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IDENTIFICATION OF PROTEINS FROM CAMBIUM TISSUES OF THE CHINESE WHITE POPLAR (*POPULUS TOMENTOSA*) SAMPLED DURING THE GROWING SEASON

JIN XIE[1], SHUXIN LIU[1], QI QI[1], JIAYIN HOU[1], YING GAI[1,2*] AND XIANGNING JIANG[1,2*]

[1]*College of Life Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, P.R. China*
[2]*The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of Chinese Forestry Administration, National Engineering Laboratory for Tree Breeding, Beijing 100083, P.R. China*
[*]*Correspondence authors(e-mails): gaiying@bjfu.edu.cn, jiangxn@bjfu.edu.cn; Tel: +86-010-62338063*

Abstract

Various protein extraction methods have been used to investigate Chinese white poplar (*Populus tomentosa*) proteomics. However, extracting and characterizing proteins from woody plants remains a challenge. Two-dimensional gel electrophoresis is a powerful, widely used method for the analysis of complex protein mixtures extracted from biological samples. The technique separates mixtures of proteins along two dimensions, by isoelectric point and molecular weight, and can resolve thousands of different proteins. Here, we report a new application of two-dimensional gel electrophoresis to investigate the proteomics of *P. tomentosa* cambium tissues over the course of a growing season. Of three protein extraction methods that we compared (the Tris-phenol method, trichloroacetic acid-acetone method, and trichloroacetic acid-acetone-phenol method), trichloroacetic acid-acetone was the most efficient approach for protein extraction from cambium tissues of *P. tomentosa*. After extraction, the proteins were separated using two-dimensional gel electrophoresis. The protein quantities of six spots changed over the course of the growing season from February to July. Five spots were identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry, and the sixth spot was identified by liquid chromatography–mass spectrometry. The proteins included enolase, class Ia chitinase, and four unnamed proteins. Our results show the best approach to proteomics in *P. tomentosa* and reveal trends in protein activities during a growing season in this tree species.

Introduction

Growth and development in trees initiate from cell division in the cambium tissues and involve many regulatory factors affecting gene expression, production of phytohormones, signal transduction, and enzymatic activities. Understanding gene expression and protein profiles in cambium tissues can aid our understanding of the growth, development, and formation of wood in trees. The poplar genome project has made great progress (Tuskan *et al*., 2004; Tuskan *et al*., 2006), and its findings on poplar genomics (Tuskan *et al*., 2004) are opening new lines of inquiry. Proteins are well known to function as the final executors of biological functions, so proteomics is an extremely important field (Wasinger *et al*., 1995; Wilkins *et al*., 1995). Compared with other areas, such as medical and basic biology, tree proteomics has made relatively slow progress; however, researchers in this field have still made dramatic achievements.

There are various traditional and modern effective techniques for separating, detecting, and sequencing proteins and peptides in proteomics studies. Techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970), two-dimensional gel electrophoresis (2-DE) (Raymond & Aurell, 1962), high-performance liquid chromatography (HPLC) (Unger *et al*., 2000), capillary electrophoresis (Landers, 1993; Karger *et al*., 1995), and two-dimensional difference gel electrophoresis (Ünlü *et al*., 1997; Yamaguchi *et al*., 2010) are used to separate mixtures of proteins (Shah *et al.,* 2011). Blue silver staining (Candiano *et al*., 2004) and silver staining are used for staining. Ultraviolet detection is used for detection, mass spectrometry (Yates III, 2000) is used for sequencing, and nuclear magnetic resonance spectroscopy (Kainosho *et al*., 2006) and Fourier transform infrared spectroscopy (Travo *et al*., 2012) are used to discern protein structure.

The major obstacle to progress in tree proteomics studies remains the extraction of proteins (Fido *et al*., 2004; Conlon & Salter, 2007; Flengsrud, 2008) from tree samples, due to the abundance of secondary metabolites, such as polyphenols, pigments, and polysaccharides, which affect protein preparation and purification (Damerval *et al*., 1986). Only highly purified proteins may be used for proteomics studies. The most effective separation technology for proteomics research is two-dimensional electrophoresis (Raymond & Aurell, 1962; Görg *et al*., 2004). It is necessary to develop methods for extraction and purification of proteins from samples of tree species.

In this paper, we compared three routinely used protein extraction methods, Tris-phenol extraction (Maldonado *et al*., 2008), trichloroacetic acid (TCA)-acetone extraction (Sheoran *et al*., 2009), and TCA-acetone-phenol extraction (Xiang and Ning et al., 2010). The approaches were compared and optimized for protein extraction and pre-purification of cambium tissues from the Chinese white poplar (*Populus tomentosa*) over the course of a growing season. The change of protein volume over the course of the season indicated growth and offered insight into the function of proteins during plant growth.

Materials and Methods

Plant materials and sample preparation: The cambium tissues were harvested in February, April, and July 2011, from a 10-year-old *P. tomentosa* tree on the campus of Beijing Forestry University. Plant materials were weighed, immediately frozen in liquid nitrogen, and then stored in a -80°C freezer.

Protein extraction: The cambium tissues (0.5 g fresh weight) were harvested and ground to powder in a pre-cooled mortar by using liquid nitrogen. The sample powder was then transferred into Eppendorf tubes for immediate protein extraction or stored in a -80°C freezer for later use.

Tris-phenol extraction protocol: First, 2 ml extraction buffer (500 mM Tris, 50 mM EDTA, 700 mM sucrose, and 100 mM KCl, at pH 8.0) was added to Eppendorf tubes for protein extraction. The tubes were vortexed for 10 min at 4°C. Then, one volume (v/v) phenol was added (Tris buffered to pH 7.9), and the solution was vortexed for 30 min at 4°C and centrifuged at 12,000 rpm for 30 min. The upper phenol layer was transferred into another tube. The lower layer was re-extracted with 1 ml each of phenol and extraction buffer, vortexed, and centrifuged, and the phenol layer was combined with the volume collected earlier. Next, 3–5 volumes (v/v) of 0.1 M ammonium acetate in methanol were added, and the mixture was maintained at -20°C overnight. The tube was centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed twice with 0.1 M ammonium acetate in methanol and twice with acetone containing 0.07% 2-mercaptoethanol (ME). In each case, the pellet was completely suspended by vortexing and centrifuged at 12,000 rpm for 15 min at 4 °C. Finally, the pellet was vacuum-dried.

TCA-acetone extraction protocol: Five volumes of ice-cold acetone (containing 0.07% 2-ME and 10% TCA) were added to an Eppendorf sample tube. The tube was vortexed for 30 min at 4°C, and proteins were extracted at -20°C overnight. The tube was centrifuged at 12,000 rpm for 30 min at 4°C. The pellet was washed 3 to 5 times with acetone containing 0.07% 2-ME. In each case, the pellet was completely suspended by vortexing and centrifuged at 12,000 rpm for 15 min at 4°C. Finally, the pellet was vacuum-dried.

TCA-acetone-phenol extraction protocol: The steps for this protocol were the same as for the TCA-acetone extraction, and then follow the Tris-phenol extraction.

Protein lysis: The pellets were dissolved in a lysis buffer (containing 8 M urea, 2 M Thio urea, 4% CHAPS, 65 mM DTT, and 2% Ampholyte), vortexed for 3 h, and centrifuged at 15,000 rpm for 30 min at 25°C. The protein concentration in the supernatant was quantified by Bradford (Bradford, 1976), using bovine serum albumin as the standard.

Two-dimensional electrophoresis: Equal amounts of protein (200 µg) extracted using each of the three methods were loaded on 7-cm, 3–10 pH linear range strips through re-hydration, and isoelectric focusing was performed using the Bio-Rad Protean IEF Cell system. Electrofocusing was carried out at 20°C with gradually increasing voltage: 0–100 V for 1 h, 250–500 V for 3 h, and 500–4000 V for 4 h, up to 40,000 Vh with a maximum voltage of 4000 V. After isoelectric focusing was complete, immobilized pH gradient strips were equilibrated (Görg *et al*., 1988) by immersing them first in 375 mM Tris–HCl, pH 8.8, containing 6 M urea, 2% SDS, 20% glycerol, and 2% DTT, and then in the same solution containing 2.5% IAA instead of DTT. For the second dimension, SDS-PAGE, 15% polyacrylamide gel (Laemmli, 1970) was utilized. Electrophoresis was carried out at 15 mA per gel for 30 min and 25 mA per gel until the dye reached the bottom of the gel. The gels were stained using Candiano'method (Candiano *et al*., 2004).

The comparison of techniques showed that the optimized protein extraction method was the best technique for the extraction of protein from the cambium tissues of *P. tomentosa*. The sample was loaded on 17-cm, 3–10 pH linear range strips through re-hydration, and isoelectric focusing was performed using the Bio-Rad Protean IEF Cell system. Electrofocusing was carried out at 20 °C with a gradually increasing voltage: 0–100 V for 5 h, 100–250 V for 3 h, 250–500 V for 5 h, 500–1000 V for 2 h, 1000–10,000 V for 6 h, and so on, up to 99,999 Vh with a maximum voltage of 10,000 V. After isoelectric focusing was performed, the same protocol as above was followed.

Image scan and analysis: After staining was achieved, we used a Model GS-800 (Bio-Rad) Calibrated Imaging Densitometer to scan the gels. To analyze the protein spots, PDQuest software was used.

In-gel digestion: We used ExQuest spot cutter (Bio-Rad) to excise a protein spot of interest from a 2-DE gel. The pieces were placed in an Eppendorf tube to which destaining solution (50% acetonitrile in 100 mM ammonium bicarbonate) was added. The sample was incubated at room temperature for 30 min. The procedure was repeated once or twice until the gel pieces were totally destained. The sample was dried in a vacuum centrifuge. Proteins were rehydrated with 10 µl of 50 mM ammonium bicarbonate containing 50 ng trypsin, and the sample was incubated for 10 min; then, 200 µl of 50 mM ammonium bicarbonate was added, and the sample was incubated at 37°C overnight. The solution was extracted from the digestion tube and transferred to a new Eppendorf tube for collection. Next, 200 µl of 60% acetonitrile containing 0.1% trifluoroacetic acid was added to the gel, and it was maintained at room temperature for 30 min, after which the solution was transferred to a collection tube. The sample was dried in a vacuum centrifuge and stored at -80 °C.

MALDI-TOF-TOF: Peptide mass fingerprints of trypsin digests were acquired with a 4800 Plus MALDI TOF/TOF mass spectrometer (AB SCIEX), operated in positive-ion reflector mode by using CHCA as the matrix. Spectra were calibrated using the trypsin autolysis peaks at m/z 850 and 4000.

HPLC conditions: The elution gradient was performed at a constant flow rate of 0.1 ml/min: 0–5 min, 90% A (0. 1% glacial acetic acid in 98% water/2% ACN) and 10% B (0. 1% glacial acetic acid in 98% ACN/2% water); 5–90 min, 15% A and 85% B.

Mass spectrometry parameters: An LCQ DECA XP MAX ion trap mass spectrometer system (Thermo-Finnigan), coupled with an electron spray ionization source, was applied with the following parameters: positive ionization mode, sheath gas: nitrogen, 25 abi units, Aux gas: nitrogen, 5 abi units (ca. 3.33 L/min), Capillary voltage +4.5 kV, Tube Lens Offset 30 V, Multipole RF Amplifier 400 Vp-p, Multipole 1 Offset -6.80 V, Multipole 2 Offset -9.50 V, Intermultipole Lens Voltage -16.00 V, Entrance Lens -50 V, Trap DC Offset Voltage -10 V, Capillary temperature 240 °C, Collision energy 35.

Results and Discussion

Comparison of protein extraction methods using 2-DE gels: Representative CCB-stained gels for each method of extraction (Tris-phenol, TCA-acetone, and TCA-acetone-phenol) are shown in Fig. 1.

The electrophoresis gels revealed that the Tris-phenol and TCA-acetone-phenol methods produced darker backgrounds than did the TCA-acetone method when applied to samples from *P. tomentosa* cambium tissues. In addition, the TCA-acetone method revealed more protein spots than did the other two methods.

As shown in Table 1, the three extraction methods yielded different concentrations of protein extracts: 3.88 ± 0.316 mg/g (for Tris-phenol), 4.386 ± 0.749 mg/g (for TCA-acetone), and 2.858 ± 0.137 mg/g (TCA-acetone-phenol). The percentage of the pellet from cambium tissues that was composed of protein was relatively low using the TCA-acetone method. However, in terms of protein yield from either buds or cambium tissues, the protein content produced using the TCA-acetone method was higher than that produced using the other methods. This result is attributed to the low quality of pellets yielded using the Tris-phenol and TCA-acetone-phenol methods.

Although the TCA-acetone extraction method did not yield the greatest quantity of protein, it did produce more spots for counting. Used in combination with PDQuest software, the TCA-acetone method provided the most protein spots and the clearest protein images, although some amounts of protein measured by this technique were lower than the amounts measured by other methods. We concluded that the TCA-acetone extraction method was the most effective method of these three for extraction of protein from cambium tissues.

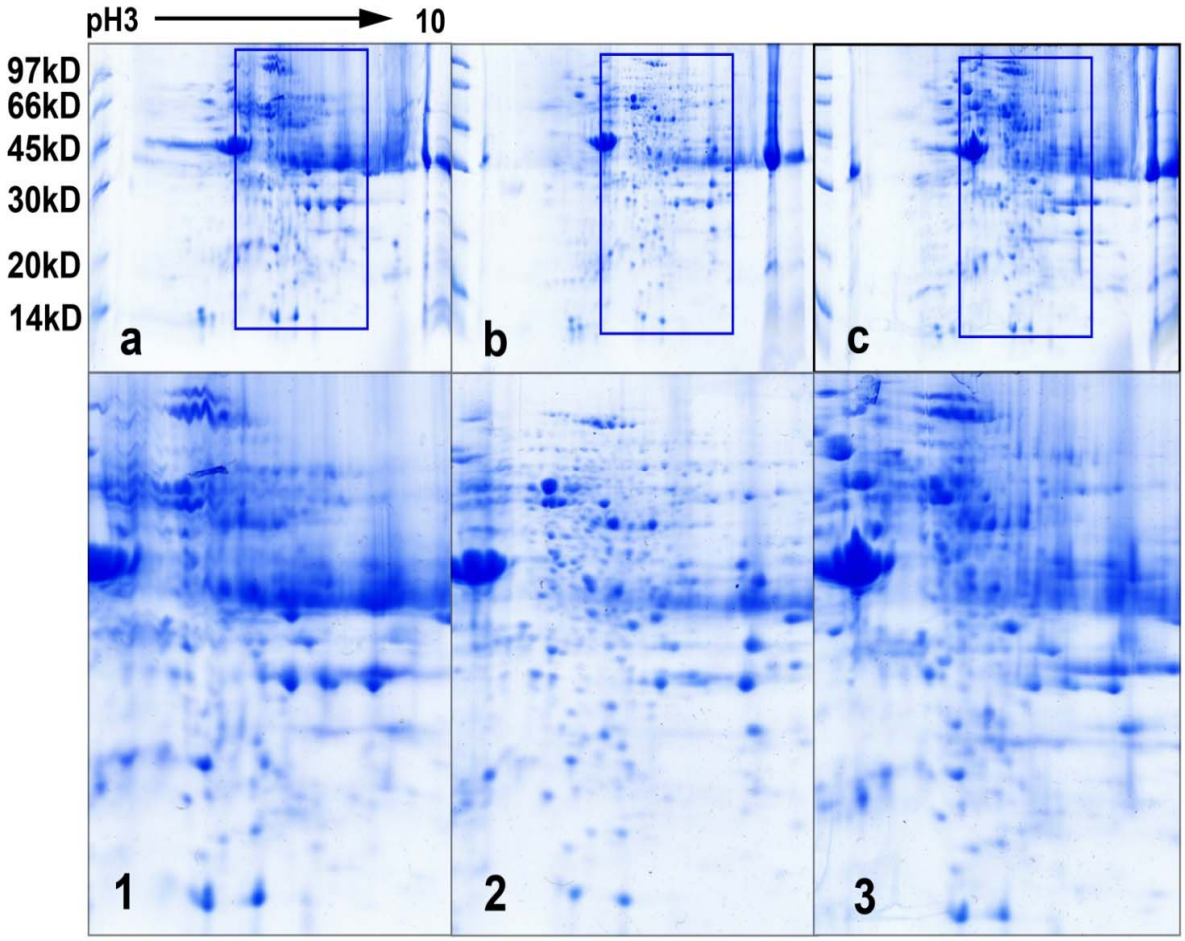


Fig. 1. Three protein extraction methods for *Populus tomentosa* cambium tissues are compared using two-dimensional gel electrophoresis: the Tris-phenol method (a), trichloroacetic acid-acetone method (b), and trichloroacetic acid-acetone-phenol method (c). Selected views of the two-dimensional gel electrophoresis gels are also shown (1, 2, 3).

Table 1. The yields and spots of proteins from poplar cambium produced using different extraction procedures.

Protocol	Pellet (mg/g protein powder)	Protein yield (mg/g fresh weight plant powder)	Number of spots
TCA-acetone	4.39 ± 0.749	7.84 ± 0.974	234 ± 3
Tris-phenol	3.88 ± 0.316	387.67 ± 0.917	189 ± 24
TCA-acetone-phenol	2.858 ± 0.137	285.95 ± 0.384	189 ± 10

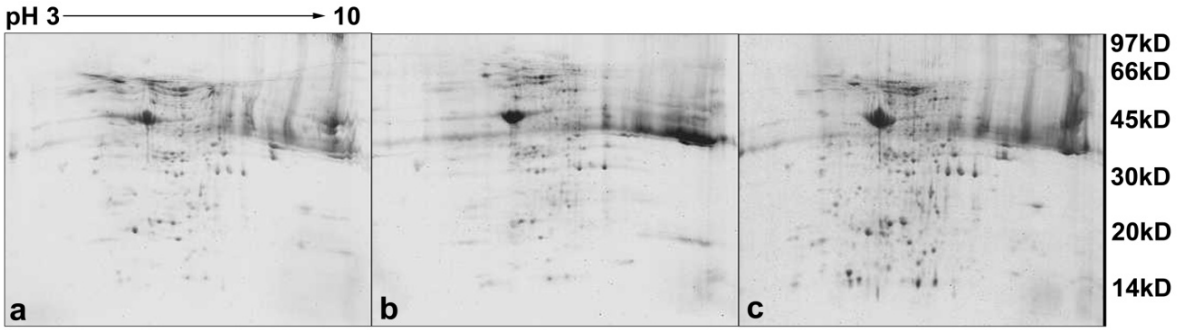


Fig. 2. Two-dimensional electrophoresis gels of *Populus tomentosa* cambium tissues sampled in February (a), April (b), and July (c).

Fig. 2 shows the 2-DE gels for the protein of *P. tomentosa* cambium tissues over the course of the whole growth period. The sample collected in July contained more protein and better quality than the others. Details are provided in Table 2.

Table 2 shows the data trends. The spots on the gels were counted using PDQuest software, and 6 spots were chosen for further analysis using mass spectrometry. Each spot represents the volume of protein during a particular growth period. Fig. 3 shows the normalized volumes of the six spots in February, April, and July.

Table 2. The yields and number of spots of protein from *Populus tomentosa* cambium tissues over the course of a growing season.

Sampling date	Pellet (mg/g protein powder)	Protein yield (mg/g fresh weight plant powder)	Number of spots
February	10.91 ± 0.47	6.39 ± 0.56	407 ± 13
April	13.35 ± 0.55	7.54 ± 0.20	412 ± 15
July	11.37 ± 1.37	6.53 ± 0.22	415 ± 15

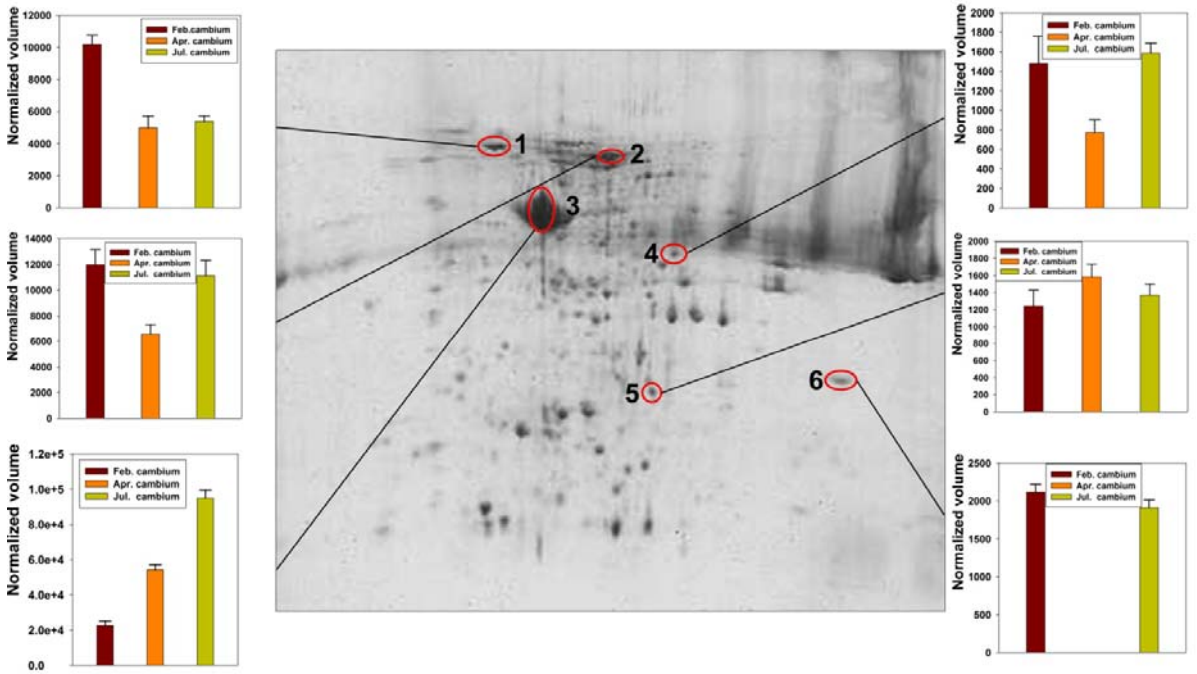


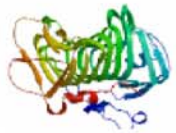
Fig. 3. The positions of the protein spots analyzed using mass spectrometry.

Table 3. Identification of Chinese white poplar *(Populus tomentosa)* spots using two-dimensional gel electrophoresis.

Spot No.	Theoretical PI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE value	Expected value	Accession number	Mass spectrometry method
1.	4.95/56042	putative endomembrane protein precursor [*Medicago sativa*]	3	8	43	0.008	gi|166418	MALDI-TOF-TOF
2.	5.84/51525	RecName: Full=Enolase [*ENO_ORYSJ*]	5	14.3	-	3e-05	gi|90110845	LC-MS/MS
3.	5.77/39869	Cup s 1 pollen allergen precursor [*Cupressus sempervirens*]	8	41	57	4e-20	gi|8101713	MALDI-TOF-TOF
4.	8.05/34967	class Ia chitinase [*Galega orientalis*]	3	20	65	5e-26	gi|33414052	MALDI-TOF-TOF
5.	7.11/20525	hypothetical protein SELMODRAFT [*Selaginella moellendorffii*]	4	22	50	3e-17	gi|302776324	MALDI-TOF-TOF
6.	9.72/24909	putative protein [*Oryza sativa*]	6	43	69	1e-17	gi|14140166	MALDI-TOF-TOF

Protein spots 1, 3, 4, 5, and 6 were identified by MALDI-TOF-TOF mass spectrometry. The proteins were identified on the basis of the peptide fingerprint of the mass spectrum. Spot 2 was identified by LC-MS/MS mass spectrometry. The MASCOT algorithm and a search of the NCBInr databases provided the information on these six proteins.

Table 4. Prediction of the structure of proteins.

Spot No.	Modeled residue range	Based on template	Sequence identity [%]	Model 3D structure
1	26 to 505	3boaA	28%	
2	4 to 443	2psn	70%	
3	45 to 386	1pxzB	53%	
4	22 to 310	2dkvA	68%	
5	21 to 215	1fi2A	47%	

The structures of the proteins were analyzed using Swiss-Mold. These structures reveal part of the proteins in Chinese white poplar (*P. tomentosa*)

In spot 1, the protein volume was about twice as high in February as in the other two months. In spots 2 and 4, the protein volume in April was almost half the volume found in February and July. Spot 3 showed a trend of steadily increasing protein volume over the months. In spot 5, the April volume was higher than the volume from February and July. In spot 6, there was no protein in April.

The function of class Ia chitinase is similar to that of chitinase. It is the enzyme that catalyzes chitin to transform into *N*-acetyl glucosamine. Enolase is one of the key enzymes in glycolysis which catalyzes 2-phosphoglycerate to form the energetic compound phosphoenolpyruvate. The 2-D gel showed that the volumes of enolase (Reed *et al*., 2009) and class Ia chitinase were higher in February and July than in April.

Protein sequencing allowed us to predict the structure of identified proteins. The structures of the proteins were analyzed using Swiss-Mold. (Guex & Peitsch, 1997; Schwede *et al*., 2003; Arnold *et al*., 2006), the result was showed in Table 4.

Conclusion

Our analysis identified six protein spots, including proteins identified as enolase and class Ia chitinase. The trends in protein volumes over the course of a season's growth in the Chinese white poplar (*P. tomentosa*) may be related to plant growth, development, and metabolism. In February, the plant stores resources and energy to provide material for growth in the spring. In April, a period of vigorous growth, many materials are consumed, including enolase and class Ia chitinase. The extraction of proteins is critically important for proteomics studies, but woody plants like P. tomentosa contain abundant secondary metabolites that could interfere with 2-DE. The extraction procedure not only needs to get rid of as many secondary metabolites as possible, but also needs to retain the proteins in the tissue sample. In this study, samples were obtained from *P. tomentosa* cambium tissues and buds to compare and optimize extraction procedures. We found that the TCA-acetone protein extraction procedure was relatively simple, highly efficient, and provided the greatest quantity of protein extracted and number of spots on the 2-DE gel. The method uses 10% TCA to inhibit protease activity, 0.07% 2-metaethanol to protect the proteins from oxidation and degradation, and cold acetone to collect the protein as sediment and clean the phenolic and quinonoids compounds and other secondary metabolites.

Although the TCA-acetone extraction procedure was the most suitable for poplar tree samples, it may not be the best technique for other plant species. For example, previous studies have reported that for turfgrass root and tomato pollen grains, the best protein extraction method is TCA-acetone-phenol, and for leaves, TCA-acetone is best (Chenping Xu, 2008; Sheoran *et al*., 2009).

In summary, in order to conduct proteomics experiments, the protein extraction protocol needs to be optimized and adapted for the particular species of interest. Proteomics can reveal changes in the levels of proteins during different stages of growth, providing insights into fundamental biological processes.

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