Quercus suber Transcriptome Analyses: Identification of Genes and SNPs Related to Cork Quality †

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Abstract: Cork is an ancestral natural material derived from the cork oak tree (Quercus suber L.) with multiple industrial applications. During the recent years, this material has been the subject of several studies. The recent sequencing of the Q. suber genome opened the possibility to make new studies regarding cork quality. In this study, the transcriptomes of cork with superior and poor quality are compared to highlight new molecular pathways and identify SNPs that can be associated to cork traits, which remain one of the main concerns of the cork industry.

Keywords: cork oak; cork quality; transcriptome; SNPs

1. Introduction

Cork oak (Quercus suber L.) is an evergreen broad-leaved tree that belongs to the genus Quercus (oaks) of the Fagaceae family and is one of the most important Mediterranean forest tree species. It plays an important environmental, social, and economic role in the Mediterranean ecosystems known as “Montado” in Portugal and “Dehesa” in Spain. Cork is obtained from the extraction of the outside layer of the cork oak tree, which is composed by suberized cells. The first cork extraction occurs when the tree is between 20–25 years old. After that, cork is extracted at regular intervals of at least nine years [1]. The chemical cork composition has already been extensively described, and it is known that it can change depending on environmental and genetic conditions [2,3]. Parameters such as thickness and structural discontinuities have been used to evaluate the cork quality [4]. Based on quality, the cork’s economic value can vary, which is one of the main concerns in the cork industry. Scientific studies have been performed to identify conditions and processes that can help to stabilize the cork quality and, therefore, ensure more profitable and sustainable production [5,6].

Recent advances in sequencing technology, and subsequent sequencing of the cork oak genome, open the opportunity to perform new studies regarding the cork formation process [7]. The aim of this study was to analyze the dynamic profile of differential expressed genes associated with cork quality, highlighting for the first time, to our knowledge, a set of SNPs that could be involved in cork differentiation.
2. Materials and Methods
2.1. Plant Material and RNA Isolation

Amadia cork planks were collected from physiologically active cork oak during the period July-August. Cork samples from eight genotypes were classified into Good and Bad quality cork (GQ and BQ, respectively) according to the main traits of thickness and structural discontinuities (porosity and inclusion of woody cells). GQ and BQ cork samples were harvested in the Barranco Velho and Cercal regions of Portugal, respectively. The inner part of harvested cork planks, corresponding to phellogen and phellem cells, was scraped and stored according to Soler et al. 2007 for RNA extractions (Figure 1) [3]. Total RNA from GQ and BQ samples (four cork oak trees per condition) was isolated according to Almeida et al. (2013) and sequenced by Illumina HiSeq 2000, producing PE reads of 100 bp in length [8].

Figure 1. Collection of sample material: the inner part from cork planks was harvested, corresponding to phellogen and phellem cells. Photographer: Leandra Rodrigues (www.liarodriguesphotography.com, accessed on 28 November 2021).

2.2. Reads Pre-Processing and Mapping

The raw reads were preprocessed keeping only reads with a minimum quality of 20 and minimum length of 80 bp using Trimmomatic (v0.38) [9]. Then, the pre-processed reads were mapped against the Q. suber genome with STAR (version 2.5.2 b) using the multi-sample 2-pass mapping mode, according to its user guidelines [10]. The unique mapped reads (UMR) were then filtered and extracted using SAMtools [11].

2.3. Differential Expression Analysis

The differential expression analyses were performed using edgeR, a Bioconductor package [12]. To avoid issues with the incompatibility of some tools such as edgeR in using biological and technical replicates at the same time for differential expression analyses, technical replicates were merged using the function “sumTechReps” in edgeR. Then, genes with low counts were excluded and a Trimmed Mean of M-values (TMM) normalization was applied. In the end, only genes with a log fold change (logFC) ≥|2| and with a False Discovery Rate (FDR) ≤ 0.05 were considered as differentially expressed genes (DEGs). At last the interactions between DEGs associated with KEGG pathways and Gene Ontology (GO) terms were visualized and analyzed with Cytoscape. Additionally, BinGO (Cytoscape plugin) was used to identify GOs overrepresented over the set of genes up- or down-regulated against the GO database [13,14].
2.4. Variant Calling and Annotation

For Single Nucleotide Polymorphism (SNP) identification, a variant calling analyses was performed using mpileup from SAMtools. The raw variants were then filtered by SNP quality (≥30) and minimum depth coverage per genotype (≥8); indels were removed and only bi-allelic SNPs were maintained, resulting in a high-quality SNP set which was annotated using ANNOVAR [15].

3. Results and Discussion

The sequencing of RNA from good and bad quality cork samples resulted in a total of 708,803,510 reads. After pre-processing, 98% of the reads were kept. Pre-processed reads were then mapped against the cork oak genome [7] and the UMR obtained, representing 78% of the mapped reads, and used for the differential gene expression analyses.

3.1. Differential Expression Analysis

A total of 172 genes were differentially expressed, of which 66 were more expressed in GQ, and 106 in BQ. Within the set of the identified DEGs, only 73 were associated with at least one GO term. For each sub-ontology (biological process (BP), molecular function (MF) and cellular components (CC)), a total of 50, 20 and 18 terms were associated with the DEGs (Table 1). After performing the over-represented analyses with BinGO, a total of 23 GO terms were over-represented in the set of genes more expressed in GQ (BP:19; CC:1; MF:3), while only one GO term (BP: response to stress process) was over-represented in the set of genes more expressed in BQ.

Table 1. Number of GO terms, for category, that were associated only with genes more expressed in BQ (gBQ) and genes in GQ (gGQ), and in common.

| Category         | gBQ | gGQ | Common | Total |
|------------------|-----|-----|--------|-------|
| Biological Process | 24  | 34  | 7      | 50    |
| Cellular Component | 8   | 17  | 5      | 20    |
| Molecular Function | 13  | 11  | 6      | 18    |

Several proteins such as thiamine thiazole, late embryogenesis abundant protein lea5, and dehydrin erd10 were associated with the response to stress process in BQ (Table 2). Additionally, heat shock proteins were also found differentially expressed: HSP17.5-E, HSP17.6C, HSP26.5 and HSP22.7 more expressed in BQ and HSP70-15 more expressed in GQ. This is not the first time that proteins from the heat shock group were identified in phellem of cork oak trees [5]. Regarding the heat shock proteins more expressed in BQ, the only information available so far is that its expression confers resistance to heat stress. [16,17]. Contrarily, HSP70-15, which was more expressed in GQ, belongs to the 70-kDA heat shock proteins group, a well-known group frequently found highly expressed in tissues under stress [18]. This group of proteins is actively involved in the folding of de novo synthesized proteins, translocation of precursor proteins into organelles and degradation of damaged proteins under stress conditions [18].

During the differential gene expression analysis, genes uniquely expressed in both conditions were identified. For instance, SRG1, which is involved in oxidation-reduction processes, and PIP2-2, an important aquaporin involved in the transport of water and other small solutes across the cell membrane [19], were the only genes exclusively expressed in BQ. On the other hand, 14 genes were found uniquely expressed in GQ. It is important to highlight the presence of genes such as EMF2 and FYPP-3 that are involved in regulation of the flowering process [20,21], and KUA1, which is a transcript factor from the MYB-like protein family that acts as a repressor and promotes response to auxin, ethylene, and abscisic acid [22]. These plant growth regulators (PGRs) are involved in the regulation of plant growth and development, and abscisic acid is also associated with the increase of resistance of plants to different stresses. Likewise, EMF2 and FYPP-3 are also associated with the regulation of abscisic acid [20,23]. These results could indicate a correlation...
between hormonal regulation and cork quality; however more studies are necessary to assess its influence.

Table 2. List of genes referred to in the results and discussion section. Genes with negative values of logFC are more expressed in GQ while genes with positives values are more expressed in BQ.

| Annotation                                             | LogFC | Exclusive Expression |
|--------------------------------------------------------|-------|----------------------|
| thiamine thiazole synthase, chloroplastic (THI 1)        | 5.53  | NO                   |
| late embryogenesis abundant protein (LEA5)              | 2.46  | NO                   |
| dehydrin (ERD10)                                        | 2.56  | NO                   |
| 17.5 kda class I heat shock protein (HSP17.5-E)         | 3.73  | NO                   |
| 17.6 kda class I heat shock protein 3 (HSP17.6C)        | 2.27  | NO                   |
| 26.5 kda heat shock protein, mitochondrial (HSP26.5)     | 2.18  | NO                   |
| 22.7 kda class iv heat shock protein (HSP22.7)          | 2.57  | NO                   |
| heat shock 70 kda protein 15 (HSP70-15)                 | −7.18 | NO                   |
| Protein srg1 (SRG1)                                     | 8.21  | YES                  |
| Aquaporin PIP2.2 (PIP2-2)                               | 8.32  | YES                  |
| Polycomb group protein embryonic flower (2EMF2)         | −8.63 | YES                  |
| Phytochrome-associated serine/threonine-protein phosphatase 3 (FYPP-3) | −9.57 | YES                  |
| Transcription factor KUA1                               | −9.56 | YES                  |
| Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (AccD) | −4.31 and −4.52| NO                   |

Two genes, both annotated as acetyl-coenzyme A carboxylase carboxyl transferase (beta subunit) (AccD), were found more expressed in GQ. The AccD protein is responsible to produce malonyl-CoA from acetyl-CoA, a starting unit of the fatty acid biosynthetic process. The fatty acid pathway is responsible for very long and long-chain fatty acids synthesis, which are important precursors of waxes and some suberin monomers, two important compounds of cork. The expression of genes involved in fatty acids biosynthesis in phellogen cork tissue from GQ have already been reported [5], which reinforce the hypothesis that genes involved in the regulation and activity of cell wall assembly can affect cork quality [24].

3.2. SNPs analysis

The variant calling resulted in the identification of 1,296,640 raw variants, of which 159,248 were considered high-quality SNPs (Table 3). The high-quality SNPs were further evaluated to confirm if some of them were located in genes identified as differentially expressed. As a result, 8078 SNPs were identified in 148 DEGs from which only the exonic and non-synonymous SNPs (879) were analysed.

Table 3. Summary of the number of SNPs identified.

| Nr. of SNPs                                      |
|--------------------------------------------------|
| SNPs                                             | 1,296,640|
| SNPs filtered Q30-DP7 (High-quality SNPs)        | 159,248  |
| SNPs in DEGs                                     | 8078 (in 149 genes)|
| Exonic and Non-synonymous SNPs                   | 879 (in 124 genes)|
| SNPs in DEGs in BQ                               | 469 (in 67 genes)|
| SNPs in DEGs in GQ                               | 410 (in 40 genes)|

The identification of exclusive SNPs—an SNP is considered as exclusive if it is only present in at least 75% of the individuals from one group, GQ or BQ—was performed. Following this criterion for GQ, 121 exclusive SNPs were found in a total of 49 genes, while in BQ 68, exclusive SNPs were found among 44 genes. Regarding the SNPs found in DEGs, 18 were exclusive in 8 genes more expressed in GQ, while 5 SNPs were exclusive in 5 genes more expressed in BQ.
4. Conclusions

In this study, we identified a set of candidate genes for cork quality in *Quercus suber*. Some mechanisms associated with cork quality were revealed that allow us to hypothesis that the observed differences in cork quality could be directly related to specific PGRs increasing resistance to stress and involved in cell wall assembly. Additionally, several exclusive SNPs for individuals of contrasting phenotypes for cork quality were identified, although further studies will be needed to assess their phenotypic influence and potential usage as genetic markers for cork quality.

Author Contributions: This study was conceived by S.G. and A.R.; collection and identification of field material was performed by T.A., T.C. and S.G.; sample preparation and nucleic acid isolation were performed by T.A. and T.C.; bioinformatics data analyses were conducted by B.M. (Bruna Mendes), A.U., B.M. (Brígida Meireles) and A.R.; biological interpretation of the results was conducted by B.M. (Bruna Mendes), A.U., A.R. and L.M.; the manuscript was written by B.M. (Bruna Mendes), A.U. and L.M. All authors have read and agreed to the published version of the manuscript.

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