# The poplar root transcriptome: analysis of 7000 expressed sequence tags

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**Abstract To date, most poplar expressed sequence tags (ESTs) are from above-ground tissues such as wood, leaf and buds. Here, we present a large-scale production of ESTs from roots of the hybrid cottonwood, *Populus trichocarpa*×*deltoides*. cDNA libraries were generated from the root system of 2-month-old rooted cuttings, and roots of 2.5-month-old cuttings water-stressed for 19 days. Partial sequences obtained from 7013 clones were assembled into 1347 clusters and 3527 singletons. This set of ESTs represents 4874 unique transcripts expressed in roots. Putative functions could be assigned to 3021 (62%) of the transcripts. A significant portion of the ESTs encode proteins of common metabolic pathways; energy and metabolism represented 5% and 8% of total transcripts, respectively. Of specific interest to root functions are the 6% of ESTs involved in signalling pathways and hormone metabolism, and 4% encoding transporters and channels. The current poplar root ESTs and the aspen root ESTs present in public databases represent 6700 unique transcripts. The Unigene set was selected from the ESTs and used to generate nylon microarrays. Changes in aquaporins and transporter transcripts were then studied during adventitious root development.**



*Key words:* Expressed sequence tag; Adventitious root; Nutrient uptake; Aquaporin; *Populus trichocarpa*×*deltoides*

## 1. Introduction

Poplars and aspens are major crop trees worldwide and several species are widely used in the wood industry. Poplars are fast-growing trees that can be propagated vegetatively; various genotypes are readily transformed, genetic maps are available, and quantitative trait loci for growth and disease resistance have been identified [1–3]. In recent years, poplar species have emerged as model systems for genomic approaches to wood formation and tree physiology [4]. Currently, both functional and structural genomics approaches are being pursued [5,6]. Sequencing of the relatively small *Populus* genome (550 Mb) has been initiated ([5]; http://genome.jgi-psf.org/poplar1/poplar1.home.html). Sterky et al. [7] reported the sequencing of 5692 expressed sequence tags (ESTs) of wood-forming tissues of *Populus tremula*×*P. tremuloides* and *P. trichocarpa*. This resource has grown to a point where over 107 000 ESTs have been sequenced from different cDNA libraries and from a wide range of above-ground tissues (e.g. seeds, wood tissues, leaf, and flowers) and developmental stages [5,8]. Analysis of these ESTs suggests that they derive from about 15 000 to 20 000 genes [5]. Over many years, there has been a great deal of effort focused on unraveling the nature of the molecular events underpinning wood formation in poplars and aspens [6,7,9]. In contrast, very little is known about the genes controlling root development and functions. ESTs have become an effective means of gene discovery in focused metabolic situations [10]. In forest trees, this concept was first applied to the isolation of genes involved in wood formation in pine [11] and poplar [7]. The root system is committed to nutrient acquisition and assimilation, and interactions with rhizospheric microbes (e.g. mycorrhizal fungi) [12]. Despite these considerable metabolic and developmental capabilities, EST profiling has not been carried out on root tissues.

In an effort to create a resource for gene discovery and to understand processes which are related to (1) water and nutrient uptake and assimilation and (2) interactions with rhizospheric microorganisms, we have generated 7013 ESTs from roots of the hybrid cottonwood *Populus trichocarpa*×*P. deltoides*.

## 2. Materials and methods

### 2.1. Plant material

Dormant hardwood cuttings from the hybrid poplar (*P. trichocarpa* Torr. and Gray×*P. deltoides* Bartr. ex Marshall, cv. Beaupré) were collected from stoolbeds in January 1998 and grown in the greenhouse in a controlled environment. Cuttings (25 cm length) were rooted in a peat–vermiculite mix in 2 l pots and the plants were watered for 2 min twice daily, with a solution containing Nutricote T100 (6 g/l), provided with 16 h of light (natural sunlight supplemented with metal halide lamps; light intensity: 1070 $\mu E/m^2/s$) daily and 60% relative humidity. Greenhouse temperature was maintained at 21–23°C during the day and 14–18°C at night. The plants were grown for 2 months to a height of approximately 80 cm with approximately 20 leaves prior to sampling or dehydration treatment. For dehydration treatment, 2-month-old plants were further grown for 19 days under restricted irrigation to reach 50% of the transpiration rate of fully watered plants. Adventitious roots of 2-month-old cuttings and water-stressed root systems were harvested, washed using tap water and fixed in liquid nitrogen. For the analysis of adventitious root development, 25 cuttings (25 cm length) were placed in a plastic box containing 30 l of the following liquid medium: 7.91 mM $KNO_3$, 7.96 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2.69 mM $NaH_2PO_4{\cdot}2H_2O$, 3 mM $MgSO_4{\cdot}7H_2O$ and micronutrients. The aeration (250 l/h) was provided by aquarium pumps and the trees were kept in a controlled environment growth chamber (17 h light period at 1000 $\mu E/m^2/s$ at 25°C). Developing root tissues of cuttings were harvested at six different stages (dormant bark tissues, root primordium, root callus, emerging roots, primary roots, and lateral root tips), frozen in liquid $N_2$, and stored at −80°C for RNA isolation. Photos of the different developmental stages are available at: http://mycor.nancy.inra.fr/poplardb/index.html.

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### 2.2. RNA extraction and cDNA library construction

Two cDNA libraries were made each with either the adventitious root system of 2-month-old rooted cuttings (library I) or the root system of water-stressed cuttings (library II). Total RNA was extracted from 1.5 g fresh weight of root tissues according to Bugos et al. [13] with a final phenol/chloroform precipitation. Full-length cDNA libraries were constructed from 1.0 μg of total RNA using the SMART cDNA synthesis kit in λTriplEx2 (Clontech, Palo Alto, CA, USA). The resulting cDNA was packaged into λ phages using the Gigapack III Gold packaging kit (Stratagene, La Jolla, CA, USA). From the initial plating the libraries were estimated to contain $1\times10^7$ (adventitious root systems) and $4\times10^9$ (water-stressed roots) recombinant clones. The average insert size was 1.0 kb. The pTriplEx2 phagemid clones in *Escherichia coli* were obtained by using the mass in vivo excision protocol according to the manufacturer's instructions (Clontech).

### 2.3. DNA sequencing

An aliquot of each excised, amplified library was used for infecting *E. coli* BM25,8 cells of $OD_{600}$ 1.0 and subsequently plated on Luria–Bertani (LB) agar containing ampicillin. About 8000 and 2000 bacterial clones were randomly picked from libraries I and II, respectively, and inoculated into 96 well plates containing selective LB medium and grown overnight without agitation at 37°C. Glycerol was added to a final concentration of 40% and a backup plate was created. Plates were stored at −80°C. Polymerase chain reaction (PCR, 50 μl) was set up on the Biomek 2000 automated laboratory workstation (Beckman Coulter, Fullerton, CA, USA) and amplified cDNA inserts suitable for sequencing were generated from 3.0 μl of defrosted bacterial glycerol stock as template and FORNAT (5′-AAGCGCGCCATTGTGTTGGTACCC-3′) and REVALEX (5′-CGGCCGCATGCATAAGCTTGCTCG-3′) as primers (present in the pTriplEx2 vector arms). The PCR consisted of 95°C for 3 min, 95°C for 60 s, 60°C for 30 s, 72°C for 3 min for 30 cycles and a final extension at 72°C for 15 min (GeneAmp System 9700; Perkin Elmer, Boston, MA, USA). A 5 μl aliquot of each reaction was analyzed on a 1% agarose gel and stained with ethidium bromide for the control of size and quality of the PCR products. Excess primers and nucleotides were removed by ultrafiltration using the Montage 96 well plate system (Millipore MAHV N45). The resulting purified PCR products were then rearrayed in 96 well plates (Sarstedt) and used as a template for a sequence reaction using 5 pmol of the FORNAT primer, 50 bp upstream of the 5′ end of cDNA insert, and the CEQ Dye-labelled Dideoxy-Terminator Cycle Sequencing kit (Beckman Coulter). For each reaction, 3 μl of PCR product (20–50 ng DNA) and 4 μl of the sequencing kit mix were mixed in a total volume reaction of 20 μl using the Biomek 2000 robotic workstation. Sequencing reactions were subjected to 95°C for 20 s, 50°C for 20 s, 60°C for 4 min for 30 cycles (GeneAmp System 9700). The products of the sequencing reaction were purified by ethanol precipitation using 96 well plates and a benchtop centrifuge according to the manufacturer's instructions (Beckman Coulter). The pellets were resuspended in the sample loading solution (DTCS; Beckman Coulter) prior to capillary electrophoretic separation (96 analyses/day) and detection by the automated multicapillary sequencer CEQ 2000XL (Beckman Coulter).

### 2.4. Sequence processing and analysis

Raw sequence data (7440 and 1368 for libraries I and II, respectively) were edited using the CEQ Sequence Analysis program (Beckman Coulter). All sequence outputs obtained from the automated sequencer were scanned visually to confirm overall quality of peak shape and correspondence with base calls. A custom APPLESCRIPT script trimmed sequence file names automatically. Sequence data were then uploaded in the SEQUENCHER (version 4.1.2) (Gene Codes Corporation, Ann Arbor, MI, USA) program for Macintosh. Leading and trailing vector and polylinker sequences, and sequence ends with more than 3% ambiguous base calls were removed by filters. Ribosomal RNA in the data sets was identified and removed and sequences shorter than 100 bases were discarded. Edited sequences were exported as FASTA text files for further processing. The average read length was 575 bp. A suite of Mac OS X-compatible scripts, MacESTtools (http://mycor.nancy.inra.fr/PoplarDB/), was used for batch execution of BLASTN and WU-BLASTX against the non-redundant nucleic acid sequence and EST GenBank databases at the Baylor College of Medicine Web server [14]. Another script was used to retrieve best matches from the output BLASTX html files to generate datatables. Sequences with an *E*-value $\leq 10^{-5}$ were considered to identify known genes or have partial similarity to known genes. MacESTtools was also used to upload BLAST results in a searchable MySQL database containing raw sequences and BLAST results (http://mycor.nancy.inra.fr/PoplarDB/). The site also provides the opportunity to search the EST sequences using BLAST. A MacESTtools script was used for batch execution of BLASTN against the *Populus* ESTs deposited at the PopulusDB Web site (http://poppel.fysbot.umu.se/blastsearch.html) (Umeå Plant Science Center [UPSC], Umeå University, Sweden). Assembly of the individual ESTs into groups of tentative consensus sequences (TCs) representing unique transcripts was performed by using the contig routine (95% similarity over 40 nt length) of SEQUENCHER. Assembly of the current set of ESTs with either the 7169 poplar root ESTs or 8751 ESTs of *Pinus taeda* roots, deposited in GenBank (28/02/2003), in TCs was performed using SEQUENCHER (80% similarity over 40 nt length). The root cDNA library of the UPSC was constructed from the whole root system from young *P. tremula*×*tremuloides* grown in sterile conditions (S. Jansson, UPSC, personal communication).

The functional assignment of ESTs was based on the results of a comparison to the non-redundant protein database of GenBank using the BLASTX algorithm (see above). EST were annotated manually following the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de) role categorization.

All of the EST sequences have been deposited in the GenBank dbEST at the National Center for Biotechnology Information

Table 1
Most abundant transcripts in poplar roots as determined by clustering of ESTs

| TC number | Accession number | Number of ESTs in TC | Strongest BLASTX hit | Species | *E*-value |
|---|---|---|---|---|---|
| TC_R28G12 | CA823610 | 24 | metallothionein-like protein type 2 | *Pyrus pyrifolia* | 2.4e-27 |
| TC_R28F07 | CA823596 | 23 | metallothionein-like protein | *P. pyrifolia* | 1.5e-27 |
| TC_R28E08 | CA823588 | 20 | metallothionein-like protein | *P. pyrifolia* | 1.4e-34 |
| TC_R45E06 | CA824613 | 16 | plasma membrane aquaporin PIP | *Glycine max* | 2.6e-57 |
| TC_R01A10 | CA821923 | 16 | cationic peroxidase | *G. max* | 1.5e-80 |
| TC_R61H03 | CA825578 | 16 | tonoplast aquaporin | *Arabidopsis thaliana* | 2.7e-64 |
| TC_R49A07 | CA824822 | 16 | NADH-ubiquinone (acyl carrier protein) oxidoreductase | *A. thaliana* | 2.4e-43 |
| TC_R04H05 | CA822196 | 16 | histone H3 | *A. thaliana* | 2.3e-37 |
| TC_R20C06 | CA823068 | 15 | hypothetical protein | *A. thaliana* | 6.0e-08 |
| TC_R22H05 | CA823238 | 13 | histone H2A | *Euphorbia esula* | 3.5e-67 |
| TC_RSH01B07[a] | CA821296 | 13 | extensin | *Populus nigra* | 1.5e-57 |
| TC_R26C09 | CA823447 | 12 | Cu/Zn-superoxide dismutase | *P. tremuloides* | 5.0e-77 |
| TC_R21A01 | CA823107 | 12 | 40S ribosomal protein S19 | *A. thaliana* | 5e-17 |
| TC_R20D04 | CA823076 | 12 | auxin-repressed protein | *A. thaliana* | 8.5e-41 |

Assembly of the individual ESTs into groups of TCs representing unique transcripts was performed using the contig routine (95% similarity over 40 nt length) of SEQUENCHER.
[a]Most abundant transcript in water-stressed roots.

(NCBI) (accession numbers CA820716 to CA826472, CB239327 to CB239875 and CB307008 to CB307015).

*2.5. Generation and analysis of cDNA arrays*

The Unigene set was selected from the ESTs from adventitious roots (library I) using the SEQUENCHER cluster analysis (95% similarity over 40 nt length) and 4608 PCR-amplified cDNA inserts were spotted onto nylon membranes by using the *Bio*Grid arrayer (*Bio*Robotics, Cambridge, UK) according to the manufacturer's instruction (Eurogentec, Seraing, Belgium). The transcript population of adventitious root tissues was amplified, labelled, and hybridized to nylon microarrays as described [15]. Phosphorimages of hybridized membranes were analyzed in XDOTREADER (Cose, Paris, France) to obtain raw spot intensity data. After background subtraction, the raw data were normalized by expressing individual spot intensities as a fraction of the sum of all spot intensities in each image and the data were analyzed using Microsoft EXCEL [15]. Hierarchical clustering [16] was performed using the program EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST) [17].

## 3. Results and discussion

*3.1. EST analysis*

A total of 8808 sequences was obtained from the 5′ end of cDNAs from primary and lateral roots of rooted cuttings of the hybrid poplar, *P. trichocarpa*×*P. deltoides*. This included 1368 ESTs from water-stressed roots. After vector sequences, ESTs identified as rRNA, and sequences shorter than 100 bases were removed, the final number of ESTs was 7013. This set of ESTs represents up to 4874 unique transcripts (also referred to as TC sequences) expressed in poplar roots, 1347 clusters assembled from two or more ESTs and 3527 singletons. This corresponds to a redundancy of 49% (number of ESTs in clusters/total number of ESTs) and implies that continued sequencing of random cDNA from the two libraries still has considerable potential to uncover novel sequences. The number of ESTs in each cluster ranged between two (523 clusters) and 24 (one cluster); only three included $\geq 20$ EST sequences (0.25% abundance).

After processing ESTs were grouped into functional categories. In non-stressed roots, 62% of the ESTs could be assigned a cellular role on the basis of sequence similarity to proteins with known function in the non-redundant GenBank database by using a BLASTX with a *P*-value $\leq 10^{-5}$, with the remaining 38% falling into the 'unclassified/hypothetical' (16%), or 'no hit' (22%) categories (Fig. 1A). In water-stressed roots, the proportion of unclassified proteins was higher (25%). These proportions of unknown proteins were similar to those observed in wood tissues [7], and young and autumn leaves of poplar leaves [8]. The total number of proteins found in the two libraries with known function was 3021, taking into account that many of the transcripts represent isoforms of the same proteins (e.g. metallothioneins). About 40% of the ESTs showed a best match with *Arabidopsis* sequences. A complete list of genes identified with *E*-values is posted on the PoplarDB Web site (http://mycor.nancy.inra.fr/poplardb/index.html).

Over 107 000 ESTs have been sequenced from different cDNA libraries from a wide range of tissues (e.g. seeds, wood tissues, leaves, roots and flowers) of western balsam poplar (*P. trichocarpa*, 24 067 ESTs), European aspen (*P. tremula*, 14 100 ESTs), hybrid aspen (*P. tremula*×*tremuloides*, 56 190 ESTs) and quaking aspen (*Populus tremuloides*, 11 597) (GenBank dbEST, 17/02/2003). Analysis of these ESTs suggests that they derive from about 15 000 to

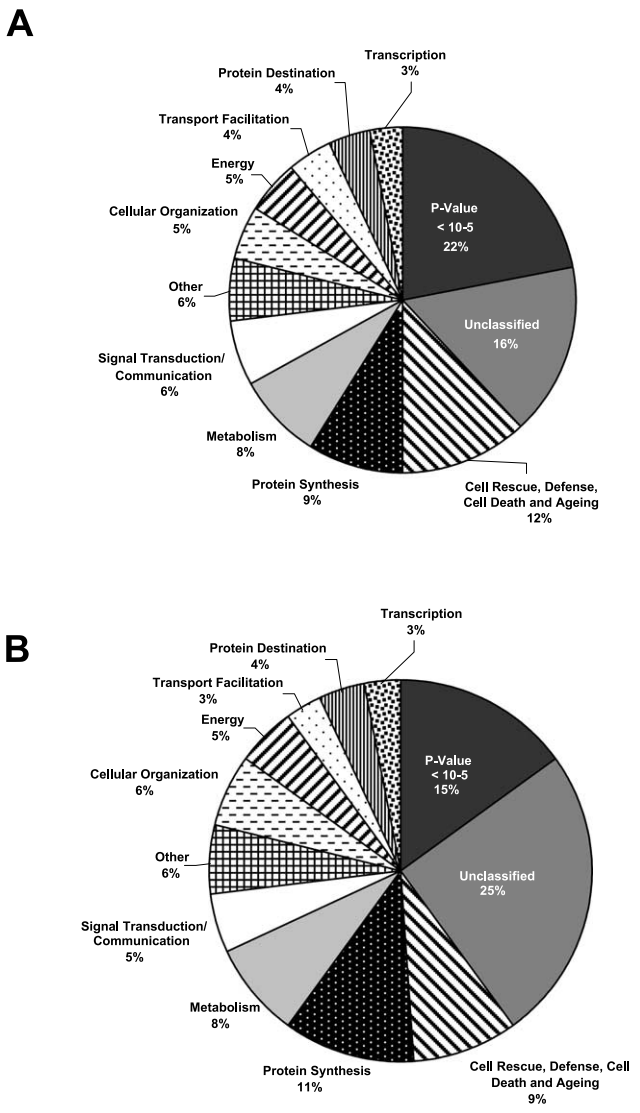


Fig. 1. Distribution of root transcripts from *P. trichocarpa*×*deltoides* by functional categories. Classification was performed for (A) 5943 ESTs of adventitious primary and lateral roots and (B) 1070 ESTs from water-stressed roots following the MIPS classification schemes.

20 000 genes [5]. BLASTN search against these ESTs (http://poppel.fysbot.umu.se/blastsearch.html) showed that 80% of our root ESTs shared significant nucleotide sequence similarity (>85%) with known ESTs. Of particular interest is the set of 20% of the ESTs in roots from *P. trichocarpa*×*deltoides* that showed weak or no sequence similarity to known *Populus* sequences[1]. For example, they encode proteins such as lysine/histidine-specific transporter (GenBank accession number CA822109), high affinity nitrate transporter (CA822323), and ferrochelatase (CA822369). This result emphasizes the complementarity of the various *Populus* EST sequencing programs.

In non-stressed roots, the largest proportion of functionally assigned sequences fell into four role categories: protein syn-

[1] Supplementary material can be found at http://mycor.nancy.inra.fr/poplardb/index.html.

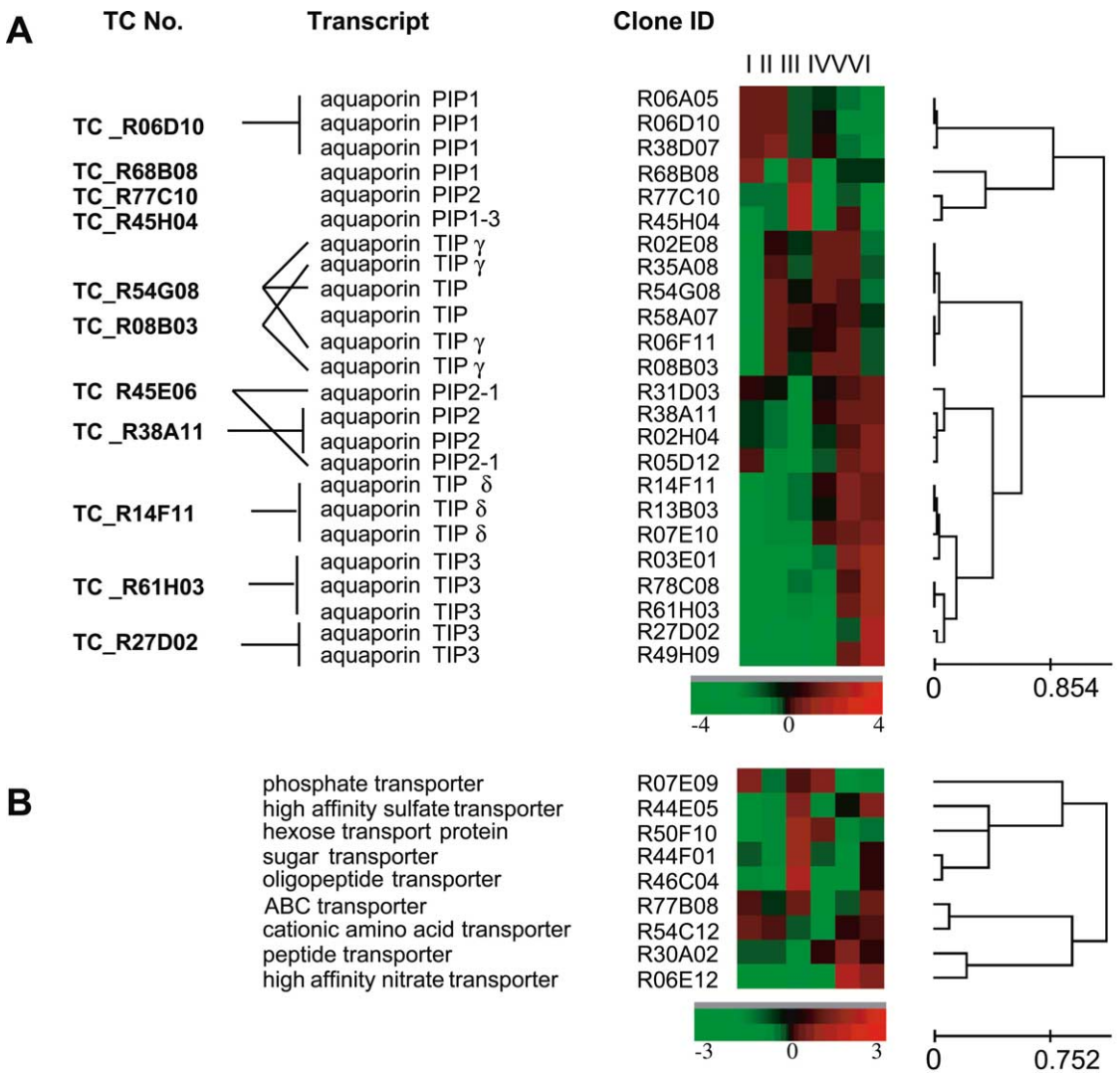


Fig. 2. Hierarchical cluster of root ESTs encoding aquaporins (A) and transporters (B) with two-fold or more changes in transcript abundance during adventitious root development. Each gene is represented by a single row of colored boxes, and a single column represents a developmental stage (I, bark tissues of dormant cuttings; II, root primordium; III, root callus; IV, emerging roots; V, primary roots; and VI, lateral root tips). Induction (or repression) ranges from pale to saturated red (or green) with a fold change scale bar (in log2) shown below the clusters. Clustering was based on the uncentered correlation measure base distance [15,16] and was carried out using EPCLUST [17]. Experiments were carried out in triplicate and representative clusters are shown.

thesis (9%), metabolism (8%), cellular communication/signal transduction (6%) and energy (5%). Together, these classes accounted for 28% of the assignable transcripts (Fig. 1A). This distribution was slightly affected by water stress (Fig. 1B), protein synthesis being higher (11%) in stressed roots as a result of a drastic accumulation of ribosomal proteins. The impact of water stress on root gene expression will be discussed elsewhere (Kohler et al., unpublished results). A major purpose of the present EST analysis of root tissues was to generate a global view of the water and nutrient uptake and biosynthetic capacity of this organ; ESTs playing a role in these functions are published as supplementary information[1]. Genes involved in nutrient uptake (transporters and channels) made up 4% of the total and comprised the Pi, $NO_3^-$, and cationic amino acid transporters, together with several metal transporters and the different members of the aquaporin gene families. Table 1 lists the 14 most highly expressed transcripts (TCs) observed amongst the randomly picked EST clones from both libraries. Three root TCs (R28G12, R28F07, and R28E08) encode three different type 2 and 3 metallothioneins (MTs) known for their metal-binding capacities and predicted to have a defense or stress function [18]. ESTs encoding aquaporins, histones, cationic peroxidase, NADH-ubiquinone oxidoreductase, superoxide dismutase, and a 40S ribosomal protein S19 were also very abundant (Table 1). In water-stressed roots, transcripts encoding an extensin (TC_RSH01B07) and a member of the heat shock hsp20 protein family (e.g. RSH10C04) were very abundant and not found in non-stressed adventitious roots.

The high expression of these genes was confirmed by cDNA microarray analysis (Kohler et al., unpublished results). These predominant transcripts are different from those identified in the cambial region of *P. tremula*×*tremuloides* [7] and developing xylem of *P. trichocarpa* [7] where genes implicated in lignin biosynthesis were the most abundant. Genes encoding MTs were the most highly expressed ESTs in autumn leaves

of *P. tremula*×*tremuloides* [8]. This difference in representation is likely the result of tissue/species differences and ecophysiological status of trees.

Merging the 14 182 root ESTs of *P. tremuloides* (C.-J. Tsai, Michigan Technological University, unpublished results), *P. tremula*×*tremuloides* (R. Bhalerao, UPSC, Sweden), and *P. trichocarpa*×*deltoides* (current set) represented a total of 8603 unique transcripts (3406 contigs and 5197 singletons). Interestingly, the most abundant transcripts found in the quaking aspen (*P. tremula*×*tremuloides*) ESTs (e.g. polyubiquitin, glutathione *S*-transferase, proline-rich protein) were not similar to those identified in *P. trichocarpa*×*deltoides* (Table 1). Assembly of the *P. trichocarpa*×*deltoides* ESTs with the 8751 GenBank ESTs of *Pinus taeda* roots was not able to detect common TCs (80% similarity over 40 nt length).

### 3.2. Transcript profiling

We analyzed the transcript abundance of genes involved in water and nutrient absorption during adventitious root development using microarrays with the Unigene set of ESTs (i.e. 4608 cDNAs, ~20% of the total poplar genes [5]). To identify transcripts which were co-regulated according to the developmental stage, hierarchical cluster analysis of aquaporins and transporters was performed [16,17]. Cluster analysis revealed several groups of cDNA clones with similar behavior during adventitious root development (Fig. 2). Aquaporins belonging to the PIP family (TC_R06D10) were highly expressed in the bark of dormant cuttings and root primordium, whereas their expression was downregulated in the primary and lateral roots. In contrast, transcripts encoding tonoplastic aquaporins were mostly expressed in root callus and emerging roots (TIPγ; TC_R54G08, TC_R08B03) or primary and lateral roots (TIP3 and TIPδ; TC_R61H03, TC_R72A08, TC_RSH07G07). The various transporters analyzed also showed a differential expression during the time course of root development. Sulfate, hexose, and oligopeptide transporters were upregulated during callus formation, whereas nitrate and the peptide transporter R30A02 were mainly expressed in primary and lateral roots. Changes in gene expression during adventitious root development will be discussed in detail elsewhere (Kohler et al., unpublished results).

## 4. Conclusion

The data presented here provide a first picture of the set of poplar genes expressed in roots. The current root database has many genes, such as channels, transporters and transcriptional factors, which hold great promise in the elucidation of nutrient uptake and assimilation pathways. The functional characterization of several transporters (phosphate, amino acid, zinc) has already been carried out by yeast complementation (M. Chalot, UMR IaM, INRA-Nancy, personal communication) and transcript profilings of root and ectomycorrhiza development using cDNA arrays have been analyzed (present data; Kohler et al., data not shown). This study, like others [7,8], shows that it is fruitful to characterize a number of different cDNA libraries, and thus the coordinated international effort on *Populus* species [5] is providing the community with valuable data for gene identification, protein prediction and cDNA array production.

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