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RESEARCH PAPER

Molecular changes associated with the setting up of secondary growth in aspen

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Abstract

Vascular secondary growth results from the activity of the vascular cambium, which produces secondary phloem and secondary xylem. By means of cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis along aspen stems, several potential regulatory genes involved in the progressive transition from primary to secondary growth were identified. A total of 83 unique transcript-derived fragments (TDFs) was found to be differentiated between the top and the bottom of the stem. An independent RT-PCR expression analysis validated the cDNA-AFLP profiles for 19 of the TDFs. Among these, seven correspond to new genes encoding putative regulatory proteins. Emphasis was laid upon two genes encoding, respectively, an AP2/ERF-like transcription factor (PtaERF1) and a RING finger protein (PtaRHE1); their differential expression was further confirmed by reverse northern analysis. *In situ* RT-PCR revealed that *PtaERF1* was expressed in phloem tissue and that *PtaRHE1* had a pronounced expression in ray initials and their derivatives within the cambial zone. These results suggest that these genes have a potential role in vascular tissue development and/or functioning.

Key words: AP2/ERF, aspen, cDNA-AFLP, *in situ* RT-PCR, RING-H2, secondary growth, vascular cambium.

Introduction

Plant growth originates from meristems, localized tissues with stem cell features that are at the origin of all organs of the plant (Bäurle and Laux, 2003). Apical meristems, contained in shoot and root tips, are involved in the extension of the components of the primary plant body. During this primary growth stage, water and sap conduction are ensured, respectively, by the primary xylem and the primary phloem that originate from procambial initials (Esau, 1965). In plants undergoing secondary growth (gymnosperms and dicotyledonous angiosperms), secondary vascular tissues that originate from cambium provide mechanical strength and allow long-distance transport of water and sap. Secondary growth, which results in the increase in the girth of stems, branches and roots, may have originated in the Middle Devonian, and cambium is present in several unrelated groups of vascular plants from the Upper Devonian (Kenrick and Crane, 1997; Lachaud *et al.*, 1999).

The cambium has typically two morphologically distinct cell types, or initials: the axillary elongated fusiform initials leading to the axial system (including tracheids, vessel elements, fibres, axial parenchyma cells, sieve elements, and companion cells) and the smaller isodiametrical ray initials giving origin to the radially orientated parenchymatous rays (Iqbal and Ghouse, 1990). Anticlinal divisions of the cambial initials cause enlargement of the circumference

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of the cambial cylinder whereas periclinal divisions produce either a phloem or a xylem mother cell (also called derivatives), leaving initial cells in the meristem. Fusiform and ray derivatives may divide several times before differentiating into vascular tissues (reviewed by Lachaud *et al.*, 1999; Dengler, 2001; Mellerowicz *et al.*, 2001). Because most anatomical criteria are often not sufficient to discriminate between cambial initials and derivatives, the term cambial zone is used to denote these cell types. However, differences in cell wall composition (Catesson *et al.*, 1994) or ultrastructural characteristics (Arend and Fromm, 2003) between cells of the cambial zone suggest that differentiation occurs early in cambial derivatives.

The vascular cambium has to maintain a balance between cell proliferation and cell differentiation, and, as for the apical meristem, this process is most likely under strict developmental control (reviewed by Dengler, 2001). For instance, in the shoot apical meristem, it has been demonstrated that WUSCHEL (WUS) promotes stem cell division whereas CLAVATA 1–3 (CLV1–3) repress mitotic activity and promote the commitment to leaf primordia formation (Brand *et al.*, 2000; Schoof *et al.*, 2000). The expression of *CLV1* is up-regulated in *Arabidopsis* stems undergoing secondary growth, but no expression was monitored for *CLV3* and *WUS* in the cambium (Ko and Han, 2004). Accordingly, the putative orthologue *PttCLV1* has been shown to be expressed in the cambial zone of *Populus*, but not *PttWUS* or *PttCLV3*, suggesting that similar but not identical mechanisms and/or genes are involved in the identity and maintenance of the vascular meristem, compared with the apical meristem (Schrader *et al.*, 2004; Ko and Han, 2004).

In *Arabidopsis*, the onset of body weight-induced secondary growth has been investigated by microarray analysis. Several up-regulated genes in wood-forming stems were shown to possess auxin responsive *cis*-acting elements in their promoter region, indicating auxin-mediated regulation of secondary growth (Ko *et al.*, 2004). However, although the *Arabidopsis* system has been suggested to be a good model to study wood formation, an apparent major structural difference in secondary xylem between *Arabidopsis* and poplar is the lack of rays in *Arabidopsis* (Chaffey *et al.*, 2002). Throughout the life of woody plants, the transition from primary to secondary growth is progressive and may require a specific regulatory network of molecular interactions.

The aim of the present study was to investigate the spatio-temporal transition from primary to secondary vascular growth in hybrid aspen. A cDNA-AFLP approach, which permits distinction between highly homologous genes among families, allowed the identification of novel putative regulatory genes associated with secondary growth in aspen. Particular attention was paid to two genes, the expression of which was localized in vascular tissues, as assayed by *in situ* RT-PCR.

Materials and methods

Plant growth conditions and sampling

Populus tremula×*P. alba* (clone INRA 717 1B-4) was micropropagated *in vitro* on half-strength MS medium (Duchefa Biochemie, Haarlem, The Netherlands). Six-week-old plants were acclimatized in the greenhouse. For cDNA-AFLP analysis, stem samples were harvested in May 2000 from three 6-month-old trees (approximate height of 150 cm). Top samples (T) consisted of the 15 cm of the stem below the shoot apical meristem and the bottom samples (B) were harvested at a height of between 30 and 50 cm above the soil. The bark was peeled away and the samples were immediately frozen in liquid nitrogen. For RT-PCR analysis stem samples were harvested in March 2003 from a 6-month-old greenhouse-grown aspen tree. The sampling was the same as for the cDNA-AFLP analysis (B, T) with an additional two middle samples (M1 and M2) in between. Leaf samples consisted of mature leaves from which main veins were removed (L) and of entire unexpanded young leaves (YL).

Safranin-stained cross-sections

Top and bottom stem samples were soaked in polyethyleneglycol (PEG) 1500 for 7 d at 65 °C. Thin cross-sections (15–30 μm) were made using a sledge microtome armed with a C-profile solid knife (Reichert, Vienna, Austria) and soaked in 75% (v/v) ethanol. Cross-sections were treated successively with 8% (v/v) commercial bleach, 10% (v/v) acetic acid, 50% (v/v) ethanol, and 0.1% (w/v) safranin. Progressive dehydration (from 50% to 100% ethanol) was performed, before washing thin sections in parasolve overnight and mounting on glass-slides. Pictures were taken with an Olympus PM-20 camera system coupled to an Olympus BX60 microscope (Olympus, Tokyo, Japan).

cDNA-AFLP analysis

Total RNA was extracted from the plant samples according to Verwoerd *et al.* (1989). cDNA-AFLP was performed according to Bachem *et al.* (1996) and Durrant *et al.* (2000), with some minor modifications. Double-strand (ds) cDNA was synthesized using a cDNA Synthesis Module (Amersham Biosciences, Uppsala, Sweden). Ds cDNA was digested with *Eco*RI (Promega, Madison, WI, USA) and *Mse*I (New England Biolabs, Beverly, MA, USA) and specific adaptors were ligated with T4-DNA ligase (Amersham Biosciences). Adaptors were made from oligos synthesized by Proligo (Paris, France): 5′-GACGATGAGTCCTGAG-3′ and 3′-TACTCAGGACTCAT-5′ for *Mse*I sites and 5′-CTCGTAGACTGCGTACC-3′ and 3′-CTGACGCATGGTTAA-5′ for *Eco*RI sites. Pre-amplification was performed at an annealing temperature of 60 °C for 28 cycles with the following primers: 5′-GACTGCGTACCAATTC-3′ (*Eco*RI-adaptor primer) and 5′-GATGAGTCCTGAGTAA-3′ (*Mse*I-adaptor primer). Selective amplification was performed with 176 combinations of *Eco*RI-adaptor(+NN) and *Mse*I-adaptor(+NN) primers. Differential bands were cut out of the gels and DNA was eluted either in TE or by electro-elution on 1% low-melt agarose (Bio-Rad, Hercules, CA, USA) gels. The eluted DNA was then re-amplified, using the same primer set used for the selective amplification. cDNA fragments were cloned in the PCR4®-TOPO vector (Invitrogen™, Carlsbad, CA, USA) and sequenced. Similarity searches were carried out with WU-BLASTX+BEAUTY (http://searchlauncher.bcm.tmc.edu/seq-search/nucleic_acid-search.html). NCBI BLAST 2.0 analyses were also performed against the EST databases PopulusDB (http://poppel.fysbot.umu.se/), PoplarDB (http://mycor.nancy.inra.fr/BLAST/blast.html), and AspenDB (http://aspendb.mtu.edu/), against the *P. trichocarpa* draft genome sequence database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and against the TAIR database (http://www.arabidopsis.org/Blast/)(Altschul *et al.*, 1997).

RT-PCR analysis

Total RNA was prepared using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) then treated with DNase I (DNAfree™ from Ambion, Austin, TX, USA). RNA concentrations were estimated spectrophotometrically at 260 nm and were further adjusted using the RiboGreen® reagent (Molecular Probes, Eugene, OR, USA). Single-stranded cDNA was synthesized using a Reverse Transcription System (Promega). PCR reactions were performed using a Master Mix from Promega, with various PCR conditions depending on the sequences and the primer pairs (Table S1: supplementary data that can be found at JXB online). *18S* rRNA was used as internal control. Primer pairs used for RT-PCR analysis were designed either on cDNA-AFLP TDFs, or on highly similar ESTs (identity ⩾93%) when those were available (Table S1: supplementary data that can be found at JXB online).

Reverse northern analysis

cDNA was amplified from each cDNA-AFLP clone using M13 Forward (F) 5′-TGACCGGCAGCAAAAT-3′ and M13 Reverse (R) 5′-GGAAACAGCTATGACCATG-3′ primers. Genomic DNA and *EF1-α* cDNA were used as internal controls, and *PM43* (a plasmid containing a set of cDNA-AFLP adaptors) and TE buffer were used as negative controls to monitor background. PCR products were arrayed on nitrocellulose filters using a MicroGrid II robot (Biorobotics, Cambridge, UK). Each cDNA-AFLP TDF was spotted 12 times. Genomic DNA, *EF1-α*, *PM43*, and TE buffer controls were spotted 70, 70, 94, and 114 times, respectively. Total RNA was prepared as described above from samples corresponding to those used for cDNA-AFLP. For each sample, 5 μg total RNA was reverse-transcribed with ABgene Reverse-iT RTase Blend (Epsom, UK) and labelled with dCTP α[^{33}P] (Amersham Biosciences). Membranes were prehybridized for 3 h and hybridized overnight in 3× SSC, 0.5% SDS, 10% PEG 6000, and 0.2% skimmed milk at 60 °C. Membranes were washed twice with 3× SSC, 0.5% SDS and once with 1× SSC, 0.5% SDS and exposed in a PhosphorImager cassette (Molecular Dynamics) for 72 h. PhosphorImager screens were then scanned with a Storm 850 scanner (Amersham Biosciences). Detection and quantification of the signals were performed using the imaging software ImageQuant 5.0 (Amersham Biosciences). The mean background of each membrane (estimated as the mean pixel intensity of TE buffer spots) was subtracted from each spot. A Wilcoxon paired-sample test was then performed on the 12 repetitions to identify TDFs that showed a significant differential pattern (P <0.01).

In situ *RT-PCR analysis*

Transverse sections were hand-cut on stems of 1-month-old acclimatized aspens. Liquid phase *in situ* RT-PCR was performed according to Pesquet *et al.* (2004) with modifications for nucleotide-based epitopic detection. Genomic DNA restriction was performed with one or several of the following enzymes, depending on the sequence of the cDNA to be amplified: *Taq*I, *Msp*I, *Hin*fI, *Hae*III, or *Rsa*I (Promega). For RT-PCR, the following pairs of primers were used: F 5′-TCAACACAGCATTCGAGAGG-3′ and R 5′-ATGCAGCGCATTCAATTACA-3′ for *CAD* cDNA (accession no. AF217957), F 5′-CTTCTGGAAGGGATGCATTT-3′ and R 5′-CGAACCCTAATTCTCCGTCA-3′ for *18S* rRNA gene (accession no. AJ236016), F 5′-TCTCCACCTTTACGCTCGTT-3′ and R 5′-CACTCGGTGTGGAAACTGTG–3′ for *PtaRHE1*, and F 5′-TTCTATCCCGGTCAATGGAG-3′ and R 5′-GCTTCCACAAAGACGGATTC-3′ for *PtaERF1*. During PCR, products were labelled with Dig-11-dUTP (Roche, Mannheim, Germany) at 10 pmoles per reaction (for a total content of 1 μmole dNTP per reaction). After PCR, cross-sections were washed twice in 100 mM TRIS–HCl pH 7.5, 150 mM NaCl, then incubated for 1 h in 100 mM TRIS–HCl pH 7.5, 150 mM NaCl, 5% (w/v) BSA. Anti-digoxigenin-alkaline phosphatase Fab fragments (Roche) were then added and the reaction was allowed to proceed for 30 min. Cross-sections were then washed three times in 100 mM TRIS–HCl pH 9.5, 150 mM NaCl, and 0.2% (v/v) Tween. Subsequently, cross-sections were incubated for 15–30 min in alkaline phosphatase substrate solution prepared from NBT/BCIP tablets (Roche). The staining reaction was stopped by washing the cross-sections in TE buffer pH 8. Pictures were taken with a light-microscope (Nikon Eclipse E800M, Tokyo, Japan) equipped with a digital still camera-based system (DXM1200 Nikon).

Cloning of PtaRHE1 and PtaERF1 coding sequences

Poly(A) mRNA was isolated from aerial parts of 3-month-old aspen using a PolyATtract®mRNA Isolation System III (Promega). The 5′ end of the *PtaRHE1* cDNA sequence was isolated using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA) with the following gene-specific primer: 5′-AAGCCGATCCCAAATGATCCTCCTC-3′. The 3′ end of *PtaRHE1* was recovered from a 96% identical overlapping homologous sequence (scaffold_77: 1287648:1288442) of the *P. trichocarpa* draft genome sequence. The 987 bp sequence of *PtaRHE1* was amplified by PCR using gene-specific primers F 5′-TGCTAAACCAAACCCATTATCC-3′ and R 5′-CTAACCGGGTCAAGAATTGC-3′. The 5′ and the 3′ ends of the *PtaERF1* cDNA sequence were isolated using a Marathon cDNA Amplification Kit with the following gene-specific primers 5′-GGATGCCTTGCTGAAGCACATGGAG-3′ and 5′-CGTGGCAAGAAAGCGAAGGTGAACT-3′, respectively. The 1409 bp sequence of *PtaERF1* was amplified by PCR using the gene-specific primers F 5′-ATAATCACCATCAATCA-3′ and R 5′-CTAAATAAGTCACACCACATT-3′. Both cDNAs were subsequently cloned in a PCR4®-TOPO vector and sequenced.

The sequences of *PtaRHE1* and *PtaERF1* were analysed with the BLAST programs on the NCBI and PopulusDB databases, as well as on the TAIR database (http://www.arabidopsis.org/Blast/) (Altschul *et al.*, 1997). Protein sequences alignment was made with Clustal W (http://www.ebi.ac.uk/clustalw) (Thompson *et al.*, 1994), and page setup was made with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Molecular weight was determined with the software Pepstats (http://bioweb.pasteur.fr/seqanal/interfaces/pepstats.html).

Nucleotide sequence accession numbers

Sequences of *PtaRHE1* and *PtaERF1* are deposited in the GenBank nucleotide sequence database under Accession numbers AY780430 and AY780431, respectively. Sequences of the 83 TDFs are registered under the Accession numbers CV555406-CV555488.

Results

Anatomical features of aspen stem tissues used for cDNA-AFLP analysis

The goal was to identify genes encoding regulatory proteins involved in the progressive transition from primary to secondary growth in aspen. Towards this end, cDNA-AFLP analysis was performed on stem tissues corresponding to two different developmental stages along a stem of a 6-month-old aspen tree (150 cm) (Fig. 1A). The first sample (top sample) consisted of the 15 cm of stem located immediately beneath the apical meristem. In the upper part of this sample (Fig. 1B, section 1), the vascular system consists of primary xylem and phloem, whereas the lower part of this stem sample is characterized by the presence of

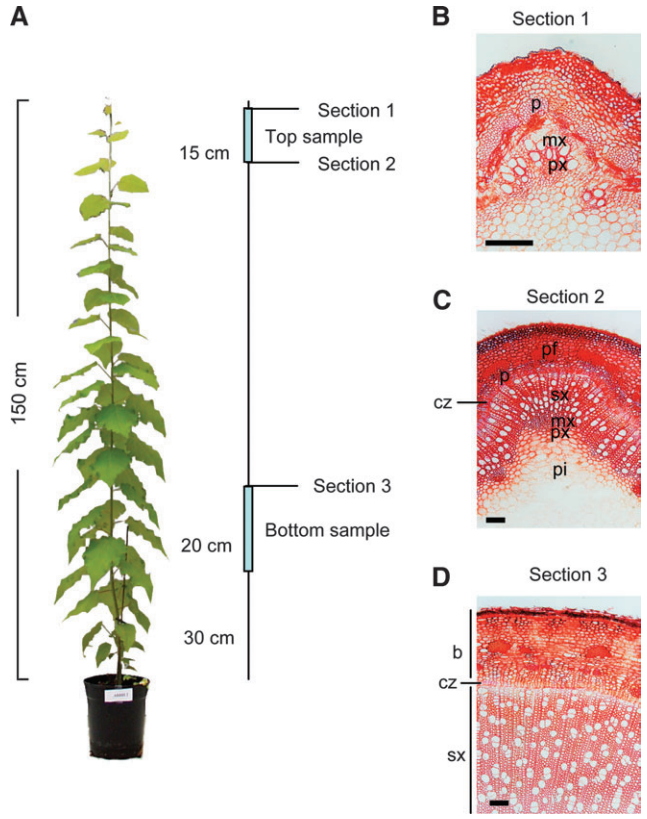

Fig. 1. cDNA-AFLP sampling and anatomical features of cross-sections along an aspen stem. (A) Top and bottom sample positions along the 6-month-old aspen stem. (B) Section 1 made in the upper part of the top sample. (C) Section 2 made in the lower part of the top sample. (D) Section 3 made in the upper part of the bottom sample. b, Bark; cz, cambial zone; mx, metaxylem; p, phloem; pf, phloem fibre; pi, pith; px, protoxylem; sx, secondary xylem. Scale bars=100 μm.

a cambium producing secondary vascular tissues (Fig. 1C, section 2). This stem sample represents therefore a spatio-temporal progression of vascular tissue differentiation, from primary xylem to young secondary xylem. The second sample (bottom sample), located at the bottom of the stem (30–50 cm above soil level), comprises a well-structured secondary xylem separated from the bark by the vascular cambium (Fig. 1D, section 3).

For the cDNA-AFLP analysis, the bark region was removed and the total remaining stem tissue was used for RNA extraction. An exception was made for the upper part of the top sample (corresponding to Fig. 1B), where the cortex was gently peeled off from the stem sample. Compared with the bottom sample that is mainly constituted of secondary xylem, the top sample is enriched in primary vascular tissues, and contains a significant proportion of pith.

Isolation and identification of genes differentially expressed during the transition from primary to secondary growth by cDNA-AFLP analysis

In order to ensure the reproducibility of the gene expression profiles, cDNA-AFLP was performed independently on three individual trees (T1–3). cDNA fragments were amplified using 176 selective primer combinations out of the 256 possible. Figure 2A shows the reproducibility of the expression pattern among the three individual trees. Figure 2B shows an example of differential gene expression between the top and the bottom samples. An estimated 26 000 TDFs were screened. In order to avoid artefacts due to the variability of the biological material, only the bands that were differential in all three individual trees were selected for further investigation. A total of 106 TDFs, with a length between 51 bp and 456 bp, were differentiated between the top and the bottom samples. The TDFs were excised from the gels, re-amplified with the corresponding cDNA-AFLP primer combinations, cloned, and sequenced. After removal of 17 chimeric sequences (clones containing two or more ligated TDFs) and 6 redundant TDFs, a set of 83 unique TDFs was retained for further analysis. Most of the TDFs (55 out of 83) showed a higher expression in the top sample ('top TDFs') rather than in the bottom sample ('bottom TDFs'). Table 1 displays the sequence similarities for the 83 TDFs. The poplar origin was confirmed for all TDFs after BLASTN analysis with the draft poplar genome sequence. Subsequently, a BLASTX search against GenBank database and a BLASTN search against poplar EST databases were performed and, according to their similarities, TDFs were classified into four different groups (Group 1, Regulation and signalling; Group 2, Cell wall biogenesis; Group 3, Various metabolic processes; and Group 4, Hypothetical/unknown proteins). Of the top TDFs (Table 1A), 11 were classified in Group 1, including five putative transcription factors (*Pta011*, *Pta070*, *Pta075*, *Pta086*, and *Pta091*), two translation-related factors (*Pta004* and *Pta097*), two receptor-like protein kinases (*Pta043* and *Pta050*), one ankyrin-like protein (*Pta041*), and one RING (Really Interesting New Gene) zinc finger protein (*Pta099*). Group 2 comprises a xyloglucan endotransglycosylase/hydrolase (XTH) precursor (*Pta026*) and a pectinesterase-like (PME) gene (*Pta101*). Group 3 consists of 19 TDFs similar to genes involved in various metabolic processes. Group 4 is composed of 23 TDFs similar to hypothetical/unknown proteins or for which no significant similarity (E values above 0.2) could be found, probably due to their small size or to their possible location within the non-coding regions in the cDNAs. The same classification was made for the bottom TDFs (Table 1B). Table 2 shows the relative distribution of the 83 TDFs within the four different groups. The TDFs classified in Group 1 are clearly more abundant in the top than in the bottom (11 out of 13 TDFs). Only four genes (two top and two bottom TDFs) related to cell wall biogenesis were identified in this study, indicating that the comparative approach used (top versus bottom) allowed the subtraction of main cell wall-related genes. Among them, the two top TDFs are similar to enzymes modulating cell wall expansion, cellular adhesion, and cell wall cross-linking (PME

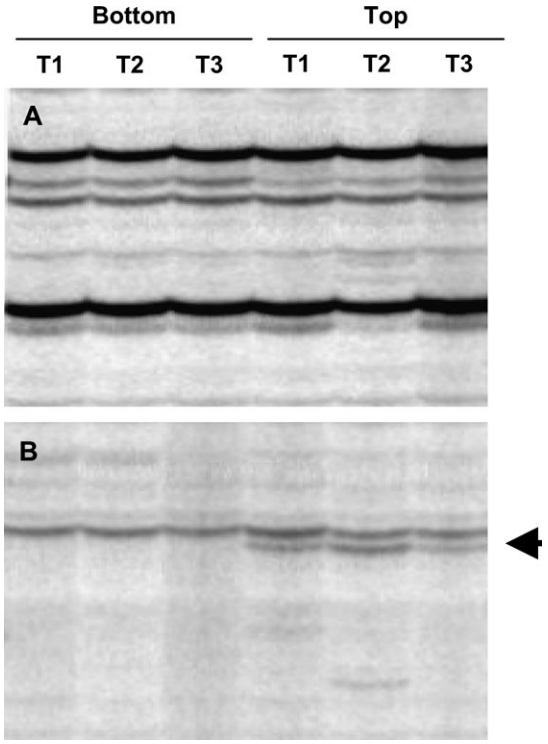

Fig. 2. Details of cDNA-AFLP autoradiograms showing accumulation of TDFs in the top and bottom samples in three individual trees (T1-T3). (A) Reproducibility of the expression pattern within the three individual trees. (B) Example of a selected differential top TDF (arrow).

and XTH). The two bottom TDFs, encoding cinnamyl alcohol dehydrogenase (CAD) and 4-coumarate coA:ligase (4CL), are associated with lignin biosynthesis, a characteristic of secondary cell wall formation (reviewed by Boerjan *et al.*, 2003). Almost 50% of the TDFs correspond to hypothetical or unknown proteins and, for about half of the TDFs, no close ESTs/contigs (identity >90%) were found. The availability of the poplar genome sequence will help to annotate these TDFs further.

Expression analysis of top TDFs along a poplar stem and in leaf tissues

To validate the cDNA-AFLP results, an independent expression study was performed for 25 TDFs by RT-PCR (Table 1). The expression analysis included four xylem samples harvested along the stem of 6-month-old aspen trees [one base sample (B), two intermediate samples (M1 and M2), and one top sample (T)] and two leaf samples [one consisting of dissected interveinal tissue of mature leaves (L), the other consisting of entire young unexpanded leaves, mainly constituted of vein tissues (YL)]. As the objective of this study was to identify genes potentially involved in the setting up of secondary growth, a focus was made on the top TDFs. Figure 3 shows RT-PCR profiles of 19 top TDFs for which the cDNA-AFLP profile was confirmed. For the six remaining TDFs, the differential expression was not validated by RT-PCR (data not shown). As shown in Figure 3A, the RT-PCR profiles are in agreement with the cDNA-AFLP results. Globally, a progressive decrease of the expression was observed from the top to the base samples. In the leaf samples, the RT-PCR expression profiles were variable. As shown in Fig. 3B, the expression levels for most of the TDFs were either similar in both leaf samples or higher in young leaf samples, except for *Pta041* that was not detected in either of the leaf samples. *Pta050* and *Pta058* had a pronounced expression in interveinal tissue compared with that in the young leaf samples and are, therefore, not likely to be associated with vascular tissue.

To assess the differential expression with a non PCR-based method, an independent reverse northern analysis was done. The 106 TDFs were spotted on two nitrocellulose membranes. These membranes were hybridized with labelled cDNA synthesized from total RNA, extracted either from the top or from the bottom part of a bark-free stem of a 6-month-old aspen. The TDFs from Group 1 that revealed significantly different hybridization signals (P <0.01) were *Pta075* and *Pta099* (Fig. 4). The signals of the other top TDFs from Group 1 were either too low or not different. These results may be due to the fact that homologous cDNAs hybridize and average the signal.

In situ *RT-PCR localization of gene expression in aspen stem sections*

To investigate the localization of gene expression at the tissular and cellular levels, *in situ* RT-PCR was performed on hand-made stem transversal sections of 1-month-old acclimatized aspen plants. Young stem tissues were best suited for *in situ* RT-PCR analysis since experiments with older tissues were hampered by phenolics that inhibit enzyme activities (data not shown). Figure 5A shows the negative control in which all the steps of the experimental protocol were carried out with a mock RT reaction (without reverse transcriptase) for *PtaRHE1*. While no cytoplasmic signal was observed in mock RT reactions, a nuclear staining was often observed, presumably due to incomplete DNA digestion. In order to validate the *in situ* RT-PCR protocol, the expression of two control genes, *CAD* and *18S*, was investigated. *CAD* expression was localized in the primary xylem cells and in ray parenchyma cells surrounding vessels (Fig. 5B), a localization that is in agreement with the expression pattern conferred by a *Eucalyptus gunnii EgCAD2* promoter-*GUS* fusion in transgenic poplar (Hawkins *et al.*, 1997; Lauvergeat *et al.*, 2002). The *in situ* expression analysis for the *18S* ribosomal gene, regarded as constitutive (Pesquet *et al.*, 2004), shows that this gene is expressed in almost all cell types including cortex parenchyma cells and in both differentiating phloem and xylem (Fig. 5C), with a more pronounced and homogenous expression in the cambial zone (Fig. 5D).

Table 1. *Homologies of top (A) and bottom (B) TDFs to sequences in the databases*

According to their homology, the TDFs were classified into four groups: regulation and signalling, cell wall biogenesis, various metabolic processes, and hypothetical/unknown proteins.

TDF number	Length (bp)	Accession number	BLASTN of the TDF with the *P. trichocarpa* draft genome sequence[a]	BLASTX of the TDF	Closest poplar EST/contig[c]	
			Scaffold/LG (coordinates in bp–identity)	Description (plant species, accession number[b], E value)	Accession number [length, identity (%), BLASTN E value]	Poplar EST description[d] except [$] for which BLASTX was made in GenBank database
(A) Top TDFs						
Group 1. Regulation and signalling						
Pta004	163	CV555408	scaffold_97 (826953: 827115 – 98%)	Translation initiation factor 4A2 (*Zea mays* – U17979 – 1.4 e-22)	POPLAR.614.C1, [741 bp, 163/163 bp (100%), 1.7 e-32]	Eukaryotic initiation factor 4A-8 (eIF-4A-8) (*Nicotiana tabacum*)
Pta011*	432	CV555412	LG_III (9688735: 9689700 – 98%)	bZIP DNA-binding protein CPRF4b (*Petroselinum crispum* – Y10810 – 5.7 e-13)	UL77PB12 [412 bp, 267/294 bp (90%), 5.0 e-50]	bZIP transcription factor 2 (*Phaseolus vulgaris*)
Pta041*	203	CV555430	LG_X (18443026: 18443228 – 98%)	Ankyrin-like protein (At3g12360- 1.6 e-21)	–	–
Pta043*	162	CV555431	LG_XIII (4400461: 4400621 – 94%)	Ser/Thr protein kinase (*Z. mays* – AJ001486 – 0.15)	CA924604 [711 bp, 76/76bp (100%), 3.0 e-37]	Receptor-like protein kinase 1[$] (At5g60900)
Pta050*	116	CV555434	LG_XII (10838870: 10838985 – 96%)	Putative receptor-like protein kinase (At2g31880 – 1.4 e-13)	P057E08 [881 bp, 116/116 bp (100%), 6.0 e-22]	Receptor-like protein kinase
Pta070*	253	CV555440	LG_XVI (11076300: 11076832 – 98%)	Nucleic acid binding protein-like (At5g05610 – 4.3 e-30)	–	–
Pta075*	68	CV555441	LG_III (6664683: 6664750 – 98%)	AP2 domain containing protein (*Prunus armeniaca* – AF071893 – 0.060)	X023D11 [850 bp, 68/68 bp (100%), 1.7 e-10]	Ethylene responsive element binding protein – EREBP1 (*Fagus sylvatica*)
Pta086*[e]	337	CV555450	LG_IV (2240260: 2242170 – 98%)	Similar to transcription factor Z46606 (At1g61140 – 0.017)	–	–
Pta091*	262	CV555453	LG_X (3825580: 3826270 – 95%)	Squamosa promoter binding protein-like 7 (At5g18830 – 1.1 e-20)	–	–
Pta097*	431	CV555456	LG_VII (1469889: 1476704 – 98%)	Protein translation factor SUI1 homologue (*Salix bakko* -AB003378 – 5.6 e-53)	POPLAR.4772.C1 [699 bp, 427/431 bp (99%), 2.3 e-91]	Protein translation factor SUI1 homologue (*S. bakko*)
Pta099*	238	CV555458	scaffold_77 (1287715: 1287952 – 98%)	RING zinc finger (At3g16720 – 3.3 e-14)	POPLAR.9452.C1 [1268 bp, 236/238 bp (99%), 7.6 e-49]	RING zinc finger protein
Group 2. Cell wall biogenesis						
Pta026*	301	CV555423	LG_III (14326775: 14327367 – 99%)	Xyloglucan endotransglycosylase precursor (XET16A) (*P. tremula*× *P. tremuloides* – AF515607 – 4.0 e-64)	POPLAR.875.C1 [1307 bp, 297/301 bp (98%), 2.8 e-62]	Xyloglucan endotransglycosylase precursor (*Actinidia deliciosa*)
Pta101*	156	CV555459	LG_XII (1903759: 1903914 – 97%)	Pectinesterase-like protein (At3g49220 – 1.6 e-17)	POPLAR.1474.C1 [1150 bp, 154/156 bp (98%), 2.7 e-30]	Pectinesterase-like protein
Group 3. Various metabolic processes						
Pta002	244	CV555406	scaffold_57 (601645: 601888 – 96%)	Putative thaumatin-like protein (At4g38660 – 2.7 e-28)	POPLAR.7553.C1 [801 bp, 239/245 bp (97%), 2.5 e-48]	Thaumatin-like protein
Pta003	80	CV555407	LG_II (13273455: 13273534 – 99%)	Putative cellular apoptosis susceptibility protein (*O. sativa* – AC006418 – 0.072)	–	–
Pta005*	175	CV555409	LG_III (10847978: 10848151 – 93%)	–	POPLAR.3057.C1 [1099 bp, 163/174 bp (93%), 1.1 e-30]	Acid invertase (*Citrus unshiu*)

Table 1. *Continued*

TDF number	Length (bp)	Accession number	BLASTN of the TDF with the *P. trichocarpa* draft genome sequence[a]	BLASTX of the TDF	Closest poplar EST/contig[c]	
			Scaffold/LG (coordinates in bp–identity)	Description (plant species, accession number[b], E value)	Accession number [length, identity (%), BLASTN E value]	Poplar EST description[d] except $ for which BLASTX was made in GenBank database
Pta006	133	CV555410	LG_III (13126811: 13126895 – 97%)	–	G059P94.3pR [213 bp 121/131 bp (92%), 1.4 e-19]	Transposase (*Methanosarcina frisia*)
Pta009	95	CV555411	scaffold_11842 (826:920 – 98%)	–	V049F12 [686 bp, 93/95 bp (97%), 2.7 e-16]	Catechol *O*-methyltransferase (EC 2.1.1.6) (*N. tabacum*)
Pta013	254	CV555414	LG_XVI (10889343: 10889595 – 97%)	Chlorophyll *a*/*b* binding protein CP29 (*Vigna radiata* – AF139466 – 7.1 e-37)	BI068847 [444bp, 251/253bp (99%), 9.6 e-100]	Chlorophyll *a*/*b* binding protein CP29 (*V. radiata*)
Pta017	225	CV555416	LG_XVI (6821378: 6821720 – 97%)	Chain A, Structures of adenylosuccinate synthetase from *Triticum aestivum* and *A. thaliana* (1DJ2_A - At3g57610 – 2.7 e-28)	–	–
Pta019	179	CV555417	scaffold_3435 (6395: 6573 – 97%)	Amino acid transporter (At5g23810 – 0.13)	–	–
Pta023	69	CV555420	LG_XIV (1257409: 1257477 – 97%)	Probable methionine aminopeptidase 1 (METAP1)(At2g45240 – 0.099)	POPLAR.8135.C1 [695 bp, 68/69 bp (98%), 1.3 e-10]	Methionine aminopeptidase
Pta024*	83	CV555421	LG_I (7335632: 7335704 – 97%)	–	POPLAR.9769.C1 [884 bp, 83/83 bp (100%), 4.2 e-14]	Monodehydroascorbate reductase (*Brassica juncea*)
Pta035*	65	CV555427	LG_XII (275918: 275982 – 97%)	Lysophospholipase homologue, putative (At1g73480 – 0.028)	UM54TG07 [559 bp, 63/65 bp (96%), 2.0 e-09]	Lysophospholipase (At1g73480)
Pta038	61	CV555428	LG_X (19072014: 19072071 – 96%)	–	C012P62 [559 bp, 60/61 bp (98%), 6.3 e-09]	Phosphoglucomutase-like protein
Pta040*	268	CV555429	LG_V (17593970: 17594333 – 99%)	Glyceraldehyde 3-phosphate dehydrogenase B, chloroplast precursor (EC 1.2.1.13) (At1g42970 – 7.6 e-40)	–	–
Pta046	68	CV555432	LG_IV (15330405: 15330472 – 100%)	Auxin-induced protein X10A (*Glycine max* – S44176 – 0.0013)	–	–
Pta078*	374	CV555442	scaffold_122 (948266: 948714 – 98%)	Glutamine synthetase cytosolic isozyme (GS1) (*Pinus sylvestris* – X69822 – 5.4 e-39)	POPLAR.102.C2 [811 bp, 367/375 bp (97%), 7.0 e-77]	Glutamine synthetase nodule isozyme (EC 6.3.1.2) (Glutamate–ammonialigase) (*V. aconitifolia*)
Pta080*	343	CV555444	scaffold_64 (864061–864624 – 93%)	Cytochrome P450-like protein (At5g10600 – 6.1 e-17)	CA933374 [649 bp, 267/277 bp (96%), 1.0 e-129]	Cytochrome P450 (At3g28740)[$]
Pta081	344	CV555445	LG_XVIII (5176741: 5177239 – 96%)	Putative ABC transporter (At1g15520 – 1.1 e-40)	–	–
Pta092*	260	CV555454	scaffold_28 (272466: 272632 – 99%)	Cysteine protease (*P. armeniaca* – U93166 – 4.8 e-05)	POPLAR.5498.C2 [1265 bp, 177/184 bp (96%), 3.6 e-34]	Senescence-associated cysteine protease (*B. oleracea*)
Pta094	212	CV555455	scaffold_107 (832108: 832412 – 97%)	Protochlorophyllide reductase, chloroplast precursor (EC 1.3.1.33) (*Cucumis sativus* – D50085 – 7.6 e-12)	POPLAR.6661.C1 [1458 bp, 210/212 bp (99%), 4.9 e-43]	Protochlorophyllide reductase, chloroplast precursor (EC 1.3.1.33) (*C. sativus*)
Group 4. Hypothetical/unknown proteins						
Pta012	181	CV555413	scaffold_40 (2756571: 2756650 – 87%)	–	–	–
Pta015	120	CV555415	LG_I (33771707: 33771826 – 97%)	Hypothetical protein F7L13.80 (At4g10500 – 0.0032)	–	–

Table 1. *Continued*

TDF number	Length (bp)	Accession number	BLASTN of the TDF with the *P. trichocarpa* draft genome sequence[a]	BLASTX of the TDF	Closest poplar EST/contig[c]	
			Scaffold/LG (coordinates in bp–identity)	Description (plant species, accession number[b], E value)	Accession number [length, identity (%), BLASTN E value]	Poplar EST description[d] except [$] for which BLASTX was made in GenBank database
Pta020	75	CV555418	LG_V (2269375: 2269449 – 100%)	–	–	–
Pta022	112	CV555419	LG_VI (7415542: 7415647 – 96%)	Unknown protein (At3g08840 – 0.00061)	–	–
Pta025*	51	CV555422	scaffold_2425 (757:805 – 100%)	–	–	–
Pta027	192	CV555424	LG_XIV (4595108: 4595202 – 100%)	–	POPLAR.2124.C3 [856 bp, 101/104 bp (97%), 9.7 e-18]	Mitochondrial ribosomal protein, putative
Pta029	153	CV555425	LG_V (10548796: 10548874 – 95%)	–	–	–
Pta030	129	CV555426	LG_IV (1777763: 1777891 – 98%)	Putative protein (At4g05140 – 4.2 e-12)	–	–
Pta047	112	CV555433	scaffold_130 (315389: 315461 – 97%)	–	–	–
Pta051	106	CV555435	LG_XIV (3993360: 3993464 – 99%)	–	–	–
Pta058*	452	CV555436	scaffold_200 (260929: 261511 – 93%)	F28K20.16 (At1g31200 – 9.8 e-24)	POPLAR.5702.C1 [626 bp, 391/421 bp (92%), 1.1 e-77]	Expressed protein
Pta062	186	CV555437	LG_XVI (2062241: 2062411 – 100%)	Hypothetical protein T3K9.8 (At2g41150 – 6.2 e-19)	UB63CPD12.3pR [POPLAR.6011] [916 bp, 183/187 bp (97%), 2.7 e-35]	Expressed protein
Pta064	227	CV555438	LG_VIII (8866272: 8866516 – 98%)	Unknown protein (At2g01690 – 2.1 e-11)	–	–
Pta066	346	CV555439	LG_XIII (1396821: 1396901 – 100%)	Contains similarity to human diaphanous 1-gene_id:FID9.13 (At2g25050 – 5.4 e-10)	–	–
Pta079*	53	CV555443	LG_XVIII (5718745: 5718791 – 100%)	Putative protein (At5g11150 – 0.060)	POPLAR.5483.C1 [1054 bp, 52/53 bp (98%), 2.0 e-06]	Vesicle-associated membrane protein 7C (At VAMP7C)
Pta082	127	CV555446	scaffold_28 (3466743: 3466869 – 97%)	–	–	–
Pta083	250	CV555447	LG_VI (12013329: 12013578 – 97%)	Hypothetical protein (At4g3090 – 3.0 e-29)	–	–
Pta084	137	CV555448	scaffold_131 (73009: 73126 – 91%)	–	–	–
Pta085*	107	CV555449	LG_I (16997945: 16998035 – 98%)	–	UL83TH05 [595 bp, 100/100 bp (100%), 3.6 e-18]	–
Pta087*	204	CV555451	LG_XV (1841436: 1841622 – 99%)	–	POPLAR.6966.C1 [433 bp, 200/204 bp (98%), 6.7 e-40]	Expressed protein
Pta090	366	CV555452	LG_III (17154003: 17154368 – 96%)	Hypothetical protein T14E10.140 (At3g54570 – 0.00027)	–	–
Pta098	83	CV555457	LG_III (17153914: 17153996 – 98%)	–	–	–
Pta105*	168	CV555460	scaffold_152 (76225: 76392 – 95%)	–	–	–
(B) Bottom TDFs						
Group 1. Regulation and signalling						
Pta031	117	CV555461	LG_XIII (2403802: 2403918 – 97%)	Putative mitogen activated protein kinase kinase (At3g04910 – 0.076)	–	–
Pta042	89	CV555463	LG_VIII (3379407: 3379495 – 100%)	Putative protein kinase (At5g02070 – 0.00019)	–	–

Table 1. *Continued*

TDF number	Length (bp)	Accession number	BLASTN of the TDF with the *P. trichocarpa* draft genome sequence[a]	BLASTX of the TDF	Closest poplar EST/contig[c]	
			Scaffold/LG (coordinates in bp–identity)	Description (plant species, accession number[b], E value)	Accession number [length, identity (%), BLASTN E value]	Poplar EST description[d] except $ for which BLASTX was made in GenBank database
Group 2. Cell wall biogenesis						
Pta036	66	CV555462	LG_IX (4269828: 4269891 – 97%)	Cinnamyl alcohol dehydrogenase (EC 1.1.1.195) (*Populus deltoides* – Z19568 – 7.8 e-05)	POPLAR.6.C1 [819 bp, 65/66 bp (98%), 1.0 e-09]	Cinnamyl alcohol dehydrogenase (*P. tremuloides*)
Pta056	138	CV555464	scaffold_1790 (117:252 – 97%)	4-Coumarate:CoA ligase (EC 6.2.1.12) (*P. tremuloides* – AF041049 – 3.3 e-16)	POPLAR.98.C1 [608 bp, 136/138 bp 98%), 6.0 e-26]	4-coumarate:CoA ligase (EC 6.2.1.12) (*P. tremuloides*)
Group 3. Various metabolic processes						
Pta008	130	CV555473	LG_II (2217046: 2217155 – 97%)	–	FL_GENBANK_64 [3079 pb, 129/131 bp (98%), 2.5 e-23]	Isoflavone reductase homologue (PILH gene) (*P. trichocarpa*)
Pta028	124	CV555465	scaffold_40 (1652019: 1652127 – 98%)	Amino acid transporter-like protein 1 (AATL1) (At1g47670 – 6.5 e-16)	–	–
Pta037	178	CV555466	LG_X (15895116: 15895293 – 95%)	Transport protein homologue YUP8H12R.2 (At1g79360 – 9.3 e-13)	–	–
Pta049	149	CV555467	LG_XVII (3351115: 3351263 – 97%)	Thaumatin-like protein (At5g40020 – 3.4 e-14)	F118P15.3pR [POPLAR.2682] [812 bp, 143/149 bp (95%), 6.3 e-27]	Thaumatin-like protein
Pta054	149	CV555468	LG_XVI (4036998: 4037248 – 98%)	Kinesin heavy chain, putative (At3g12020 – 4.1 e-07)	CA927379 [640bp, 149/149bp (100%), 7.0 e-81]	Kinesin heavy chain-like protein (At5g06670)$
Pta060	137	CV555469	scaffold_204 (389882: 390131 – 98%)	Putative ABC transporter (At2g47000 – 3.7 e-10)	–	–
Pta073	283	CV555470	LG_XV (8172067: 8172423 – 99%)	Superoxide dismutase (EC 1.15.1.1) (Fe) (*N. plumbaginifolia* – M55909 – 1.4 e-33)	–	–
Pta089	456	CV555471	scaffold_44 (2595417: 2594963 – 98%)	Flavonol 3-*O*-glucosyltransferase-like protein (At3g16520 – 1.6 e-37)	POPLAR.4016.C1 [1002 bp, 450/455 bp (98%), 1.6 e-96]	Flavonol glucosyltransferase (*O. sativa*)
Group 4. Hypothetical/unknown proteins						
Pta007	176	CV555472	LG_XIV (491453: 491628 – 100%)	Hypothetical protein F14N23.3 (At1g10150 – 4.0 e-11)	–	–
Pta010	83	CV555474	LG_XIV (6745418: 6745463 – 100%)	–	POPLAR.6442.C1 [415 bp, 83/83 bp (100%), 3.6 e-14]	P0506E04.8 protein (*O. sativa*)
Pta014	185	CV555475	scaffold_40 (2756565: 2756751 – 98%)	–	POPLAR.104.CB1 [1599 bp, 182/185 bp (98%), 2.7 e-36]	Expressed protein
Pta032	107	CV555476	scaffold_142 (289449: 290443 – 98%)	–	–	–
Pta033	96	CV555477	scaffold_86 (1070505: 1070596 – 96%)	–	POPLAR.734.C1 [637 bp, 81/82 bp (98%), 1.0 e-13]	Expressed protein
Pta039	74	CV555478	LG_VIII (12747341: 12747410 – 93%)	–	–	–
Pta048	200	CV555479	LG_VII (8357316: 8357514 – 98%)	Hypothetical protein F3A4.200 (6.0 e-21)	CA931704 [651 bp, 193/200bp (96%), 3.0 e-93]	Hypothetical protein F3A4.200(At3g50120)$
Pta052	89	CV555480	scaffold_86 (1070505: 1070589 – 98%)	–	POPLAR.734.C1 [637 bp, 78/81 bp (96%), 1.1 e-12]	Expressed protein

Table 1. *Continued*

TDF number	Length (bp)	Accession number	BLASTN of the TDF with the *P. trichocarpa* draft genome sequence[a]	BLASTX of the TDF	Closest poplar EST/contig[c]	
			Scaffold/LG (coordinates in bp–identity)	Description (plant species, accession number[b], E value)	Accession number [length, identity (%), BLASTN E value]	Poplar EST description[d] except [$] for which BLASTX was made in GenBank database
Pta053	150	CV555481	scaffold_44 (1545226: 1545367 – 93%)	–	–	–
Pta057	172	CV555482	scaffold_57 (838168: 838339 – 98%)	Hypothetical protein (At2g17970 – 0.024)	–	–
Pta069	108	CV555483	LG_XIII (1396821: 1396900 – 97%)	–	–	–
Pta072	287	CV555484	LG_XI (13641067: 13641343 – 95%)	–	–	–
Pta088	51	CV555485	LG_VI (18046259: 18046299 – 95%)	–	–	–
Pta095	114	CV555486	LG_XVI (11846416: 11846523 – 95%)	–	POPLAR.4916.C1 [636 bp, 106/108 bp (98%), 2.8 e-19]	Expressed protein
Pta104	128	CV555487	scaffold_232 (173885: 174006 – 93%)	–	–	–
Pta106	78	CV555488	LG_VI (7689981: 7690058 – 96%)	–	POPLAR.7178.C2 [823 bp, 78/78 (100%), 5.4 e-13]	Non-green plastid inner envelope membrane protein

[a] BLASTN was made in the draft poplar genome sequence http://genome.jgi-psf.org/poplar/.
[b] For *Arabidopsis* sequences, the Arabidopsis Gene Index (AGI) numbers are given (http://www.arabidopsis.org/Blast/).
[c] BLASTN was done in the poplar EST databases http://poppel.fysbot.umu.se/, http://aspendb.mtu.edu/ and http://mycor.nancy.inra.fr/.
[d] Annotation given in http://poppel.fysbot.umu.se/.
[e] Pta086 was 93 bp long and this 337 bp sequence was obtained by RACE-PCR; LG, linkage group; –, no hit (E value >0.2 or identity <90%).
*TDFs for which RT-PCR was carried out.

Table 2. *Relative distribution of TDFs among the different groups*

Groups	Number of top TDFs[a]	Number of bottom TDFs[a]	Total of top and bottom TDFs[a]
1. Regulation and signalling	11 (4*)	2 (2*)	13 (6*)
2. Biogenesis of cell wall	2	2	4
3. Various metabolic processes	19 (6*)	8 (4*)	27 (10*)
4. Hypothetical/unknown proteins	23 (17*)	16 (9*)	39 (26*)
Total	55 (27*)	28 (15*)	83 (42*)

[a] (*)TDFs for which no EST with identity ⩾90% was found in poplar EST databases (http://poppel.fysbot.umu.se/, http://mycor.nancy.inra.fr/BLAST/blast.html and http://aspendb.mtu.edu/).

Subsequently the localization of gene expression corresponding to the two selected top TDFs from Group 1 (*Pta099 and Pta075*), was examined. Their expression profile was associated with the vascular system (Fig. 5E, H). *Pta099* expression was associated with the cambial zone and to a lesser extent with differentiating phloem, primary xylem, and ray parenchyma cells of secondary xylem (Fig. 5E). A closer observation (Fig. 5F) revealed that within the cambial zone, *Pta099* gene expression was localized in the ray initials and derivatives and not in their fusiform counterparts when compared to the *18S* gene expression pattern (Fig. 5D). *Pta075* expression was confined to the phloem tissue (Fig. 5G, H).

PtaERF1 (Pta075) cDNA cloning and sequence analysis

A *PtaERF1* cDNA of 1409 bp, including a 19 bp 5′ untranslated region (UTR) and a 250 bp 3′ UTR, was cloned. The 1140 bp open reading frame (ORF) predicts a 380 amino acid polypeptide with a calculated molecular weight of 42 kDa. This ORF shows a 97% identity with the scaffold LG_III at the coordinates 6664108:6666668 in the poplar draft genome sequence, and a 99% identity with the contig POPLAR.406.C2 in the PopulusDB. This polypeptide is homologous to AP2/ERF (Apetala2/Ethylene responsive factor) transcription factors. Figure 6 shows the alignment of the deduced amino acid sequence of PtaERF1 with the four most similar deduced proteins, including that of *Nicotiana tabacum* NtDRF1 (AY286010) (52% identity, 62% similarity over 380 amino acids), *Lycopersicon esculentum* LeJERF1 (AY044235) (47% identity, 61% similarity over 380 amino acids) and LeJERF3 (AY383630) (48% identity, 59% similarity over 311 amino acids), and *Fagus sylvatica* ERF1 (FsER1—AJ420195) (55% identity, 68% similarity over 370 amino acids). The most similar polypeptide of *A. thaliana* is At1g53910 (RAP2-12) (43% identity, 52% similarity over 380 amino acids). As shown in Fig. 6, sequence comparison revealed a conserved N-terminal region (domain I), a putative nuclear localization signal (NLS) KRKRK

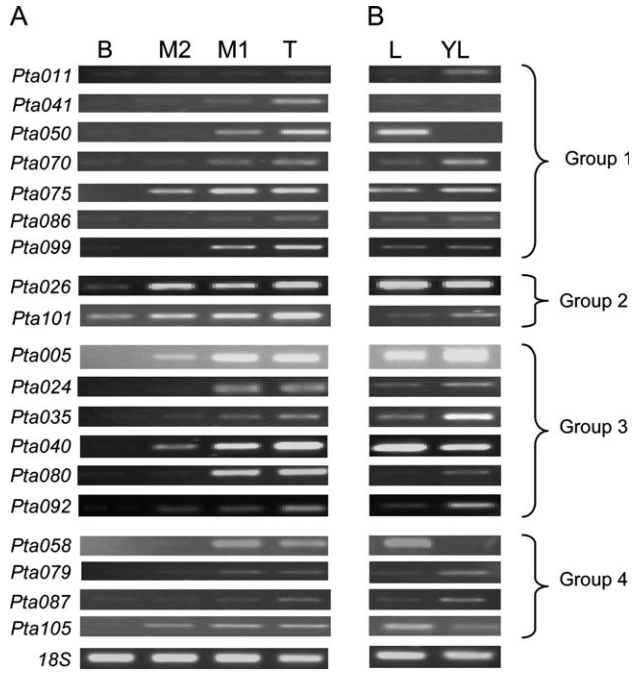

Fig. 3. Expression analysis of top TDFs by RT-PCR. (A) Expression patterns along the aspen stem (B, base; M1-2, middle samples; T, top). (B) Expression patterns in leaf tissue (L, interveinal tissue of mature leaf; YL, young unexpanded leaf). Nineteen top TDFs have been analysed and classified into four groups (Group 1, Regulatory and signalling; Group 2, Cell wall biogenesis; Group 3, Various metabolic processes; and Group 4, Hypothetical/Unknown proteins). The *18S* gene was used as internal control.

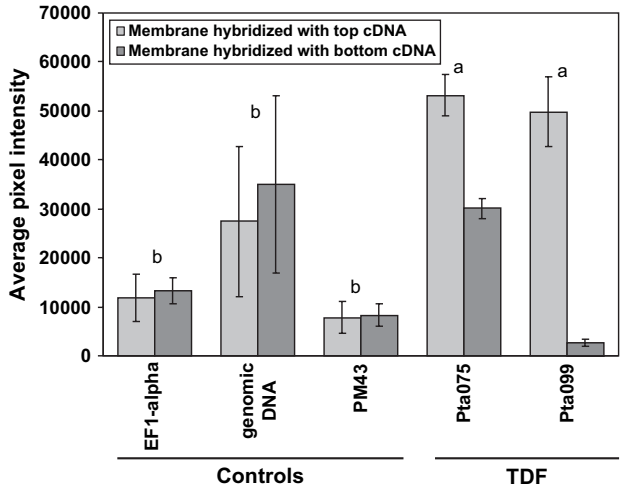

Fig. 4. Reverse northern analysis of two top TDFs from Group 1 (Pta075 and Pta099). Membranes were hybridized either with top or with bottom cDNA. Three controls were included, *EF1-α*, genomic DNA, and *PM43*. a, $P < 0.01$; b, not significant.

(domain II), and a conserved ERF DNA binding domain (domain III). Domain I, of unknown function, typifies a new class of ERF proteins, named class IV (Tournier *et al.*, 2003).

PtaRHE1 *(*Pta099*) cDNA cloning and sequence analysis*

A *PtaRHE1* cDNA of 987 bp, including a 74 bp 5′ UTR and a 34 bp 3′ UTR, was cloned. The 879 bp ORF predicts a 293 amino acid polypeptide with a calculated molecular weight of 33 kDa. This ORF shows a 98% identity with the scaffold_77 at the coordinates 1287428:1288306 in the poplar draft genome sequence, and a 99% identity with the contig POPLAR.9452.C1 in the PopulusDB. Homology throughout the sequence indicates that PtaRHE1 belongs to the RHE subgroup of RING-H2 proteins described by Jensen *et al.* (1998). Figure 7 shows the alignment of the deduced amino acid sequence of PtaRHE1 with the four most similar deduced proteins including the *Arabidopsis* At3g16720 (ATL2) (54% identity, 65% similarity over 280 amino acids), At3g62690 (ATL5) (41% identity, 64% similarity over 234 amino acids), At2g47560 (49% identity, 69% similarity over 144 amino acids), and the *N. tabacum* AF211532 (Avr9/Cf-9 rapidly elicited-132, ACRE-132) (39% identity, 58% similarity over 228 amino acids). The predicted polypeptides have four conserved regions, including a putative transmembrane hydrophobic region at the N terminus (region I), a region rich in basic amino acids (region II), a region with highly conserved amino acid sequences (region III), and a RING-H2 zinc finger domain (region IV) (Salinas-Mondragón *et al.*, 1999) (Fig. 7). The latter conforms to the stringent RING-H2 consensus sequence of the RING-H2 domain ($CX_2CX_{14-15}CXHX_2HX_2CX_3WX_{6-8}CPXC$ (X means other amino acid) (Jensen *et al.*, 1998).

Discussion

During the last decade, the analysis of secondary growth by various new molecular tools has allowed the identification of a large number of differentially expressed genes in both woody (Whetten *et al.*, 2001; Hertzberg *et al.*, 2001; Yang *et al.*, 2003; Sterky *et al.*, 2004) and non-woody species (Zhao *et al.*, 2000; Fukuda, 2004; Ko *et al.*, 2004). Until now, few of these genes have been functionally characterized and data on cell-specific expression is lacking for most of them.

In this study, genes encoding putative regulatory proteins that could be involved in the transition from primary to secondary growth in aspen were identified. Poplar is the model tree species in molecular biology, for which there is a large poplar EST resource (Sterky *et al.*, 2004) and for which the genome sequence has been released (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).

The experimental strategy consisted of gene expression comparison, by cDNA-AFLP, between the top and bottom stem samples from 6-month-old aspen trees. The top sample corresponds to tissues in which secondary growth is being set up whereas in the bottom sample, the secondary

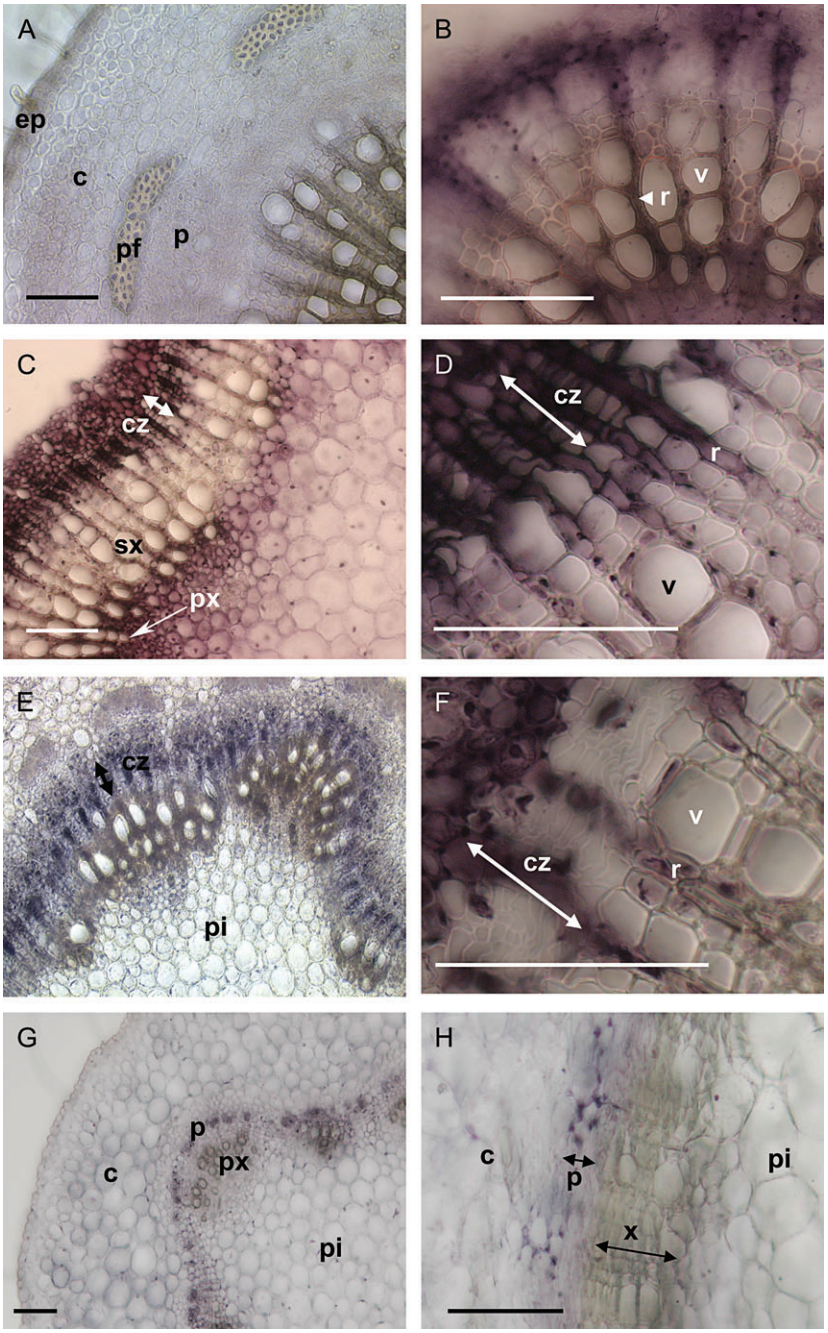

Fig. 5. *In situ* RT-PCR localization of transcripts in hand-made transverse sections of 1-month-old aspen stems. (A) Negative control (mock reaction with *PtaRHE1* primers); (B) *CAD*; (C, D) (D=close-up of C), *18S*; (E, F) (F=close-up of E), *Pta099* (*PtaRHE1*); (G, H) (H=close-up of G), *Pta075* (*PtaERF1*). c, Cortex; cz, cambial zone; ep, epiderm; p, phloem; pf, phloem fibre; pi, pith; px, primary xylem; r, ray; v, vessel; sx, secondary xylem; x, xylem. Scale bars=100 μm.

growth is well established (Fig. 1). In this context, the bottom sample was used as a subtractive control for typical secondary growth gene expression (e.g. genes involved in cell wall biosynthesis and modifications).

In order to limit false positives due to biological variation, only TDFs that showed an identical expression pattern for three independent trees were considered (Fig. 2). Consequently, a restricted number of 83 differential TDFs was selected (Table 1). A focus was made on TDFs that showed a higher expression level in the top than in the bottom sample. RT-PCR expression analysis validated the cDNA-AFLP profiles for 19 top TDFs and revealed a clear decreasing expression level along the aspen stem from the top to the base (Fig. 3). At the biological level and considering the vascular tissue development, three hypotheses can be considered to explain this decreasing gene

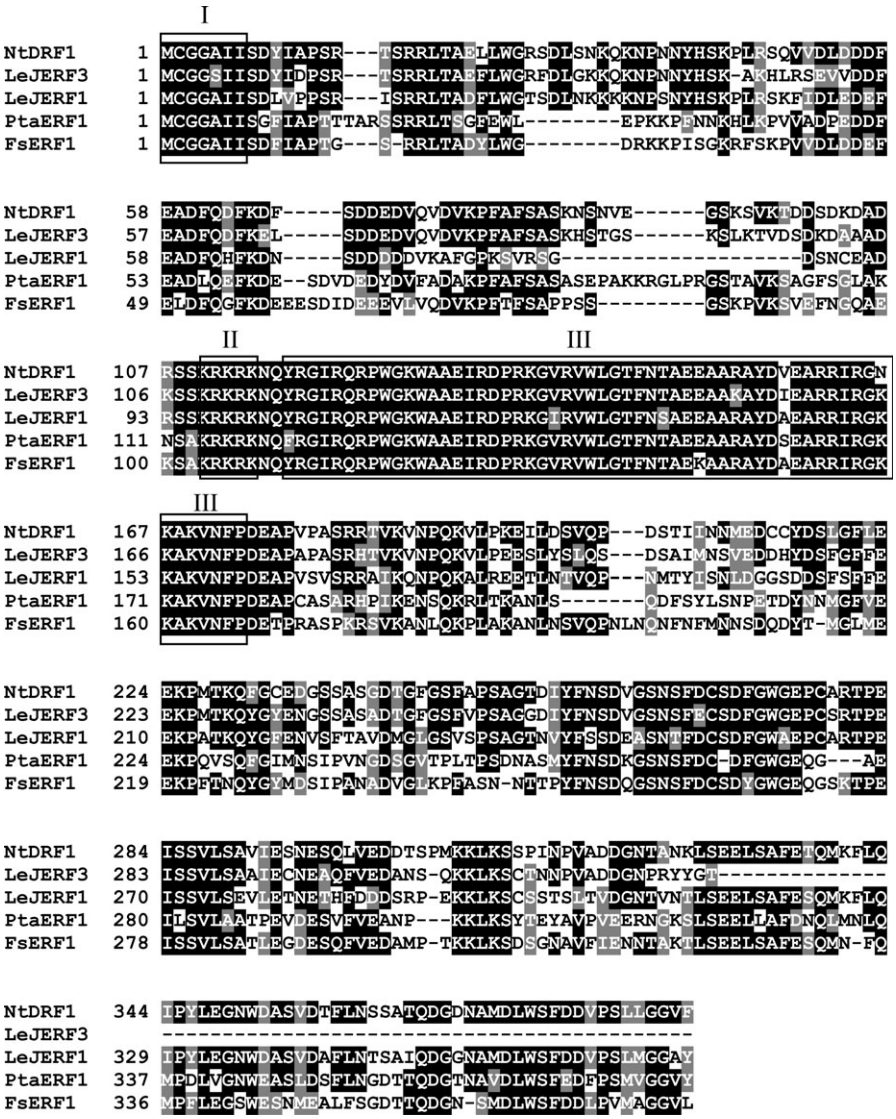

Fig. 6. Alignment of PtaERF1 amino acid sequence with the four most similar predicted proteins found in the databases, the tobacco NtDRF1 (AY286010), the tomato JERF3 (AY383630) and JERF1 (AY044235), and the beech FsERF1 (AJ420195). I. Conserved region in class IV ERF proteins. II. Putative NLS. III. ERF DNA binding domain (Tournier *et al.*, 2003). Black and grey boxes indicate strictly conserved amino acids and closely related amino acids, respectively.

expression pattern: (i) the TDFs correspond to genes associated with primary xylem but not with the transition to secondary growth, (ii) the TDFs correspond to genes specific for the transition from primary to secondary growth, a process that is linked to vascular cambial activity, and (iii) the TDFs correspond to genes associated with both situations. To document these hypotheses further, particular attention was given to six TDFs (*Pta011*, *Pta041*, *Pta070*, *Pta075*, *Pta086*, and *Pta099*) classified in the 'Regulation and signalling' group (Figs 3, 4). To the authors' knowledge, the function of the corresponding genes has not been revealed yet. For *Pta041* and *Pta070*, only sequence data are available. *Pta041* is homologous to *At3g12360*, encoding an ankyrin-like protein and *Pta070* is homologous to *At5g05610*, a gene coding for a plant homeodomain (PHD) finger protein. For the four remaining TDFs, a possible involvement of their corresponding genes in plant developmental processes can be suggested. One TDF (*Pta011*) is similar to CPRF4b (Common Plant Regulatory Factors 4b), a parsley basic region/leucine zipper (bZIP) DNA-binding protein. CPRF4b is 86% identical to CPRF4a, which has been suggested to be involved in light signal transduction (Kircher *et al.*, 1998). Light is known to control several central plant developmental processes, such as germination and transition from the vegetative to the reproductive stage. The role of light in the transition from primary to secondary growth is worth further investigation.

Another TDF (*Pta086*) is similar to *At1g61140*, a gene coding for a SWI2/SNF2 (Switch 2/Sucrose Non Fermenting 2)-like protein. SNF2-like proteins are ATPases

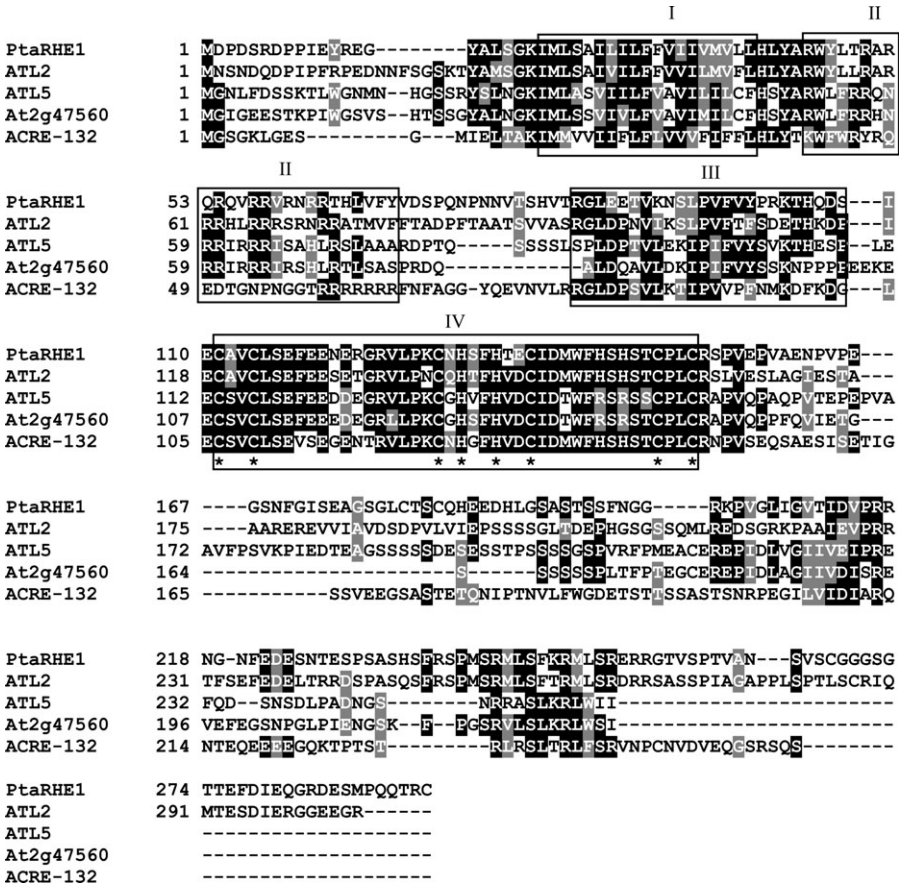

Fig. 7. Alignment of the PtaRHE1 amino acid sequence with the four most similar predicted proteins found in the databases, the *Arabidopsis* ATL2 (At3g16720), ATL5 (At3g62690), and At2g47560, and the tobacco ACRE-132 (AF211532). I. Hydrophobic region. II. Basic region. III. Conserved region. IV. RING-H2 zinc finger domain (Salinas-Mondragón *et al.*, 1999). Black and grey boxes indicate strictly conserved amino acids and closely related amino acids, respectively. Asterisks indicate the position of cysteine and histidine residues of the RING-H2 domain.

involved in ATP-dependent chromatin remodelling machines that are multisubunit complexes altering DNA-histone interactions via ATP hydrolysis. Loss of the SNF2-like function in plants affects cytosine methylation of genomic DNA, transcriptional activity and development, such as in the *Arabidopsis pickle* mutant in which embryonic traits are expressed after germination (reviewed by Reyes *et al.*, 2002).

Pta075 (PtaERF1) belongs to the subfamily of the AP2/ERF domain plant-specific transcription factors, characterized by an ERF DNA binding domain, and represented by 124 members in the *Arabidopsis* genome (Riechmann, 2002). ERF genes are induced by abiotic (Sakuma *et al.*, 2002) and biotic (Berrocal-Lobo *et al.*, 2002) stresses, but are also reported to play a role in plant growth and development (Kirch *et al.*, 2003). PtaERF1 is homologous to the tomato JERF3 (Fig. 6), a transcriptional activator that binds to the GCC box, an element responsive to ethylene and jasmonic acid as well as to dehydration responsive element (DRE) (Wang *et al.*, 2004). These authors showed that transgenic tobacco plants expressing *JERF3* ectopically have an enhanced salt tolerance and an increased expression of several pathogenesis-related genes. *In situ* RT-PCR showed that *PtaERF1* is expressed in phloem tissue (Fig. 5G, H). Further functional studies of *PtaERF1* should reveal its potential role in phloem functioning and/or development.

The last TDF, *Pta099* (*PtaRHE1*), shows homology to RING-finger proteins. These proteins are involved in diverse regulatory processes such as protein–protein interactions (Borden, 2000), proteolysis as ubiquitin E3 ligases (Joazeiro and Weissman, 2000), and signal transduction (Saurin *et al.*, 1996). The RING finger motif is a zinc binding domain defined by a conserved pattern of cysteine and histidine residues capable of co-ordinating two atoms of zinc within a characteristic cross-brace structure (reviewed by Saurin *et al.*, 1996). *PtaRHE1* is most homologous to the *Arabidopsis ATL2* (Fig. 7), a gene involved in the early response to elicitors such as chitin and inactivated cellulase preparations (Martínez-García *et al.*, 1996). Promoter–*GUS* fusion expression studies in *Arabidopsis* have shown that *ATL2* is expressed in the root of etiolated seedlings and in the shoot apical meristem of light grown-seedlings, with the strongest expression in the leaf primordia

and in the stipules (Salinas-Mondragón *et al.*, 1999). The function of ATL2 has still to be elucidated. However, for other RING-H2 proteins, a role in various plant developmental processes has been reported. For instance, the expression of the alfalfa *MsRH2-1*, which is related to the *ATL* family, is correlated with the differentiation of vascular tissues in lateral roots and symbiotic nodules (Karlowski and Hirsch, 2003). *In situ* RT-PCR localization studies in stem cross-sections indicated that *PtaRHE1* is expressed in primary xylem, in ray parenchyma cells, and exhibits a differential pattern in vascular cambium (Fig. 5E, F). Expression of several aspen genes has also been localized in the cambial zone, including the MADS-box gene *PtM5*, (Cseke *et al.*, 2003), the homeobox genes *PttHB1* and *PttHB2* (Hertzberg and Olsson, 1998), and *PttRPS18* encoding the S18 ribosomal protein (Johansson *et al.*, 2003). By contrast, *PtaRHE1* exhibits a sharp differential pattern of expression between the two initial cell types within the cambial zone (Fig. 5E, F). Although caution should be taken when interpreting these *in situ* RT-PCR signals in secondary vascular tissues due to differences in cell size (Gray-Mitsumune *et al*., 2004), the *PtaRHE1* signal in the cambial zone (Fig. 5F) seems to be located only in ray initials and their derivatives, when compared to the ubiquitous 18S signal (Fig. 5D). A potential role for this gene in the determination and/or the maintenance of cambial cell identity can therefore be suggested. Reverse genetics should confirm or refute this hypothesis.

In the cambial zone, at least three patterns of cell differentiation can be distinguished. The first pattern is related to the differentiation of the cambial derivatives to either xylem or phloem through periclinal segmentations. The second differentiation pattern is related to the specification of the different components of either xylem or phloem tissue within the cambial zone. Little is known about the genetic control of cell type identity leading to the differentiation of vessels, fibres, axial parenchyma cells, sieve elements, or companion cells. Nevertheless, comprehensive genomic approaches have resulted in the identification of genes, the expression of which is associated with particular tissues or cell types in phloem (Vilaine *et al.*, 2003; Ivashikina *et al.*, 2003) and xylem (Hertzberg *et al.*, 2001; Demura *et al.*, 2002; Milioni *et al.*, 2002). *PtaERF1* is apparently expressed mainly in phloem tissue. A suggestion for the functional significance of PtaERF1 as a transcription factor in phloem is its involvement in cellular processes related to phloem identity, functioning (e.g. defence signalling) and/or development.

The third differentiation pattern concerns the identity of the two cambial initials, either fusiform or ray initials. Rays are essential determinants for secondary growth in plants because they ensure the translocation of nutrients between phloem and xylem and the transmission of messenger molecules (Lachaud *et al.*, 1999). Until now, the induction and the regulation of vascular ray differentiation have received only limited attention. As *PtaRHE1* expression is localized in ray initials and derivatives within the cambial zone, a role for this gene in the identity of this cell type and/or in the communication between xylem and phloem can be postulated.

In conclusion, the approach used to analyse the setting up of secondary growth in aspen has allowed the identification of several regulatory genes. The expression of two of them was localized in particular cell types within the vascular system. *PtaRHE1*, encoding a RING-H2 finger protein, is expressed in ray initials within the cambial zone and *PtaERF1*, encoding an AP2/ERF transcription factor is expressed in phloem tissue. Further work will focus on the determination of the role of these genes in vascular tissue differentiation by transgenic approach.

Supplementary material

Supplementary information (Table S1: Primer pairs used for RT-PCR analysis) can be found at JXB online.

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