# Gene expression in relation to growth traits in *Pinus pinaster* Ait.

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## Introduction and Objectives

Selection of elite tree individuals for superior biomass production is labour —and time— consuming. Phenotypic traits as plant diameter and height are regulated by a number of genes, which limits the application of biotechnological tools to the selection of elite genotypes. Understanding the processes of plant growth through the study of the gene expression patterns can contribute to the improvement of plant production.

The aim of this study was the identification of candidate genes relative to differential in diameter and height growth phenotypes in *Pinus pinaster* Ait.

To achieve this goal, we analyzed the relative expression level of 12 genes potentially related to growth in height and diameter using quantitative PCR (qPCR). These genes were previously selected after comparative transcriptomic analysis among individuals showing contrasted phenotypes.

## Material and Methods

Adult vegetatively propagated plants of *Pinus pinaster* Ait. were used as plant material. The plants were grouped into 5 morphotypes according to their annual growth in height and diameter: Group 1 (high diameter, high height); group 2 (high diameter, low height); group 3 (low diameter, high height); group 4 (low diameter, low height); control group (intermediate diameter and height). From each group, 3 representative plants were selected, and fully developed needles were collected from them for expression analysis.

Total RNA was extracted from 40 mg of needles grinded under liquid nitrogen following the protocol of Yang *et al.* (2008), followed by genomic DNA digestion using RQ1 RNase-Free DNase (Promega) for 30 min at room temperature. RNA was purified using the NucleoSpin® Gel PCR Clean-up kit (Macherey-Nagel) following the manufacturer's instructions. cDNA was obtained from 0.7 µg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad) also following the manufacturer's instructions.

The quantitative PCR (qPCR) reactions were carried out on an iCycler iQ Real-Time thermal cycler (Bio-Rad). Reactions were performed in a 10 µL volume containing 5 µL of 2x reaction mix (SsoFastTM EvaGreen Supermix, Bio-Rad), 400 µM of each primer and 7 ng of cDNA. A total of 12 different genes (Table 1) were analysed using Actin and Ubiquitin as the reference genes for relative expression calculations. The PCR efficiency for each primer pairs were calculated using the LingRegPCR (Ruijter *et al.*, 2009) software, and the relative expression of each gene was determined using the REST-2009© software (Pfaffl *et al.*, 2002). The expression values were transformed using a base-2 logarithm in order to obtain a symmetric scale.

| Gene | Morphotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 (High diameter, High height) | | 2 (High diameter, Low height) | | 3 (Low diameter, High height) | | 4 (Low diameter, Low height) | |
| | Relative expression | P value | Relative expression | P value | Relative expression | P value | Relative expression | P value |
| **PAT** | 0,729 ± 0,45 | 0,152 | 0,924 ± 0,10 | 0,045 | 1,256 ± 0,09 | 0,002 | 1,265 ± 0,13 | 0,003 |
| **AGT** | -0,804 ± 0,18 | 0,028 | -0,447 ± 0,05 | 0,178 | -1,268 ± 0,27 | 0,000 | -0,678 ± 0,01 | 0,025 |
| **AMP1** | -1,648 ± 0,00 | 0,081 | -0,599 ± 0,00 | 0,525 | -2,458 ± 0,00 | 0,028 | 0,324 ± 0,00 | 0,803 |
| **C4H** | 1,651 ± 0,10 | 0,006 | 0,866 ± 0,15 | 0,013 | 1,264 ± 0,16 | 0,012 | 2,126 ± 0,30 | 0,001 |
| **Chalcone synthase** | 0,059 ± 0,01 | 0,905 | - 0,705 ± 0,22 | 0,027 | 0,310 ± 0,31 | 0,433 | -0,094 ± 0,01 | 0,838 |
| **DAHP** | 3,633 ± 0,01 | 0,000 | 2,562 ± 0,35 | 0,001 | 2,733 ± 0,11 | 0,003 | 1,728 ± 0,00 | 0,015 |
| **GS1a** | 0,247 ± 0,07 | 0,543 | -0,065 ± 0,43 | 0,913 | 0,187 ± 0,21 | 0,668 | 0,355 ± 0,19 | 0,390 |
| **GS1b** | -0,236 ± 0,03 | 0,466 | 0,252 ± 0,39 | 0,551 | -0,501 ± 0,41 | 0,114 | -0,835 ± 0,27 | 0,007 |
| **NADP-IDH** | 1,327 ± 0,09 | 0,005 | 1,502 ± 0,37 | 0,009 | 1,365 ± 0,18 | 0,003 | 1,429 ± 0,02 | 0,000 |
| **PAL1** | 3,428 ± 0,34 | 0,001 | 2,143 ± 0,24 | 0,011 | 3,515 ± 016 | 0,002 | 4,199 ± 0,08 | 0,000 |
| **Sucrose synthase** | 2,861 ± 0,14 | 0,005 | 0,239 ± 0,07 | 0,674 | 2,122 ± 0,06 | 0,013 | 2,279 ± 0,01 | 0,018 |
| **Myb8** | 0,522 ± 0,00 | 0,560 | -1,400 ± 0,00 | 0,077 | 1,735 ± 0,00 | 0,128 | 1,578 ± 0,00 | 0,072 |

Table 1. Values of relative expression for the 12 analyzed genes in each of the four morphotypes in respect to the control group (intermediate diameter and height). Values represent the means of two independent experiments ± S.E., with the significance value. In red color, data statistically significant ($p < 0.05$) are presented.

## Results

The relative expression analysis (Table 1) of the 12 tested genes showed that from them, 10 genes presented significant differences in respect to the control group (intermediate diameter and height) in at least any of the 4 morphotypes. Most relevant results were that of the DAHP and PAT genes (Figure 1).

DAHP gene encodes the enzyme responsible for the first step in the shikimate pathway (Figure 2), initial step for the synthesis of the aromatic amino acids Tyrosine, Tryptophan and Phenylalanine. These amino acids are precursors of molecules such as phytohormones, anthocyanins, antimicrobial compounds and lignin. About 20% of the carbon fixed through the photosynthesis is estimated to be channeled through the shikimate pathway. The other enzyme, encoded by the PAT gene connects the shikimate pathway with the metabolism of the Phenylalanine (Figure 2), where the phenylpropanoid skeleton needed for the synthesis of lignin is generated.

DAHP showed a decreasing expression pattern (Figure 1A), in which the highest level of expression in respect to the control was obtained within the morphotype 1 (high diameter and high height), and the lowest level of expression within the morphotype 4 (low diameter and low height). Intermediate expression levels of DAHP were obtained for the morphotypes 2 and 3 (see description in Materials and Methods). High DAHP gene expression values suggest a high flow through the shikimate pathway, maybe generating substrate for the synthesis of structural compounds as lignin, quantitatively abundant in tall trees.

PAT showed the opposite expression pattern (Figure 1B) compare to DAHP, with the lowest expression levels for the morphotype 1, the highest for the morphotype 4, and again with intermediate expression values for the morphotypes 2 and 3. The lack of correlation with DAHP level suggests that not all the shikimate produced is channeled to lignin biosynthesis in the tree, or even an inhibition can take place due to substrates generated in excess.

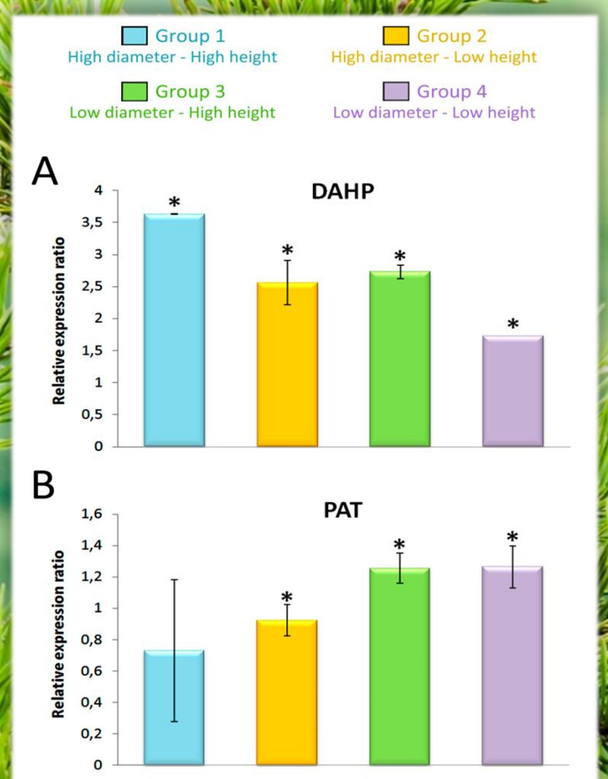


Figure 1. Relative expression of the genes DAHP (A) and PAT (B) in the four morphotypes in respect to the control group (intermediate diameter and height). Values represent the means of two independent experiments ± S.E. Asteriscs indicate significant differences ($p < 0.05$) between the control group and each of the morphotypes analyzed.

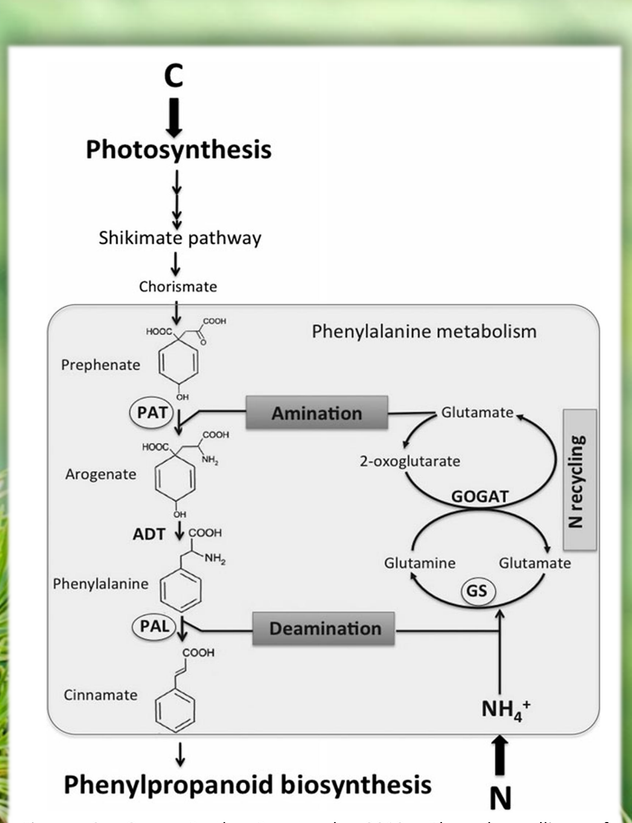


Figure 2. Craven-Bartle B *et al.*, 2013. The channelling of photosynthetic carbon for phenylpropanoid biosynthesis and the associated nitrogen metabolism. The central role of the phenylalanine metabolism is highlighted. Critical steps in the pathway are indicated, including amination, deamination and nitrogen recycling. The enzymes PAT, PAL and GS1b are circled.

## References

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