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Plasticity of maritime pine (*Pinus pinaster*) wood-forming tissues during a growing season

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Summary

• The seasonal effect is the most significant external source of variation affecting vascular cambial activity and the development of newly divided cells, and hence wood properties. Here, the effect of edapho-climatic conditions on the phenotypic and molecular plasticity of differentiating secondary xylem during a growing season was investigated.

• Wood-forming tissues of maritime pine (*Pinus pinaster*) were collected from the beginning to the end of the growing season in 2003. Data from examination of fibre morphology, Fourier-transform infrared spectroscopy (FTIR), analytical pyrolysis, and gas chromatography/mass spectrometry (GC/MS) were combined to characterize the samples. Strong variation was observed in response to changes in edapho-climatic conditions.

• A genomic approach was used to identify genes differentially expressed during this growing season. Out of 3512 studied genes, 19% showed a significant seasonal effect. These genes were clustered into five distinct groups, the largest two representing genes over-expressed in the early- or late-wood-forming tissues, respectively. The other three clusters were characterized by responses to specific edapho-climatic conditions.

• This work provides new insights into the plasticity of the molecular machinery involved in wood formation, and reveals candidate genes potentially responsible for the phenotypic differences found between early- and late-wood.

Key words: early-wood, late-wood, metabolome, molecular plasticity, *Pinus pinaster*, transcriptome, wood formation.

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Introduction

Trees are long-lived organisms that develop in a variable environment. These perennial plants synthesize wood (secondary xylem), which confers mechanical strength and provides a long-distance path for water, minerals and hormones.

Wood formation is a complex and dynamic process that consists of the successive addition of secondary xylem which differentiates from the vascular cambium. Wood formation (xylogenesis) involves four major steps: cell division, cell expansion, cell wall thickening, and programmed cell death, which are temporally and spatially regulated. Environmental conditions and the developmental stages of the plant play

crucial roles in the rate and duration of each of these steps (Wodzicki, 1971; Dodd & Fox, 1990). As a consequence, wood-forming tissues retain environmental and developmental signatures of cellular organization, as well as the individual cell (size, shape, cell wall structure and chemical composition), resulting in a highly variable raw material.

The seasonal effect is among the most significant external sources of variation affecting cambial activity and the development of newly divided cells, and therefore influencing wood characteristics (Zobel & van Buijtenen, 1989). In temperate regions, climatic variation occurring during the annual course of cambial activity gives rise to two types of wood: early-wood (EW) formed early in the growing season, when temperature, rainfall and photoperiod are favourable for active growth, and late-wood (LW) formed in the summer or fall, when the rate of cambial cell division and expansion decline. The transition between EW and LW coincides approximately with the cessation of terminal shoot extension and the reduction of soil moisture (Larson *et al.*, 2001), when the change in climatic conditions slows down cell division in the cambial meristem, and extends the duration of secondary cell wall thickening (Uggla *et al.*, 2001). EW and LW show obvious differences at the anatomical and chemical levels. Compared with LW, EW tracheids are generally shorter, and have thinner walls with lower cellulose content and higher microfibril angles in the S2 layer of the secondary cell wall. EW is also characterized by a higher lignin content and larger radial diameter (Barnett & Jeronimidis, 2003). The morphology of tracheary elements is determined by the rate and duration at which the developing cambial derivatives expand and form their secondary walls. The formation of LW, in particular, is a result of slower rates of cell division, decreases in the rate and period of cell expansion, and a longer duration of secondary wall thickening (Wodzicki, 1971; Dodd & Fox, 1990).

Few efforts have been made to understand the molecular mechanisms involved in the environmental and developmental regulation of wood formation, and the underlying anatomical, structural and chemical differences observed between different types of wood (Plomion *et al.*, 2001; Boerjan, 2005; Yeh *et al.*, 2006). Gion *et al.* (2005) compared the proteomes expressed in wood-forming tissues associated with different types of wood and found that the seasonal effect (EW vs LW) was more important as a factor affecting protein accumulation than ontogenic (juvenile vs mature wood) and gravitational (opposite vs compression wood) effects. More recently, Paiva *et al.* (2008) described cell wall chemical composition, transcriptome, and proteome variation from the base to the crown in maritime pine wood-forming tissue. Moderate variations were found at these different levels of integration.

In this study, our objective was threefold: to describe the variation of metabolites and cell wall composition of wood-forming tissue during a growing season; to study the transcriptome of differentiating xylem collected along this gradient; and to interpret the observed plasticity in terms of reaction to edapho-climatic conditions.

Materials and Methods

Tissue sampling

Samples of differentiating xylem were collected on straight maritime pine (*Pinus pinaster* Ait.) trees planted at the Forest Research Unit of INRA-Pierroton (Cestas, France), over two growing seasons, as follows.

Five samples (T1–T5) were taken at the same time of day during the 2003 growing season from grafted copies (at internodes of year 1991 to year 1993) of a single 17-yr-old Corsican clone (accession #520) on 4 April (T1), 18 April (T2), 12 May (T3), 27 May (T4), and 23 June (T5). Additional samples collected on 30 July and 4 September corresponded mainly to fully developed wood and were not considered in the analysis. It should be noted that a severe drought period occurred in late July and August 2003 in southwest France (Rebetez *et al.*, 2006), resulting in growth cessation. These five samples were used to describe variation in the fibre morphology, cell wall chemical composition and metabolites of developing tracheids, and to characterize their transcriptome during the seasonal gradient.

To confirm the differential accumulation of selected transcripts between EW- and LW-forming tissues, and to assess genotypic effects on transcript accumulation, differentiating xylem was also collected on two additional Corsican genotypes (accessions #4015 and #3006), during the 2006 growing season (every 15 d starting in April and ending in August). These trees were located in the same trial as the trees sampled in 2003. After bark removal, the layer of phloem with attached cambium was removed with a knife. Exposed secondary differentiating xylem was then collected from the trunk by scraping the tissues with a knife as described in Paiva *et al.* (2008). All the samples were collected at the same time of day (10:00 h) to minimize diurnal variation in gene expression. The differentiating xylem samples were immediately frozen in liquid nitrogen and stored at –80°C until use. Upon microscopic observation, the majority of cells were found to be in the process of cell expansion or secondary cell wall thickening, as reported by Paiva *et al.* (2008).

Phenotypic characterization of wood-forming tissues

The methodology used to characterize the different samples at the chemical level is summarized in Fig. 1. Automated fibre analysis, Fourier-transform infrared (FTIR) spectroscopy and analytical pyrolysis were based on the methods described by Paiva *et al.* (2008). Metabolite analysis was performed by GC/MS of dichloromethane, methanol and water extracts.

For dichloromethane extract analysis, aliquots (2 ml) of a 20-ml initial sample of extract solution were dried and

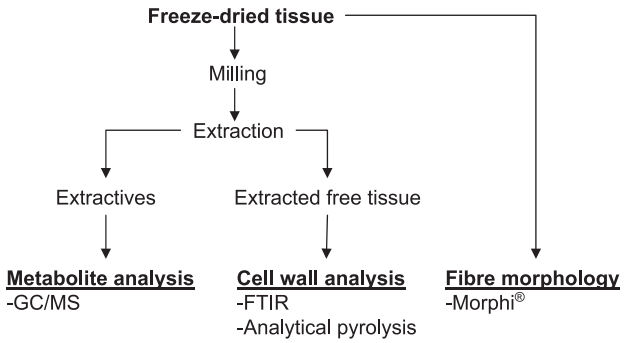

Fig. 1 Procedures followed to characterize the chemical composition of differentiating xylem samples of maritime pine (*Pinus pinaster*). GC/MS, gas chromatography/mass spectrometry; FTIR, Fourier-transform infrared spectroscopy.

weighed. These samples were derivatized with pyridine/N,o-Bis(Trimethylsilyl)trifluoroacetamide (BSTFA) (*c.* 40 µl of derivatization solution per mg of dry mass). These solutions were analyzed by GC/MS in an Agilent 5973MSD (Agilent Technologies, Santa Clara, CA, USA). Chromatographic conditions were as follows:

- column DB5-MS (60 m × 0.25 mm internal diameter × 0.25 µm thickness of film);
- initial temperature 150°C (5 min); temperature increment of oven 6°C min^{-1} until a temperature of 250°C was reached, followed by a temperature increment of 4°C min^{-1} until a temperature of 300°C was reached (15 min);
- injector and interface to mass spectrometer, 300°C;
- electron ionization spectra (EIMS) obtained at 70 eV.

For methanol and water extract analysis, aliquots (1.4 ml) of a 20-ml initial sample of extract solution were dried and weighed. These samples were derivatized with pyridine/BSTFA (*c.* 80 µl of derivatization solution per mg of dry mass). These solutions were analyzed by GC/MS in an Agilent 5973MSD. The chromatographic conditions were the same as described above for dichloromethane extractives.

In order to take into account the variation in absolute quantities injected into the GC and to allow sample comparison, raw data were corrected for the total signal (integrated area) of the chromatogram.

Transcriptome analysis

Reverse northern Total RNA preparation, production of macroarrays, hybridization and signal quantification followed the procedures described by Paiva *et al.* (2008).

Experimental design and statistical analysis Three replicated hybridizations (including labeling, overnight hybridizations, washing and screen exposure) were performed, resulting in a total of six data points for each spotted probe (3 replicated hybridizations × 2 spots per probe). After background noise removal, the average density for each membrane set and hybridization replicate was calculated. Then, to normalize the data, all data points from a membrane were divided by the corresponding average mean membrane density to account for technical effects. The following ANOVA model was then applied to each probe *i* on corrected density values:

$$Y_{ijk} = \mu + T_{ij} + \varepsilon_{ijk}$$

(Y_{ijk}, the corrected density of probe *i* in sample *j* for replicate *k*; µ, the general mean; T_{ij}, the seasonal effect ($j = 1–5$); ε_{ijk}, the residual of the declared model ($k = 1–6$).) Statistical analyses were performed with SAS version 6.12 (Statistical Analysis System version 6.12; SAS Institute, Cary, NC, USA) under a fixed model using proc GLM.

Criteria used to select differentially expressed genes were based on ANOVA results. A transcript was classified as differentially expressed between the different samples if: (1) the seasonal effect showed a *P*-value < 10^{-4}, which corresponds approximately to a *P*-value of 5% for each test after applying the Bonferroni correction for multiple testing (in our case 3512 tests); (2) the main effect explained more than 50% of the total sums of squares, and (3) it displayed a normal residual distribution. Together these three criteria ensured a low rate of false positives.

For differentially expressed genes, centred-reduced data were analyzed using EXPANDER (Shamir *et al.*, 2005). We used the Click algorithm in this software (Sharan & Shamir, 2000) to cluster genes by their expression profiles. This clustering algorithm uses graph theory to cluster genes by their expression homogeneity. It has the advantage over other clustering methods, such as *k*-means or a self-organizing map, of not having to predetermine the final number of clusters.

Quantitative real-time PCR (qPCR) assay The qPCR profile of a glycine-rich protein previously reported as 'specifically' up-regulated during the formation of LW (Le Provost *et al.*, 2003) was first used to choose contrasting samples during the 2006 season (Supporting Information Fig. S2a). Based on this screening step, sampling dates corresponding to 28 April and 7 August were selected. PCR primer pairs (Supporting Information Table S1) were designed using the PRIMER3 software (Rozen & Skaletsky, 2000) for seven genes and a control gene corresponding to a maritime pine superoxide dismutase copper chaperone (accession number BX677784). Primers were designed to have an optimal size of 22 bp (18–24 bp), a GC content of 40–60%, and an annealing temperature (TM) of 58–62°C. Other criteria such as primer self-annealing were also taken into account. The predicted fragment size ranged from 100 to 224 bp. Olignonucleotides were synthesized by Eurogentec (Liege, Belgium). For each sample, total RNA was isolated three times from 0.1 to 0.3 g of xylem using a micro-extraction method (Le Provost *et al.*, 2007) based on the Chang *et al.* (1993) protocol. Genomic DNA was removed as described by Le Provost *et al.* (2003). The quantity and

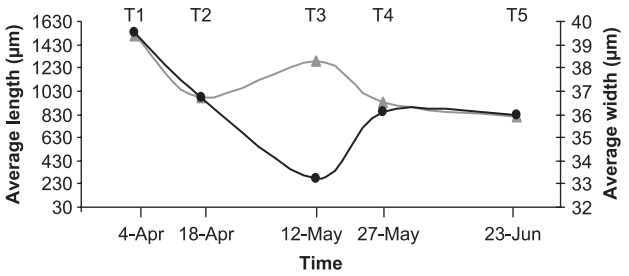

Fig. 2 Variation of fibre width (circles) and fibre length (triangles) of differentiating tracheids of maritime pine (*Pinus pinaster*) during the growing season in 2003.

quality of pooled RNA were analyzed by spectrophotometry and visual inspection on 2% agarose gels. Reverse transcription (RT), qPCR and data analysis were performed following Paiva *et al.* (2008).

Recording of meteorological data and simulation of ecophysiological data

Meteorological data were recorded every 30 min at the Forest Research Unit of INRA Pierroton. Daily or weekly means were computed, except for precipitation for which cumulative data were obtained for periods of 1 and 2 wk before the sampling date. In addition to the descriptive variables of the forest stand where the trees were grown (e.g. soil nature, plantation density and silvicultural practices), climatic variables were introduced into the GRAECO model (Loustau *et al.*, 2005) to simulate the functioning of a *P. pinaster* stand typical of the French forest region of Les Landes de Gascogne. GRAECO is a physiologically process-based model designed to predict fluxes of water, carbon and energy, and growth of an even-aged monospecific *P. pinaster* forest ecosystem. Resulting parameters included, among others, soil water content (SWC), soil moisture deficit (SMD), stomatal conductance (G_s) and transpiration (Supporting Information Table S2).

Results

Characterization of environmental conditions

The annual variations in temperature, precipitation, transpiration, SWC, G_s, and SMD are presented in Supporting Information Fig. S1 for the 2003 and 2006 growing seasons. Year 2003 started with a very low SMD and very high SWC. A lack of precipitation for long periods, in particular after mid-May, associated with high temperatures from June to August, induced a rapid and severe decrease in SWC. Occasionally heavy precipitation occurred in spring. Year 2006 was quite different, but EW and LW samples were taken under similar edapho-climatic conditions to those of samples T2 and T5 in 2003 (Supporting Information Fig. S1).

Variability of fibre morphology and cell wall composition during the season

Fibre morphology Figure 2 shows the variation of fibre (mainly tracheid) width and length during the 2003 growing season. Fibre width showed a rapid decrease from T1 to T3 and then increased to reach a plateau at T4 and T5. Fibre length showed a similar trend except for a marked peak at T3.

FTIR spectroscopy and analytical pyrolysis Figure 3 shows the relative proportion of protein (amide I and II peaks as described in Paiva *et al.*, 2008) content for the samples collected during the growing season. Protein content decreased between T1 and T2 and then suddenly increased, reaching its highest value at T3, and then slowly decreased from T4 to T5.

Pyrograms contained 57 pyrolysis products typical of polysaccharide and lignin origin (see also details in Supporting Information Table S3). The variation in five groups of pyrolysis products (aa1, g, cP, c and cH) during this seasonal gradient is presented in Fig. 4.

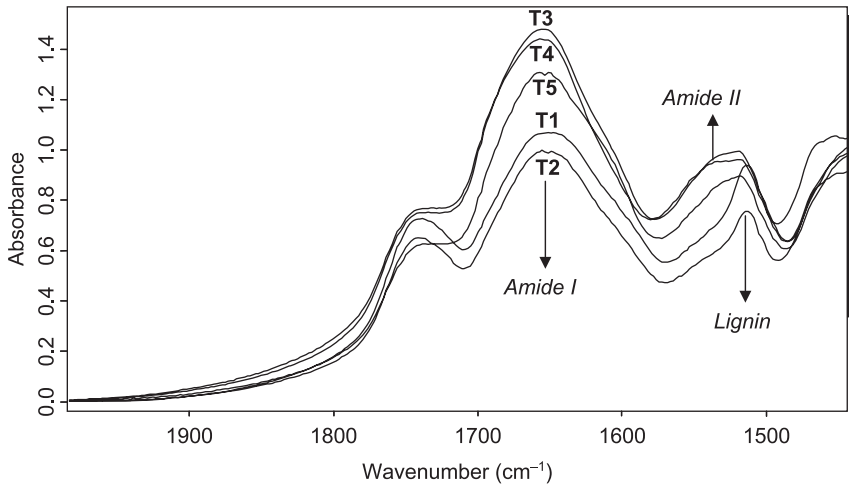

Fig. 3 Variation of minimum–maximum normalized spectra during the growing season in 2003, showing the broad amide I and amide II bands.

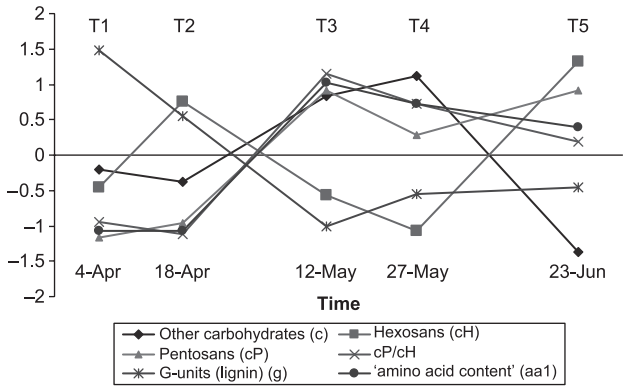

Fig. 4 Variation of main classes of pyrolysis products of maritime pine (*Pinus pinaster*) during the growing season in 2003: aa1, toluene (amino acid content); cP, pentosans (hemicellulose origin); cH, hexoxans (mainly from cellulose); c, other carbohydrates (mainly of hemicellulose origin); g, guaiacyl lignin (G) units. Data for each pyrolysis product were normalized to have a mean of 0 and standard deviation of 1.

• Toluene (aa1), a pyrolysis product derived from phenylalanine (Moldoveanu, 1998), showed the same profile as the amide I peak described in the previous section, being low at T1 and T2, rapidly increasing between T2 and T3, and then decreasing slowly from T3 to T5.

• Lignin is mainly composed of guaiacyl (G-units) and a few per cent of p-hydroxyphenyl (H-units) phenylpropanoid units. However, the main pyrolysis products of these H-units, phenol and cresol, are also pyrolysis products of tyrosine (Faix *et al.*, 1991; Moldoveanu, 1998), therefore when proteins are present part of the H-unit products are from lignin and part are from proteins. Indeed, H-units followed the same pattern as phenylalanine (the same pattern as aa1 (data not shown)). For this reason we preferred not to use the H-units as a reliable measure of lignin composition but to rely more on lignin composition determined from G-units (g). G-unit lignin decreased linearly from T1 to T3 and then slowly increased from T3 to T5.

• Pentosans (cP) increased from T1 to T3 and remained quite stable until S6.

• Carbohydrate pyrolysis products 'c' other than pentosans and hexoxans (mainly of hemicellulose origin) and 'cH' (mainly of cellulose origin), presented inverted profiles. While 'c' increased gradually to reach a maximum value at T4 and then dropped drastically to T5, 'cH' showed its lowest and highest values at T4 and T5, respectively.

The EXPANDER software was used to cluster the differentiating xylem samples according to their metabolic profiles obtained from the 57 individual pyrolysis products. This analysis revealed two distinct sub-trees (Fig. 5): group A included the differentiating xylem samples derived from the earliest samples (T1 and T2). These samples had lower amino acid (aa1), higher lignin (g), and lower hemicellulose (c and cP) contents. Group B consisted of the differentiating xylem samples derived from the latest samples (T3–T5). They were characterized by higher amino acid (aa1) and higher hemicellulose contents. The particular position of sample T5, which had a higher cellulose (cH7, levoglucosan) content than samples T3 and T4, should be noted.

Metabolite composition Significant metabolite quantities were found in dichloromethane and methanol, while water extraction did not produce any detectable compounds. The relative quantities of each type of metabolite extracted by these two solvents (indicated in Supporting Information Table S4) were found to be highly variable among the studied samples.

Figure 6(a) illustrates the variation of major metabolites extracted in dichloromethane. Three major metabolites showed striking variations: cyclitols (30.3 ± 11.4%, mean ± SE), total resinic acids (25.4 ± 20.8%, consisting of 81.8% dehydroabietic acid), and disaccharides (only sucrose derivates; 23.6 ± 17.1%). It should be noted that sucrose was not detected in the dichloromethane fraction. The amount of sucrose derivates and cyclitols increased to reach a maximum at T3 and T4, and then decreased at T5. Conversely, total resinic acids, in particular dehydroabietic acid, showed an opposite profile. It should also be noted that hexapyranoses were barely detectable. Fig. 6(b) illustrates the variation of the major metabolites extracted in methanol. The major extracted compounds were hexapyranoses (26.6 ± 2.5%), fructose-type compounds (24.3 ± 3.2%), nonidentified compounds (NIDs) (21.7 ± 1.4%), and sucrose (14.8 ± 4.1%). Hexafuranoses were not detected. The pattern of variation of disaccharides (i.e. a gradual decrease from T1 to T5) contrasted with that of fructose and hexapyranose types which presented inverted profiles.

Relationships between variables

Principal component analysis (PCA; Fig. 7) was used to summarize and explore the relationships between phenotypic variables: synthetic variables computed from individual pyrolysis products, metabolites and fibre morphology. The main plane of the PCA (PC1 × PC2) explained 86% of the variation among the five xylem samples (Table S5), with 57.45% for PC1 alone. The correlation circle in Fig. 7(a) shows that the PC1 axis was well correlated with fibre width, resinic acid content and lignin (g) content (negative correlation) and protein (aa1 and h pyrolysis products) and hemicellulose (cP) contents (positive correlation). The PC2 axis was highly positively correlated with cellulose content (cH7) and negatively correlated with fibre length (see also Supporting Information Table S6). In Fig. 7(b), samples of this temporal series are connected by successive sampling dates. Their position in the main PCA plane could be explained by structuring them into three groups. The first group comprised the two samples collected in April (T1 and T2). They had higher G-lignin and resinic acid contents, and wider fibres. The second group comprised samples T3 and T4, collected in May. They were

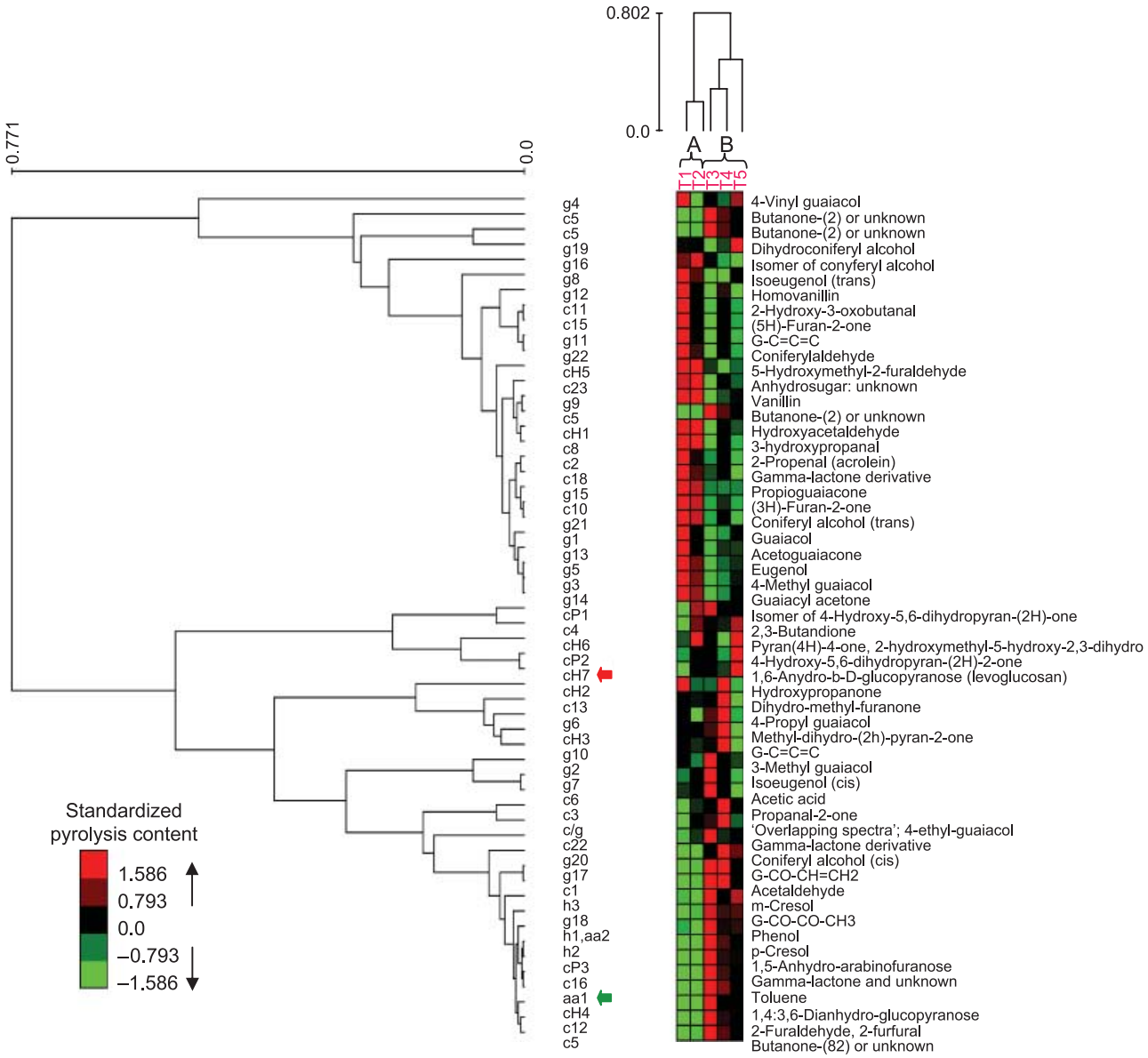

Fig. 5 Clustering of differentiating xylem samples (T1–T5) according to their metabolic profiles and clustering of pyrolysis products. cH7, marker for cellulose content; aa1, marker for amino acid content (toluene). The scale bar adjacent to each dendrogram represents the distance measurement used in the EXPANDER software algorithm ((1 – Pearson correlation)/2). The color scale bar represents the standardized content of pyrolysis products. For each pyrolysis product, data were standardized to give a mean of 0 and a standard deviation of 1.

characterized by higher hemicellulose (cP) and protein (aa1 and h pyrolysis products) contents. The third and final group comprised sample T5, collected at the end of June. While this sample was not well explained by PC1 (see PC contributions in Supporting Information Table S7), it was extremely well represented on PC2, with cellulose (cH7) content and fibre length providing the largest contributions to this axis.

To facilitate biological interpretation of the relationships among the samples, edapho-climatic and eco-physiological variables were projected on the main PCA plane as supplementary data (Fig. 7a; see correlations in Supporting Information Table S8). Relative atmospheric humidity (HR), precipitation (Pi) and accumulated precipitation (accum-Pi) were best positively correlated with PC1, whereas most other variables were best correlated with PC2, either negatively (G_s, light use efficiency (LUE), and SWC) or positively (mean temperature (Ta-moy), leaf area index (LAI) and SMD). Samples of group 2 were positively correlated with HR and Pi; the heavy rainfall occurring just before T3 probably explains this grouping relative to samples of group 1. The unique sample belonging to group 3 was positively correlated with SMD and Ta-moy, and negatively correlated with SWC and G_s.

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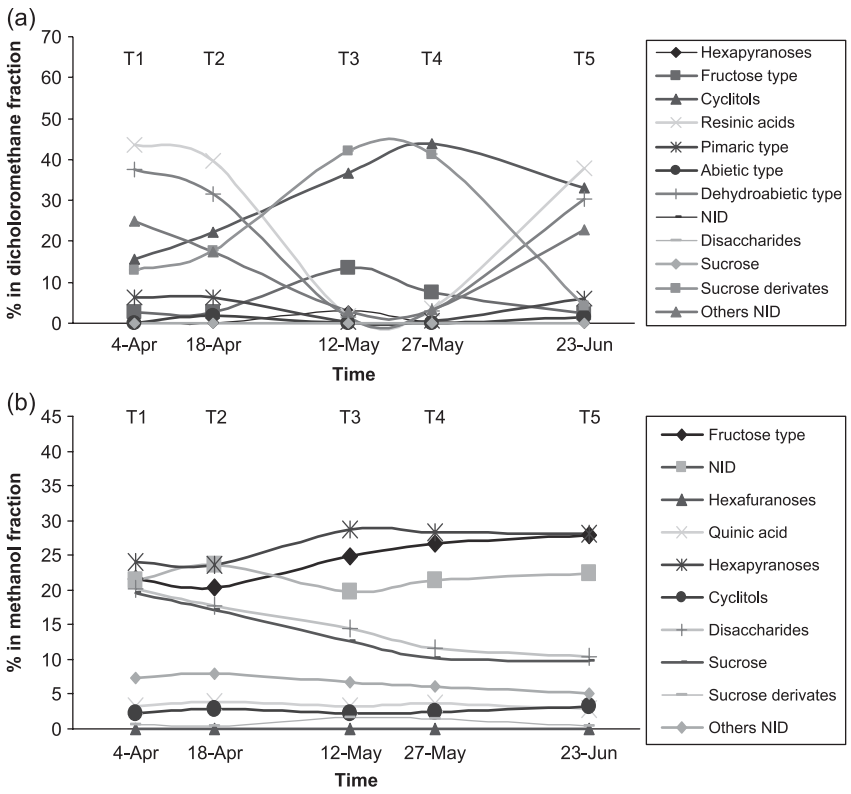

Fig. 6 Variation of metabolites from maritime pine (*Pinus pinaster*) extracted in (a) dichloromethane and (b) methanol during the 2003 growing season. Metabolite quantification and identification were by gas chromatography/mass spectrometry (GC/MS). NID, nonidentified compound.

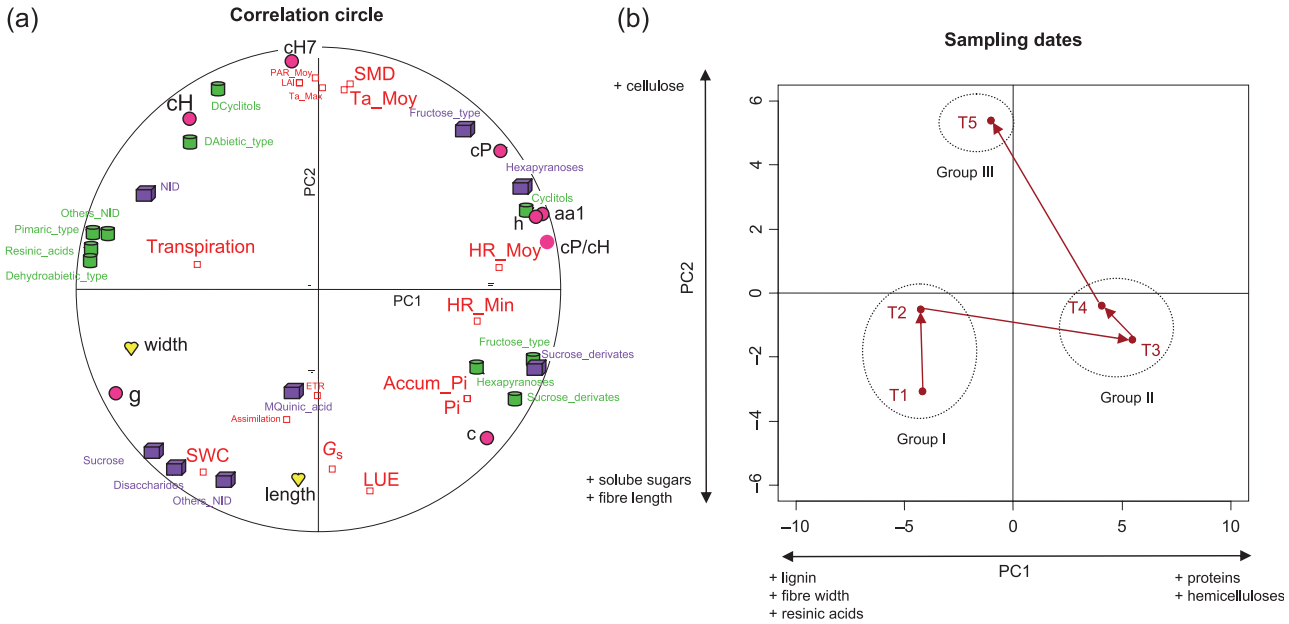

Fig. 7 Principal components (PC) analysis (PCA). (a) Correlation circle showing the position of the variables (synthetic pyrolysis products, metabolites and fibre morphology) on the PC1–PC2 plane. Edapho-climatic and ecophysiological variables were projected as supplementary variables (in red). In the correlation circle pyrolysis products are represented in pink, dichloromethane-extracted metabolites in green, methanol-extracted metabolites in purple, and fibre length and width in yellow. (b) Main plane (PC1–PC2) showing the position of the differentiating xylem samples collected during the growing season. PCA was performed using the ade4 library of the R package (Chessel *et al*., 2004). aa1, toluene (amino acid content); c, other carbohydrates (mainly of hemicellulose origin); cH, hexosans (mainly cellulose origin); cP, pentosans (hemicellulose origin); g, guaiacyl lignin (G) units; G_s, stomatal conductance; LUE, light use efficiency; SMD, soil moisture deficit; SWC, soil water content.

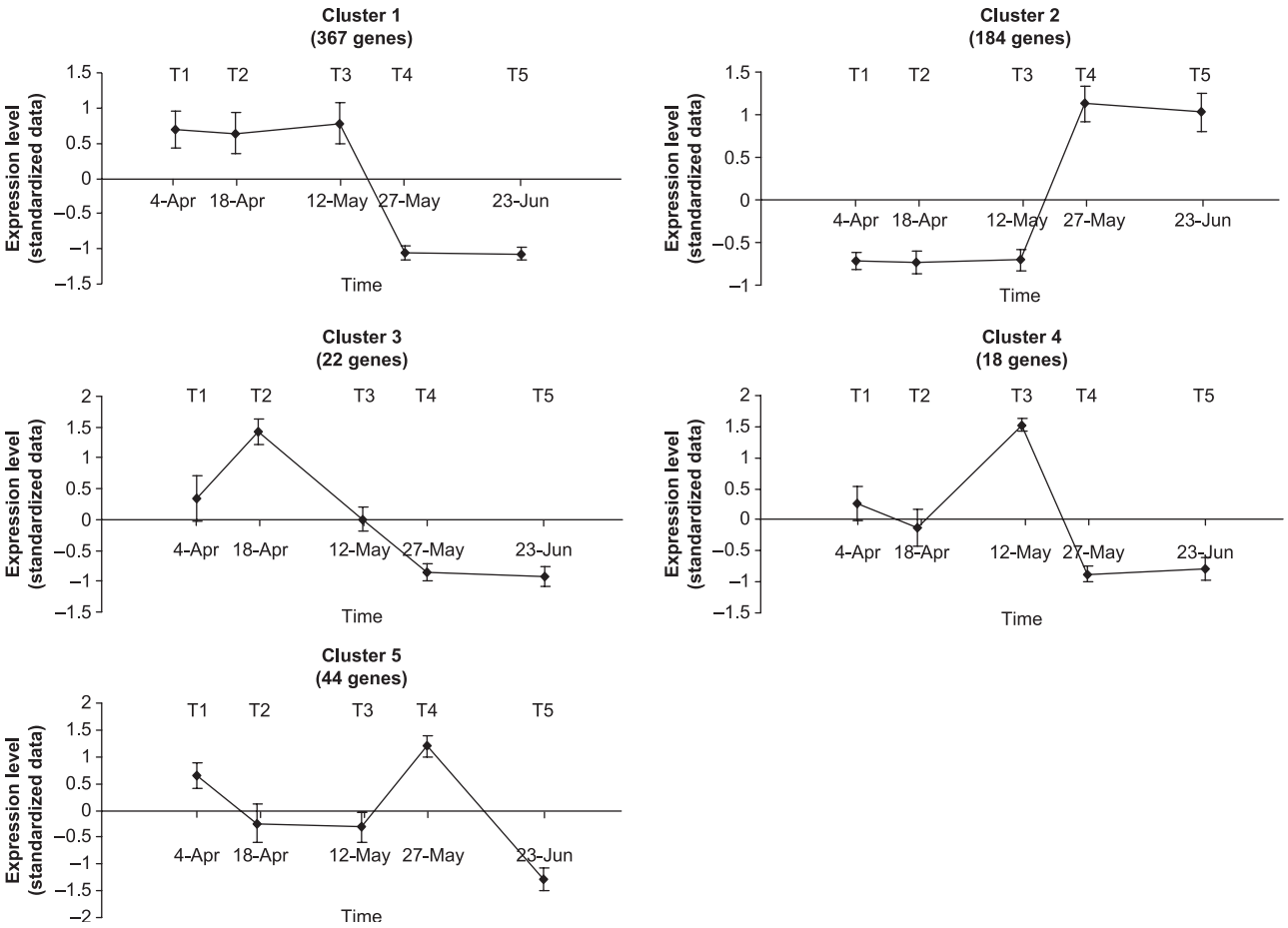

Fig. 8 Clustered mean expression profiles of differentially expressed genes during the 2003 growing season. Clusters were obtained using the 'Click function' of the EXPANDER software on standardized data (mean 0 and standard deviation 1). Error bars represent the standardized expression level variation within each level.

Transcriptome analysis

To evaluate the molecular changes taking place in wood-forming tissues during the growing season, we analyzed the transcript accumulation in the five samples collected in 2003. Their transcriptome profiles were assessed using high-density filters containing 3512 unique cDNA clones. These clones were derived from a nonnormalized xylem cDNA library and their sequences are publicly available (Paiva *et al.*, 2008). Statistical analysis of expression levels of each transcript allowed us to identify genes whose accumulation levels significantly differed in response to variation in environmental conditions. A total of 667 genes (19%) were found to be differentially expressed during this seasonal gradient (see Supporting Information Table S9). It was possible to attribute a functional role to *c.* 49% of these genes by sequence homology with publicly available gene products of known function. The remaining genes were classified as follows: 29.8% putative proteins (BLASTX > 10^{-5} or 'no hit category') and 21.1% homologs of Arabidopsis proteins of unknown function (BLAST < 10^{-5}). Such a high proportion of unidentified expressed sequence tags (ESTs) can be in part attributed to a lower sequence read length for the 'putative protein' category (433 bp on average vs 601 bp for the proteins of known function), but it is also likely to result from a general lack of information about the molecular mechanisms involved in wood formation for the 'unknown function' category (mean length of 603 bp).

Gene expression profiles In order to reduce the complexity of the data, differentially expressed genes were clustered according to their expression profiles. Of the 667 genes, 635 transcripts were clustered into five groups (whose average profile is represented in Fig. 8) with an average cluster homogeneity of 0.971, and an average cluster separation score of −0.336. Initially, 14 functional categories were attributed to the ESTs spotted onto the microarray (European Molecular Biology Laboratory (EMBL) accessions and annotations sorted by functional categories are provided in Supporting Information Table S9). The equiproportional partitioning of differentially

Table 1 Number of differentially expressed genes according to their cluster and functional category

Functional category	% EST spotted onto the array	Cluster 1	2	3	4	5	Total	G-test (*P*-value)
Communication/signal Transduction	2.7	16	1	0	0	3	20	0.00
Cell division and growth	2.3	9	3	0	0	1	13	0.16
Protein fate	3.0	13	2	4	0	1	20	0.01
Energy	2.6	13	4	0	1	1	19	0.00
Metabolism	10.2	55	15	2	1	4	77	0.00
Cellular organization	2.1	10	3	1	1	1	16	0.58
Stress response	2.8	12	2	4	1	3	22	0.65
Protein synthesis	4.5	39	5	1	7	0	52	0.00
Intracellular traffic	1.5	10	1	0	1	0	12	0.01
Transcription	4.0	15	6	0	1	1	23	0.00
Transport	2.3	10	3	0	0	0	13	0.00
Not classified								
Putative protein	36.9	81	82	6	2	18	198	0.00
Unknown protein	21.6	67	52	3	3	9	134	0.00
Others	3.4	17	5	1	0	2	25	0.00
Total	100	367	184	22	18	44	635	
G-test (*P*-value) (%)*		0.00	0.00	0.51	2.94	0.00		

*Log-likelihood tests of independence and goodness of fit. R (Ihaka & Gentleman, 1996) function available at www.psych.ualberta.ca/~phurd/cruft/g.test.r
EST, expressed sequence tag.

expressed transcripts across these different categories was assessed using G-tests with Williams correction: (1) within clusters and between categories (null hypothesis: the different categories are represented in a given cluster in proportions equivalent to their representation among spotted ESTs, i.e. are distributed independently of the observed clusters), and (2) within categories and between clusters (null hypothesis: the different clusters are represented in a given category in the same proportions as the total number of genes in each cluster, i.e. clusters contribute independently to any category). These statistics allowed us to take into account the different numbers of ESTs in each cluster/functional category that would bias a classical G-test. As shown by the *P*-values listed in Table 1, categories and clusters were not distributed within clusters and categories, respectively, in an independent manner.

The majority of the transcripts (87%) were grouped into two clusters: cluster 1 (367 transcripts) and cluster 2 (184 transcripts), which had contrasting expression profiles. Cluster 1 comprised genes up-regulated at the beginning of the season (samples T1–T3), but down-regulated at the end of the gradient (samples T4–T5). Among the genes of known function, the most represented categories were 'metabolism' (25.2%, including glutamate-ammonia ligase (accession BX253698), a cellulose synthase (UDP-forming) (BX250234), and glucan endo-1,3-beta-D-glucosidase (BX251908)), 'protein synthesis' (17.9%, including mainly ribosomal proteins), 'communication/signal transduction' (7.3%, e.g. a GTP-binding protein SAR (secretion-associated and Ras-related) 1 (BX249833)), and 'transcription' (6.8%, e.g. a glycine-rich RNA-binding protein 7 (BX252406)). Cluster 2 presented an opposite profile, with genes down-regulated at the beginning of the season, but up-regulated at the end of the gradient. This cluster comprised 73% of the transcripts coding for putative or unknown proteins. 'Metabolism' (30% of known genes in this cluster, including 3-dehydroquinate dehydratase (BX250768)), 'transcription' (12% of known genes, e.g. zinc finger protein 216 (BX248938)), and 'protein synthesis' (10%, e.g. the ribosomal proteins) were the main functional categories of this cluster.

The other three clusters only comprised 13% of the transcripts, half being of known function. A common characteristic of these clusters was the presence of a marked peak preceded and/or followed by a much lower expression level. These peaks were observed at T2, T3 and T4 in clusters 3, 4 and 5, respectively. Apart from these different profiles, these clusters also differed in terms of their composition.

In cluster 3, the 'stress response' category comprised four transcripts (two small molecular weight (*smw*) heat-shock proteins (BX249170 and BX255667), an abscisic-stress-ripening protein (BX249387), and a chitinase (BX254191)). The 'protein fate' category also comprised four transcripts (peptidylprolyl isomerase (BX251374), aspartic proteinase (BX249116), heat-shock cognate 70-kDa protein (BX249170), and dynamin GTPase (BX250255)). This cluster also included two transcripts coding for cell wall biosynthesis enzymes (cellulose synthase (UDP-forming) (BX249248) and caffeate-O-methyltransferase (BX254093)) and two transcripts coding for two other proteins (a proline-rich protein (BX250093) and a eukaryotic initiation factor (BX253807)).

In cluster 4, the 'protein synthesis' category comprised seven transcripts (mainly ribosomal proteins). This cluster also included GTP-binding nuclear protein RAN (BX249436), ethylene-responsive element binding factor 3 (BX250239), hydrophobic protein RCI2A (BX251900), histone H2B (BX248896), cytochrome c oxidase copper chaperone (BX255452), and L-ascorbate oxidase (BX251790).

In cluster 5, genes of known function were classified into three main categories: 'metabolism' (glycine hydroxymethyltransferase (BX250275), tryptophan synthase (BX250574), protein phosphatase 2C-like (BX251682) and lipase (BX254252)), 'stress response' (putative dehydrin (BXBX249564), putative plasma membrane-associated protein (BX255221) and nucleotide binding site and leucine-rich repeat domains (NBS/LRR) proteins (BX254260)), and 'communication/signal transduction' (Ras-related protein serine/threonine-protein mitogen-activated kinase MAK (BX249205) and putative transmembrane kinase-like protein TMKL1 (BX250142)). The other genes of known function in cluster 5 corresponded to genes encoding an arabinogalactan protein (BX255488), ubiquitin ligase restorer-of-fertility nuclear protein RNF8 (BX251836), a pyruvate kinase (BX253452), a tubulin beta-3 chain protein (BX249322), and a 28-kDa ribonucleoprotein (BX251694).

Amplitude of variation For each of the differentially expressed genes, the amplitude of variation between the minimum and maximum values during the seasonal gradient was computed (Supporting Information Table S9). Briefly, 29% of the genes presented a maximum:minimum expression ratio of < 3, 49.2% a ratio of between 3 and 10, and 21.8% a ratio of > 10.

A total of 10 genes presented a ratio > 100, including, among the genes of known function, an arabinogalactan/proline-rich protein (100-fold; BX249981), a glycine-rich RNA-binding protein 7 (101-fold; BX252406), an iron transport multicopper oxidase FET5 (126-fold; BX252150), a tubulin beta chain (β-tubulin) (129-fold; BX249177), a glutamate-ammonia ligase (202-fold; EC 6.3.1.2; BX253698), a metallothionein-like protein (302-fold; BX249412), and a peptidylprolyl isomerase (469-fold; EC5.2.1.8; BX251374).

Gene expression analysis by qPCR Using a different and more powerful transcriptome technology (qPCR), several genes that were detected as differentially expressed between the extreme samples of the 2003 growing season were re-analyzed in 2006 (Supporting Information Fig. S2). This investigation was carried out for two biological replicates, on seven genes: a metallothionein-like protein (BX249412), a glutamine ammonia-ligase (BX253698), an arabinogalactan/proline-rich protein (BX249981), a putative protein (BX249755), a glycine-rich RNA-binding protein 7 (BX252406), an iron transport multicopper oxidase FET5 (BX252150), and a fructose-bisphosphate aldolase (BX249425). These genes were initially found to be up-regulated during EW formation in 2003. For five genes, we found that qPCR expression profiles displayed the same trend as that observed in 2003 for both genotypes (Supporting Information Fig. S2b–f), that is, they were found to be up-regulated in the EW-forming tissue. However, the maximum:minimum ratio of expression levels was much lower for the 2006 samples. Using the primer pairs presented in Table S1, we were not able to obtain qPCR expression profiles for glycine-rich RNA-binding protein 7 (BX252406) and iron transport multicopper oxidase FET5 (BX252150), because of low PCR efficiency.

Discussion

Variability of tracheid dimension and cell wall chemical composition in response to variations in edapho-climatic conditions

Anatomical, chemical and physiological studies have shown that the consequences of environmental fluctuations such as drought stress can be tracked within the secondary xylem of forest tree species (Zahner, 1968; Liphschitz & Waisel, 1970; Barber *et al.*, 2000). Wood can therefore be considered as a bio-marker of environmental changes. In this report, the analysis of wood properties of developing xylem (cell wall chemical composition, fibre morphology and metabolites) sampled during the 2003 growing season gave us a unique opportunity to study the phenotypic plasticity of differentiating xylem in a single *P. pinaster* genotype in response to changes in edapho-climatic conditions.

PCA (Fig. 7) suggested that differences in climatic conditions strongly influenced the observed annual pattern of cell wall chemistry and fibre morphology. Higher relative atmospheric humidity and precipitation promoted the accumulation of proteins and hemicelluloses, while less water promoted lignin and resinic acid biosynthesis. Moreover, higher SMD and temperature induced an increase in cellulose (cH7) accumulation.

Tracheid dimension also responded to variation in edapho-climatic conditions (see Fig. 2). A negative relationship was found between fibre length and SMD. The shorter tracheids of sample T5 also had higher cellulose content, probably reflecting a thicker cell wall, characteristic of LW-forming fibre. Pittermann *et al.* (2006a) reported that an increase in cell wall reinforcement was associated with a decrease in tracheid length, implying that stronger tracheids tended to be shorter. Pittermann *et al.* (2006b) also found that tracheid diameter was nearly optimized to achieve the greatest hydraulic efficiency for a given tracheid length. In contrast, the positive relationship that was found between lignin content and fibre width is probably the result of a higher proportion of primary cell wall (more lignin-rich) in EW-forming tracheids (i.e. in samples T1 and T2), rather than a causal correlation.

Resinic acids accumulated early in the season (T1 and T2 samples) and then dropped to very low levels for samples T3 and T4, and finally increased at T5. We attributed the reduction

at T3 and T4 to the heavy rainfall before sampling date T3. This suggestion is supported by the finding that climatic conditions affect the accumulation of secondary metabolites and particularly resinic acids in developing xylem. In conifers, it is well known that water deficit causes the accumulation of constitutive terpenes and flow of constitutive resin (Lombardero *et al.*, 2000; Turtola *et al.*, 2003), as well as an increase in the number of specialized terpene secretory structures (Lewinsohn *et al.*, 1991).

Molecular plasticity during the growing season

Using our stringent statistical criteria, we found that 19% of genes (667 genes) were differentially expressed during the 2003 growing season. A similar proportion was found by Egertsdotter *et al.* (2004) in *Pinus taeda*. Among these genes, 21.8% displayed a maximum:minimum ratio > 10, showing that the transcriptome of differentiating xylem was greatly affected during the annual course of wood formation.

Based on their expression profiles, differentially expressed genes were clustered into five groups. The two most important clusters (1 and 2) contained 83% of the genes, corresponding to genes differentially regulated in EW- and LW-forming tissues. Three minor clusters (3, 4 and 5) included fewer genes whose expression profiles could be interpreted as responsive to particular edapho-climatic conditions encountered during the study period.

In the following sections we will discuss some of the genes found in the contrasting clusters, referred to as 'EW' and 'LW' responsive genes for clusters 1 and 2, respectively, as well as genes belonging to clusters 3, 4 and 5, which we refer to as 'specific edapho-climatic condition' responsive genes.

EW responsive genes A total of 95 genes were found to be over-expressed early in the season (cluster 1) with a fold-change ratio > 10, of which six (whose expression profiles were analyzed by qPCR; Supporting Information Fig. S2) presented ratios > 100. These included the following.

Three genes encoding metallothionein-like proteins (MTs; BX249412 (302-fold, validated by qPCR; Supporting Information Fig. S2b), BX249603 (32.9-fold) and BX252580 (14.7-fold)) were over-expressed in EW-forming tissues. MTs play a role in detoxification of heavy metals and in homeostasis of intracellular metal ions (Cobbett & Goldsbrough, 2002). Although their exact function is still not completely understood, it seems that MTs may be expressed as part of a general stress response. Bhalerao *et al.* (2003) and Andersson *et al.* (2004) reported that MTs are induced during leaf senescence. Xylogenesis is characterized by the genetically programmed loss of cell structure and metabolic function, leading to cell death (Fukuda, 1996). We suggest that MTs could play a crucial role as metal chelators, in protecting differentiating xylem cells from the toxic effects of metal ions released during the lignification and programmed cell death (PCD) steps.

A glutamate-ammonia ligase (also glutamine synthetase (GS); EC 6.3.1.2; BX253698; 202-fold) was also validated by qPCR (Supporting Information Fig. S2c). This enzyme is implicated in the nitrogen metabolism cycle. In actively lignifying cells the phenylpropanoid-N cycle involves the enzymes phenylalanine ammonia-lyase (PAL) (EC4.3.1.5), GS and possibly glutamine-oxyglutarate aminotransferase (GOGAT) (EC 1.4.1.14, EC 1.4.7.1), which has a major role in rapidly recycling the ammonium liberated by the PAL reaction. The high level of accumulation of GS at the beginning of the growing season is also consistent with the higher lignin content of samples T1 and T2 probably resulting from increased PAL activity. Indeed, one PAL (BX248906; 3.5-fold) was also found to be over-expressed during 'EW' formation.

An arabinogalactan/proline-rich protein (AGP; BX249981; 100-fold, validated by qPCR; Supporting Information Fig. S2d) belongs to a family of proteins in *P. pinaster* that have been found to be abundantly expressed in wood-forming tissues (Paiva, 2006). Proteome analysis of *P. pinaster* wood-forming tissues also showed that one spot corresponding to an AGP was identified as an 'EW' protein (Gion *et al.*, 2005). Numerous potential roles of AGP during xylogenesis have been proposed, including roles in cell division and expansion (reviewed by Schultz *et al.*, 2000), secondary cell wall initiation (Kieliszewski & Lamport, 1994), and PCD (Schindler *et al.*, 1995, Greenberg, 1996). The over-expression of this putative AGP-like protein could be related to the higher rate of fusiform initials differentiating in spring.

A gene encoding a putative protein (BX249775; 153-fold, validated by qPCR; Supporting Information Fig. S2e) was also highly expressed in EW-forming tissues. It should be noted here that the length of this EST was very small (132 bp) which may explain its lack of homology with a gene of known function.

Two other genes, encoding a glycine-rich RNA-binding protein 7 (GR-RBP; BX252406; 101-fold) and an iron transport multicopper oxidase FET5 (MCO-FET5; BX252150; 126-fold), could not be validated by qPCR with the tested primer pair, because of low efficiency.

- ***Cell division-related genes***

Genes encoding a cyclin A/CDK2-associated protein (BX255795) and cyclin-dependent kinase regulatory subunit 1 (CKS-1; BX251584) were over-expressed in EW-forming tissue and showed fold-change ratios of 10.8 and 5.2, respectively. These genes are known to be involved in the control of cell cycle progression (Horvath *et al.*, 2003). This result indicates that cell division was probably highly activated at the beginning of the season, in agreement with Uggla *et al.* (2001).

- ***Energy-related genes***

Among the genes up-regulated in EW-forming tissues, and related to energy production, we found a fructose-bisphosphate aldolase (EC4.1.2.13; BX249425; 58-fold, also validated by qPCR; Supporting Information Fig. S2f), a cytochrome c1 (BX254511; 32.3-fold), a naphthoate synthase (EC 4.1.3.36;

BX250373; 11.5-fold), and a glyceraldehyde 3-phosphate dehydrogenase (G3PDH; EC 1.2.1.13; BX249100; 11-fold).

Fructose-bisphosphate aldolase (BX249425) was also found to be highly expressed in xylem compared with seven other tissues (Paiva, 2006). However, it should be noted that another transcript of fructose-bisphosphate aldolase (BX249029; 22.9-fold) was also found in the LW cluster (cluster 2), suggesting the presence of members of the same gene family with different levels of regulation in different types of wood-forming tissues.

G3PDH is implicated in primary metabolism, namely in energy production. Le Provost *et al.* (2003) previously showed that a transcript of this enzyme was up-regulated in EW-forming tissues.

• ***Genes involved in sugar transport and cell wall biogenesis***

Sucrose has been shown to be the major carbohydrate of cambial metabolism (Krabel, 2000). In the 2003 growing season, we found that the polysaccharide content in the apolar fraction reached its maximum in sample T3. We also found that fructose and hexapyranose types increased from T1 to T3 in the polar metabolic fraction, followed by a decrease of sucrose (Fig. 6 and Supporting Information Table S4). At the molecular level, four transcripts showing carbohydrate transporter activity were found in cluster 1, including a D-xylose-proton symporter (D-xylose transporter; BX251928; 26.9-fold), a putative sugar transporter (BX250728; 24.3-fold), and two plasma membrane H^+-ATPases (PM H^+-ATPases; BX249881, 9.6-fold and BX253719, 7-fold). The PM H^+-ATPase is a key enzyme that generates the proton-motive force that drives the uptake of nutrients such as sugars and ions across the plasma membrane of growing plant cells. It seems to be especially important in the uptake of potassium ions (Hoth *et al.*, 1997). This uptake is essential for osmotic regulation and cell enlargement in differentiating tissues (MacRobbie, 1977; Hsiao & Läuchli, 1986). Arend *et al.* (2002) reported increased abundance of PM H^+-ATPase in spring in cambial cells and expanding xylem. Paiva (2006) also reported that a gene coding for PM H^+-ATPase (TC51926) was abundantly represented in the differentiating xylem library of *P. pinaster*. He also showed that the corresponding tentative consensus (TC51926) of the Pine Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) was significantly over-expressed in xylem libraries when compared with other tissue libraries. A seasonal variation of PM H^+-ATPase transcripts was also observed in the bud tissue of the peach tree (*Prunus persica*; Gévaudant *et al.*, 2001) and in roots of *Pinus sylvestris* (Iivonen & Vapaavuori, 2002).

Intracellular traffic-related genes were found to be highly expressed in EW-forming tissues. These included an exportin 1 (BX249137; 94.6-fold), a GTP-binding protein SAR 1 (BX249833; 41.3-fold), and an ADP-ribosylation factor (BX253253; 26.8-fold).

We also found several genes implicated in the carbohydrate/polysaccharide metabolism of the cell wall to be strongly up-regulated in EW-forming tissues, namely, a dTDP-glucose 4,6-dehydratase (EC 4.2.1.46; BX252145; 75.3-fold), a cellulose synthase (*Ppin*CESA3; BX250234; 38.5-fold), a glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39; BX249285; 35.5-fold), and an alpha-1,4-glucan-protein synthase (UDP-forming; EC 2.4.1.112; BX250805; 34.2-fold).

To conclude, the over-expression in EW of genes involved in cell wall carbohydrate metabolism, sugar transport and intracellular trafficking suggests that a substantial mobilization of carbohydrates occurred at the beginning of the growing season. Indeed, during cambial growth, the cambial region acts as a strong axial sink, which probably competes for minerals and assimilates with other sinks such as young leaves and roots (Dünisch & Bauch, 1994; Krabel, 2000).

LW responsive genes Fold-change ratios for LW-related genes were much lower compared with EW-related genes. In addition, for > 72.5% of the LW differentially expressed genes, we could not assign a known function, a proportion that was much higher than the average of the *P. pinaster* xylem ESTs, which was 53% (Paiva, 2006). A total of 37 genes were found to be over-expressed late in the season (cluster 2) with fold-change ratios > 10. Among these, 29 corresponded to putative proteins or proteins of unknown function.

Only three genes showed a maximum:minimum ratio > 100. These included two putative proteins (BX251778, 666 bp, 112-fold and BX253111, 122 bp, 165-fold) and one β-tubulin (BX249177; 129-fold). LW tracheids are characterized by a thicker secondary cell wall, which mainly consists of highly ordered cellulose deposits. Microtubules mainly consist of α-tubulin and β-tubulin, and are implicated, among other functions, in the orientation of cellulose microfibrils during the differentiation of tracheary elements (Chaffey *et al.*, 1998; Spokevicius *et al.*, 2007; Oakley *et al.*, 2007). Interestingly, one F-actin capping protein alpha subunit (BX250456; 3.1-fold), which is involved in actin assembly, was found to be co-regulated with this β-tubulin. Together these results highlight the importance of cytoskeletal proteins in LW formation. Genes of the 'transcription' category were also well represented in cluster 2, which contained six genes from this category, including a gene encoding a zinc finger protein 216 (BX248938; 49.6-fold).

Effect of particular edapho-climatic conditions on gene expression Our sampling strategy also made it possible to detect modifications of the transcriptome (identified from peaks of transcript accumulation) in response to particular changes in edapho-climatic conditions.

Sampling date T2 was preceded by a long period (30 d) without effective precipitation (daily mean 0.5 mm and 18 d without rainfall), and with high temperatures for the time of year and continuous loss of SWC. LUE was maximal at T2 and we also observed an increase in transpiration rate, probably as a result of leaf area expansion. Additionally, a pronounced

decline in stomatal conductance was predicted by the GRAECO model, suggesting that the trees were under drought stress. We therefore suggest that genes of cluster 3 (Fig. 8), which showed a peak of expression at T2, are involved in a physiological response to increasing loss of SWC. This cluster comprised genes coding for stress-responsive proteins, that is, three heat-shock proteins (BX255667, 5.4-fold; BX249170, 9.2-fold and BX251102, 42.5-fold), one protease (aspartic proteinase; BX249116; 15.8-fold), and an abscisic stress ripening protein (ASR; BX249387; 6.5-fold). A peptidylprolyl isomerase (BX2499248) showed the highest fold-change ratio of all differentially expressed genes of this study (469-fold). Peptidylprolyl isomerase is implicated in the ubiquitin-proteasome pathway. It should be noted that another peptidylprolyl isomerase (BX249687; 27.3-fold) and a ubiquitin-protein ligase (EC 6.3.2.19; BX248908; 11.1-fold) were also found in cluster 1, and were thus considered as EW-related genes. Together, these results suggest the direct recruitment of stress-responsive genes, as well as genes involved in the modification of the metabolic machinery, via the ubiquitin-proteasome pathway.

A cellulose synthase (*Ppin*Cesa1; 97% identical to *Pt*Cesa2; BX249248; 6.7-fold), a caffeate O-methyltransferase (COMT; BX254093; 3.0-fold), and a proline-rich protein (PRP; ortholog of *Pta*PRP1; 2.9-fold) were also found to be over-expressed in sample T2. *Ppin*Cesa1 is involved in cellulose biosynthesis in secondary cell walls, while COMT is implicated in lignification. PRPs have also been reported to be involved in lignification (Cassab, 1998). Indeed, PRPs may provide sites for selective complex formation with phenolic precursors, for example for initiation or polymerization of the lignin polymer (Whitmore, 1978; Zhang *et al.*, 2000). The up-regulation of these cell wall biosynthesis-related genes could be related to the reinforcement and reduced permeability of the cell wall in response to the rapid decrease of SWC.

Heavy rainfall preceded T3 sampling, temporarily increasing SWC. This physiological state was reflected by genes of cluster 4, which showed a peak of expression at T3 (Fig. 8). This cluster was characterized by a high number of transcripts related to protein synthesis, consistent with the peak of protein content measured by FTIR spectroscopy and analytical pyrolysis. The fold-change ratios of six ribosomal genes were found to vary between 2.3 and 39. Another interesting gene encoded a GTP-binding nuclear protein RAN (BX249426; 61.0-fold), which is known to regulate the karyopherins, which are involved in nucleocytoplasmic transport, also indicative of high transcriptional activity occurring at T3.

Finally, the genes included in cluster 5 showed a peak of expression at T4 (Fig. 8). Their expression profile could be related to the sudden increase of SMD and abnormally high temperatures in late spring. Ten genes with known functions were found to have a fold-change ratio > 2, including a gibberellin (GA)-stimulated 5 (GASA5)-like protein (BX249894; 3.5-fold), NBS/LRR (BX254260; 3.1-fold), an arabinogalactan protein (AGP; BX255488; 2.9-fold), and a putative plasma membrane-associated protein (BX255221; 2.6-fold). Arabidopsis GASA is homologous to the original GA-regulated tomato (*Lycopersicum esculentum*) gene GA-stimulated 1 (GAST1) (Aubert *et al.*, 1998), with GAST1 homologs implicated in cell division (Aubert *et al.*, 1998), cell elongation (Taylor & Scheuring, 1994) and radial cell expansion (Kotilainen *et al.*, 1999). Israelsson *et al.* (2005) reported a dramatic increase in expansion zones of wood-forming tissues where concentrations of bioactive GA were highest. The coincidence between a peak of this *P. pinaster* GASA5-like expression and an increase in fibre width also suggests a role for this gene in radial expansion of maritime pine wood-forming tissues. AGPs are cell wall proteins that have been implicated in many processes of plant growth, development and adaptation, including, cell proliferation, expansion and differentiation, and plant defence (reviewed by Majewska-Sawka & Nothnagel, 2000). AGPs have been reported to be among the most expressed genes in poplar (*Populus* spp.) and pine stems (Sterky *et al.*, 1998, 2004; Paiva, 2006).

Concluding remarks and perspectives

In this paper, the metabolic profiling and cell wall chemical composition of wood-forming tissues and fibre morphology were assessed during a growing season. We found that differences in edapho-climatic conditions (mainly air temperature and soil water availability) strongly influenced the observed annual patterns of metabolite and chemical composition. Our results show that, in favourable eco-physiological conditions, trees channel carbon and energy towards growth and cell division, and increase protein and hemicellulose contents. Conversely, water deficit induced an increase in the allocation of carbon to cellulose and lignin biosynthesis, probably reflecting an increase in cell wall thickening for hydraulic conductance protection. In addition, transcript profiling provided us with new hints about the molecular players involved in wood formation, and their plasticity during the growing season. A step forward is to ask whether this plastic response has an adaptive value. A recent study in *P. pinaster* (Eveno *et al.*, 2008) identified, among differentially expressed candidate genes for the drought stress response, genes that showed a departure from neutrality and demographic equilibrium, which is a first indication of the adaptive value of these genes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Weekly mean temperature, accumulated precipitation, stomatal conductance, transpiration, daily soil water content and soil moisture for years 2003 and 2006.

Fig. S2 Quantitative real-time PCR (qPCR) expression profiles in the 2006 samples (genotypes 4015 and 3006).

Table S1 List of primer pairs used in the quantitative real-time PCR (qPCR) assay for earlywood up-regulated genes

Table S2 Edapho-climatic and predicted ecophysiological variables obtained using the GRAECO model (Loustau *et al.*, 2005)

Table S3 (1) Identification of pyrolysis products of differentiating xylem samples collected during the 2003 growing season. (2) Synthetic cell wall chemical composition variables

Table S4 Quantification of metabolites extracted in dicholoromethane and methanol

Table S5 Eigenvalues and percentage of variation explained by principal components (PCs)

Table S6 Quality of the representation of fibre morphology, synthetic pyrolysis product and metabolite variables

Table S7 Quality of sample representation

Table S8 Correlation of supplementary edapho-climatic and ecophysiological variables with principal components (PCs)

Table S9 List of 3512 cDNA clones used for high-density filter preparation and statistical analysis of expression levels

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