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Gene Expression Analysis of *CWPO-C* from Poplar's Isolated Cells obtained by Cryosection followed by Laser Microdissection

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1 Introduction

A unique class III peroxidase, CWPO-C, which that has wide substrate spectra, from Poplar (*Populus alba*), has been studied in the relation with lignification in our laboratory (1-3). The *CWPO-C* gene expression analyses by Real time PCR (RT-qPCR) at organ levels such as developing xylem, leaf and shoot, indicated some new insights about the CWPO-C functions besides lignification. Plant organs like stems possess different tissue types with different functions. Such in the case, xylem is composed of tracheids, vessels, parenchyma, and fibers. Therefore, it is necessary to determine the more detailed spaciotemporal expression of the gene at tissue/cell level, in order to understand the role of *CWPO-C*.

Laser microdissection (LMD) has been used to collect specific tissue and cell types widely in herbaceous plants, but it has not been used in woody perennials (4). LMD microscopy possess a cutting laser mounted over the microscope lens which enables to micro-dissect and collet the target tissue/cell. LMD is often used for transcripts studies but occasionally used for protein, enzyme and metabolite analyses. Because the gene expression analysis requires the intact preservation of transcripts through all the procedure, it is essential to keep RNase free and cold conditions when manipulating the samples. Sections must be prepared by cryo-condition prior to the microdissection. The microdissection of the cells from woody plants is a challenging step due to the rigidity of the thickened cell wall. Furthermore, the presence of proteins, phenolics and other contaminants in plant cells frequently cause blockage of the extraction and enzyme inhibition. These reasons may explain the lack in reports in terms of gene expression analyses at tissue/cell level in woody perennials so far (4-5).

Temperature while RNA being extracted and purified has a considerable effect over RNA quality and yield. During RNA manipulation samples must be handled with RNase free solutions and RNase free instruments. But, it is also essential to conduct the experiments under low temperature in order to avoid the RNA degradation by endogenous RNases localized inside the cytosol. It is known that any biological activity is stopped under cold conditions, which inhibits the activity of these enzymes at -80°C (6).

This report describes establishing a robust method to study the CWPO-C gene expression in specific tissues/cells in poplar plant, but it will further serve as a methodology for the gene expression studies in woody perennials.

2 Experimental and Results

2.1 Plant Material and Cryosection

Explants from two-month-old plantlets of poplar (*Populus alba*) were used, and 0.5 cm explants were processed by cryo-sectioning for laser microdissection. The stem tip and middle stem were mounted in a plastic cryomold (Tissue-Tek, The Netherland) with embedding medium Cryomatrix (Thermo scientific, USA), snap-frozen in liquid nitrogen, then stored at -80°C for cryostat sectioning overnight. Before cryosection samples were acclimatize at -15°C for 15 minutes inside the cryostat. Twenty-five µm thick sections were made with a Thermo Scientific HM 525 Cryostat (VWR International, PA, USA) at -15°C, and mounted on PEN-membrane 2.0 µm glass slides (Leica, Germany). Glass slides were placed inside a 50 ml Falcon tube and acclimatize to room temperature to avoid dew condensation on the slides. Prior to the microdissection operations, the sections were fixed in cold RNase free 99.5 % ethanol at -20°C for 10 sec, deprived of Cryomatrix medium with RNase free water for 2 min, and refixed in cold RNase free 99.5% ethanol at -20°C for 1 minute. Subsequently the sections mounted on the glass slides were air-dried and microdissected with a LMD instrument at room temperature (6).

Despite cryosectioning is a rapid and cell conservative method to prepare plant sections without degradation of RNA, obtained sections sometimes showed inferior morphology when contrasting to traditional paraffin embedding. To avoid this problem, explants and Cryomatrix were solidified together forming a block, by "flash-freeze" immersing into liquid nitrogen. But, by cause liquid nitrogen boils irregularly, Cryomatrix occasionally freezes uniformly. However, "flash-freeze" is still prompt and simple method to perform forming block. Also, cells must be vacuum infiltrated with a cryopreservant which protects the intact morphology. A high concertation (30%) of sucrose was selected as cryopreservant agent, according to the knowledge that sucrose prevents the formation of ice crystals within the cytosol, leading to keep the cellular structure intact (Fig. 1). Cryo-sectioning is recommended to be conducted at -20°C, but it could be changed within ±10°C range depending on the stiffness of the explants, by increasing the temperature when explants are more rigid or decreasing the temperature when explants are softer. The Cryomatrix attaches better with explants, between -10°C to -20°C. It is considerably important to remove any bubbles from surface of explants to ensure the tight attachment of the embedding compound to keep intact morphology in the sections.

Inside the cryostat, dew condensation on the slides should be avoided by a constant cold dry air outflow current, therefore it is critical to acclimatize the RNase free slide in a close air tight container, such as a Falcon tube.

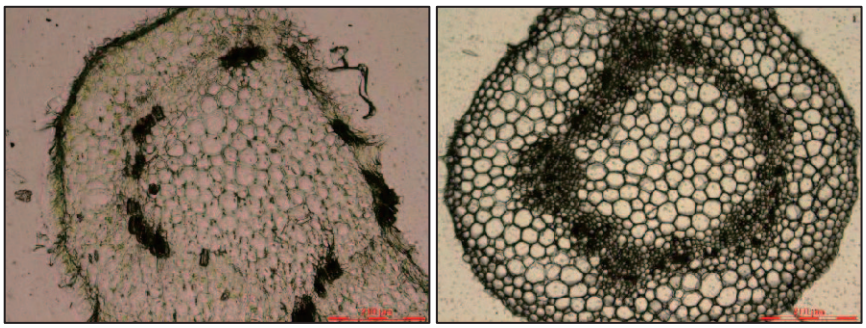

Fig. 1. Cryo-cross section (25 μm thickness) of poplar stem. Both embedded in the Cryomatrix and flash-freeze with liquid nitrogen. Left: without 30% sucrose treatment, Right: treated with 30% sucrose and vacuum infiltration. Bar. 200 μm.

2.2 Laser microdissection

Laser microdissection (LMD) is a type of microscope that uses a cutting laser to isolate tissues/cells of interest. The most common application of laser-microdissection is for RNA isolation and transcript analysis by Real time PCR (RT-qPCR). In woody perennials, LMD has been scarcely used but it has the potential to improve the sensitivity of spatial gene expression assays.

In this study LMD was performed as described by Abbot et al. (5) with adaption to the subjected cell and tissue types. The longitudinal-cryo-sections 25 μm thickness from stem tip and middle stem were allowed to dry at room temperature for 5 min prior to micro-dissection with a LMD7 Laser Micro-dissection Microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). For optimal dissection we used the maximum laser intensity and a slow cutting speed. Different micro-dissected tissues were collected individually into the cap of nuclease free 0.5 ml PCR tubes (Axygen, Union City, CA, USA) containing 60 μl of lysis buffer. The tubes were then closed and centrifuged at low speed (3000 rpm) for 20 s to sediment the LMD samples, additional 20 μl of lysis buffer was added and centrifuge for 10 s twice, to a final volume of 100 μl, finally the tubes were placed on liquid nitrogen.

Specific tissue regions were micro-dissected from the stem tip. Cell types selected for LMD at 20x magnification for SAM (Stem apical meristem), ground meristems and pith. In addition, cambium and collenchyma were dissected at 4x magnification. The total cross-sectional area dissected from cryosections was approximately 100 x10^3 μm² for SAM and 1000 x10^3 μm² for ground meristem and pith. When different

cell types and tissue regions were micro-dissected from the same cross-section, dissection of one type was always completed before starting the another type to avoid cross-contamination.

The latent risk of RNase contamination increases during microdissection procedure, because the LMD microscope doesn't offer any protection from environmental RNase contamination. As our empirical rule to avoid RNA degradation, microdissection time must not exceed 1 hour/slide, all dos and don'ts for RNA manipulation are compulsory at any moment. The lysis buffer must be kept at 4°C prior to microdissection collection, and the collected dissection samples must be kept in liquid nitrogen until they are subjected to further analyses. It is also necessary to calibrate the laser beam alignment before cutting in order to conduct an accurate dissection.

2.3 Micro RNA extraction and Gene Expression Quantification by Real Time Polymerase Chain Reaction

RNA was extracted independently from the candidates tissue type by following the protocol for the RNAqueous-Micro RNA Kit (Ambion, Inc,, Austin, TX, USA). Special care when handling RNA tubes was taken. Samples that were transported in liquid nitrogen got successfully amplified. Because RNA was collected from a limited number of cells, it is difficult to confirm the RNA quantity by spectroscopic method. So, the downstream application, RT-qPCR, is usually performed even with the uncertain possibility that RNA is degraded in some samples during the steps between the cryo-sectioning and laser micro-dissection. However, the RNA quality can be examined by determining the melting curves of the reference gene, *ubiquitin* (*UBQ*, *Genebank*: BU879229), and RNA quantity can be also estimated by the fluorescence intensity of melting curves (Fig. 2).

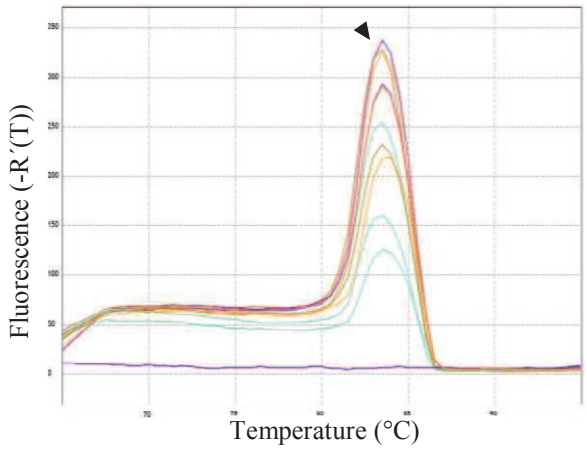

Fig. 2. Melting curve analysis of RNA samples prepared from the laser micro-dissected cell in poplar tree. Amplifying reference gene UBQ. Black arrow: Specific amplification at specific Tm value indicates a good quantity and quality of extracted RNA for each sample.

The target genes were *UBQ* and *CWPO-C*. Amplification data was analyzed using the Agilent Mx Real time PCR software (Agilent, US). Relative transcript abundance was calculated and then normalized to *UBQ* expression (7-8). Reactions with non-discrete melting curves or other anomalies were excluded from analysis.

Low quantity of RNA extracted from tissues was used for gene expression analysis. Apical meristem, cambium and collenchyma tissue samples were compared each other in terms of *CWPO-C* relative expression. Amplification of *CWPO-C* in three different tissue types with low standard deviation values demonstrates a successful procedure in every stage, explants freeze embedding cryosection, and microdissection, unto RNA extraction and reverse transcription. The differences of *CWPO-C* expression among the collected tissue types, suggests that this method is also useful to analyze the gene expression at the tissue/cell level (Fig. 3).

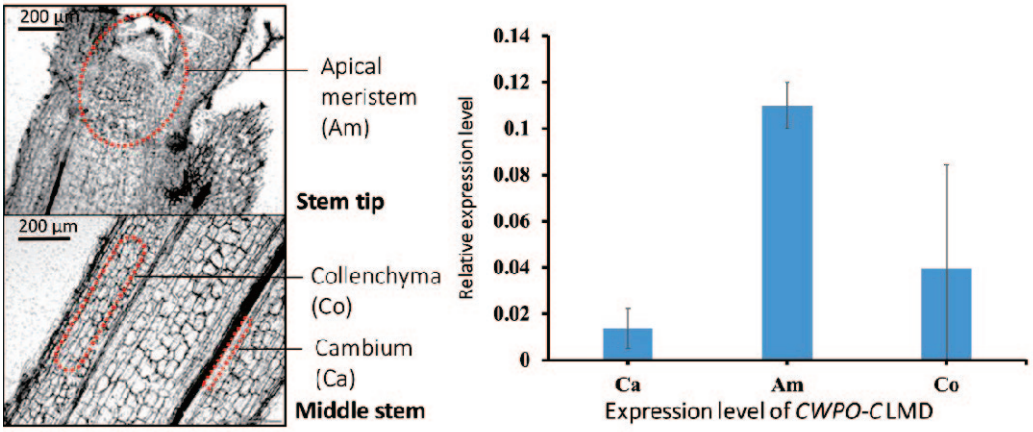

Fig. 3. *CWPO-C* expression in the different tissues. RT-qPCR analyses were performed with the RNAs those were prepared from the different tissues collected by the LMD. LMD was conducted with the cryo-section of poplar shoot tip. Standard error represents three biological replicates. Am: Apical meristem, C: Cambium, Co: Collenchyma. Red dotted lines delimit LMD collected area. Scale bar 200 µm.

3 Conclusion

Spatial expression of *CWPO-C* was determined at tissue level in woody perennial poplar tree. To analyze the gene expression in the specific cell types, special techniques and care were required. During the preparation of sections from explants, RNA protection was achieved under low temperature throughout all the procedure, fixation, embedding, and sectioning. The usage of Cryomatrix and "flash-freeze" in liquid nitrogen was effective for RNA protection. Cryo-sectioning at around -15°C was suitable for not only RNA protection but also keeping morphology of the sample. It is also definitively required the use of liquid nitrogen for the preservation of samples, such as explants, sections, and laser micro-dissected cells. In this study, it was demonstrated the feasibility to analyze the expression of the gene of interest in the specific tissue/cell level in woody perennials like poplar. The process includes special techniques and care for cryo-fixation, -embedding, -sectioning. Therefore, it would serve as a reference for the further gene expression studies at the specific tissue/cell level in woody perennials.

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