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Comparative proteomic analysis of *Populus trichocarpa* early stem from primary to secondary growth

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ABSTRACT

Wood is derived from the secondary growth of tree stems. In this study, we investigated the global changes of protein abundance in *Populus* early stems using a proteomic approach. Morphological and histochemical analyses revealed three typical stages during *Populus* early stems, which were the primary growth stage, the transition stage from primary to secondary growth and the secondary growth stage. A total of 231 spots were differentially abundant during various growth stages of *Populus* early stems. During *Populus* early stem lignifications, 87 differential spots continuously increased, while 49 spots continuously decreased. These two categories encompass 58.9% of all differential spots, which suggests significant molecular changes from primary to secondary growth. Among 231 spots, 165 unique proteins were identified using LC–ESI-Q-TOF-MS, which were classified into 14 biological function groups. The proteomic characteristics indicated that carbohydrate metabolism, oxidoreduction, protein degradation and secondary cell wall metabolism were the dominantly occurring biochemical processes during *Populus* early stem development. This study helps in elucidating biochemical processes and identifies potential wood formation-related proteins during tree early stem development. It is a comprehensive proteomic investigation on tree early stem development that, for the first time, reveals the overall molecular networks that occur during *Populus* early stem lignifications.

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1. Introduction

The emergence of the vascular system of land plants results in plant development to increase stature and photosynthetic output, as well as to colonize an expanded range of environmental habitats [1]. The vascular system connects all of the parts of land plants, expending from the root system through the stem and into the leaves. Two distinct phases of vascular systems are recognized in land plant primary and secondary growth. Primary vascular tissue, which consists of xylem and phloem, are constructed in the bundles of leaves, stems and roots, while perennial tree species further develop a considerable secondary vascular system in the stems and roots [2]. Tree secondary vascular tissues (i.e., wood) are derived from stem secondary growth and begin with the development of cell wall thickening and lignifications of secondary xylem cells from a ring of vascular cambium in the young developing stem. The wood is used industrially as timber for construction and pulp for paper manufacturing; additionally, wood is also the most environmentally renewable source of energy. Currently, the increasing global wood consumption demand necessitates greater understanding of the molecular basis of tree stem development to enhance artificial tree breeding.

Several molecular determinants of vascular development in the herbaceous model plant *Arabidopsis* have been examined in depth during the primary growth phase [3–6]. *Arabidopsis* has also been proposed as a genetic model for secondary xylem development (i.e., vessels and fibers) [7–9], and many transcription factors such as NACs and MYBs have been documented to be important regulators during secondary wall formation [10]. Nevertheless, trees constitute the optimal model for investigating plant secondary vascular development. Tree wood is manufactured by a succession of major steps including cell division, cell expansion, cell wall thickening (cellulose, hemicellulose and lignin biosynthesis and deposition), programmed cell death, and heartwood formation [11–15]. However, there is limited understanding of the molecular basis of tree wood formation thus far.

The apical portion of the expanding shoots of trees is soft and green, while the basal portion becomes stiff and woody, which indicates the transition from primary to secondary growth. The terminal region of

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the developing shoot provides a gradual developmental gradient from primary to secondary growth that facilitates the identification of genes with important functions during each of these growth phases. Based on the cDNA-AFLP approach, two studies have used this unique system to identify several differentially expressed genes during the secondary growth of poplar stems, including regulatory and structural genes that are involved in secondary growth and secondary wall biogenesis [16,17]. Another study presented a microarray gene expression profile of the developmental transition from primary to secondary growth in Sitka spruce shoots [18]. A recent report described the global change patterns for all of the gene transcripts that occur during the early stem transition from primary to secondary growth in *Populus*[19]. Although these studies extensively described the transcription pattern of the genes that are involved in tree early stem development, there is little data concerning the global changes in tree early stem proteome.

Proteomic analysis is one of the best strategies for understanding plant growth and development or responses to environmental stimuli. *Populus trichocarpa* was the first model tree that was used for studies on woody plant-specific growth characteristics (including wood formation), and that has had its genome-published [20,21]. In this study, a comparative proteomic approach was used to ascertain the changes that occur in *Populus* early stem proteome during primary to secondary growth. Our data reveals that carbohydrate metabolism, oxido-reduction, protein degradation and secondary cell wall metabolism were the dominant biochemical processes that significantly changed during the transition from primary to secondary growth. These results provide a comprehensive overview of tree early wood formation networks.

2. Materials and methods

2.1. Plant materials

P. trichocarpa trees (clone Nisqually-1) were selected for this study. The *Populus* seedlings were clonally propagated using tissue culture as described in Song et al. [22]. Seedlings that had developed to the same extent were then potted in soil and were grown under a 16 h/8 h day/night photoperiod at 25 °C. More than 700 trees that were 90-days old were used for the following experiments: i) the length measurement of each internode (IN) from the apical bud to the base of the shoot; ii) histological analysis of the shoot segments of successive IN1 to IN9 below the apex; iii) proteomic experiments of the tissues of IN1, IN3 and IN5, excluding the nodes. The experimental designs are provided in Supplementary Fig. S1; iv) quantitative RT-PCR (qRT-PCR) analysis of the tissues of the apical bud, IN1, IN3, IN5 and IN9; and v) analysis of monolignin biosynthetic enzyme total activities in IN1, IN3, IN5 and IN7. Three independent biological replicates were performed for each of the above experiments.

2.2. Sectioning and histology analysis

Approximately 3-mm-long segments of each internode sample were fixed in FAA buffer (50% ethanol, 5% acetic acid and 3.7% formaldehyde) and embedded in paraffin. Transverse sections that had an 8-μm thickness were cut with a sliding microtome (HM340E, MICROM, Germany) from the embedded tissues. Sections were stained with 0.025% (m/v) toluidine blue O for 30 s and, then were washed with dH_2O, mounted and examined using bright field microscopy (BX43, Olympus, Japan). For lignin detection in the secondary walls, the sections were stained with 2.5% phloroglucinol in 12 N HCl for 3 min and were observed using a microscope (SteREO Lumar.V12, Zeiss, Germany). For lignin autofluorescence visualization in the secondary walls, the sections were examined using a UV fluorescence microscope (Axioimager A1, Zeiss, Germany). The cellulose in the secondary walls was stained with 0.25 μg/mL Calcofluor white for 2 min and was visualized using a UV fluorescence microscope [23].

2.3. Protein sample preparation for 2-DE

The samples of different internode tissues were ground to a fine powder in liquid nitrogen and were suspended in ice-cold buffer that contained 10% (w/v) TCAand 0.07% (v/v) 2-mercaptoethanol in acetone. The homogenates were precipitated overnight at −20 °C. Each mixture was centrifuged at 40,000 *g*, at 4 °C for 1 h and the pellets were washed with cold acetone that contained 0.07% (v/v) 2-mercaptoethanol, 1 mM PMSF and 2 mM EDTA for 1 h. The pellets were washed three times and then dried for 20 min using vacuum freeze-drying. The pellets were re-suspended in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, and 1% (v/v) proteinase-inhibitor (GE, USA), and then the solution was centrifuged at 40,000 *g* at 4 °C for 1 h. The subsequent supernatant was collected and further purified using the 2D Clean-Up Kit according to the manufacturer's instructions (GE, USA). The protein concentration of every stem internode extract was evaluated using BSA as the calibration standard according to the manual of Quant-kit (GE, USA).

2.4. 2-DE and image analysis

The protein samples were separated and visualized using the 2-DE approach according to Dai et al. [24]. An Ettan IPGphor 3 apparatus (GE, USA) was used for isoelectric focusing with immobilized pH-gradient strips (pH 4.0–7.0 linear gradient, 24 cm). After the IPG strips were rehydrated, each of all sample proteins was loaded for IEF electrophoresis. The second dimension was performed using 12.5% SDS-PAGE at 20 mA/block until the dye front reached the end of the gel. The gels were stained with Coomassie Brilliant Blue (CBB) G-250. Images of the stained gels were captured using a scanner (ImageScanner III, GE, USA) and were analyzed using ImageMaster 2D Platinum Software (Version 6.0, GE, USA). Three well-separated gels of each sample were used to create "replicate groups". Spots were considered reproducible when they were well resolved in the three biological replicates. The average vol.% values of the protein spots were normalized from three technical replicates to represent the final vol.% values of each biological replicate. Comparison analysis was performed using the values of three biological replicates among the different samples. The differentially abundant spots were selected on the basis of a greater than 1.5-fold difference in vol% and a p-value of <0.05 when comparing spot size between groups with one-way ANOVA using the statistical software SPSS 17.0. The molecular mass (kDa) of each protein was estimated using a standard set of protein markers, and the isoelectric points (pIs) were determined by the spot positions along the immobilized pH gradient strips.

2.5. Protein identification and database searching

The differentially abundant spots were excised from the gels and digested with trypsin. MS and MS/MS spectra were acquired on an ESI-Q-TOF MS (QSTAR XL) as described by Yu et al. [24].The MS/MS spectra were searched against the NCBInr protein databases (http://www.ncbi.nlm.nih.gov) and *Populus* databases (http://www.phytozome.net) using Mascot software (Matrix Sciences, UK). The searching criteria included a mass accuracy of 0.3 Da, with one missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as a variable modification. To obtain a highly confident identification, the proteins had to meet the following criteria: (1) top hits in the database searching report, (2) a probability-based MOWSE score of greater than 43 ($p < 0.01$), and (3) more than two peptides matched with a nearly complete y-ion series and complementary b-ion series present. According to the MASCOT probability analysis, only significant hits were accepted for the identification of each protein sample.

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