

A *Populus* EST resource for plant functional genomics

Fredrik Sterky^{*†}, Rupali R. Bhalerao^{*†‡}, Per Unneberg^{*}, Bo Segerman[‡], Peter Nilsson^{*}, Amy M. Brunner[§], Laurence Charbonnel-Campaa[¶], Jenny Jonsson Lindvall[‡], Karolina Tandre^{¶||}, Steven H. Strauss[§], Björn Sundberg[¶], Petter Gustafsson[‡], Mathias Uhlén^{*}, Rishikesh P. Bhalerao[¶], Ove Nilsson[¶], Göran Sandberg[¶], Jan Karlsson[‡], Joakim Lundeberg^{*}, and Stefan Jansson^{*†**}

^{*}Department of Biotechnology, Kungliga Tekniska Högskolan Royal Institute of Technology, AlbaNova University Center, SE-106 91 Stockholm, Sweden; [†]Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-901 87 Umeå, Sweden; [‡]Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden; and [§]Department of Forest Science, Richardson Hall, Oregon State University, Corvallis, OR 97331-5752

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Trees present a life form of paramount importance for terrestrial ecosystems and human societies because of their ecological structure and physiological function and provision of energy and industrial materials. The genus *Populus* is the internationally accepted model for molecular tree biology. We have analyzed 102,019 *Populus* ESTs that clustered into 11,885 clusters and 12,759 singletons. We also provide >4,000 assembled full clone sequences to serve as a basis for the upcoming annotation of the *Populus* genome sequence. A public web-based EST database (POPULUSDB) provides digital expression profiles for 18 tissues that comprise the majority of differentiated organs. The coding content of *Populus* and *Arabidopsis* genomes shows very high similarity, indicating that differences between these annual and perennial angiosperm life forms result primarily from differences in gene regulation. The high similarity between *Populus* and *Arabidopsis* will allow studies of *Populus* to directly benefit from the detailed functional genomic information generated for *Arabidopsis*, enabling detailed insights into tree development and adaptation. These data will also be valuable for functional genomic efforts in *Arabidopsis*.

After the completion of the *Arabidopsis* genome sequence (1) and the publication of near-complete sequences of indica and japonica rice (2, 3), plant researchers have been able to scan these genomes to identify and compare genes of interest. *Arabidopsis* and rice represent the two major angiosperm phylogenetic groups, dicotyledons and monocotyledons, respectively. They diverged ≈ 170 million years ago (4) and differ in numerous physiological traits. Within these groups, however, great diversity also exists in life history and plant structure. Some of the most striking differences observed are those between woody (trees and shrubs) and herbaceous species. Trees and shrubs form hard, long-lasting structures that are distinct from the soft stems and branches of herbs, especially annuals. The lignocellulosic cell walls of trees and shrubs are critical for their survival, stature, competitive ability, and provision of habitat, and they have a dramatic influence on ecosystem cycles. Trees and shrubs are found intermixed with herbaceous plants in many phylogenetic groups within the angiosperms, showing that the tree growth habit has been lost or acquired many times during evolution (5). The herbaceous life form is often considered to be the derived state, evolving numerous times from tree-like ancestors (6).

The tree life form imposes several different physiological and morphological constraints compared with those of herbaceous plants. Many of the processes that distinguish trees from herbs take years to fully develop and express themselves (e.g., wood formation, vegetative phenology, maturation, and the juvenility/maturity transition) and are therefore not easily studied in herbs. The need for a tree model system for functional genomics has therefore become evident (7). The genus *Populus*, consisting of ≈ 40 species distributed in diverse habitats throughout the northern hemisphere, best fulfills the criteria for a good model tree. It has a small genome (slightly bigger than rice), diploid inheritance, facile clonal propagation, and rapid growth, and it is amenable to transformation by *Agrobacterium* (8). Its genome has now been shotgun sequenced (to >7 times coverage) by the U.S. Department of Energy (www.jgi.

doe.gov), and the assembly and annotation is expected to be ready during 2004. Modeled after similar efforts in *Arabidopsis*, the International *Populus* Genome Consortium (www.ornl.gov/ipgc) (9) was established to provide coordination of postsequence research activities and education throughout the world. A critical remaining need for *Populus*, as a model organism, is high-quality sequence annotation. We describe EST resources that are essential for this goal.

Materials and Methods

EST Sequencing, Clustering, and Annotation. In total, >140,000 sequence reads were retained for clustering and assembly. In addition to sequences presented previously (10, 11), ESTs were generated from 16 new cDNA libraries. Sequences were run on Beckman SEQ2000, MegaBACE, ABI3700, and ABI377. The 5' and 3' end sequences from the confirmatory resequencing of the clones selected for microarray production (12, 13) were also included in the assembly. Sequence reads obtained from the ABI3700 and ABI377 sequencers were base-called by using TRACETUNER (www.paracel.com). Because of incompatibility problems with TRACETUNER, sequences obtained from the Beckman and MegaBACE sequencers were base-called with PHRED V. 000925.C (www.phrap.org). All sequence information has been submitted to GenBank.

Filtering, clustering, and assembly of EST sequences were performed with the PARACEL TRANSCRIPTASSEMBLER program (www.paracel.com), which integrates quality filtering, clustering, and assembly into one single pipeline. The filtering step includes masking of vector sequence and low-quality regions and annotation of low-complexity regions, repeats, and poly(A) regions. A hash search algorithm was used to remove contaminating sequences. The algorithm initially uses a hash lookup, followed by a gapless hit extension. Several hits to the same subject were tiled to obtain a final raw score. Four reference database collections were searched, with score parameters match = 1, mismatch = -9, to remove unwanted sequences. *Arabidopsis* mitochondrial and chloroplast sequences (score threshold = 100), *Escherichia coli* sequences (threshold = 40), and *Arabidopsis* rRNA sequences (threshold = 35) were removed. Finally, ESTs that passed the filters but had an unmasked sequence <70 bases were discarded. After filtering, assembly, and clustering, the output contained 102,019 ESTs in

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Abbreviations: MIPS, Munich Information Center for Protein Sequences; AFC, assembled full clone.

Data deposition: All EST sequences reported in this paper have been deposited in the GenBank database (accession nos. can be found in Table 2, which is published as supporting information on the PNAS web site).

[†]F.S. and R.R.B. contributed equally to this work.

[¶]Present address: Department of Natural Sciences, Södertörn University College, SE-141 89 Huddinge, Sweden.

^{**}To whom correspondence should be addressed. E-mail: stefan.jansson@plantphys.umu.se.

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addition to 10,782 3' sequences and 8,140 5' sequences derived from confirmation of microarray clones. In the clustering step, each sequence was initially sorted into a unique cluster. The reads were pairwise compared, and clusters were merged if the sequences showed sufficient similarity (85.7% identity, which corresponds to threshold >50, match = 1, mismatch = -6). A total of 11,891 clusters and 12,767 singletons (clusters with one sequence) were obtained. The assembly step uses CAP4, which is a refinement of the CAP3 algorithm (14). Each cluster was assembled into contigs representing unique transcripts, producing 15,574 contigs and 6,804 singlets (contigs containing only one sequence). The resulting sequences (contigs, singlets, and singletons) were annotated according to our annotation pipeline (11) as noted in POPULUSDB.

Assembled Full Clone (AFC) Sequences. All contigs covered by both the 5' and the 3' end of the same clone were extracted. In many cases, the 5' and 3' sequences did not overlap, but other ESTs spanned the region between these sequences. The resulting 4,166 sequences were compared against the *Arabidopsis* proteome by using BLASTX (15) with default parameters.

Library Comparisons. Dendrograms and clustered correlation maps were prepared following procedures described by Ewing *et al.* (16). First, an expression profile was constructed by counting the number of ESTs in each cluster. The ESTs were classified according to their libraries, giving 18 counts for each cluster. Clusters containing <10 ESTs were removed from the subsequent analysis, giving a final data matrix with 1,475 rows, corresponding to clusters, and 18 columns.

The similarity between clusters and libraries was estimated by Pearson's correlation coefficient, giving matrices of correlation values. From these matrices, the Euclidean distance between each pair of object was calculated. Dendrograms were constructed from the pairwise distances with the UPGMA algorithm. The original data set was reordered based on the ordering in the dendrograms, so that the most similar objects were adjacent to one another in the correlation map. The entire procedure was performed with the R software package (www.r-project.org).

Calculation of Codon Usage. From the AFC sequences, a high-quality data set suitable for determination of codon usage was derived. A set of 2,000 sequences with a BLASTX score against the *Arabidopsis* proteome higher than 450 were chosen, and each alignment was

manually inspected. Criteria for inclusion in the high-quality data set were the following: (i) the translated *Populus* sequence should have high similarity up to the C terminus of an annotated *Arabidopsis* protein (maximum 10 amino acids of the *Arabidopsis* protein unaligned by BLAST); (ii) the *Populus* sequence should end with an in-frame stop codon at a position similar to the *Arabidopsis* gene; (iii) a maximum of 50 amino acids should be unaligned at the N terminus of the protein; and (iv) the *Populus* sequence should start with a start codon at a position similar to the *Arabidopsis* gene, unless the *Populus* sequence was clearly not full-length. The reason for allowing more discrepancy at the N terminus was that signal peptides typically have much lower conservation than mature polypeptides. All sequences fulfilling these criteria should lack reading frame errors. They were put in a database and a codon usage table was created by using CODONW (www.molbiol.ox.ac.uk/cu).

Results

EST Sequencing and Clustering. When defining gene sequences in a genome, ESTs are important for the training of gene prediction algorithms, for species-specific codon usage, and for the characterization of splice-site preferences. Predicted exons can be linked together into genes, and splice variants can then be detected. In addition, if EST data sets are large and have been derived from multiple nonnormalized libraries, they can be used to obtain digital expression profiles, providing information on the tissues and conditions in which genes are expressed.

We reported 5' sequences of three cDNA libraries from different tissues of *Populus* (10, 11) and set up an annotation pipeline for the ESTs (11). Here, we have extended this to reach a total of 18 nonnormalized cDNA libraries, plus one partially subtracted library. These libraries represent either different tissues or different experimental treatments (Table 1). The libraries were derived from several taxa of *Populus*, aspen (*Populus tremula*), a hybrid aspen (*P. tremula* × *tremuloides* T89), and black cottonwood (*Populus trichocarpa*). A detailed description of the source material for the libraries can be found in Table 3, which is published as supporting information on the PNAS web site.

A total of 102,019 EST sequences were clustered by using PARACEL TRANSCRIPTASSEMBLER to generate a nonredundant set of genes that could be used to select clones for microarray construction. To improve the clustering, several sequences were added to the EST data set. First, sequences from confirmatory sequencing

Table 1. Overview of the EST data set

Tissue	Code	<i>Populus</i> genotype	ESTs, <i>n</i>	Average length,* bp
Cambial zone	A + B	<i>tremula</i> × <i>tremuloides</i>	6,326	367
Active cambium	UB	<i>tremula</i>	4,647	366
Dormant cambium	UA	<i>tremula</i>	3,655	403
Tension wood	G	<i>tremula</i> × <i>tremuloides</i>	5,723	408
Wood cell death	X	<i>tremula</i> × <i>tremuloides</i>	4,867	548
Young leaves	C	<i>tremula</i> × <i>tremuloides</i>	5,013	351
Senescing leaves	I	<i>tremula</i>	5,726	366
Cold-stressed leaves	L	<i>tremula</i> × <i>tremuloides</i>	4,066	448
Dormant buds	Q	<i>tremula</i>	5,815	558
Petioles	P	<i>tremula</i>	6,443	559
Virus/fungal-infected leaves	Y	<i>tremula</i>	1,395	438
Floral buds	F	<i>trichocarpa</i>	6,760	351
Female catkins	M	<i>trichocarpa</i>	6,112	553
Male catkins	V	<i>trichocarpa</i>	4,855	485
Apical shoot	K	<i>tremula</i> × <i>tremuloides</i>	5,380	481
Shoot meristem	T	<i>tremula</i> × <i>tremuloides</i>	8,371	535
Bark	N	<i>tremula</i> × <i>tremuloides</i>	4,891	548
Roots	R	<i>tremula</i> × <i>tremuloides</i>	5,786	593
Imbibed seeds	S	<i>tremula</i>	6,188	502

*The part of the sequences that passed the PARACEL TRANSCRIPTASSEMBLER filters. The average read length has increased substantially during the course of this work.

of earlier microarrays (12, 13) were added. The clustering output contained 10,782 3' sequences and 8,140 5' sequences with that origin. Second, *Populus* sequences in public databases and several full-length sequences from work in our laboratories were also included. The clustering resulted in 12,759 singletons and 11,885 clusters, further divided into 15,574 contigs and 6,804 singletons. Clustering did not discriminate between *P. trichocarpa* and *P. tremula/tremuloides* sequences; of the 4,456 clusters containing *P. trichocarpa* ESTs, 86% also contained ESTs from aspen/hybrid aspen. Manual inspection of species-specific contigs within the same clusters, presumably originating from orthologous genes from the two species, showed that coding sequences were almost identical (typically >98%), whereas UTRs could show much higher sequence variation. Most of the 11,885 clusters appear to correspond to single genes, whereas the contigs and singletons within clusters appear to largely consist of splice variants, paralogs, alleles, or cloning artifacts. However, very closely related genes also clustered together in several cases. Our experience from the 3' sequencing of 11,175 ESTs demonstrated that the true number of genes represented is substantially lower than the number of clusters and singletons. The complete unigene set is currently undergoing 3' sequencing, which in addition to the genome sequence will allow more accurate estimates of the true number of genes covered by our data set.

Comparison of *Populus* and *Arabidopsis* Gene Content. For a reliable comparison between *Populus* and *Arabidopsis* genes, we extracted a set of 4,166 contig sequences where the full insert of one clone was covered by ESTs. Although cDNAs could be incomplete, this set represents a data set with multiple sequence coverage and therefore higher quality than the ESTs alone. These AFC sequences (see Table 4, which is published as supporting information on the PNAS web site) had an average length of 820 bases and were compared with the *Arabidopsis* proteome by using BLASTX. Sequences <300 bp (typically representing only the 3' UTR) showed, as expected, very low similarity to *Arabidopsis* proteins (Fig. 1). For sequences exceeding 300 bp, a strong correlation occurred between sequence length and BLASTX score. For example, if only sequences >1,000 bp were considered, 97.9% had a BLASTX score > 100, and 95.0% were >200 (1,089 and 1,056 of 1,112, respectively). For sequences >1,500 bp, 98.8% (237 of 240) had a BLASTX score >200. Eight of the 23 AFC sequences >1,000 bp with least similarity to *Arabidopsis* (BLASTXscore <100) were false positives. Four of them were found in other annotations of the *Arabidopsis* genome, and four others were in a cluster where another contig had a strong hit to an

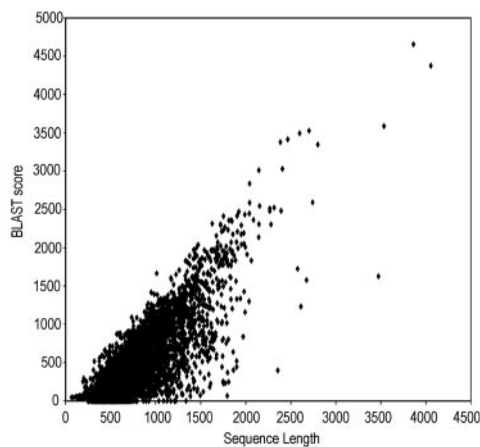


Fig. 1. Relation between sequence length of *Populus* contigs and similarity to the best scoring *Arabidopsis* sequence. AFC *Populus* sequences (4,166) were compared by using BLASTX with the *Arabidopsis* proteome, and the score for each sequence was plotted against sequence length.

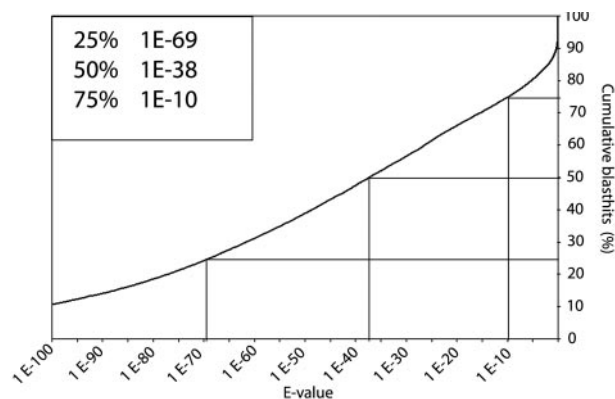


Fig. 2. Homologs to most *Arabidopsis* gene families are represented in POPULUSDB. The cumulative curve indicates the percentage of genes in the *Arabidopsis* genome that has sequence similarity better than the value given to a sequence in POPULUSDB. For example, 50% of the genes have a hit with an *E* value < 10^{-38} and 75% have a hit with an *E* value < 10^{-10} .

Arabidopsis protein. In summary, only 15 (1.3%) of the AFC sequences (Table 5, which is published as supporting information on the PNAS web site) lacked an apparent homolog in *Arabidopsis*. However, all of them were present in the *Populus* genomic sequence (<http://genome.jgi-psf.org/poplar0/poplar0.home.html>)

The 2,506 AFC sequences with BLAST scores <100 toward the *Arabidopsis* proteome were also compared with the *Arabidopsis* genomic sequences by using TBLASTX. Only five sequences scored higher to the translated genome than to the proteome, indicating that the number of novel *Arabidopsis* genes that could be predicted by using *Populus* ESTs is low, but we have found cases where *Arabidopsis* gene models probably could be improved by using the *Populus* data. We also compared all genes in the *Arabidopsis* genome with our data set by using TBLASTN. Nearly 50% of all predicted *Arabidopsis* proteins had a match with an *E* value < 10^{-38} , and 75% had a match with an *E* value < 10^{-10} (Fig. 2). No established criterion of similarity (BLAST scores or *E* values) can distinguish orthologs within gene families, but these figures show that most *Arabidopsis* genes or gene families have a homolog represented in our database.

Taken together, a very low percentage of genes in *Populus* do not have a close homolog in *Arabidopsis*, and the majority of the *Arabidopsis* gene families have a counterpart in *Populus*. The genome size of *Populus* is \approx 500 Mbp, but the gene density is not yet known. Manual inspection of selected gene families that are well represented by ESTs indicates that some, but far from all, *Populus* gene families are larger than those of *Arabidopsis*. For example, one of the best characterized gene families in plants, the *Lhc* genes

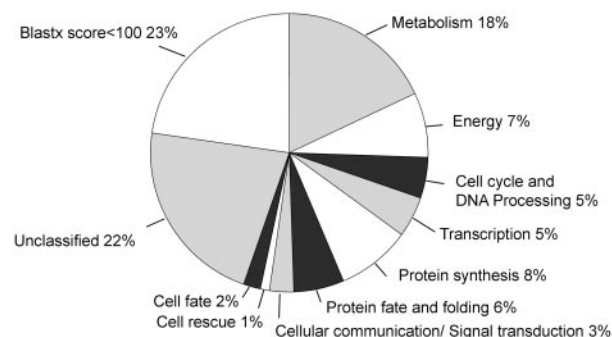


Fig. 3. Functional classification of the *Populus* EST data set according to the Umeå Plant Science Center-MIPS classification schedule

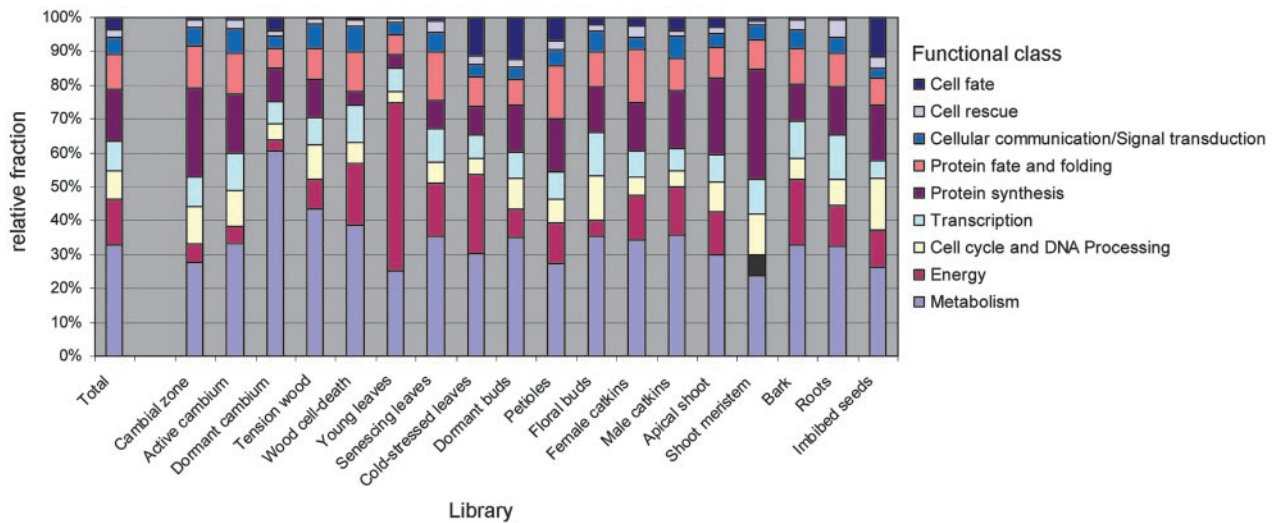


Fig. 4. Relative abundance of clones in functional classes (excluding clones without significant homology to a protein with a predicted function (classes 98 and 99) in the different libraries.

(including PsbS and ELIP) contain 23 copies in *Arabidopsis* and at least 27 copies in *Populus*.

The Coding Content of the *Populus* Genome. We annotated the ESTs by using a previously developed annotation pipeline (11), where information is retrieved from several databases and integrated into POPULUSDB. From the best *Arabidopsis* match, a functional classification is retrieved from the Munich Information Center for Protein Sequences (MIPS) database (mips.gsf.de). The list of sequences, with annotations and functional classifications, is found in Table 6, which is published as supporting information on the PNAS web site. Both the annotations and functional classifications will be subjected to manual curation, according to the modified MIPS functional classification scheme Umeå Plant Science Center-MIPS (www.populus.db.umu.se). POPULUSDB should be continuously updated as the curation of annotations and classifications progress.

Because of low sequence similarity (BLAST score <100), 23% of our ESTs were not classified (class 99), and another 22.5% were most similar to an *Arabidopsis* protein of unknown function (class 98). From the whole data set (Fig. 3), classifications of subsets can be extracted: We previously compared the functional classifications of ESTs from young and senescing leaves (11). In Fig. 4, the frequency of clones (excluding the noninformative classes 98 and

99) in each main Umeå Plant Science Center-MIPS category in the different libraries, are shown. Most libraries had a distinct pattern of gene expression. For example, genes from the class “protein synthesis” were most abundant in the libraries from the shoot meristem, cambial zone, and apical shoot, whereas genes from the class “energy” dominated the young leaf library, and genes from the class “cell cycle and DNA processing” were most frequently detected in the imbibed seeds library.

An Expression Catalog of *Populus* Tissues. EST frequencies approximate message abundance in the mRNA population used to construct a cDNA library (11). Clusters containing >10 ESTs were subjected to a cluster correlation analysis (16) to compare expression profiles in the different libraries. One of the libraries (Y, from virus- and fungus-infected leaves) is a partially normalized library and was excluded from this analysis. When the result was displayed in the form of a dendrogram (Fig. 5), many libraries with similar origins clustered together (e.g., young and senescing leaves, and apical shoot and shoot meristem). Three of the four libraries derived from the wood-forming zone of the stem (cambial zone, active cambium, and tension wood) clustered together, but the fourth (dormant cambium) was most similar to the bark library. Genes that have similar functions were generally clustered together

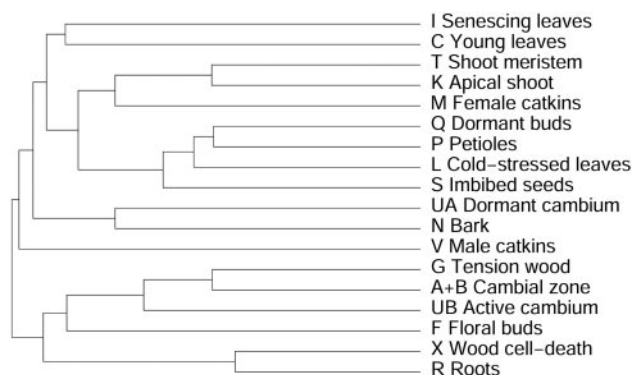


Fig. 5. Dendrogram representation of the similarities in expression profiles for the *Populus* libraries, based on a clustered correlation map calculated according to Ewing et al. (16).

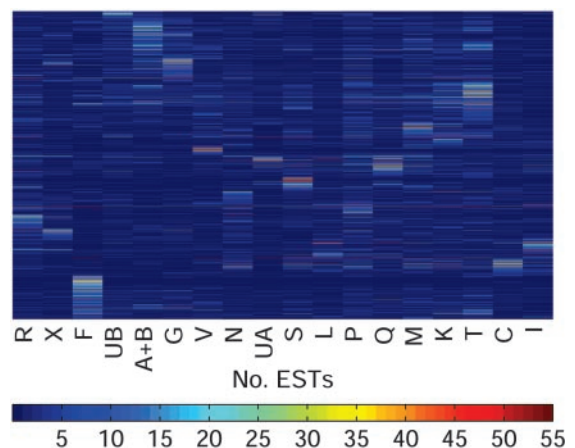


Fig. 6. The complete clustered correlation map calculated according to Ewing et al. (16). Only clusters with >10 ESTs were included.

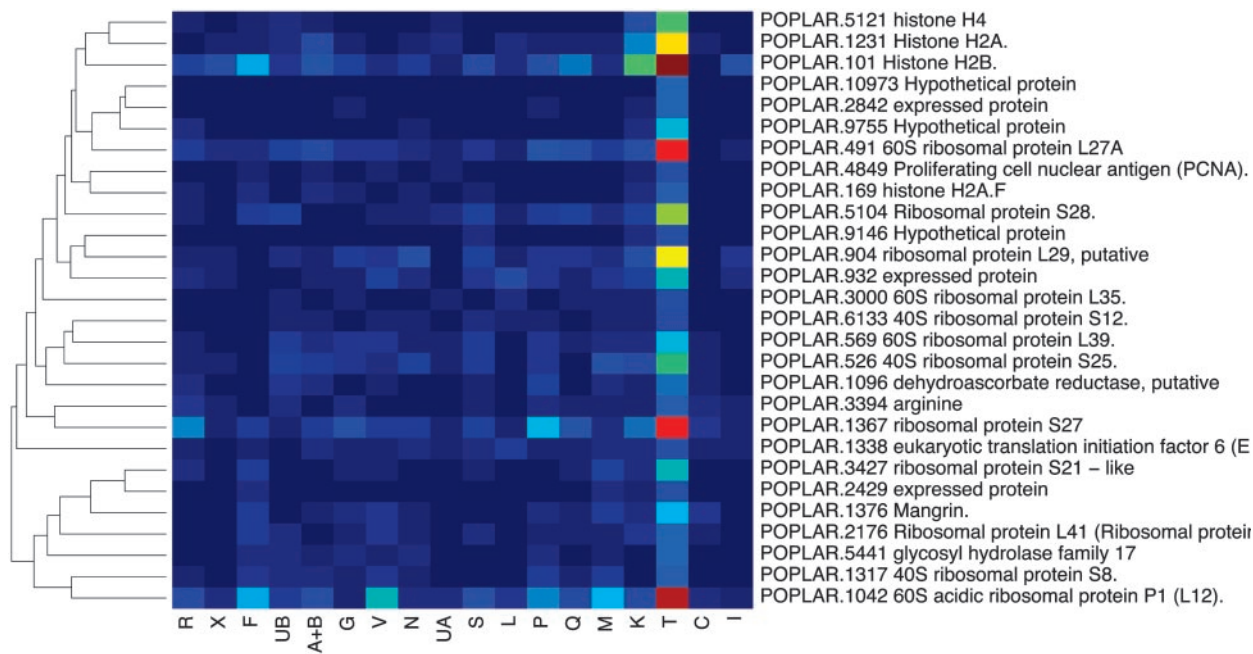


Fig. 7. Subsection of the clustered correlation map, showing a cluster mainly containing genes encoding histone and ribosomal proteins.

in the cluster correlation map (Fig. 6). One cluster, most enriched in apical meristems, contained genes encoding histone and ribosomal proteins (Fig. 7), whereas another, most enriched in young leaves, contained mainly genes encoding proteins involved in photosynthesis (Fig. 8). Evidently, mining of POPULUSDB gives an estimate of the tissue specificity and expression level for most genes with high or moderate expression. Comparison of the different libraries revealed only 26 genes that were found in all 18 libraries. These “housekeeping genes” (see Table 7, which is published as supporting information on the PNAS web site) included, for example, four ribosomal proteins, polyubiquitin, CuZn-SOD, calmodulin, and one storage protein (annotated as pollen coat pro-

tein) and, somewhat surprising, one sequence most similar to an *Arabidopsis* protein with unknown function related to the systemic acquired resistance-related protein SRE1a from potato.

Codon Usage. Accurate gene prediction based on genome sequence requires knowledge of codon usage. To generate a high-quality data set of correct sequences for this purpose, we selected from the AFC sequences a smaller data set containing only sequences highly similar to an *Arabidopsis* protein throughout its whole length. The criterion was used to ensure that no sequences containing reading frame errors were included in the analysis. From this data set, containing 873 sequences (206,763 codons), ORFs were defined

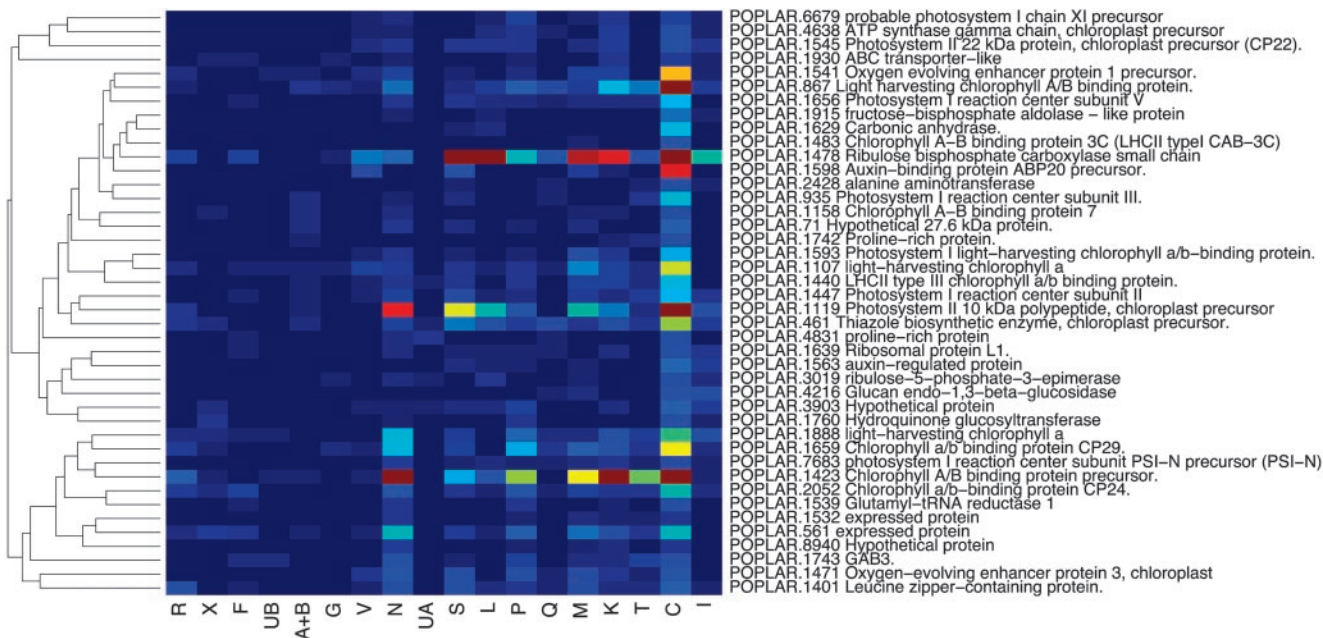


Fig. 8. Subsection of the clustered correlation map, showing a cluster mainly containing genes encoding photosynthetic proteins.

and a codon usage table was created (see Table 8, which is published as supporting information on the PNAS web site).

As expected, the codon usage of *Populus* shared many similarities with that of *Arabidopsis* and other dicots. For example, T is the preferred base in the third codon position for all amino acids except glycine, and TGA is the preferred stop codon occurring in 44% of the sequences. However, one difference was evident. *Arabidopsis* shows a very mild suppression of the CG dinucleotide in the last two codon positions ($XCG/XCC = 0.92$), whereas the corresponding figure in *Populus* was 0.38. CG suppression is most likely a consequence of methylation of C in the CG dinucleotide, resulting in an increased mutation rate. CG suppression is very common in dicot genomes; tomato has an XCG/XCC ratio of 0.58, pea 0.51, soybean 0.37, potato 0.48, and spinach 0.42. The GC content in third base position was similar in *Populus* and *Arabidopsis* (44 vs. 42%).

Discussion

Large-scale EST sequencing provides a gateway into the genome of an organism. The ESTs give important information about its coding content and expression patterns in different tissues and environments. Our data set comprises >100,000 ESTs and a unigene set with 11,885 clusters and 12,759 singletons. These sequences represent a substantial part of the complete gene content in *Populus*, although it would be premature to estimate the total number of genes represented. We provide a data set of >4,000 full-clone sequences for training of gene prediction algorithms and a codon usage table based on 873 ORFs. All these data are essential for accurate annotation of the *Populus* genome sequence.

The EST resource and POPULUSDB will play important roles when the genomic sequence is complete and released. By using a common gene nomenclature and direct links, researchers will be able to rapidly learn about tissue specificity and expression level of a considerable fraction of *Populus* genes. Mining of POPULUSDB for specific orthologs will also give researchers working with other plant species information about tissue specificity. We have established a platform for transcript analysis with cDNA microarrays that contain >13 000 clones (13); and using the data set presented here, we have increased the number of clones on microarrays to almost 25,000 (the list of selected clones is found in Table 9, which is published as supporting information on the PNAS web site). We have also shown that *Populus* species are highly conserved in DNA sequence, and in preliminary studies have found that the DNA microarrays can be effectively hybridized with RNA prepared from different *Populus* species and related genera, including *Salix viminalis* and *Salix caprea* (Salicaceae). This finding means that these genomic tools can be used within the entire family Salicaceae and genus *Populus*, both of which contain extensive genetic and adaptive diversity (7). The *Populus* genome sequence, POPULUSDB, and the rapid progress in *Populus* metabolomics, proteomics, and reverse

genetics are establishing *Populus* as one of the most versatile model systems for plant genomics (7, 9).

Populus and its genomic resources will aid in fundamental genomic studies of plants. We demonstrated that gene content is very similar between *Populus* and *Arabidopsis*. Nearly all gene families found in one of the species have a homolog in the other. This high degree of similarity means that *Arabidopsis* may not have lost as many genes during its evolution as had been suggested (5–10%) (17). However, analyses of gene content based on ESTs are inherently difficult because ESTs cover only the nonconserved parts of genes and may include large numbers of contaminant sequences; $\approx 1\%$ of the *Arabidopsis* ESTs in public databases do not have a match in the *Arabidopsis* genome (18). The extensive similarity between the *Populus* and *Arabidopsis* genomes will make it possible to use these two plant model systems in parallel when gene function is studied. The rapid life cycle and amendable genetics of *Arabidopsis* makes it a superior system for the majority of fundamental genomic studies. However, the unique features of a tree system (e.g., the large size that makes tissue separation easier, wood formation, seasonal growth/hardiness patterns, longevity, and phase change) means that studies in *Populus* could complement, and will, in some cases, replace studies in *Arabidopsis* where organismal traits are concerned. In gene families with a complicated structure, orthologs may be hard to identify between *Populus* and *Arabidopsis*. However, for most genes, we expect that phylogenetic analysis will make relationships clear. Moreover, with the *Populus* physical map now being generated (7), microsynteny (19) will provide important independent clues to orthology.

Populus genomic resources provide major new opportunities to study adaptive evolution in plants. Because *Populus* species have extensive natural populations and some of the widest distributions of any plant species (7), they contain enormous natural genetic variation within and between species, populations, and genotypes. Some taxa grow in extremely hot and arid conditions, whereas others survive extreme frost in arctic regions. *Populus* is an outcrossing (largely dioecious) tree that shows very little population differentiation (20), whereas *Arabidopsis* is an annual, often inbreeding species that shows fine-scale ecological differentiation. Their comparison should give fresh insights into the diversity of mechanisms for adaptive evolution.

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