

# Changes in Gene Expression in Needles and Stems of *Pinus radiata* Rootstock Plants of Different Ontogenic Age

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## Abstract

A major problem in forest clonal productivity is the loss of morphogenetic capability with the increasing age of plants. However, despite of the importance of loss of morphogenetic competence, very little research has been done about the underlying mechanisms involved in this process. For this reason, a gene expression analysis using dot blot technique was performed in needles and stems of 1- and 3-year old *Pinus radiata* rootstock plants with a proved decrease in morphogenetic competence. Needles of one year old rootstock plants showed a higher number of up-regulated in genes mainly corresponding to photosynthesis and protein synthesis, degradation and modification, reflecting a higher number of active pathways in younger hedges, contrary to the older ones. Gene expression profiles found in stems are in agreement with those found in needles, indicating more active pathways in younger rootstock plants than in older ones. Several transcripts regulating transcription and translation were up-regulated in young competent tissues. Three-year-old stems presented an increase in the expression of an ethylene response factor, involved in plant organ senescence, indicating that pathways involved in senescence and ageing might inhibit the adventitious root formation, as in the older cuttings.

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## Keywords

**Plant Ageing, Gene Expression, Morphogenetic Competence, *Pinus radiata***

## 1. Introduction

In terms of annual wood production, *Pinus radiata* D. Don is one of the most important forestry species in the world, with almost 25% of the world's production. In Chile, due to its importance, rooting of woody cuttings is being used to capture the genetic gain and produce large amounts of plant material from “superior clones”, which are selected from control-pollinated orchards [1]. Unfortunately, this practice is severely affected by the age of rootstock plants; being widely known that tree maturation is one of the most important obstacles in the development of selected clones [2] [3]. Thus, in woody species, the loss of morphogenetic competence is a consequence of maturation and ageing, this implies a barrier for plant multiplication or regeneration. Therefore, upgrading forestry programs face the problem of selecting interesting traits during the mature age, while vegetative propagation is only possible during juvenile phases of development [2]. In relation to the latter, four phases of maturation have been recognized 1) embryonic phase, 2) post-embryonic juvenile vegetative phase 3) adult vegetative phase and 4) the adult reproductive phase [4]. In gymnosperms, maturation is associated with decrease growth rates during phases 2 - 4, increased plagiotropism, and changes in reproductive competence, branching characteristics and foliar morphology, with the most pronounced changes occurring between ages 1 and 4 years. This indicates that, loss of morphogenetic competence or ageing is not strictly associated to the onset of flowering, and in the specific case of *Pinaceae* these changes are abrupt, irreversible and can occur in the early stages of the plant life cycle [5] [6].

The most consistent evidence of the maturational process is the decrease in the ability of cuttings to produce adventitious roots, decreasing the rooting rates with the increase of rootstock age [7] [8]. Research in *Pinus taeda* [6] [9] in hedges from 1 to 2 year old presented a marked decrease in rooting compared with hypocotyls cuttings of 20 to 25 days old; and only after 50 days after germination in response to auxin. The same results were found in *Pinus banksiana* [10]. These results indicate that adventitious rooting decline and ageing are highly correlated processes. Although it has been suggested that this decline could be due to the loss in competence at the cellular level [11], the intrinsic mechanism behind the loss of morphogenetic competence due to ageing has not been researched in pine species. Most studies have been focused mainly in morphological traits [1] [12] and hormone action [13]-[15], but due to maturation implies a loss in morphogenetic capability, it is highly likely that this phenomenon causes changes in gene and protein expression. Some genes have been proposed as regulators of the rooting process in *Pinus contorta* hypocotyls: two S-adenosylmethionine genes (*PcSAMS1* and *PcSAMS2*) [16], a *PINHEAD/ZWILLE* gene [17], *SCARECROW-LIKE* gene [18] and a *SHORT-ROOT* gene [19]. However, little has been reported about the molecular mechanisms involved in plant ageing and in woody species.

Only one research was reported, concerning changes in gene expression in relation to loss of morphogenetic capability [20]. Combined proteomic and transcriptomic techniques described changes in mRNA and protein species that characterize the needle maturation developmental process in *Pinus radiata*. Since maturation of the complete tree is a very complex process, the first step was to study individual organs. Because needles are the main source employed for tree cloning and *in vitro* micropropagation, they are ideally suited to study maturation. According to the above, 176 genes obtained from a SSH library were differentially expressed between *P. radiata* immature (competent tissues) and mature needles (incompetent tissues), and may explain differences in morphogenetic competence. Mature needles were characterized by an overexpression of energy-related and photosynthetic pathways, while immature needles overexpressed genes related to stress and defense mechanisms.. Based on these results, we evaluated changes in gene expression in needles and stems of *P. radiata* woody cuttings, of ages typically used as rootstock plants in forest nurseries. Additionally, we also evaluated rooting rates and anatomy. We hypothesized that genes differentially expressed through needle maturation can be altered due to ontogenic ageing in needles and stems of 1 and 3-year-old *P. radiata* rootstock plants. To prove this hypothesis, we select 176 genes that cover different areas of metabolism, which, were tested in needles and stems of 1 and 3-year-old *P. radiata* rootstock plants with decrease in morphogenetic capability. This research represents the first approach describing the main metabolic pathways involved in *P. radiata* ontogenic ageing.

## 2. Materials and Methods

### 2.1. Plant Material

Plant material was obtained from Proplantas Nursery S.A located in Bio-Bio region in central Chile, with coordinates, 36°37'25.87"S and 72°21'23.80"W. Plant material corresponds to rootstock plants of 1 and 3-year-old from the same full-sib family, and cultivated under the same conditions of fertilization and watering. In brief, the collection of plant material was performed in August of 2012, 250 mg of fresh material from needles and the base of the stem cuttings were collected with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until needed for RNA extraction.

For the stem cutting rooting procedure, the cuttings were rooted on the nursery of the Forestry Science Faculty of the Universidad de Concepción according to Proplantas S.A. nursery protocol for container rooting. In brief: cuttings were washed with a 0.5 g/l benomilo solution to avoid fungal contamination. Then, cuttings were placed in containers type Arauco (88 cavities, 130 cm<sup>3</sup>) with pine bark compost as substrate. Cuttings were irrigated three times a day to maintain foliage and substrate wet at field capacity. Rooting percentage was evaluated until younger cuttings reached 100% of rooting.

### 2.2. Needle and Stem Anatomy

Fully developed needles and the basal portion of 1 and 3-year-old *P. radiata* cuttings were analyzed microscopically using OLYMPUS CX31. Plant material was previously fixed in formaldehyde, acetic acid and ethanol (FAA), for 72 h and preserved in 70% ethanol (v/v). Transverse sections of 10  $\mu\text{m}$  were obtained using a microtome. The sections were stained with safranin-fast green and mounted in water-glycerol for microscopic observation [21]. Photographs were analyzed using Image J software. In needles, the width of epidermis, hypodermis and epidermis, percentage of vascular cylinder and resin canal diameter were measured. In stems, the diameter of whole stem, xylem and phloem and periderms width were measured.

### 2.3. RNA Extraction and cDNA Synthesis

RNA extraction was performed following a CTAB protocol [22]. Total RNA was purified with the RNeasy Clean Up kit from Qiagen [23]. Purified RNA was diluted with RNase-free water and stored at  $-80^{\circ}\text{C}$  until use. One  $\mu\text{g}$  of total RNA was reverse transcribed with Superscript III first strand synthesis kit (Invitrogen) to obtain target cDNA, which was marked with digoxigenin (Roche) and Terminal Transferase kit (Roche).

### 2.4. Probes Synthesis for Dot Blot

Differential gene expression was assessed using dot blot technique with samples of plant material described above. Probes correspond to candidate genes associated to *P. radiata* needle maturation and loss of morphogenetic competence, which were previously obtained using a suppressive subtractive (SSH) library [20]. The probes correspond to a 176 cDNA (supplementary material), belonging to the following functional categories obtained according to KEGG: photosynthesis, mitochondria (electron transfer), carbohydrates and carbon metabolism, cell wall, lipid metabolism, nitrogen and aminoacid metabolism, transcription and DNA replication, protein translation folding modification and degradation, secondary metabolism cytoskeleton, stress and defense related proteins, signaling, transport and cell cycle and morphogenesis. cDNA was PCR amplified directly from *E. coli* clones employing SP6 (primer sequence: ATTTAGGTGACACTATAG) and T7 primers (primer sequence: TAATACGACTCACTATAGGG) (PCR program:  $94^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 70 s) and a final extension of 7 min at  $72^{\circ}\text{C}$ . Resultant PCR products were mixed with 1.5 M NaOH, 300 mM EDTA pH 8.2 to reach a final concentration of 400 mM NaOH, 80 mM EDTA. Three  $\mu\text{L}$  of each cDNA clone were spotted into a positively charged nylon Hybond N+ membrane (GE healthcare).

### 2.5. Dot Blot Procedure

Membranes were pre-hybridized with Church buffer (0.5 M phosphate buffer, 10 mM EDTA, 7% SDS and 1% BSA) for an hour. Target cDNA was added to a fresh buffer to reach a final concentration of 50 ng/ml and hybridized over night at  $55^{\circ}\text{C}$ . After three consecutive washes in  $2\times$  SSC-0.1% SDS,  $2\times$  SSC-0.1% SDS and  $1\times$  SSC-0.1% SDS, the membranes were treated with CSPD ready to use (Roche) chemiluminescence subtract ac-

cording to the manufacturer instructions. Blots were imaged exposing them to a CL-Xposure film (Thermo Scientific) for 30 minutes. Images were analyzed using a dot blot analyzer for Image J. Global normalization was adopted for normalizing the difference of signal intensity of each nylon filter: the intensity of all the spots on the filter was averaged and the relative signal intensity was calculated as the ratio of each signal to the average intensity of each filter.

## 2.6. Quantitative RT-PCR (qRT-PCR)

To validate results found with dot blot, five genes that might be related to ageing and that were differentially expressed were selected for qRT-PCR. The qRT-PCR was performed as follows: extraction of RNA, purification, quantification and cDNA synthesis (as previously described). The primer pairs were design with Primer3Plus software. qRT-PCR was performed with 300 nM of each forward and reverse primer and 12.5 µL of 2X Power SYBR Green PCR master mix (Applied Biosystems) to reach a final concentration of 1×, according to the manufacturer instructions. The PCR reaction was performed in a Step-One Plus real-time PCR system (Applied Biosystems). Polymerase chain reaction efficiency was estimated by template dilutions and the equation  $E = 10^{(-1/\text{slope})}$  [24]. Five biological replicates and three technical replicates were used. Specific primers are listed in Table 1; the ubiquitin gene was used as internal control. Expression ration were obtained using the method of  $2^{-\Delta\Delta C_t}$ .

## 2.7. Statistical Design and Analysis

After global normalization (as described above) a Student's *t* test was carried out to compare between 1-and 3-year-old rootstock plants, results were considered to be statistically significant a  $P < 0.05$ . Gene expression of needles and stems were evaluated by separate. The TTEST procedure was used for Student's *t* test with SAS statistical software. The same approach was used to compare for histological measurements and morphological competence assessment. Clustering analysis was done using the cluster 3.0 software and Java Tree View software for cluster visualization; the gene tree was created using the average linkage clustering method (Pearson correlation).

## 3. Results

### 3.1. Effect of Rootstock Age on Morphogenetic Capability, Needle and Stem Anatomy

The rooting capability of cuttings from 1 and 3 year old rootstock plants was evaluated to assess the morphogenetic capability of rootstock plants. After two months from the beginning of the rooting procedure almost 100% of 1-year-old cuttings had root primordium, while in 3-year-old cuttings only 20% showed this response (Table 2). Cuttings from 1-year-old rootstock plants formed a thickening at the base of the cutting within 15 days from the initiation of the rooting process, at this time point it is possible distinguish the initial formation of root primordium. Cuttings from 3-year-old rootstock plants presented the same response at 30 days from the initiation of the rooting process.

**Table 1.** List of primers used in quantitative real time PCR (qRT-PCR), forward and reverse primer sequence are written in direction 5' to 3'. Gene name, NCBI accession number and primers melting temperatures are described.

Gene name	NCBI accession number	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	Tm (°C)
<i>CAB</i>	GO096311	AGAGCTGTTGACTAAGTTGGG	AGATTGGATCGGTGTTGACTG	54
<i>DI19</i>	GO096128	ATAGATGCCCATGCTGTGTAG	CTTCCCTCTGTTCCCACTTG	54
<i>CAOMT</i>	GO096097	CTTCGTCTCTGTCAGGTGTG	TACAGTCCTCATCGTCCAG	56
<i>TRPS</i>	GO096137	AGATGGAGGACCACAGATTT	CCGATGTGTTTAGCAAGAAG	56
<i>SPD</i>	GO096159	GCAATAAGTTGAGGCGATCT	CAAACCATCCAGGAATAACA	55

Abbreviations: CAB—Chlorophyll a/b binding protein; DI19—Dehydration induced protein 19; CAOMT—Caffeic acid O-methyltransferase; TRPS—Tryptophan synthase  $\beta$  subunit; SPD—Spermidine synthase.

**Table 2.** Anatomical characteristics of needles and stems of 1 and 3-year-old *Pinus radiata* rootstock plants, and cuttings rooting capability after 2 months. Values  $\pm$  standard deviation, different letters correspond to significant differences according to Student's *t* test for  $P < 0.05$ .

Rootstock plant age		
	1-year-old	3-year-old
Needles anatomical characteristics		
Mesophyll width ( $\mu\text{m}$ )	181.3 $\pm$ 20.7 (a)	76.6 $\pm$ 7.6 (b)
Epidermis width ( $\mu\text{m}$ )	18.6 $\pm$ 1.9 (a)	16.2 $\pm$ 2.3 (b)
Hypodermis width ( $\mu\text{m}$ )	0.03 $\pm$ 0.04 (b)	15.7 $\pm$ 1.9 (a)
% Vascular cylinder	14.5 $\pm$ 0.5 (b)	41.6 $\pm$ 0.4 (a)
Resin canal ( $\mu\text{m}^2$ )	2013.0 $\pm$ 235.4 (b)	2741.7 $\pm$ 763.6 (a)
Stems anatomical characteristics		
Total diameter ( $\mu\text{m}$ )	2355.6 $\pm$ 209.2 (b)	3717.5 $\pm$ 170.7 (a)
Xylem diameter ( $\mu\text{m}$ )	461.7 $\pm$ 74.2 (b)	811.4 $\pm$ 13.4 (a)
Phloem diameter ( $\mu\text{m}$ )	119.6 $\pm$ 10.2 (a)	149.1 $\pm$ 12.0 (a)
Periderm width ( $\mu\text{m}$ )	81.5 $\pm$ 8.5 (b)	117.5 $\pm$ 1.3 (a)
Rooting capability		
% of rooting	96.66 $\pm$ 1.44 (a)	18.81 $\pm$ 0.98 (b)

One-year-old needles (**Figure 1(a)**) presented less developed tissues, showing only one vascular bundle while 3-year-old needles (**Figure 1(b)**) presented two vascular bundles. Moreover, needles from 3-year-old rootstock plants formed a hypodermis, unlike 1-year-old that lacked this formation. On the other hand, younger rootstock plants showed higher values of mesophyll and epidermis width (**Table 2**) and lower values of hypodermis width and percentage of vascular cylinder (**Table 2**). In the same way stem tissues were significantly more developed in older rootstock plants; total diameter, xylem and cortex widths were greater in 3-year-old stems compared to younger 1-year old stems (**Figure 1(c)** and **Figure 1(d)**, **Table 2**).

### 3.2. Effect of Rootstock Plant Age on *Pinus Radiata* Needle Gene Expression

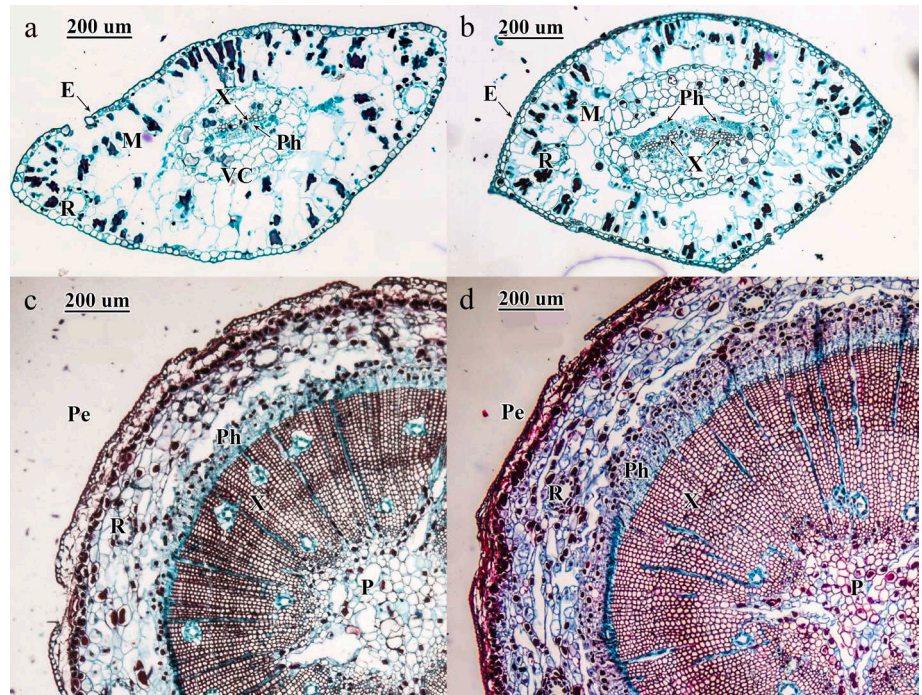
Changes in gene accumulation patterns with rootstock plants ageing was evaluated, by comparing 1 and 3-year-old of *P. radiata* needles. From the 176 tested cDNAs, 36 were differentially expressed between both ages, from which 32 increase their accumulation in needles from 1-year-old rootstock, and 4 increased their accumulation in 3-year-old (**Figure 2**). Cluster analysis generated two clearly differentiated clusters; differentiating genes with an increase in gene expression in three year old cuttings in the minor cluster and genes up-regulated in younger cuttings in the mayor cluster.

The four accumulated genes in older cutting needles belong to several functional categories such as: stress and defense, carbohydrate metabolism, signaling and transport. Genes related to photosynthesis were mainly up-regulated in 1-year-old cuttings such as: type II chlorophyll A-B binding protein precursor, Rubisco small subunit, photosystem II D2 (*PSBD*), photosystem II CP43 (*PSBC*) proteins, and photosystem II core complex *PSBY* protein. Also, this tissue presented an increase in expression of genes related to transcription and DNA replication such as the transcription elongation factor 1-alpha.

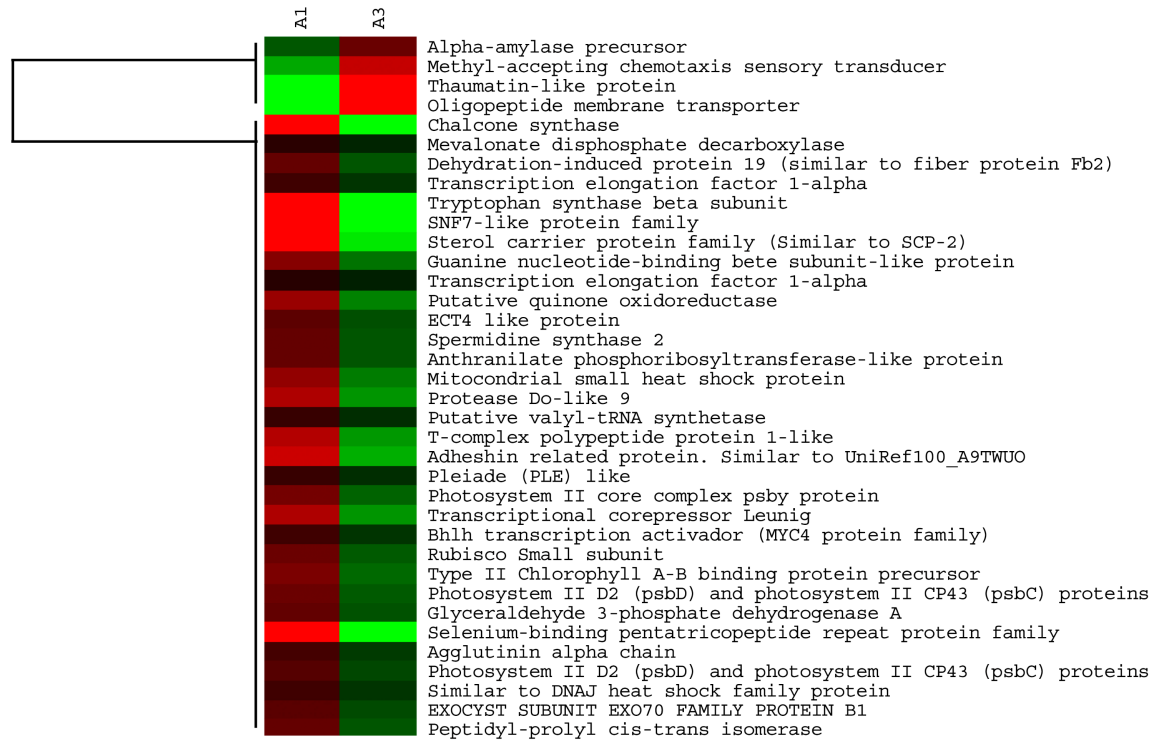
### 3.3. Effects of Rootstock Plant Age on *Pinus Radiata* Stem Gene Expression Patterns

Changes in gene expression patterns were assessed in the stem of *P. radiata* cuttings from 1 and 3-year-old rootstock plants. From the 176 cDNAs tested only 30 were differentially expressed between both types of rootstock plants; similar to the results from pine needles, the majority of genes presented increased gene expression in younger cuttings. Out of the 30 differentially expressed genes, 22 presented higher accumulation in 1-year-old





**Figure 1.** Cross section of needles and cuttings of *Pinus radiata* rootstock plants. Needles from 1-year-old; (a) and 3-year-old; (b) rootstock plants, and cross section of cuttings of stems from 1-year-old; (c) and 3 year-old; (d) rootstock plants. M: mesophyll, E: epidermis, R: resin canal, X: xylem, Ph: phloem and VC: vascular cylinder, P: pith, Pe: periderm. Bar represent 200  $\mu$ m. Figures were obtained with a 10 $\times$  magnification.

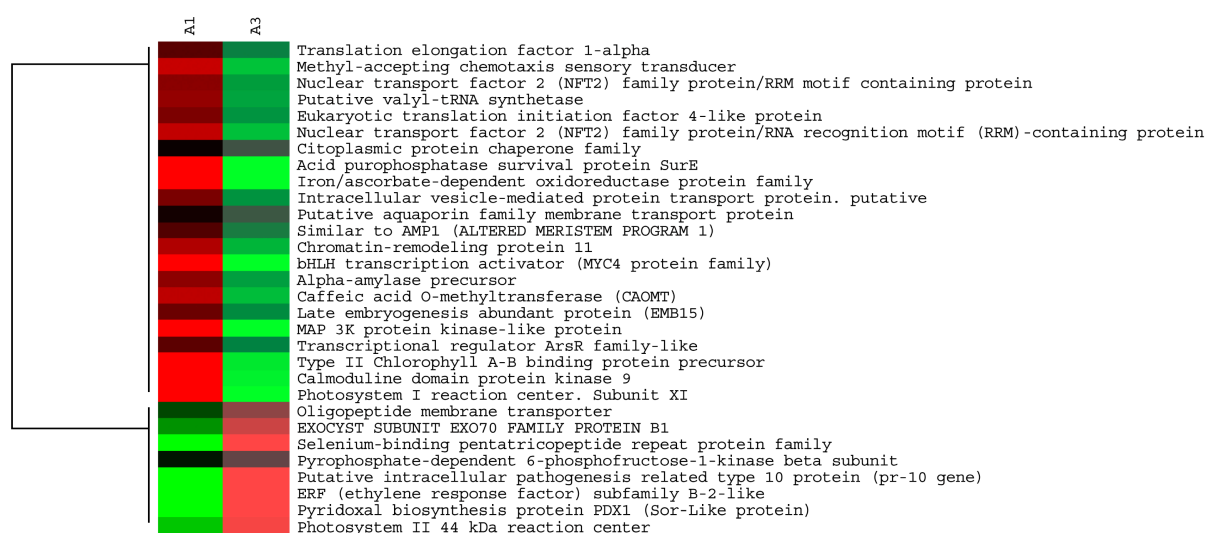


**Figure 2.** Hierarchical clustering of 36 genes differentially expressed between needles of 1 and 3-year-old *Pinus radiata* rootstock plants. The gene tree was created using the average linkage clustering method (Pearson correlation). Red and green represents up and down gene expressions, respectively. A1: 1-year-old rootstock plants, A3: 3-year-old rootstock plants.

*P. radiata* stems, while only 8 increase their accumulation in the 3-year-old (Figure 3). Also, the cluster analysis generated two clearly differentiated clusters according to changes in accumulation between both rootstock ages. The first cluster includes genes that have higher mRNA levels in younger stems, while the second cluster presents genes with higher expression in older stems.

The eight genes with higher accumulation in 3-year-old plants belong to several functional categories such as: carbon metabolism, secondary metabolism, stress and defense. Among these genes there are two genes belonging to the category of transport like oligopeptide membrane transporter and exocyst subunit *EXO70* family protein B1. On the other hand, genes with higher gene expression in 1-year-old stems have functions related to protein folding, modification and degradation such as: translation elongation factor 1-alpha, putative valyl-tRNA synthetase and eukaryotic initiation factor 4-like protein. Transcripts involved in transcription and DNA replication are also represented among genes up-regulated in 1-year-old stems.

Gene expression results are in agreement with genes tested through real time qRT-PCR (Table 3), the type II chlorophyll a/b binding protein, spermidine synthase and tryptophan synthase  $\beta$ subunit presented the same tendency in dot-blot and qRT-PCR measurements. Caffeic acid-O-methyltransferase did not show a significant change in neither dot blot nor qRT-PCR for needle results. Also, the *DI19* gene presented a significant increase in needles and stems from 1-year-old rootstock plants, in qRT-PCR. On the other hand, the *DI19* gene did not



**Figure 3.** Hierarchical clustering of 30 genes differentially expressed in stems of 1 and 3-year-old rootstock of *Pinus radiata* plants. A gene tree was created using the average linkage clustering method (Pearson correlation). Red and green represents up and down gene expressions, respectively. A1: 1-year-old rootstock plants; A3: 3-year-old rootstock plants.

**Table 3.** Quality control of dot-blot experiments. Fold  $\pm$  SE change differences of five different cDNAs were confirmed by real time qRT-PCR. Gene name is indicated. Negative and positive ratios indicate decreased and increased mRNA accumulation respectively, results are shown relative to 1 year-old rootstock plants. Fold changes were considered significant over 2-fold.

Gene name	Fold change $\pm$ SE	
	Needles	Stems
<i>CAB</i>	+2.78 $\pm$ 0.260	+2.65 $\pm$ 0.634
<i>SPD</i>	+6.56 $\pm$ 0.392	+1.92 $\pm$ 0.127
<i>TRPS</i>	+2.73 $\pm$ 0.284	+1.85 $\pm$ 0.086
<i>DI19</i>	+3.11 $\pm$ 0.360	+4.43 $\pm$ 0.222
<i>CAOMT</i>	+1.61 $\pm$ 0.286	+3.16 $\pm$ 0.149

Abbreviations: CAB—Chlorophyll a/b binding protein; DI19—Dehydration induced protein 19; CAOMT—Caffeic acid O-methyltransferase; TRPS—Tryptophan synthase  $\beta$  subunit; SPD—Spermidine synthase.

showed significant differences on dot blot results. However, in dot blot this gene presented a tendency to an increase accumulation in younger needles and stems but without statistical significance. Thus, it is well known that hybridization techniques, as in the case of dot blot, tend to underestimate qRT-PCR values.

#### 4. Discussion

Changes in gene expression between *P. radiata* rootstock plants of 1 and 3-years-old with differences in their morphogenetic capability were evaluated. Our results showed that rootstock plant age induces changes on rooting capability and in needles and stems anatomy. Quicker and earlier response in younger 1-year-old cuttings and higher rooting rates, are in agreement with several researches that indicate that capability for rooting is lost between 3 and 5 year old in gymnosperms [6] [10] [11] [25] [26]. Anatomical changes observed in both types of needles are in agreement with reports in Douglas-fir and *P. halepensis* [27] [28], indicating anatomical differences between young and old needles. Needles from younger trees presented a smaller vascular cylinder and increased mesophyll area. These anatomical characteristics are often related with decrease in photosynthesis observed in increased-age individuals [29]. In this regard, changes in mRNA levels in genes related to photosynthesis were also found between both types of needles. Genes such as: type II chlorophyll A-B binding protein (*CAB*) precursor, photosystem II D2 (*PSBD*) and photosystem II CP43 (*PSBC*) proteins, photosystem II core complex PSBY protein, RuBisCO small subunit (*RBCS*) and glyceraldehyde 3-phosphate dehydrogenase A, decreased their accumulation in 3-year-old needles. This is in agreement with reports indicating that photosynthesis parameters are reduced with the increasing age of shrubs and trees [29]–[31]. At mRNA level, the *CAB* gene mRNAs are accumulated in juvenile needles of larch and young leaves of *Arabidopsis* [32] [33]. Additionally, *RBCS* gene is highly regulated at transcriptional level during developmental processes and differentiation in tomato [34] [35]. On the contrary, genes related to photosynthesis as the ones mentioned above, presented an opposite tendency they were up-regulated in mature needles and down-regulated in immature ones [20], indicating that these genes reveal differential gene expression for needle maturation process and needle ontogenic ageing.

According to the analysis of differentially expressed genes, we found that from 176 genes tested only 36 were differentially expressed between 1 and 3-year-old rootstock plants. Most of the age-regulated genes (32) were accumulated in the younger rootstock needles. Also, changes in gene expression between stems from juvenile 1-year-old and 3-year-old rootstock plants were determined. Similar to the results in needles, 1-year old stems showed 22 highly accumulated genes against the 8 accumulated transcripts in older stems. These results are in agreement with a previous study [20], where most of genes and protein evaluated were overexpressed/accumulated in immature needles, (Figure 2 and Figure 3), and two main branches can be distinguished separating 1 year-old rootstock plants from the 3-year-old ones.

Among differences determined, our results showed an increase in spermidine synthase (*SPD*) transcript abundance in needles of 1-year-old rootstock plants. Transcripts of this gene are more accumulated in younger than older apple leaves, similar to the results obtained in alfalfa for enzymatic activity levels [36]–[38]. Polyamine levels, such as spermidine, spermine and putrescine, have been reported as a physiological marker of ageing, maturation, and loss of morphogenetic capability [39] [40]. Tryptophan synthase b subunit (*TRPS*) gene, responsible for the synthesis of the amino acid tryptophan whose biosynthetic pathway produces the hormone indole-3-acetic acid (IAA) [41], had also greater accumulation in needles from 1-year-old rootstock plants. In this regard, an increase in IAA contents in younger needles and a decrease of this hormone during changes from juvenile to adult vegetative phases occur in *P. radiata* and *P. pinea* [14] [42]. This agrees with the higher levels of the anthranilate-phosphoribosyl transferase transcript determined in 1 year-old-needles, enzyme that is also part of tryptophan biosynthetic pathway [43].

Several genes involved in transcription activation and translation regulation were accumulated in needles and stems from younger 1-year-old rootstock plants. In needles, the transcriptional corepressor leunig, two-transcription elongation factor 1-alpha, a putative valyl-tRNA synthase were modified. Also, a *bHLH* transcriptional activator (*MYC4*) presented increased transcript levels in needles and stems from 1-year-old rootstock plants. This transcription factor has been found associated to multiple cellular differentiation pathways in many plant species indicating that the *bHLH* transcription factor is accumulated in immature needles with high morphogenetic potential [20] [44]. According to our results, transcripts related with translation were accumulated in stems from 1-year-old rootstock plants, a translation elongation factor 1-alpha, putative valyl tRNA synthase and eukaryotic translation initiation factor 4-like proteins are among these. Two nuclear transport factor 2 (*NTF2*) family



protein/RNA recognition motifs (*RRM*) presented higher transcript levels in 1-year old stems. *NTF2* is a protein required for efficient import of nuclear protein in to the nucleus [45]. Additionally, *RRM* are involved in most post-transcriptional gene expression processes (*i.e.* mRNA and rRNA processing, RNA export and stability) [46], indicating that younger stems would be highly regulated and active at protein synthesis and transport levels.

Ethylene response factor (*ERF*) presented higher levels of mRNA in stems from 3-year-old rootstock plants. The *ERFs* are transcription factors that regulate the expression of several downstream target genes by binding to *cis*-regulatory elements in their promoters [47], and act in the ethylene perception and signal transmission cascade [48]-[50]. *ERFs* has been induced at transcriptional level by ethylene treatment in hybrid aspen (*Populus tremula* x *Populus tremuloides*), indicating that the presence of this hormone can induce an increase in the transcription of *ERFs* [51], as occurred in 3-year-old stems. Ethylene act in response to a diverse group of stimuli, especially organ senescence [52] [53], this could be related to the increased accumulation of an *ERF* in older *P. radiata* stems. However, ethylene and *ERFs* are also involved in wood formation process and lignin deposition [54] and the application of exogenous ethylene can enhance xylem growth [51] [55]. This is in agreement with the results observed regarding to anatomical changes between juvenile and aged *P. radiata* stems. An increase in the presence of xylem and periderms, indicative of secondary growth, was found in stems of 3-year-old rootstock plants.

Finally, a caffeic acid O-methyltransferase (*CAOMT*) gene, implicated in monolignols synthesis in gymnosperms [56] [57], was accumulated in 1-year-old stems, indicating that lignin biosynthesis may be more active in juvenile tissues. Also, this gene is as well implicated in plant stress response [57] [58], and these results are in agreement with Valledor et al. (2010) indicating that younger plants increase the expression of gene related to stress responses, which may be related to higher growth rate and fragility against biotic and abiotic stresses.

For non-model species like *P. radiata*, for which most of the high throughput technologies, such as microarray and next generation sequencing, and a reference genome were not available until recently, there is an increase in the usefulness of the dot blot technique used in this research. Dot blot allowed the customized expression analysis of genes of interest during the process of ageing, a poorly described process. We were able to perform the first screening analysis dealing with the main metabolic pathways involved in *P. radiata* ontogenic ageing.

## 5. Conclusion

These analyses clearly showed a differentiation between juvenile and aged rootstock plants, both in needles and stems. Thus, juvenile rootstock plants were characterized by an increase in the expression of photosynthesis genes, and genes related to activation of transcription and translation. On the contrary, aged 3-year-old rootstock plants were characterized by a low amount of genes accumulated on these tissues, and the overexpression of a senescence related gene. Although quantification of gene expression is the first step to assign gene function, additional experimental work is needed to support the conclusions of this paper. And though we indicate that there are interesting gene patterns that can be associated to plant ageing and loss of morphogenetic competence, experiments are currently being undertaken in our lab exploring this hypothesis.

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