normalized to RPKM, to quantify gene expression abundance.

3. Results

In this study, a protocol for single-cell isolation by Laser Microdissection and extraction of undegraded total RNA was optimized for phellogen, lenticels and xylem tissues of cork oak (*Q. suber*) for transcriptomic analysis.

3.1. Laser Microdissection and RNA Isolation

Several adjustments on laser microdissection and RNA extraction procedures were made in order to successfully isolate total RNA, using PicoPure RNA Isolation kit, from single-cells of xylem, phellogen and lenticels.

The total RNA with RIN higher than 6, was converted to cDNA, fragmented and adapter sequences added onto template using Picelli et al. [7] and Baym et al. [8], respectively. The paired-end sequencing libraries were sequencing through Illumina HiSeq 2500 platform.

Figure 1 illustrates electropherograms (Fragment Analyser System, Agilent) of total RNA isolated from xylemic tissue from three-year-old branches of cork oak and subsequent fragmented cDNA library size distributions. The observed peaks (Figure 1a) corresponding to 18S and 28S rRNA. The broad size distribution of the library (from 150 bp to 1000 bp) is acceptable for further sequencing.

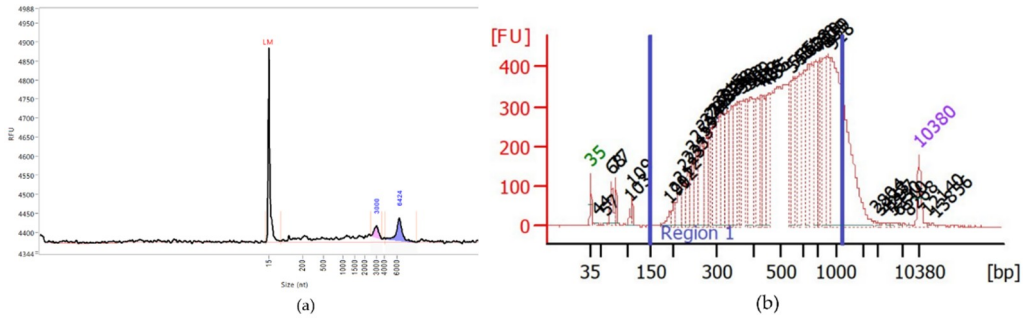

Figure 1. Representative electropherograms (Agilent) of RNA from xylem cells isolated (**a**), and cDNA library (**b**). RFU/FU: Fluorescence units.

3.2. RNA Sequencing and Differentially Expressed Transcripts

The transcriptomic profile of the three tissues (xylem, phellogen and lenticels) were obtained. Between 30 and 50 million paired reads with 125bp per library were generated from RNA-seq, with an average of 41% GC content (quality control evaluated with FastQC).

After filtering-out raw reads, in order to remove duplicates and Ilumina adapters sequences, the clean reads from each library were aligned against the cork oak genome [10]. About 25–30 M reads-pairs were mapped to the *Q. suber* genome sequence. Considering the annotation available, the reads aligned mostly in exonic and intergenic regions (Figure 2), which supports the high content of protein-coding transcripts in the samples analyzed.

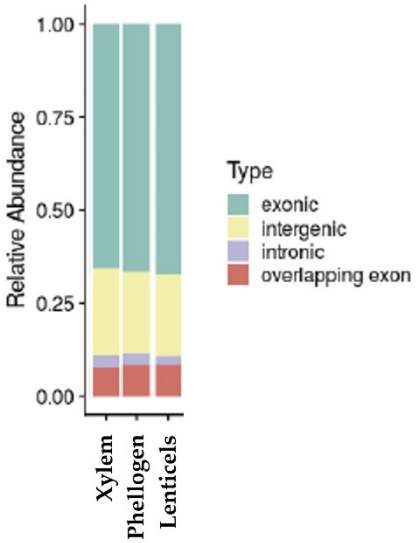

Figure 2. Genomic source of reads for each sample.

The differential gene expression analysis of the xylem, phellogen and lenticels tissues are represented as a heatmap in Figure 3. The results show different expression patterns for the tissues studied, with an evident difference between the lenticular phellogen regarding the phellogen and xylem tissues. Differential expression and GO terms analysis clearly reveal enzyme active players on lignin and suberin critical point pathways.

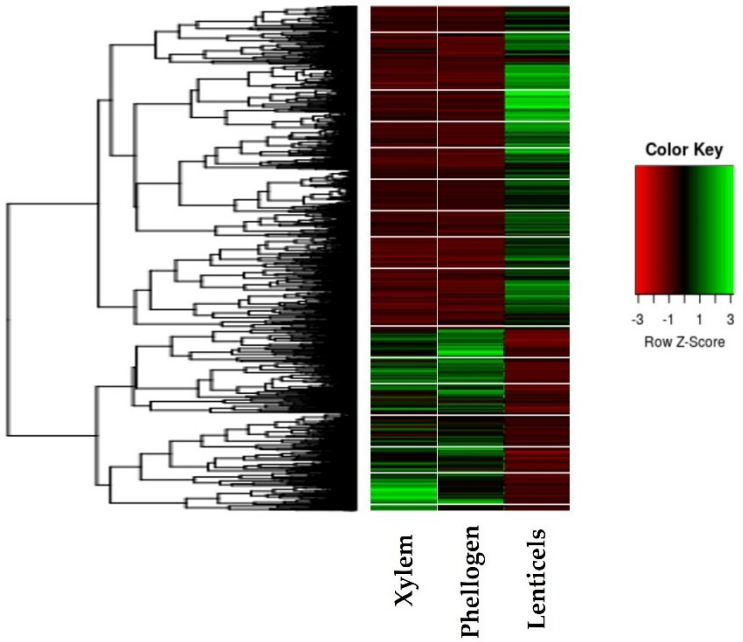

Figure 3. Heatmap of differential expressed genes of the xylem, phellogen and lenticels tissues. The code of color bars are presented. Green—upregulated, Red—downregulated genes.

4. Discussion

Plants are composed by several specialized tissues with specific biological functions, thus exhibiting different genetic, protein and metabolic profiles. The data acquired from whole organ analysis can mask valuable information at cellular level. Currently, more precise isolating individual cells techniques have made possible analyze cell or tissue type-specific transcriptome profiles, revealing intricate and unique gene networks.

In this context, we developed a combined laser microdissection and Next Generation Sequencing method in order to investigate the gene expression pattern of lignified and suberized tissues from *Quercus suber*. We successfully microdissected single-cells from three different tissues, namely xylem, phellogen and lenticels from cork oak. Isolation of undegraded total RNA, followed by cDNA libraries preparation for RNA-seq were also accomplished.

The mapping results suggest that single-cell isolation, RNA extraction, and sequencing of Illumina libraries procedures were viable for transcriptomic studies of cork oak tissues. Differential gene expression of studied tissues clearly shows variations of up and downregulated genes between the tissues, which indicates that different biological pathways are activate.

The single-cell RNA-Seq will allow gene expression analysis in individual tissues of oaks, contributing to understand the molecular mechanisms associated with the development processes and the lenticular channels formation.

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Conflicts of Interest: The authors declare no conflict of interest.

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