

Developmental Effects in Wood Formation on Transcripts' Accumulation in Maritime Pine

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Summary. Wood (secondary xylem) provides both mechanical strength and long-distance transport for water, nutrients and hormones. Despite the simplicity of secondary xylem structure (basically tracheids and rays), conifers' wood is a highly variable material in terms of anatomical, chemical and technological characteristics. To study the effect of the environmental and developmental factors on the accumulation of transcripts and proteins in wood-forming tissues of maritime pine, differentiating xylem tissue samples were collected along a cambial age gradient (samples taken every 3 years' internodes from base to top on a 30-years old adult tree). A genomic approach combining large-scale EST and protein sequencing, expression profiling (high density filters macroarray) and statistical analyses was undertaken to identify candidate genes for wood properties. Functional validation of some of these candidate genes is ongoing using association studies. This work provides us with new insights into both the identity of genes involved in the variability of wood characteristics and their possible underlying regulation mechanisms.

Resumo. O xilema secundário das lenhosas assegura quer suporte mecânico quer o transporte a longa distância de água, nutrientes e hormonas. Apesar da sua simplicidade (basicamente constituído por traqueídeos e raios), a madeira das coníferas apresenta características muito variáveis em termos das suas características anatómicas, químicas e tecnológicas. Para estudar o efeito ontogénico (idade do cambio secundário) na acumulação dos transcritos e proteínas durante a formação do lenho do pinheiro bravo, foram recolhidas amostras em diferenciação ao longo de um gradiente de idade cambial (5 amostras recolhidas da base ao topo de uma árvore com mais de trinta anos).

Utilizou-se uma metodologia que incorpora diferentes ferramentas do campo da genómica, combinando a sequenciação em larga escala de uma biblioteca de cDNA de xilema em diferenciação de pinheiro bravo (ESTs, etiquetas de sequências de transcritos), a obtenção de perfis de expressão (filtros de alta densidade), e a análise estatística dos dados obtidos. A validação funcional dos genes candidatos está decorrer através de estudos de associação. O trabalho desenvolvido possibilitou adquirir novos conhecimentos quer dos genes e quer dos possíveis mecanismos de regulação envolvidos na formação do lenho.

1. Introduction

Wood is one of our most important natural resources, and has been exploited for many hundreds of years as fuel, building material and source of paper.

Wood properties are known to vary between species and among genotypes within species (Zobel and Buijten, 1989), and are heritable (Pot et al., 2002). Wood formation (xylogenesis) is controlled by a wide variety of factors both exogenous (photoperiod and temperature) and endogenous (phytohormones), and by interaction between them. Wood (secondary xylem) is formed from the terminal differentiation of vascular cambium derived cells (reviewed by Catesson et al., 1994). It is driven by the coordinated expression of numerous structural genes involved in cell division, cell elongation, cell wall thickening, and programmed cell death (reviewed by Mellerowicz et al., 2001 and Plomion et al., 2001).

Despite numerous studies about differentiation of vascular cambium in plant models, in particular *Arabidopsis* (Ko & Han, 2004) or *Zinnia* (Demura et al., 2002; Milioni et al., 2002), little is known about the molecular mechanisms involved in process of differentiation of vascular cambium in trees. Plant models like *Arabidopsis* present some disadvantages to study wood formation like: a) small size, that makes difficult to obtain secondary-xylem samples with high resolution; b) annual growth habit that prevents further studies of xylem differentiation's seasonal variation, dormancy and cambial aging process. We also need to test the information generated in the model in our species of interest.

Wood formation would indeed better be understood using directly real trees (Taylor 2002; Lev-Yadun and Sederoff, 2000). Species from *Populus* sp. and *Pinus* sp. (in particular *Pinus taeda*) are being currently used as model plants to study wood formation. Genomic approaches including large-scale EST sequencing (Allona et al., 1989; Sterky et al., 1998, 2004, Dejardin et al., 2004, Whetten et al., 2001, Le Provost et al., 2003, Canton et al., 2004), analyses of gene expression of transcripts (Whetten et al., 2001; Hertzberg et al., 2001, Le Provost et al., 2003) or proteins (Gion et al., 2004), have been recently used to unravel wood formation. These studies allowed significant progress in the understanding of gene expression and signalling mechanisms during secondary wall differentiation in trees.

Maritime pine (*Pinus pinaster* Ait.) is an important commercial conifer species in Southwest Europe, and particularly in Portugal and France. Maritime pine wood is used both in the timber and pulp industries, which involve different actors (forest owners, timber and pulp industrials) who might be interested in different traits.

Maritime pine as a member of the *Pinus* genus is an excellent model for gymnosperms (Lev-Yadun & Sederoff, 2000), in particularly to study wood formation. First, maritime pine has been the subject of intensive molecular genetic and genomic analyses (Pot 2004; Chagne 2004; Canton et al., 2004; Le Provost et al., 2003, Gion et al., 2004). Second, only a few type of cells are present in the secondary xylem (the majority tracheids and few ray parenchyma cells; Harada and Coté, 1985). Third, it is easy to obtain large quantities of differentiating xylem during active growth (the bark and phloem can be removed leaving the immature xylem attached to wood), and finally the observation of different types of wood is possible within the same tree.

In fact, despite the simplicity of secondary xylem structure, maritime pine wood is a highly variable material, and six types of wood can be observed within the same tree: reaction and opposite woods, early and late woods, juvenile and mature wood. The formation of reaction wood in response to a non-vertical orientation of stem is caused by environmental effects (e.g. wind, snow, and slope). Reaction wood enables the stem to reorient itself, to ensure a favourable position for the tree (Timell, 1986). One of largest variation observed in wood characteristics is the transition from early wood (EW) to late wood (LW) in temperate-zone. This variation occurs within the same growth ring with EW being formed at the start of the growing season, when temperature and precipitation are favourable to active growth, and LW being initiated towards the end of the growing season, when adverse climatic conditions slow down cell divisions in cambial meristem, decrease the rate and the duration of cell expansion, and extend the duration of secondary wall formation (Uggla et al., 2001). Finally, an important wood variation is observed between the centre of the tree and the phloem, and from the base of the tree to its top (Zobel and Buijtenen, 1989). In an adult tree, juvenile wood is observed both in the center of the bole, and near the crown where it continues to form, and mature wood can be observed at the bottom of the bole (Zobel and Sprague, 1998).

The variation observed between these 6 types can be defined in terms of chemical, anatomical and technological properties.

These variations are mainly due to micro-environmental pressures which are experienced by cambial and derived cells during the life of a tree, and suggest a very plastic expression of tree genomes in wood forming tissues. If this hypothesis is correct, the relative transcript/protein accumulation must vary according to the developmental state (cambial age) and the environment (e.g. growing season) experienced by differentiating cambium cells. Here we present the preliminary results of a genomic approach looking at developmental effects (cambial age effects) on transcripts' accumulation in wood forming tissue of maritime pine.

2. Material and Methods

2.1. Tissue sampling

After removing the bark from the tree, a layer of phloem with attached cambium was collected. Exposed secondary differentiating xylem was then scraped from the trunk with a knife. It should be noticed that xylem samples consisted on a mixture of cells taken along the differentiation process, from cell in division to cell already death. Differentiating xylem samples were immediately frozen in liquid nitrogen after sampling and stored at -80°C before use. Six xylem samples were collected on 12th May 2003 (plot Y, Pierroton Forestry station) on a 30 year-old tree of the Aquitaine provenance, along a cambial 15 years old age gradient corresponding to internodes 1985, 1988, 1991, 1994, 1997, and 1999/2000.

2.2 RNA extraction

Total RNA was isolated from 3g of xylem following Chang et al., (1993). Genomic DNA was then removed as described by Le Provost et al. (2003). RNA quantity and quality were analysed by spectrophotometry and visual inspection on 2% agarose gel.

2.3. High-density filters preparation

To prepare the high-density filters we selected (based on sequence length and position in the gene) a total of 3,512 clones from the *P. pinaster* xylem cDNA library (Canton et al., 2004). Several controls (luciferase, desmin, nebulin, RAS polylinker of insertional vector of the cDNA library, and water) were also selected to be spotted. All plates (except the water control plates) were amplified using nested PCR. The first PCR (PCR-I) was prepared in 10 μL , with 1 μL of bacteria suspension added to a PCR mix containing 5mM dNTPs, 2mM MgCl_2 and 0.2 μM of Universal primers M13 (-20) and R13 and 0.2U/ μL of Taq polymerase. The second PCR (PCR-II) was prepared in 100 μL , with 2 μL of PCR-I amplification product added to a PCR mix containing 5mM dNTPs, 2mM MgCl_2 , 0.2 μM of T7 and T3 primers and 0.2U/ μL of Taq polymerase. All PCR reactions were performed using a Primus Thermoblock (MWG, Reinach, Switzerland). After an initial denaturation step of 5min. at 95°C , 15 cycles for the PCR-I and 40 cycles for the PCR-II were carried on as follows: 30s denaturation at 95°C , 45s annealing at 55°C , and 1min and 30s elongation at 72°C . A final elongation step was conducted for 10min at 72°C . All PCR-II were checked for their quality and quantified in agarose gel, re-ordered into twelve 384-well plates, and spotted in duplicates onto 50 sets of 2 nylon membranes (figure 1), each containing 1,756 xylem clones (*xyl* in figure 1), 4 control plates and one water plate (*cont*). A set of 96 xylem clones (*xyl-c*) was also spotted on both membranes to estimate the membrane effect. Membranes spotting were performed by Eurogentec (Eurogentec, Liege, Belgium).

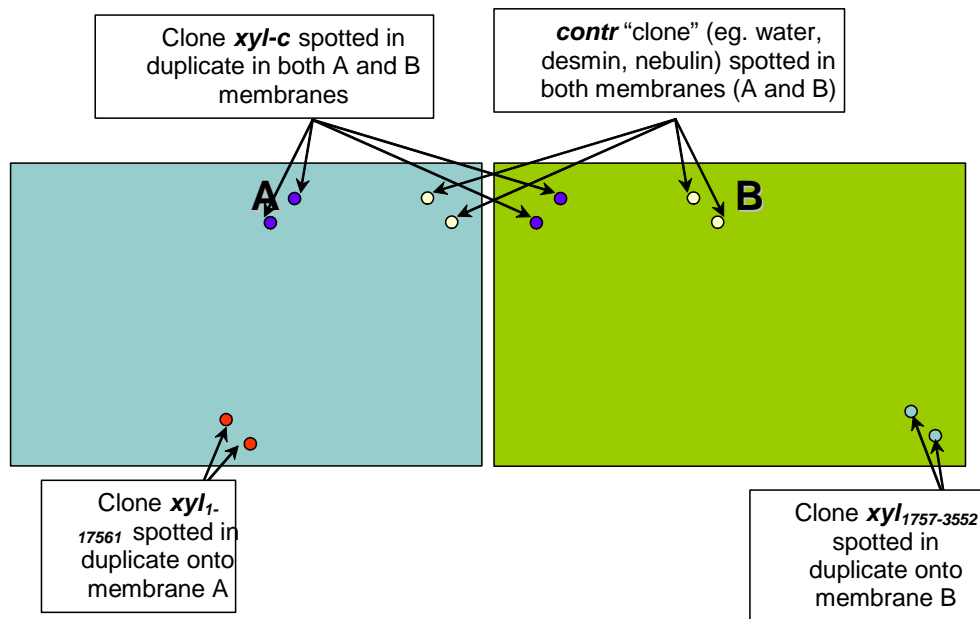


Figure 1 – Array design. Xylem clones (*xyl* and *xyl-c*) and controls (*cont*) were spotted into two membranes (A and B) as sketched below.

2.1.4. Amplification of antisense RNA

Antisense mRNA (aRNA) was obtained from 2 μ g of total RNA using the MessageAmp kit (Ambion, Inc, Austin, USA). aRNA was quantified using the “RiboGreen RNA quantitation assay” (Molecular Probes, Inc., Eugene, OR). All aRNA samples were diluted in Rnase free water to a final concentration of 50 ng/ μ L .

2.1.5. Probe labelling, hybridisation procedures and image digitalization

Probes were synthesised using 300ng aRNA supplemented with 2% desmine (human gene) aRNA using the Strip-EZ™ RT kit from (Ambion, Austin, USA). Random sequence decamers primers provided with the kit were used for priming reverse transcription. Filters (membranes A and B together in a glass tube rotating in an air incubator) were pre-hybridised for 8h in 15mL of hybridisation buffer (5X SSC, 5X Denhardt, 0.5% SDS, 10 μ g.mL⁻¹ denatured salmon sperm DNA) at 65°C. Hybridisations were carried out in high stringency conditions at 65°C overnight using 15ml of fresh hybridisation buffer supplemented with a minimum of 10⁶ cpm of purified and denatured probe, per mL of buffer. Membranes were washed twice at room temperature during 5min with 2X SSC 0.5% SDS buffer. After, they were washed twice during 15min in 2X SSC 0.1% SDS buffer, 2X 15min in 1x SSC 0.1% SDS at 65°C, followed by two washes of 15min each in 0.1X SSC 0.1% SDS buffer. Finally, they were wrapped in a plastic film, exposed to the General Propose PhosphorImager screen (Amersham Biosciences, Buckinghamshire, England) for a period of 4 days, and scanned using the Storm System (Amersham Biosciences, Buckinghamshire, England), at 50 μ resolution.

2.1.6. Signal quantification

Hybridisation signals were quantified using ArrayVision (Imaging Research, Ontario Canada). After alignment with designed template (Figure 2) several measures (spot density and background) were performed and data exported into excel format files.

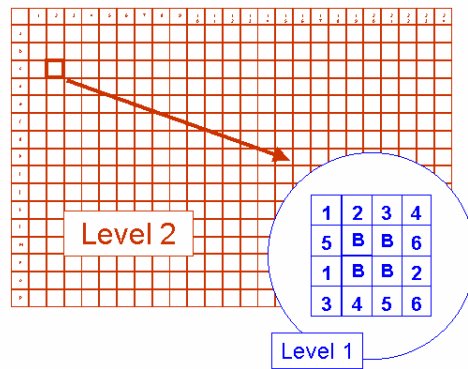


Figure 2 - Template grid used for signal quantification. A template was designed that contained two levels: in level#1, 1 to 6 refers to the six 384-well plates in which the clone was taken, and B refers to the blank spots used for background calculation. Level #2 (16*24 grid) correspond to the coordinates of a 384-well plate.

Median-based Trimmed Mean density (MTM density) available in ArrayVision software was used for spot quantification. The background values were removed from individual spots density value, and adjusted values were used for statistical analysis.

2.1.6. Experimental design

Macro-arrays were used to identify differentially expressed genes between tissues. Two batches of three hybridisations (including labelling, overnight hybridisations, washing and screen exposition) were performed: samples N1, N3, N5 correspond to batch A and N2, N4 and N6 correspond to batch B.

2.1.7. Statistical analysis

After background removal, average density for each membrane set and hybridization were calculated. Then all data points from a membrane set were divided by the corresponding average mean. For each membrane set hybridized, a group of 98 clones spotted in both membranes were similarly corrected. To account for technical and biological effects, the following ANOVA model was applied to each clone spotted in the membranes, on corrected density values:

$$Y_{ijk} = \mu + B_i + N_{j/i} + \varepsilon_{ijk},$$

where Y_{ijk} is the spotted clone corrected density with k replicates ($k=1, \dots, 6$), B_i is the batch effect ($i= A$ and B), and $N_{j/i}$ is the cambial age effect ($j=1,2,3,4,5,6$) nested in batch effect B_i , and ε_{ijk} is the error of the declared model.

Criteria used to select differentially expressed genes were based on results from the ANOVA. A EST clone was classified as differentially expressed between levels of cambial age if their P-value for cambial age effect was inferior to 1/10000, if this effect explained more than 50% of the model total variation in Sums of Squares, and if it displayed normal residuals' distribution. For differentially expressed genes, corrected data from batch effects were then analysed using Expander (<http://www.cs.tau.ac.il/~rshamir/expander/expander.html>). We used Click method to cluster genes by their expression profile. The available "Click" method in Expander software uses graph theory to cluster genes by their expression homogeneity. It has the advantage over the other clustering methods like k-means or SOM (self-organizing map) because it is not necessary pre-determine the final number of the clusters.

3. Results and discussion

From the 3512 cDNA clones spotted in HDF, 262 transcripts were selected as differentially expressed between the six xylem samples taken across cambial ages. Their corrected expression data were used to determine an expressional distance between cambial age samples using EPclust (<http://ep.ebi.ac.uk/EP/EPCLUST/>). This analysis clustered these six samples in two distinct sub-trees: one sub-tree corresponding to the older cambial age samples (N1, N2, N3), mainly mature wood forming tissue, and the other sub-tree grouping younger cambial age samples (N4, N5, and N6) corresponding to the juvenile formation zone.

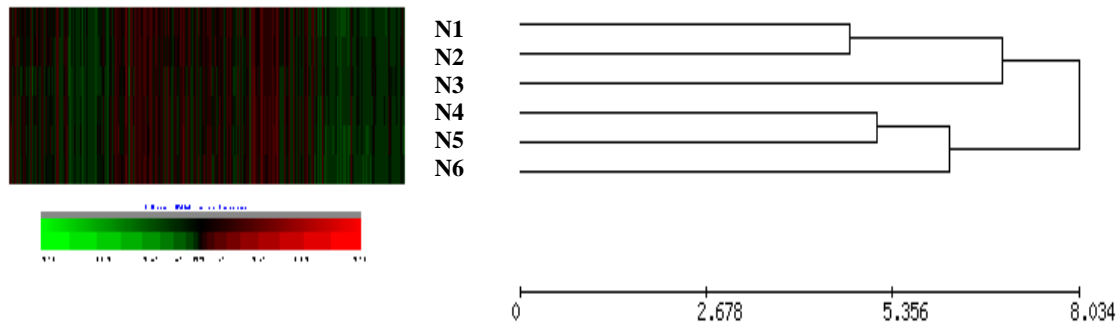


Figure 3 – Clustering of the differentially expressed clones along the cambial age gradient. In the dendrogram, two distinct sub-trees can be observed

About 15% of the transcripts belong to genes involved in protein synthesis and post-translational modifications (Figure 4), revealing a high protein synthesis and turnover during xylogenesis. Metabolism category is represented by 10% of the transcripts differentially expressed. It was not possible to attribute a functional annotation to about 45% of the differentially expressed transcripts.

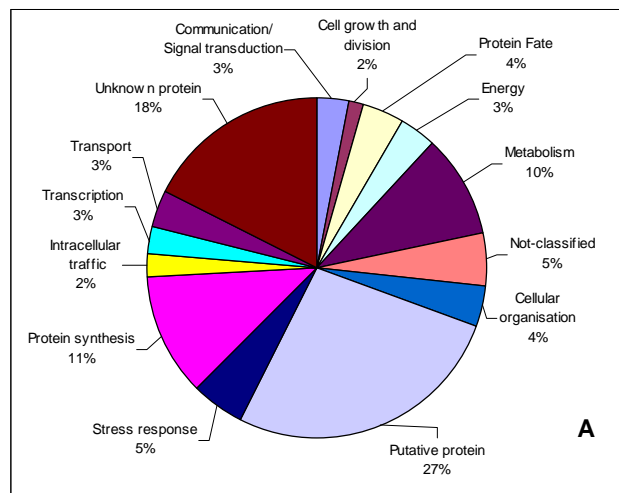


Figure 4 – Distribution of 262 differentially expressed genes by functional category

Among differentially expressed transcripts, we found genes encoding for lignification proteins (like Caffeate O-methyltransferase), cellulose synthesis (Cellulose synthase (UDP-forming)), cell wall proteins (Arabinogalactan-like protein and GRP2), programmed cell death (Cystein proteinase), stress response (stress related protein), transcription factors (Homeobox protein SHOOT MERISTEMLESS), calcium regulation (calmodulin), cytoskeleton (tubulin

alpha) or implied in ubiquitin-proteasome pathway (peptidylprolylisomerase, uiquitin-protein ligase).

Genes were further clustered using the mentioned “Click method” according to their expression profile, and 223 (out of the 262 differentially expressed transcripts) were found to cluster into 4 main groups illustrated in Figure 5.

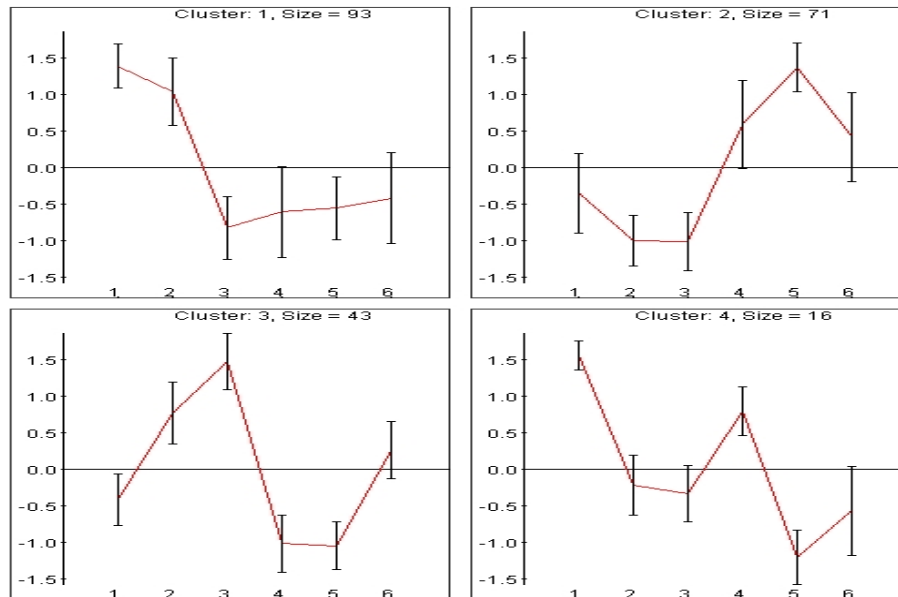


Figure 5 – Mean expression profiles of all differential expressed genes clusters along a cambial age gradient. 1-N1, 2-N2, 3-N3, 4-N4, 5-N5 and 6-N6. Clusters were obtaining using “Click function” of Expander software on standardised data.

Two of these clusters (cluster #1 and cluster #2) were particularly interesting because they show opposite expressional profiles. For cluster #1 genes were over-expressed for the older cambial ages N1 and N2, and quickly decreasing their expression at age N3, maintaining their expression at the same level than N3 for N4, N5 and N6 levels. Cluster#1 genes may thus be involved in mature wood formation. Cluster #2 profile genes were found to be over-expressed in the younger cambial age tissues, and may be involved in the formation of juvenile wood. Comparison between the functions of genes included in these two clusters (Table1) revealed that gene transcripts involved in communication/signal transduction, energy, cellular organisation, and protein synthesis categories are preferentially expressed in juvenile wood forming tissues. On the other hand, genes involved in growth and cell division, metabolism, protein fate, stress response, transcription and transport categories seem to be more expressed in mature wood. The clustering results also suggest that transition between juvenile to mature wood must occur around cambial age N3, i.e. in cambium tissues aging around 10 years.

Table 1 – Cluster distribution of functional categories of genes differentially expressed across a cambial age gradient. Singleton represents all the genes not clustered in the four main clusters

Functional category	Click Cluster				singleton	Grand Total
	1	2	3	4		
Communication/ Signal transduction	2.15%	4.23%	2.33%	0.00%	5.13%	3.05%
Growth and cell division	3.23%	0.00%	0.00%	6.25%	0.00%	1.53%
Protein fate	5.38%	0.00%	4.65%	12.50%	2.56%	3.82%
Energy	2.15%	7.04%	2.33%	0.00%	2.56%	3.44%
Metabolism	10.75%	7.04%	4.65%	37.50%	7.69%	9.92%
Not-classified	3.23%	5.63%	4.65%	0.00%	10.26%	4.96%
Cellular organisation	3.23%	5.63%	0.00%	0.00%	7.69%	3.82%
Putative protein	26.88%	22.54%	44.19%	12.50%	23.08%	27.10%
Stress response	5.38%	1.41%	0.00%	18.75%	10.26%	4.96%
Protein synthesis	8.60%	23.94%	4.65%	0.00%	7.69%	11.45%
Intracellular traffic	2.15%	2.82%	4.65%	0.00%	0.00%	2.29%
Transcription	4.30%	0.00%	6.98%	0.00%	0.00%	2.67%
Transport	5.38%	2.82%	0.00%	0.00%	5.13%	3.44%
Unknown protein	17.20%	16.90%	20.93%	12.50%	17.95%	17.56%

4. Future prospects

This study presented results on the accumulation of transcripts along a cambial age gradient. Using high-density filters, we were able to identify functional candidate genes according to their preferential expression in differentiating xylem tissues at different cambial ages. This approach provides us with a set of genes that are now being used in genetic mapping and association studies (Chagné et al., 2003), where relationships between allelic variation (Pot et al., 2005, Le Provost et al., 2003) of wood-related traits can potentially be estimated (Garnier-Géré et al., 2005).

Similar studies using high-density filters are also being used to analyse the accumulation of transcripts during the growing season. Parallel studies looking at variation at the protein level will provide us with new insights about the developmental and environmental effects on protein quantities, complementing the information already available on transcripts. Expression profiling using new microarray chips currently being developed (about 7.5K cDNA clones) has the potential to increase the efficiency of identifying genes involved in xylogenesis structure and regulation.

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