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Generation and analysis of expressed sequence tags from poplar (*Populus alba* × *P. tremula* var. *glandulosa*) suspension cells

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Abstract

We have analyzed 8962 expressed sequence tags (ESTs) obtained from poplar cell suspension culture to gain an insight into the changes in gene expression during their growth phase in suspension culture. Single pass sequencing from 5′-end of 8962 randomly selected cDNA clones resulted in 7586 high quality ESTs with average reading length of 517 bp. A total of 3378 unigenes, consisting of 1337 contigs and 2041 singletons, were formed after assembly using CAP3 program. Among them, 2091 unigenes (61.9%) were found to significantly match (BLASTX score more than 80) the proteins with known function and 949 (28.1%) matched significantly with those having unknown function in the public databases. The remaining 338 unigenes (10.0%) failed to show significant homology to any proteins in the public databases, suggesting that they represent novel sequences. A significant portion of highly expressed genes in poplar cells encodes cell wall-related proteins, such as extensin, polygalacturonase inhibiting protein and proline-rich protein. The potentiality of the ESTs resource in studying of the regulation of cell growth was proved by growth phase-dependent expression of several selected genes.

Keywords: Poplar; Expressed sequence tags; Growth phase-dependent expression; Suspension cell

1. Introduction

Trees have great values as a source of essential elements for human living. They have unique characteristics, such as perenniality, developmental phase changes, secondary growth and secondary metabolism, and also have resistance systems to extreme environmental conditions including biotic and abiotic stresses [1,2]. Among them, the genus *Populus* occupies a rather prominent place as a model system for functional genomics studies. The genus *Populus* have many positive features to make them suitable for molecular genetic examination. These include fast-growth, relatively small genome, ease of vegetative propagation, facile transgenesis, and tight coupling between physiological traits and biomass productivity [3–5]. A *Populus* genome project initiated by Department of Energy's Joint Genome Institute has recently been completed and finished sequencing of the whole genome. As a part of the ongoing *Populus* genome project, the *Populus* genome assembly 1.0 has been preliminary released on the web (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and the final draft sequence is scheduled to be available sometime in 2005. To annotate and elucidate gene function in *Populus*, massive high-quality sequences of the genes expressed in *Populus* are needed.

Expressed sequence tags (ESTs) are a relatively rapid and cost effective method to obtain massive information on gene expression and coding sequences of genome by partial sequencing of cDNA. In *Populus*, 5692 ESTs from wood-forming tissues, 7013 from roots, and 9969 from young and autumn leaves have been reported [6–8]. Recently, Sterky et al. [5] reported that the analysis of 102,019 ESTs from 19 different cDNA libraries including fore-mentioned. These ESTs have been clustered into 11,885 clusters and 12,759 singletons. However, the number represents only a fraction of a total of 58,036 predicted gene models produced through

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the collaboration for *Populus* genome assembly 1.0. So far most of the published data on *Populus* ESTs have been obtained using various parts of tissues growing in the field. There is a need to extend the ESTs set to other tissues or developmental stages, which may display different gene expression profile from those already reported.

Employing cell suspension has several advantages over using whole plants in studying physiology and gene expression. Cell culture provides a source of genetically and physiologically uniform cells that can be easily manipulated by simple elicitation with various stimuli and to study basic controls of cell cycle, proliferation and growth without developmental processes. In addition, it allows us to separate cellular response from whole plant response [9–12]. Thus, we believe that the data set obtained from cultured cells may complement those published results since a large number of genes are expected to be expressed during cell culture.

In this study, we performed an ESTs sequencing project generated from cDNA clones of poplar (*Populus alba* × *P. tremula* var. *glandulosa*) suspension cells and analyzed 8962 ESTs to gain an insight into the changes in gene expression during their growth phase in suspension culture.

2. Materials and methods

2.1. Plant material and growth conditions

We used a hybrid poplar (*Populus alba* × *P. tremula* var. *glandulosa*) since we developed a good transformation and regeneration system from cell and tissue cultures. This feature will definitely help when individual gene functions have to be determined. Poplar suspension cells were maintained by subculturing biweekly as transferring 0.4 g fresh mass of cells to 100 ml of liquid MS medium [13] containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.1 mg l^{-1} 1-naphtalene acetic acid and 0.01 mg l^{-1} 6-benzylaminopurine [14]. The suspensions were maintained at 100 rpm on a gyratory shaker in the culture room at 22 ± 1 °C under dim (20 μmol $m^2 s^{-1}$) cool-white fluorescent light. For Northern blot analysis, cells were harvested, every 2 days after subculturing until 30 days later, by vacuum filtration through a couple of 3 MM filter papers, weighed, frozen in liquid nitrogen, and kept at −80 °C for RNA isolation.

2.2. Construction of cDNA library

Total RNA was extracted from the cell suspension 8 days after subculturing by guanidine thiocyanate method [15]. Poly(A)$^+$ RNA was purified by oligo(dT) column chromatography. A directional cDNA library was constructed using a ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The library was mass excised using Ex-Assist helper phage (Stratagene). The obtained phagemid of the library was plated on LB agar plates supplemented with 100 mg l^{-1} ampicillin.

2.3. DNA sequencing

Bacterial colonies were randomly picked and inoculated into 96 well plates containing selective LB medium, and grown overnight with 600 rpm at 37 °C. Plasmid DNA was prepared automatically using AccuPrep Plasmid Extraction kit (Bioneer, Korea) on HT-Prep Automatic DNA Extractor (Bioneer). Plasmid DNA was used as a template and all sequencing reactions contained the T3 universal primer (Promega, Madison, WI), thus read into the presumed 5′-end of the each cDNA. Reaction was concentrated and washed by ethanol precipitation and the pellet resuspended in formamide prior to separation. Three thousand nine hundred and seventy two clones were sequenced using BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster city, CA) on ABI PRISM 3100 Sequencer. Four thousand nine hundred and ninety clones were sequenced using Dynamic ET Terminator Cycle Sequencing kit (Amersham, Uppsala, Sweden) on RISA-384 automatic sequencer (Shimazu, Tokyo, Japan).

2.4. Sequence analysis

Sequences were edited automatically to remove the vector sequence and ambiguous regions. Poly(A) or poly(T) tails, if present, were removed manually. Contaminants of bacterial, mitochondrial, chloroplast and ribosomal RNA genes were identified by BLASTN similarity searches and removed along with sequences less than 100 nucleotides in length. ESTs were assembled into contigs using the CAP3 program with a 75% homology and 30 base minimum overlap as assembly parameters. The assembled sequences were used for BLASTN searches against both 306,085 *Populus* ESTs deposited at the GenBank dbEST and the version 1.0 preliminary draft of the *Populus* genome sequence from DoE Joint Genome Institute and Poplar Genome Consortium (http://genome.jgi-psf.org/Poptr1/Poptr1.download.html).

The individual ESTs were subjected to similarity searches against the GenBank non-redundant (nr) protein databases using BLASTX algorithm. ESTs that hit on the same target gene were manually reassembled into a single contig. From the BLASTX results, ESTs could be classified into three categories: known, unknown, and no hit. A known ESTs was declared when the score was greater than 80 and the *E*-value was less than 10^{-14}. Unknown was defined as a score greater than 40 and less than 80, and an *E*-value of greater than 10^{-14} but less than 10^{-2}. No hit was defined as a score of less than 40 with an *E*-value greater than 10^{-2}. The functional assignment of ESTs was based on the results of comparison to the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* annotation database and the yeast functional category database (The FunCat version 2.0) using a BLASTX algorithm [16].

2.5. Northern blot analysis

Steady-state mRNA levels of selected genes during the normal growth phase of the cell suspension culture were compared by Northern blot analysis. Total RNA was isolated from the cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). Ten micrograms of the RNA were run on 1.2% formaldehyde agarose gel and then transferred to Hybond-XL nylon membrane (Amersham). The membrane was prehybridized in 1 × PerfectHYB plus hybridization buffer (Sigma–Aldrich, St. Louis, MO) and 0.1 mg ml^{-1} denatured salmon sperm DNA solution for 30 min at 68 °C. It was then hybridized with ^{32}P-dCTP labeled cDNA for 12 h. The membrane was washed in 2 × SSC and 0.1% SDS (50 °C) for 10 min and in 0.2 × SSC and 0.1% SDS (50 °C) for 30 min followed by exposure to an X-ray film at −70 °C.

3. Results and discussion

3.1. Sequencing and assembly

Total RNA extracted from 8-day-old poplar suspension culture was used for the construction of cDNA library. The growth kinetics of the culture was determined by measuring cell mass at 2-day interval over 30 days after subculture. The measurement time point corresponded to the late lag/early exponential phase (see Fig. 2A). The number of independent phages in the library was approximately 5.5×10^6 with a titer of 1.1×10^7 plaque forming units ml^{-1}. Thus, we assumed that the library had an adequate representation of the expressed genes in the cells. Insert lengths estimated by polymerase chain reaction on 96 randomly selected clones ranged from 200 to 2800 bp, with an average size of 1.0 kb.

A total of 8962 cDNAs of the library were subjected to 5′-end single pass sequencing. Of these, 7645 high quality sequences were selected after trimming both the vector sequence and the poly(A) tails. The average reading length after trimming was 517 bp. When the total ESTs were blasted against the GenBank nr database, 58 *E. coli* genomic DNA sequences and a chloroplast sequence were found. Thus, the library appeared to be reasonable quality with only less than 1% contamination by chloroplast or *E. coli* sequences. A total of 1337 contigs and 2041 singletons were formed after assembly of the 7586 ESTs from this dataset using the CAP3 program. The 1337 contigs encompassed 5545 ESTs resulting in a redundancy of 45%. Redundancy ranged from one cluster with 107 sequences to 632 clusters with 2 sequences. Approximately, 78.6% of the ESTs belonged to the sequence clusters of low redundancy (2–4 sequences per contig), while medium redundancy (4–10 sequences per contig) and high redundancy (>10 sequences per contig) ESTs represented 16.1% and 5.3%, respectively. The library summary for this analysis is shown in Table 1.

To identify the complementarity of our ESTs data, BLASTN search against 306,085 poplar ESTs deposited at the GenBank dbEST was conducted. The results showed that 9.3% of our suspension cell ESTs (313 out of 3378 assembled sequences) shared weak (BLAST score <85) or no sequence similarity to them. When our ESTs sequences were compared to the *Populus* genome sequence, 96.9% (3272 out of 3378 assembled sequences) showed high homology (BLAST score >200). The remaining 94 ESTs showed moderate homology (200> a score >80) and 12 ESTs showed weak homology (a score <80). It is appealing to postulate that the moderate and weak homology of 106 ESTs is thought to be due to: (1) existence of introns in the genome sequence, (2) the version 1.0 preliminary draft of the *Populus* genome sequence consists of 22,136 scaffolds, or (3) sequence variations among different poplar species.

EST sequences obtained have been deposited in the GenBank dbEST at the National Center for Biotechnology Information (accession numbers are CB176580 to CB176659, CB184951 to CB185048, CB240211 to CB24055 and CX653361 to CX660436).

3.2. Similarities found

BLAST searches of individual ESTs against the GenBank nr database enabled us to group the ESTs into three distinct categories based on the match significance (BLASTX score) and descriptor of their top hit. Class I is defined as having significant sequence similarity to known proteins (BLAST score >80) and consists of 2091 unigenes (61.9%) that are likely to be the transcripts of the genes with similar functions. Class II is for the sequences having significant similarity to unknown or unclassified function proteins in the database or low sequence similarity (BLAST score 40–80)

Table 1
Poplar suspension cell EST sequencing project summary

Library and EST summary	
Mean insert size	1.0 kb
Library titer	5.5×10^6 pfu ml^{-1}
Number of cDNAs sequenced	8962
Mean reading length	573.5 bases
Mean EST length[a]	516.5 bases
Number of high quality ESTs	7586
Contig assembly results	
Number of ESTs assembled[b]	7586
Number of contigs	1337
Number of singletons	2041
Number of assembled sequences[c]	3378
Contig sizes	
2–4 ESTs	1051
5–7 ESTs	161
8–13 ESTs	80
>14 ESTs	45

[a] Mean EST length following vector and end clipping.
[b] EST assembly parameters were 75% minimum match with 30 minimum base overlap.
[c] Assembled sequences are the sum of contigs and singleton.

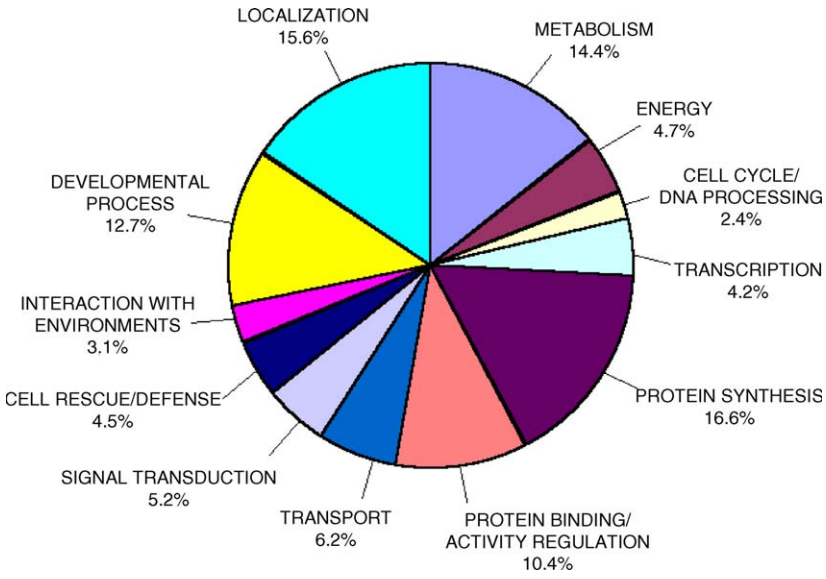

Fig. 1. The classification of genes with known functional annotation by BLASTX analysis.

and consists of 949 unigenes (28.1%) that could not be reliably assigned their cellular roles. Finally, Class III consisting of 338 unigenes (10.0%) refers to those having no matching sequences in the public database and thus could be considered as novel sequences. The proportion of those assigned to the class I with cellular function was similar to those observed in roots [8], wood tissues [6], and young leaves [7] of *Populus*.

A total of 2091 ESTs assigned to the class I were further classified into 12 putative cellular functions based on the MIPS functional categories. The percentage of clones found in the Class I ESTs is as follows: 14.4% for metabolism-related processes, 4.7% for energy, 2.4% for cell cycle and DNA processing, 4.2% for transcription, 16.6% for protein synthesis, 10.4% for protein binding/activity regulation, 6.2% for transport, 5.2% for signal transduction, 4.5% for cell rescue/defense, 3.1% for interaction with environments, 12.7% for developmental processes, and 15.6% for localization (Fig. 1). Functional classification is highly indicative of the biological and physiological status of the tissues studied. In the present study, the transcripts related to protein synthesis were most abundant in suspension-cultured cells. ESTs datasets from more than 19 nonnormalized cDNA libraries from various parts of tissues of poplar were reported [5–8]. Functional classifications of their results also showed that the ESTs for protein synthesis were most abundant in shoot meristem, cambial zone and water-stressed roots. However, with an exception of energy related genes that were most abundant in young leaves, metabolism related genes were most abundant in the remaining tissues.

3.3. The most abundant transcripts in poplar suspension cells

An EST dataset from a specific tissue or organ indicates gene expression profile under the conditions studied. Assuming that the poly(A)$^+$ RNA populations are adequately reflected in the cDNA library, then a high redundancy of a specific cDNA is consistent with an increase in the abundance of the corresponding transcripts. Therefore, it is possible to infer the physiological status of the cells at molecular level. The 21 most abundant transcripts in the poplar suspension cells are presented in Table 2. The largest representation was extensin-like protein that accounted for 107 ESTs or 1.41% of the total. Two other extensin-like proteins (64 and 51), polygalacturonase-inhibiting protein (61), an unknown protein (60), and FAD-binding domain containing protein (45) were also highly represented. Peroxidase (37), proline-rich protein (32), S-adenosylmethionine synthetase (31), 60S ribosomal protein L5 (30), and bacterial-induced peroxidase (29) were also abundant in this analysis.

Highly abundant transcripts in poplar suspension cells include cell wall-related proteins: extensins, polygalacturonase inhibiting protein, proline-rich protein and class IV chitinase. Plant cell wall, composed of polysaccharides, ions and proteins, is an extracellular matrix with both structural and growth-regulating functions. Cell division is tied to cell plate formation, which eventually leads to cell wall synthesis. Subsequent elongation of the divided cells also requires numerous changes in the composition and structure of cell wall. Thus, a number of genes are involved in the synthesis of cell wall components and in cell wall assembly [17–19]. Initially, our cDNA library was constructed using total RNA extracted from cells at 8 days after subculture to a new medium. The time point corresponded to the transition from the late lag to early exponential phase. Since the cells were not fully synchronized, some might have already divided and others about to divide during the transition. Thus, high abundance of cell wall-related transcripts reflects the cellular status. There are also other abundant genes encoding stress-related, especially biotic stress-related

Table 2
Most abundant ESTs found in the poplar suspension cell cDNA library

Cluster name	Description	No. of ESTs	*E*-value	Percentage of total ESTs
Contig197	Extensin like protein	107	2E−66	1.41
Contig766	Extensin like protein	64	1E−75	0.85
Contig710	Polygalacturonase-inhibiting protein	61	E−133	0.81
Contig681	Unknown protein	60	1E−18	0.80
Contig930	Extensin like protein	51	2E−67	0.67
Contig133	FAD-binding domain-containing protein	45	E−119	0.59
Contig317	Peroxidase	37	0.0	0.49
Contig19	Proline-rich protein precursor	32	3E−52	0.42
Contig808	S-adenosylmethionine synthetase	31	E−134	0.41
Contig712	60S ribosomal protein L5	30	E−144	0.40
Contig676	Bacterial-induced peroxidase precursor	29	E−140	0.38
Contig716	Extensin like protein	28	2E−64	0.37
Contig58	Unknown	26	8E−06	0.34
Contig386	Ribosomal protein S19	26	3E−63	0.34
Contig1045	Class IV chitinase	24	E−112	0.32
Contig151	Extensin CYC17 precursor	24	3E−69	0.32
Contig844	Receptor protein kinase	24	1E−73	0.32
Contig1054	60S ribosomal protein L31	23	5E−54	0.30
Contig1301	Glyceraldehyde 3-phosphate dehydrogenase	21	E−158	0.28
Contig1042	Proline-rich protein 1	21	1E−59	0.28
Contig34	20S proteasome beta subunit D	21	2E−95	0.28

proteins and pathogen resistance proteins. It is no surprise to see such proteins since many of the cell wall-related proteins are known to be involved in the resistance mechanisms because plant cell wall serves as the first barrier to the invading organisms. The rigidity and strength of cell wall may also be affected by pathogens since their infection leads to the production of reactive oxygen intermediates, which make the cell wall more resistant to pathogen attack [20,21].

3.4. Identification of cell growth-dependent expressed genes

The uniformity and repeatability of cell suspension culture offer an ideal model system for functional genomics approaches, and in particular for transcript profiling in relation to the regulation of normal cell growth and cell division, and cellular responses to changes in the culture conditions [12].

The ESTs dataset provided by this study could serve a powerful platform on which we could ascertain transcriptional responses of plant cells to various cellular conditions as mentioned above. To prove this possibility, several candidate genes were selected and analyzed for their growth phase-dependent expression by Northern blot analysis. As shown in Fig. 2, they showed dynamic expression pattern during different growth phases with different manners. Beta-glucanase was highly expressed after subculturing but the expression was diminished abruptly since the beginning of logarithmic phase. Both an unknown protein and a respiratory burst oxidase protein were also highly expressed after subculturing and the higher expression were prolonged to logarithmic phase. The expression of a putative peroxidase was induced in logarithmic phase. The expression of cytochrome P450 monooxygenase (P450) was increased in late logarithmic/stationary phases. Isoflavone reductase (IFR) was expressed in stationary phase only.

The expression of the selected genes in the present study appears to be sensitively and specifically regulated at the transcription level during the growth phase. Beta-glucanase hydrolyzes cell wall beta-glucans during several physiological and morphogenetic processes including vegetative cell elongation and changes in cell wall architecture [22,23]. The plant peroxidases are bifunctional enzymes involved in both the reduction of hydrogene peroxide and the production of reactive oxygen species during oxidative burst and in cell elongation [24,25]. They are encoded by a high number of superfamily genes. Expression of swpa1 and swpn1, genes for anionic and neutral peroxidases from sweet potato suspension cultured cells, were differently regulated during cell growth [26]. Swpa1 is highly expressed at both early lag phase and stationary phase whereas swpn1 is expressed at stationary phase specifically. The different regulation of peroxidase gene during growth phase suggests that they have been implicated in a wide range of physiological processes. Plant respiratory burst oxidase is a homolog of the human neutrophil pathogen-related $gp91^{phox}$ [27]. It is related to the control of steady state cellular level of reactive oxygen species and functions as signal transducer of stress and developmental responses. IFR, one of the key enzymes of isoflavonoid phytoalexin biosynthesis, is an NAD(P)H-dependent oxidoreductase [28]. Expression of the IFR genes is regulated by biotic and abiotic stress, and developmental responses. P450 is heme-dependent oxidase that catalyze a wide variety of reactions using NAD(P)H as electron donor [29]. In plant, it is known to be associated with wound healing, pest resistance, synthesis of secondary metabolites during cell proliferation and in response to environmental stresses, and detoxification of xenobiotics. However, it is

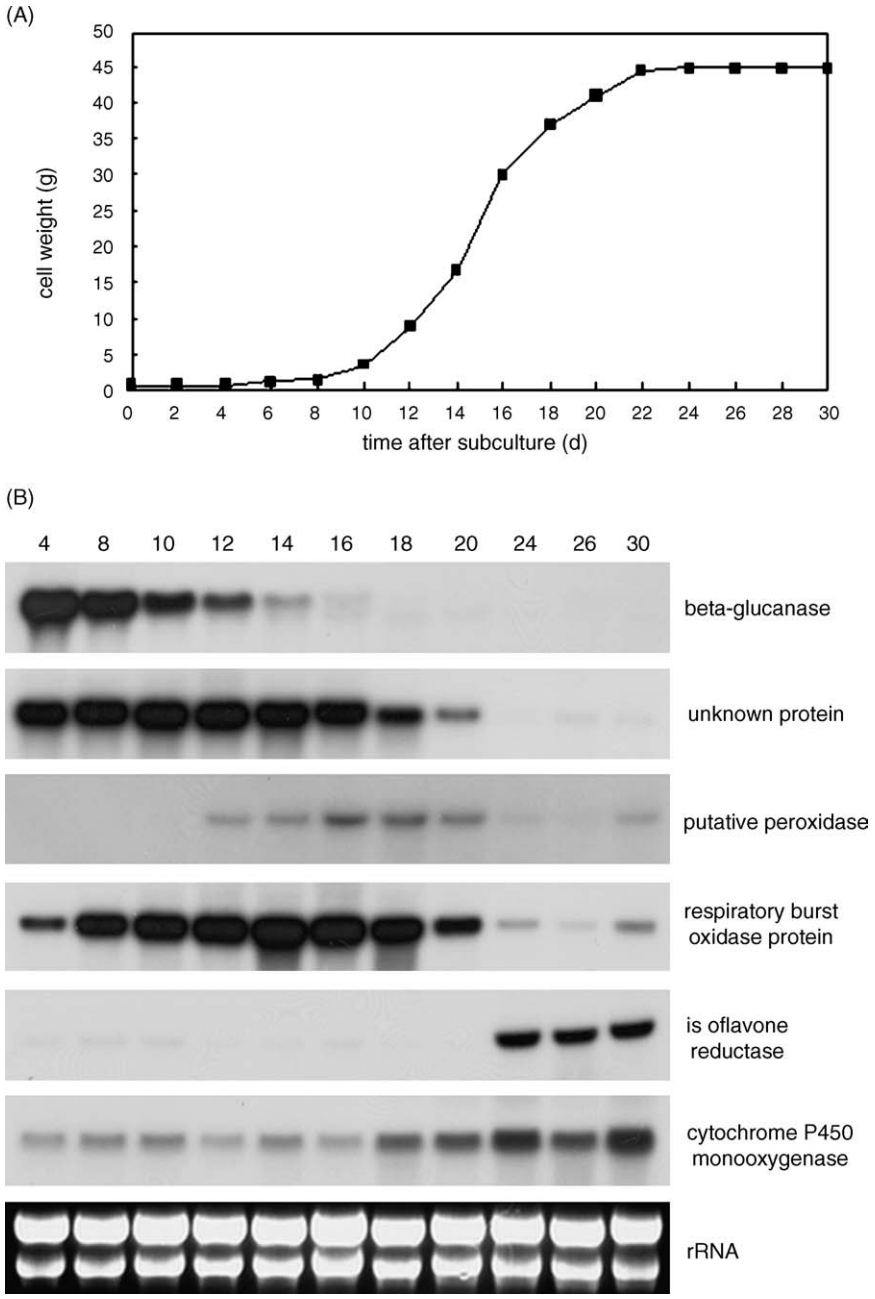

Fig. 2. Growth phase-dependent expression of the selected genes. (A) Growth kinetics of poplar suspension cells. Cell weight was determined, every 2 days after subculturing until 30 days later. (B) Northern blot analysis. Total RNA was extracted from the cells of the defined days. Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control.

unclear whether the growth phase-dependent expression of the selected genes accurately reflects the physiological status of the cells or not. Plant possesses numerous isozymes that may respond differently to different cellular environments. In *Arabidopsis*, for example, there are 246 genes for P450 and 73 genes for class III plant peroxidase whose functions are largely unknown. Furthermore, the information on growth phase-dependent expression of different isozymes is totally lacking [30,31]. Therefore, further work is needed to clarify their physiological roles in plant cell including why the gene is regulated growth phase-dependently.

In conclusion, we analyzed 8962 ESTs from poplar suspension cell cDNA library. The obtained unigene set representing poplar cell transcriptome could be used to understand the regulation mechanism of normal cell growth using cDNA microarray analysis and the potentiality has been demonstrated by growth phase-dependent expression of several selected genes in the present study. The dataset obtained will also be used to refine further identification of

genes regulated both in the environmental culture conditions and in the cell cycle through the development of efficient synchronization of poplar cell culture as in the case of tobacco Bright Yellow-2 cell suspension.

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