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Original article

Proteomics analysis reveals the molecular mechanism underlying the transition from primary to secondary growth of poplar

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ABSTRACT

Wood is the most important natural source of energy and also provides fuel and fiber. Considering the significant role of wood, it is critical to understand how wood is formed. Integration of knowledge about wood development at the cellular and molecular levels will allow more comprehensive understanding of this complex process. In the present study, we used a comparative proteomic approach to investigate the differences in protein profiles between primary and secondary growth in young poplar stems using tandem mass tag (TMT)-labeling. More than 10,816 proteins were identified, and, among these, 3106 proteins were differentially expressed during primary to secondary growth. Proteomic data were validated using a combination of histochemical staining, enzyme activity assays, and quantitative real-time PCR. Bioinformatics analysis revealed that these differentially expressed proteins are related to various metabolic pathways, mainly including signaling, phytohormones, cell cycle, cell wall, secondary metabolism, carbohydrate and energy metabolism, and protein metabolism as well as redox and stress pathways. This large proteomics dataset will be valuable for uncovering the molecular changes occurring during the transition from primary to secondary growth. Further, it provides new and accurate information for tree breeding to modify wood properties.

1. Introduction

Vascular tissue is composed of xylem and phloem, which are continually produced from stable populations of stem cells, called the procambium, during primary growth and from the cambium during secondary growth (Campbell and Turner, 2017). The vascular procambium develops during embryogenesis and determines vascular patterning in postembryonic growth (Yang and Wang, 2016). During postembryonic development, the procambium appears in the shoot apex (SAM) and root apex (RAM) to give rise to all types of vascular cells, contributing to the elongation of the primary axes. In both the shoot and root, the procambium ceases to exist and is replaced by the vascular cambium at the end of primary growth. The procambium gives rise to the entire vegetative body in ferns, most monocots, and herbaceous dicots; however, in woody dicots, the vascular cambium produces part of the secondary body of the plant, the secondary xylem (wood) and the secondary phloem (part of the bark; the cork cambium produces the rest of the bark), resulting in radial thickening (Mauseth, 1988).

In the shoot of woody dicots, such as poplar, procambial strands initiate acropetally through the residual meristem beneath the apex. Afterward, undergoing cell division, these procambial strands in turn develop into mediolaterally organized vascular bundles with centripetal xylem and centrifugal phloem cells (Rose, 2016). Following primary growth, the procambium and its neighboring cells differentiate into the "ring" vascular cambium that drives secondary, lateral growth. Secondary xylem is generally termed wood, which is an important reservoir of fixed carbon and also a useful source of raw materials for humans. Wood formation is a sequential developmental process, including differentiation of vascular cambium cells into secondary xylem mother cells, cell expansion, secondary cell wall formation, programmed cell death (PCD), and finally the formation of heartwood (Han et al., 2012; Ye and Zhong, 2015). During the development of vascular tissues, water, nutrients, RNA, and proteins are carried through the whole vascular plant. In addition, internal stimuli as well as external stimuli also control these developmental processes in vascular tissues (Plomion et al., 2001). This coordinated and environmen-

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tally responsive program of meristematic differentiation and tissue patterning involves multiple developmental processes with interacting regulatory mechanisms (Dharmawardhana et al., 2010).

Studies of primary growth have mostly focused on vascular procambium initiation in embryos, the RAM, and leaf venation systems because procambial cells are imbedded under layers of other tissues and are not easy to access (Caño-Delgado et al., 2010; Kondo et al., 2014; Růžička et al., 2015). A positive feedback loop consisting of auxin-MONPTEROS (MP)-ARABIDOPSIS THALIANA HOMEOBOX8 (ATHB8)-PIN-FORMED PROTEIN1 (PIN1) regulates the initiation and specification of procambial cells (Möller and Weijers, 2009; Yadav et al., 2009). Another well-studied regulatory mechanism is tracheary element differentiation inhibitory factor (TDIF) and its receptor, TDR/PHLOEM INTERCALATED WITH XTLEM (PXY), which promotes cell proliferation and inhibits xylem differentiation in the procambium (Etchells and Turner, 2010; Hirakawa et al., 2008; Ito et al., 2006). However, studies on the SAMs have been restricted to experiments of the entire tissue. Most of the current knowledge obtained from procambial cell initiation, specification, and regulation were derived from studies of Arabidopsis (Clark et al., 1996; Jeong et al., 1999; Ma et al., 2017; Mayer et al., 1998; Ogawa et al., 2008; Schoof et al., 2000; Yadav et al., 2009).

Arabidopsis is the best model for studying primary growth, whereas woody plants (e.g. poplar) are the most suitable models for studying secondary growth due to the advanced cambium and cork cambium in stems. In woody plants, the cambium and cork cambium (referred to collectively as cambium) increase the stem diameter, resulting in a developmental continuum of secondary xylem and phloem (wood formation). These secondary tissues provide mechanical support and a conduit for the long-distance transport of water and nutrients through the whole plant. It is of great importance to study the regulatory pathway involved in secondary growth. Although knowledge about the molecular mechanism of cambium-driven secondary growth in woody plants is still limited, good progress has been made in recent years in understanding some regulatory mechanisms, such as the high-level hormonal influence on secondary growth and the identification of downstream regulators such as PXY (Campbell and Turner, 2017; Ye and Zhong, 2015). In addition, transcriptome analyses in several tree species have revealed that a suite of genes, including receptor kinases, transcription factors (TFs), and secondary wall biosynthesis genes, are highly expressed in wood-forming cells (Aspeborg et al., 2005; Dharmawardhana et al., 2010; Pavy et al., 2008; Wilkins et al., 2009). Recently, 108 poplar genes involved in wood formation were reviewed, including 12 genes involved in vascular cambium activity, 4 genes in secondary xylem differentiation, and 92 genes in secondary wall deposition (Ye and Zhong, 2015).

Much progress has been made in the study of hormonal and transcriptional regulation of stem primary and secondary growth in recent decades. There is evidence that the functions of some genes taking part in primary and secondary meristems have overlapping regulatory mechanisms. For example, the SAM and vascular cambium both have an indeterminate cell fate, and several genes, such as *STM*, *CLV1*, *KANAD I*, and members of the *HD-ZIP III* gene family, are probably involved in the genetic regulation of both meristems (Baucher et al., 2007). However, there are still important characteristics specific to stem primary and secondary growth. Much work has focused on specific aspects of secondary growth, such as regulatory mechanisms for vascular cambium development and wood formation. Regarding global changes during the stem development process, genome-wide transcriptome analysis in poplar has provided some insights (Dharmawardhana et al., 2010; Prassinos et al., 2005). However, knowledge of large-scale proteomics analysis for understanding young stem growth is still lacking. In this study, a comparative proteomics approach based on tandem mass tag (TMT)-labeling was used to analyze the global changes in protein accumulation in stems undergoing secondary growth in *Populus alba* × *P. glandulosa* (cv. "84k"). A total of 10,816 proteins were identified and quantified in both primary and secondary stems. Of these, 3106 proteins showed differential protein abundance during primary and secondary growth. Our analysis revealed that these differentially expressed proteins are involved in many pathways, such as signaling, phytohormones, cell cycle, cell wall, secondary metabolism, carbohydrate and energy metabolism, protein synthesis and degradation, and redox and stress pathways. Moreover, we confirmed proteomics data by using histochemical staining, enzyme activity assays, and quantitative real-time PCR (qRT-PCR). These findings provide a comprehensive overview of protein networks involved during the transition from primary to secondary growth in tree stems.

2. Material and methods

2.1. Plant materials

The hybrid poplar 84k was used for all studies. Poplar seedlings were grown for one month in tissue-culture vessels on half Murashige and Skoog medium (1/2 MS), pH 5.75, containing 2% sucrose and 0.7% agar at 25 °C under a 16 h day/8 h night cycle. Primary growth-stems (PG-stems) (from 1st internode (IN1) to IN4) and secondary growth-stems (SG-stems) (from IN5 to IN8) were selected from at least 20 one-month-old poplars and frozen in liquid nitrogen, then immediately stored at −80 °C for protein extraction and quantification.

2.2. Anatomical and histochemical observation

Free-hand cross-sections, about 30 μm thick, were made from IN1 to IN8 of one-month-old fresh poplar stems. For observation of the morphology structure of the stem, cross-sections were stained with 0.1% toluidine blue (Sigma, St. Louis, MO, USA).

For detection of lignin, cross-sections were stained with phloroglucinol (Sigma, St. Louis, MO, USA). For total insoluble carbohydrates and starch staining, periodic acid-schiff's (PAS) (Sigma, St. Louis, MO, USA) and iodine-potassium iodide (I_2-KI) were used according to previous method (Riding and Little, 1984). For protein staining, amino black (Sigma, St. Louis, MO, USA) was used according to the manufacturer's protocol. Sections were observed under an OLYMPUS microscope (OLYMPUS, Japan) equipped with a computer-assisted digital camera MODEL ARTCAM-1400MI-WOM (ARTRAY, Japan).

2.3. Protein extraction and quantification

Frozen stems were ground into a fine powder under liquid nitrogen. Total protein from each sample was extracted from the ground powder using a slight modification of our previously published method (Jin et al., 2017). For each sample, 300 mg powder was extracted with 600 μL cooled Tris-phenol (pH 8.8) and 600 μL cooled extraction buffer with 0.1 M Tris–HCl, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose, proteinase inhibitor, and phosphatase inhibitor. Protein concentration was determined with a commercial protein assay (Bio-Rad Laboratories, Hercules, CA), using a spectrophotometer to measure the absorbance at 595 nm.

2.4. Protein digestion

Each sample in Coomassie Brilliant Blue-stained gel slices was subjected to in-gel tryptic digestion after reduction and alkylation. First, the gel slices were incubated at 60 °C for 30 min with 10 mM DTT. After cooling to room temperature, 20 mM iodoacetamide was added and kept in the dark for one hour to block free cysteine.