CUTTING TREES WITH LASERS: ISOLATION OF HIGH QUALITY RNA, ENZYMATICALLY ACTIVE PROTEIN AND METABOLITES FROM INDIVIDUAL TISSUE TYPES OF WHITE SPRUCE STEMS OBTAINED USING LASER MICRODISSECTION

by

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B.Sc., The University of British Columbia, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2010

ABSTRACT

Laser-assisted microdissection has been established for isolation of individual tissue types from herbaceous plants. However, there are few reports of cell- and tissue-specific analysis in woody perennials. While microdissected tissues are commonly analyzed for gene expression, reports of protein, enzyme activity and metabolite analysis are limited due in part to an inability to amplify these molecules. Conifer stem tissues are organized in a regular pattern with xylem, phloem and cortex development controlled by the activity of the cambial zone (CZ). Defense responses of conifer stems against insects and pathogens involve increased accumulation of terpenoids in cortical resin ducts (CRDs) and *de novo* formation of traumatic resin ducts from CZ initials. Woody plants are difficult to study at the level of individual tissues or cell-types and are thus good candidates for application of LMD. This thesis describes robust methods for isolation of individual tissue-types from white spruce (*Picea glauca*) stems for analysis of RNA, enzyme activity and metabolites. A tangential cryosectioning approach was important for obtaining large quantities of CRD and CZ tissues using LMD. Differential expression is reported for genes involved in terpenoid metabolism between CRD and CZ tissues and in response to treatment with methyl jasmonate (MeJA). Transcript levels of β-pinene synthase and levopimaradiene/abietadiene synthase were constitutively higher in CRDs, but induction was stronger in CZ in response to MeJA. 3-Carene synthase was more strongly induced in CRDs compared to CZ. A differential induction pattern was observed for 1-deoxyxylulose-5-phosphate synthase, which was up-regulated in CRDs and down-regulated in CZ. We identified terpene synthase enzyme activity in CZ protein extracts and terpenoid metabolites in both CRD and CZ tissues. Combined analysis of transcripts,

proteins and metabolites of individual tissues will facilitate future characterization of complex processes of woody plant development, including periodic stem growth and dormancy, cell specialization, and defense and may be applied widely to other plant species.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank Dr. Krystyna Klimaszewska for providing white spruce seedlings, Dr. Trygve Krekling for microscopy training and invaluable advice with histology, Dr. Dawn Hall for assistance with method development, Dr. Björn Hamberger and Ms. Jamie Pighin for technical assistance, Mr. Hardy Hall for initial guidance with cryosectioning, Dr. Katherine Zulak for providing microscopy images, Dr. Alfonso Lara Quesada for maintenance of plant materials, and Ms. Karen Reid for excellent support with project and laboratory management. I am also grateful for the advice and guidance of my committee members, Dr. Robert Guy and Dr. Geoffrey Wasteneys for advice and guidance, and to my supervisor Dr. Jörg Bohlmann and all past and present members of the Bohlmann Lab for contributing to a fun and dynamic lab research environment. A special thanks to my fellow grad students, friends and family for all your love and support. And finally, to the hosts, writers and producers of Radio Lab, This American Life, Wire Tap, Savage Love, The Vinyl Café, Learn French by Podcast, Coffee Break French and CBC Radio 3 for providing such fine programming during those long hours on the cryostat and LMD.

This research was supported with funding from Genome British Columbia and Genome Canada for the Treenomix Conifer Forest Health project (www.treenomix.ca) and the Natural Sciences and Engineering Research Council (EWR Steacie Memorial Fellowship and Discovery Grant awarded to Dr. Jörg Bohlmann). I was personally supported through a NSERC Canada Graduate Scholarship.

DEDICATION

L'Chaim

CO-AUTHORSHIP STATEMENT

Experiments were conceived and designed by Eric Abbott (EA), Dr. Dawn Hall (DH) and Dr. Jörg Bohlmann (JB). All white spruce seedlings used during these experiments were maintained by EA. Methyl jasmonate treatment and tissue harvesting was performed by EA and DH. Björn Hamberger (BH) contributed to the initial evaluation of different laser-assisted microdissection platforms and assessment of reference genes for qRT-PCR. All cryosectioning and laser microdissection was performed by EA. RNA extraction, quantification, qRT-PCR and analysis of gene expression was performed by EA. Protein extraction, quantification and enzyme assays were performed by DH. Metabolite extractions and all GC/MS analysis was performed by DH. Data analysis and interpretation was performed by EA, DH and JB. All writing for this thesis was completed by EA with editing contributions from DH, BH and JB.

1. INTRODUCTION

1.1 Laser microdissection: A powerful tool for tissue-specific analysis in plants

A genome sequence is an enormously powerful tool for molecular biology, but it does not fully represent the complex metabolism of a plant. In plants, many metabolic processes are spatially localized to specialized organs, tissues and cell-types and temporally regulated in response to internal or environmental stimuli. Different tissues are characterized by unique gene expression patterns that determine their metabolic state. Genome sequences of several plant species [1-9] have been complemented by transcriptome analysis including cDNA sequencing, microarray analysis and quantitative real time PCR (qRT-PCR). Proteomic and metabolomic analyses have led to a more comprehensive picture of metabolic processes in plants.

Molecular analysis of whole organs (i.e. leaves, stems, roots, flowers or fruits) is relatively easy because RNA, protein and metabolites can be extracted in large quantities. However, even individual organs are comprised of a number of different tissues and cell-types. Specialized biochemical and physiological properties can be localized to small tissues or even individual cells that are defined by unique gene expression patterns, enzyme activities and metabolite profiles. The function of larger organs is determined by the specialized processes carried out by individual tissues and cells within the heterogenous structure of the organ. For example, the quiescent center of the *Arabidopsis* root consists of a cluster of only four cells that are critical for maintaining patterns of cell differentiation in the root apical meristem [10]. Isolation of these cells for microarray analysis revealed a unique set of genes with enriched expression in the quiescent center

relative to other tissues [11]. The unique features of each cell-type are lost if analyzed within a heterogenous sample comprised of multiple tissues and cell-types. A more complete understanding of plant biochemistry and physiology requires the ability to perform molecular analysis with high spatial resolution.

There are many methods for molecular characterization of individual tissues or cell-types *in situ* without requiring purification or enrichment of the sample. Localized gene expression patterns can be observed by *in situ* hybridization using an oligonucleotide probe complementary to a specific RNA transcript. Specific proteins can be localized within a sample by immunolocalization or by fusion to a reporter gene such as green fluorescent protein. Spectroscopic techniques can also be used to localize specialized metabolites within plant tissue [12, 13]. Confocal microscopy and transmission electron microscopy are powerful tools for elucidating structural and functional relationships within intact thin tissue sections. However, these techniques can be costly and time consuming, may suffer from problems with specificity and background signal and are not scalable to high throughput analysis using current technologies.

High throughput techniques exist for analysis of RNA, protein and metabolites. Microarray analysis as well as cDNA library production combined with EST sequencing are effective tools for transcriptome analysis. Large scale protein identification can be performed using mass spectrometry-based technologies including matrix-assisted laser desorption/ionization-time of flight (MALDI/TOF) mass spectrometry. Metabolite profiles are commonly assessed using gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS). These techniques require that

the tissue of interest is isolated from the biological sample for extraction of RNA, protein or metabolites prior to downstream analysis. Most high throughput profiling techniques require relatively large sample quantities, which presents a problem for the analysis of specialized tissues or cell-types that are of low abundance within the sample. While RNA can be amplified by *in vitro* transcription and cDNA can be amplified using PCR, this is not the case for protein and metabolites.

Manual dissection of small samples is a simple and commonly used method for tissue enrichment. Small structures such as flowers, seeds, embryos, root tips and meristems can be harvested with the aid of a dissecting microscope. In woody plant species such as conifers and poplars, specific tissues of the stem are commonly enriched by separating bark from wood [14, 15]. Surface structures are more easily isolated because they are not imbedded within other tissues. For example, glandular trichomes or epidermal cells can be isolated using abrasion [16, 17] or simply by adhesion to a piece of tape. Enriched cuticle samples can be obtained by enzymatic isolation [18].

Internal tissues are more difficult to dissect from a specimen compared to surface structures. For stems of woody plants, enriched samples of cambial zone (CZ) and developing secondary xylem tissue can be obtained by taking scrapings from the xylem surface after removing the bark [19, 20]. A higher resolution approach involves taking serial tangential cryosections across the CZ [21-23]. In *Arabidopsis*, methods have been developed to create protoplasts from root tissue for isolation of individual cell-types using fluorescence activated cell sorting [24]. However, these methods are not yet widely applicable to a broad range of tissue types.

Laser-assisted microdissection is a powerful technique for isolating individual tissues or cell-types from thin sections. There are several types of laser-assisted microdissection which are outlined in Figure 1.1 and are described in detail in recent reviews [25]. Laser capture microdissection (LCM; Molecular Devices, USA) uses an infrared laser to activate a thermoplastic film to expand and adhere to target cells. Laser microdissection (LMD; Leica Microsystems, Germany) uses a UV laser to cut around the perimeter of a region of interest, which then falls by gravity into a collection tube below the sample. Laser microdissection pressure catapulting (LMPC; PALM-Zeiss, Germany) also uses a UV cutting laser, but cut regions are catapulted into a collection tube above the sample using a defocused laser pulse. Each platform has unique characteristics that may be well suited for isolation of a broad range of tissues and cell-types.

The LCM method was first described for isolation of human cell populations [26], but all platforms have since been applied to a wide range of plant species [25, 27]. There have been many reports of RNA transcript analysis using qRT-PCR [28-44], microarrays [28, 33, 35, 39, 41-43, 45-49] or transcriptome sequencing using traditional Sanger sequencing [30, 50] as well as next-generation high throughput technologies such as 454 sequencing [49, 51, 52]. Protein and metabolite analysis of laser microdissected samples is limited due to small sample sizes and an inability to amplify these molecules. However, two-dimensional gel electrophoresis and LC/MS/MS analysis of protein samples has been reported in laser microdissected plant tissues [53] and the techniques developed for proteomic analysis of laser microdissected human tissues may be applied to plants [25]. To the best of my knowledge, enzyme activity has not been reported in protein extracts from laser microdissected plant tissue and there is only a single

publication in an animal system, which describes the analysis of ornithine decarboxylase activity measured in proteins extracts from rat lung alveolar and epithelial tissue [54]. Metabolite analysis of laser microdissected tissues was recently reported in vascular bundles from *Arabidopsis* [55] and stone cells from Norway spruce [56, 57]. There have been few reports of a combined analysis of RNA, protein and metabolites from individual cell-types [31, 58], but these approaches have not been applied to laser microdissected plant tissues.

1.2 Cell- and tissue-specific aspects of constitutive conifer defense

Conifers (Coniferales) are some of the largest and longest lived organisms on Earth. Some conifers, including species of the pine family (Pinaceae), are capable of growing over 100 m in height, 10 m in diameter and living more than 1000 years. The ability to survive in a fixed location with the threat of potentially faster evolving insect and fungal pathogens necessitates a complex system of defense against biotic stress. Structural elements act as physical barriers to herbivory, whereas specialized metabolites may act as antifeedants, toxins or through other inhibitory functions. Many aspects of physical and chemical defense are associated with specific cells and tissues within a conifer stem [59].

Anatomical features of conifer defense can be observed using light microscopy, scanning electron microscopy and transmission electron microscopy. The general organization of a conifer stem is a system of concentric rings (Figure 1.2). The outer bark is comprised of the periderm, cortex and secondary phloem. The inner wood consists mainly of secondary xylem tissue, which is separated from the bark by the CZ. Axial

initials in the vascular cambium give rise to sieve cells, parenchyma cells and fibers towards the secondary phloem as well as tracheids and parenchyma cells towards the secondary xylem, while ray initials in the vascular cambium give rise to ray parenchyma cells that extend radially from the central pith into the cortex. The CZ includes the stem cell initials of the vascular cambium as well as partially differentiated cells that separate mature secondary xylem and secondary phloem tissues.

The periderm forms the first line of defense where the thick cell walls rich in lignin or thin cell walls rich in suberin are impregnated with phenolic material to form a tough physical barrier against mechanical damage [59, 60]. Calcium oxalate crystals are often distributed through the periderm, cortex and secondary phloem and may hinder tree boring insects such as bark beetles [59, 61]. Fungal inoculation on the periderm surface does not induce a defense response suggesting that the periderm is an effective barrier to microbial infection [62]. In the secondary phloem tissue, layers of compressed sieve cells and either highly lignified stone cells (sclereids) in Pinaceae, or fibers in other conifer families, form additional physical barriers [59].

In many conifer species, terpenoid-rich oleoresin plays a major role in defense against pathogens and herbivores [63, 64]. Oleoresin is carried in resin blisters, resin ducts or individual resin cells that may be preformed or produced *de novo* during the defense response depending on the species, and are particularly common in Pinaceae [14, 59, 65]. Pre-formed axial resin ducts may be present in the cortex (cortical resin duct, CRD) or secondary phloem [65, 66]. After physical wounding, oleoresin is exuded under pressure facilitating the removal of debris, fungal pathogens and insect pests from the wound site. The volatile monoterpenoid components of the oleoresin evaporate when

exposed to air leaving a solid plug that seals the wound site [67]. In addition to acting as a physical defense, terpenoid components of the oleoresin can be toxic to herbivorous insects and their associated microbial pathogens [68]. Terpenoids are produced by the activity of terpene synthase (TPS) enzymes, which are thought to be localized to the epithelial cells lining a resin duct or blister, but this has been demonstrated only for a single levopimaradiene/abietadiene synthase (LAS) [69]. Resin duct epithelial cells are also rich in lipid droplets, which have not been chemically characterized [61]. Resistance of white spruce (*Picea glauca*) to white pine weevil (*Pissodes strobi*) is correlated to the number and density of resin ducts in bark tissue [70].

In addition to terpenoid defense, phenolic compounds form a major part of the conifer defense response. Polyphenolic parenchyma (PP) cells are the most prominent cell-type in the secondary phloem tissue and are present in all conifer species [65, 66]. PP cells are produced annually as a single concentric ring in the secondary phloem [59-62]. This barrier is maintained as the stem increases diameter by cell growth and division resulting in expansion of the PP cell concentric ring. PP cells have been shown to be viable for more than 70 years [59]. PP cells are thought to accumulate large quantities of phenolic compounds, which have been extensively described using microscopy techniques [59-62]. However, the specific chemical composition of PP cells has not been well documented. Phenolic compounds in PP cells are located in vacuoles and may appear evenly distributed as soluble phenolics or condensed into globular polyphenolic bodies [61]. Calcium oxalate crystals may also be present in PP cell vacuoles with starch granules and lipid droplets appearing along the peripheral cytoplasm [61]. Resistance of Norway spruce (*Picea abies*) to inoculation with blue stain fungus (*Ceratocystis*

polonica) is correlated with PP cell number and density as well as the amount of vacuolar phenolic contents [61]. The phenolic biosynthetic enzyme phenylalanine ammonia lyase (PAL) has been shown to be constitutively expressed in PP cells and ray parenchyma cells of Norway spruce [61, 71].

1.3 Cell- and tissue-specific aspects of induced conifer defense

Biotic stress such as insect feeding, oviposition or fungal inoculation can induce both physical and chemical responses in the conifer stem. Specialized cells and tissues in the conifer stem are spatially organized and temporally regulated during the defense response. After insect feeding or fungal inoculation, a hypersensitive response occurs rapidly at the site of infection resulting in localized cell death in the cortex and secondary phloem [59, 62, 71]. This is followed by differentiation of PP cells to form callus tissue that becomes lignified, suberized and fortified with phenolics as it forms the wound periderm, which isolates the infected area [59, 62]. Young fibers and sclereids near the CZ are also strengthened by increased lignification [59, 65].

In Pinaceae, inducible traumatic resin ducts (TRDs) are formed *de novo* in the secondary xylem from initials in the CZ [64]. Rapid TRD formation is associated with increased oleoresin flow [14, 60, 65, 72, 73], resistance to insect or fungal attack [71, 74-77] and acquired resistance to subsequent attack [65, 78, 79]. In other species such as Monterey Cypress (*Cupressus macrocarpa*), monkey puzzle (*Araucaria araucana*) and Norfolk Island pine (*Araucaria heterophylla*), additional axial resin ducts are formed from dormant, incipient resin duct sites in the secondary phloem [65, 66].

As early as six days after induction, the early stages of TRD formation is indicated

by the differentiation of xylem mother cells in the CZ. This is marked by swelling, anticlinal and periclinal cell divisions and the appearance of phenolic bodies and starch grains in adjacent parenchyma cells at regular intervals within the CZ, usually adjacent to ray parenchyma cells [60-62, 71, 80]. By 18 days, TRD epithelial cells become discrete and a schizogenous lumen is formed that may be continuous with radial resin ducts [65, 71]. Association between TRDs and ray parenchyma cells or radial resin ducts may increase resin flow to the outer bark tissue [71]. At this point, TRD epithelial cells are metabolically active as they contain many plastids and enlarged nuclei [71]. After 36 days, parenchyma cells adjacent to TRDs have lost their phenolic contents and differentiated into tracheids, the lumen space is expanded, xylem mother cells have resumed normal tracheid production and TRDs form a concentric ring embedded in the secondary xylem [60, 62, 71, 80]. Multiple rings of TRDs may form during periods of sustained stress such as insect feeding or fungal inoculation [62, 77, 80]. The epithelial cells of TRDs become lignified by nine weeks or more after inoculation [62, 80]. While terpenoids are not UV-fluorescent, they can be visualized in CRD and TRD lumen by staining with copper acetate [14] or NADI reagent [81]. TRD formation in the xylem is induced by subperidermal or phloem fungal inoculations or by exogenous treatment with methyl jasmonate (MeJA), suggesting that a mobile signal is able to stimulate the differentiation in the CZ [14, 62].

Histological characterization shows that PP cells expand as they accumulate increased amounts of phenolics resulting in compression of sieve cells in the secondary phloem [60, 62, 65, 66, 80, 82]. A new concentric ring of PP cells may form in addition to the yearly PP cell layers [62]. The number and density of globular polyphenolic bodies

decreases and a greater number of PP cells have uniformly stained vacuolar phenolic contents [60, 61]. In Norway spruce, this effect is strongest in resistant trees and it has been suggested that the ability to convert globular polyphenolic bodies into free soluble phenolics and possible release from the cell may be important for defense [61].

Treatment with MeJA has been shown to induce anatomical and chemical changes that mimic the normal defense response to insect feeding or fungal inoculation including formation of TRDs, increased resin accumulation and activation of PP cells in Norway spruce [14, 15, 60]. These results are consistent between Norway spruce 30-year old mature trees and 2-year old seedlings [14, 60], suggesting that MeJA treatment is a good model for studying induced defense responses in conifers. Ethylene also induces similar responses in Douglas fir (*Pseudotsuga menziesii*) and giant sequoia (*Sequoiadendron giganteum*) and since ethylene production is stimulated by MeJA treatment it has been suggested that ethylene is the immediate signal for induced TRD defense responses [83]. However, wound periderm formation and hypersensitive response are not observed in response to MeJA or ethylene treatment, which suggests that other defense signals may be induced by tissue damage [60, 83].

The induced anatomical responses described above are associated with specific changes in the chemical composition of defense-related tissues. In Norway spruce, increased terpenoid accumulation is observed in spruce stems with distinct patterns of TPS expression, enzyme activity, protein abundance and relative terpenoid profiles observed between bark and wood tissue [14, 84]. Volatile emission of terpenoids from needle tissue increases in response to weevil feeding or MeJA treatment with a more complex monoterpene mixture in the induced emissions [15, 85]. The amount and type of

individual phenolic compounds also changes in response to attack [59, 86, 87]. It has been suggested that the specific changes in terpenoid and phenolic composition of spruce stems may be optimized for specific pathogens [59].

Induced defense is both local and systemic. In Norway spruce, the signal spreads axially through the stem at a rate of about 2.5 cm/day [80] with diminishing response strength at greater distances (up to several meters) from the inoculation site [60, 71, 77, 78]. Both anatomical and chemical defense responses are associated with resistance indicated by higher levels of PP cell activation and TRD formation in resistant clones [61, 71, 77, 82]. Pretreatment with MeJA or a sublethal fungal inoculation induces a defense response that is associated with increased resistance to subsequent attack even up to a year after the initial inoculation [78, 79, 88].

While phenolics and terpenoids have been generally localized to individual structures as described above, analysis of the specific chemical contents of individual tissues or cell-types is limited. Auxin gradients have been measured across the CZ by taking serial tangential cryosections through this tissue [21, 22], but whole cryosections still represent a heterogenous mixture of cells and this approach has not been applied broadly to other tissue types. Recently, LMD was used to isolate stone cells from Norway spruce stems for analysis of specific phenolic contents [57] and a similar micrometabolomic approach has been applied to other plant species [56].

Four enzymes involved in terpenoid, phenylpropanoid or ethylene biosynthesis have been localized to specific cell-types during the defense response using immunohistochemical staining. The phenolic biosynthetic enzyme PAL has been localized to phloem PP cells, ray parenchyma cells and TRDs of Norway spruce [61, 71].

However, there is not a corresponding accumulation of phenolics in TRD cells, which suggests that phenolics may be secreted into the contents of the lumen [71]. The same LAS enzyme shown to be localized to CRD epithelial cells in Norway spruce [69] has recently been shown to be expressed in TRD epithelial cells (Zulak *et al.*, unpublished). The ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) have been shown to be localized to CRDs, polyphenolic phloem parenchyma cells and ray parenchyma cells in the bark of MeJA- and wound-induced Douglas fir seedlings [83, 89, 90].

To date, there have been very few reports of RNA transcript analysis of individual cell types in spruce. Protocols for *in situ* hybridization in Norway spruce have been recently reported [91, 92] but have not been applied to studying the defense response. Transcriptome sequencing [93, 94] and microarray analysis [95-97] has been performed for a variety of organs and heterogenous tissues in conifer species, but there have been no reports of RNA transcript profiling in purified populations of individual tissues or cell-types. A tangential cryosectioning approach has been used to measure transcript profiles across the CZ of poplar [23], but this approach has not been widely applied to other species or tissues.

While the anatomical features of the defense response in conifer stems have been well studied with high spatial resolution, the molecular aspects of this response have only been characterized at the level of larger, heterogenous tissues such as bark and wood. A detailed understanding of conifer defense requires a molecular and biochemical characterization of specialized tissues and cell-types associated with the defense response in conifer stems.

1.4 Laser microdissection: A new tool for studying conifer defense

As described above, the products of terpenoid and phenylpropanoid metabolism feature prominently in conifer defense. The role of terpenoid and to a lesser degree phenylpropanoid biosynthetic enzymes have been well characterized during constitutive and induced defense. For terpenoid biosynthesis, this includes DXPS [84, 98], 1-deoxy-D-xylulose 5-phosphate reductoisomerase [98], 4-hydroxyl 3-methylbutenyl diphosphate reductase [98], TPS [14, 15, 84, 98] and cytochromes P450 [99, 100]. For phenylpropanoid biosynthesis, this includes peroxidase [82], dirigent proteins [101, 102], laccase [101], PAL [61, 71], chalcone synthase [82] and stilbene synthase [101]. Additional defense related genes include chitinases [103], enzyme inhibitors and uncharacterized transcription factors and transporters [101]. The defense response is regulated at least in part through octadecanoid signaling including allene oxide synthase and allene oxide cyclase [15] and ethylene signaling including ACS [90] and ACO [83, 89].

Recent efforts have been made to use a multi-level approach to the study of conifer defense by incorporating analysis of RNA transcript abundance, protein abundance, enzyme activity and metabolite profiles [84]. A need has been identified for tissue-specific analysis of RNA transcript, protein and metabolite profiling during the conifer defense response [15, 104]. Laser-assisted microdissection technology provides a means to increase the resolution of conifer defense research by focusing analysis on specific tissues. The approaches described here expand recent methods reported for laser-assisted microdissection and micrometabolomic analysis of stone cells from Norway spruce [57]

to allow analysis of RNA, protein and metabolites from a variety of spruce stem tissues. This study applies these approaches for the targeted analysis of gene expression of terpenoid biosynthetic genes, TPS enzyme activity and terpenoid metabolite profiles in CRD and CZ tissue. These methods will substantially increase the spatial resolution of conifer defense research. Additionally, these methods may be particularly well suited for the study of stem growth, wood development and seasonal dormancy in woody perennials and may also be applied to herbaceous plant species.

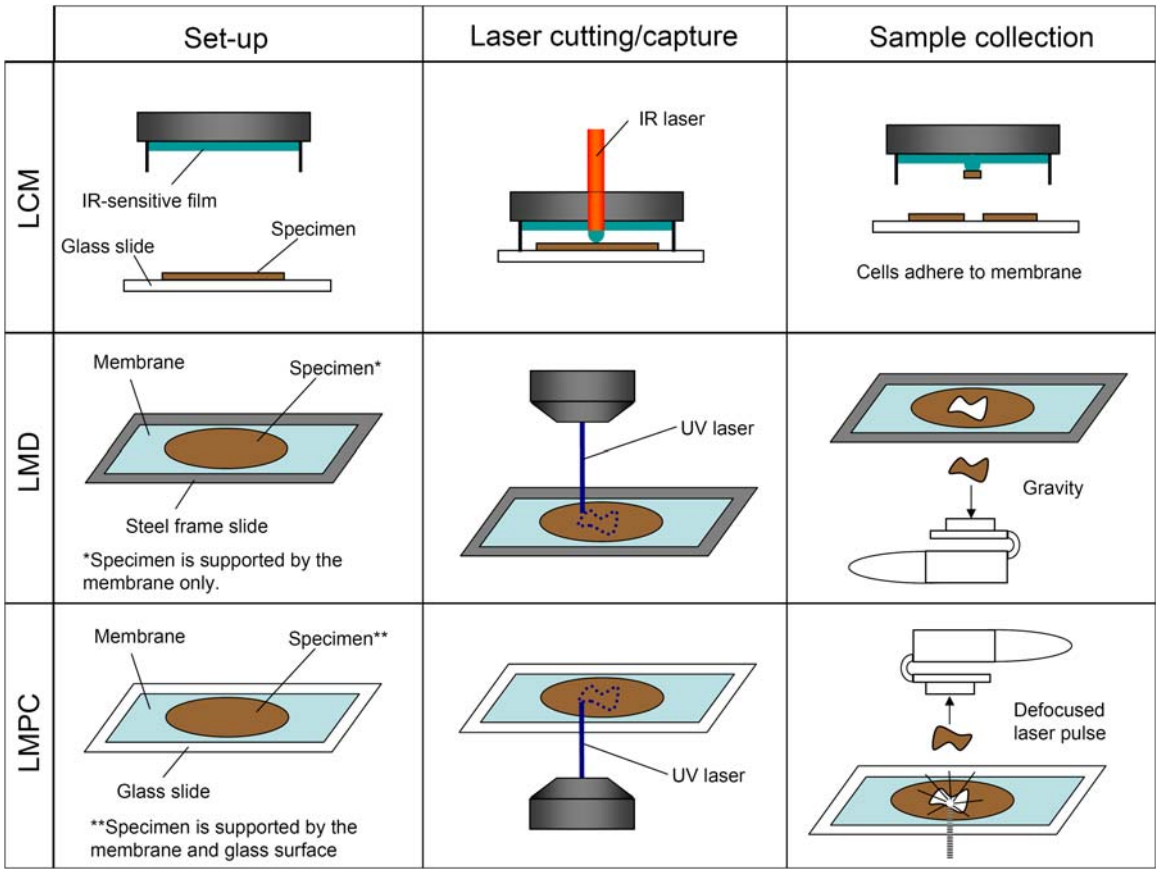

Figure 1.1 – Schematic overview of different laser-assisted microdissection platforms. LCM, laser capture microdissection; LMD, laser microdissection; LMPC, laser microdissection pressure catapulting.

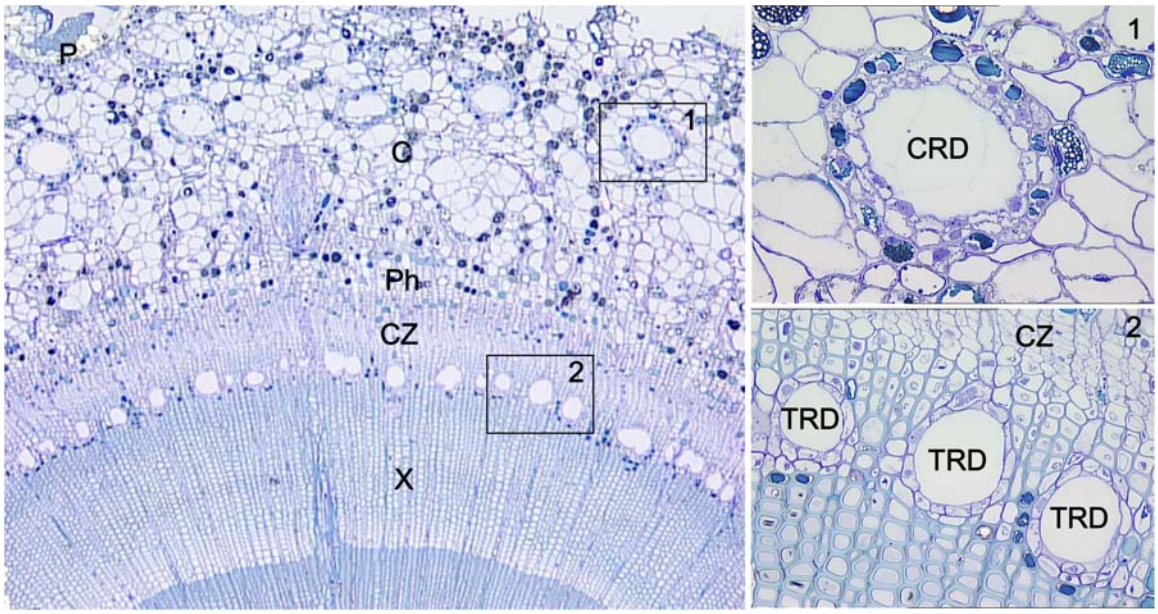

Figure 1.2 – Anatomy of a spruce stem. A Sitka spruce seedling was treated with 0.1% MeJA and harvested 24 days after induction. Stem tissue was fixed in LR white resin and cross-sections were stained with toluidine blue O. P, periderm; C, cortex; CRD, cortical resin duct; Ph, secondary phloem; CZ, cambial zone; X, secondary xylem; TRD, traumatic resin duct. Enlarged insets 1 and 2 are shown to the right. (Photo credit: Dr. Katherine Zulak)

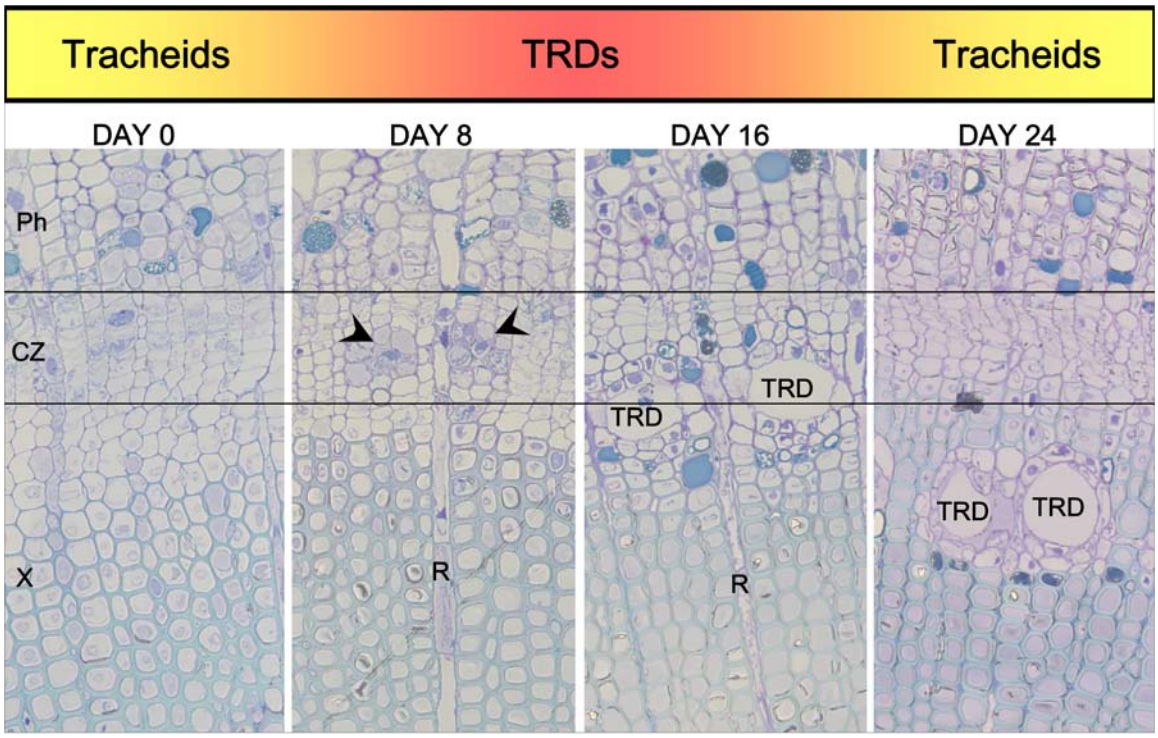

Figure 1.3 – TRD development results from a transient shift in CZ activity from normal tracheid production to TRD production. Sitka spruce seedlings were treated with 0.1% MeJA and harvested 0, 8, 16 and 24 days after induction. Stem tissue was fixed in LR white resin and cross-sections were stained with toluidine blue O. Ph, secondary phloem; CZ, cambial zone; X, secondary xylem; R, ray parenchyma; TRD, traumatic resin duct; arrowhead, immature TRD. (Photo credit: Dr. Katherine Zulak)

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2. ISOLATION OF INDIVIDUAL TISSUES FROM WHITE SPRUCE STEMS USING LASER MICRODISSECTION AND EXTRACTION OF HIGH QUALITY RNA, ENZYMATICALLY ACTIVE PROTEIN AND METABOLITES FOR ANALYSIS OF SPECIALIZED METABOLISM.[1]

2.1 Background

Complex metabolic processes in plants are often localized to specialized cells or tissues. The woody stem of a conifer contains a large number of specialized tissues that are organized in a regular pattern. The outer bark tissue (phloem, cortex and periderm) and the inner wood tissue (xylem) are separated by the cambial zone (CZ) [1]. Initial cells within the CZ give rise to sieve cells, parenchyma cells and fibers towards the phloem and parenchyma cells and tracheids towards the xylem. In spruce, large cortical resin ducts (CRDs) in the bark carry terpene-rich oleoresin that plays a role in defense against biotic stress such as insect feeding, egg deposition, or pathogen inoculation [2, 3]. In response to biotic stress, tracheid mother cells in the CZ are transiently reprogrammed to produce additional traumatic resin ducts before resuming tracheid production, which is associated with increased defense and resistance [4, 5]. Treatment of spruce stems with methyl jasmonate (MeJA) has been shown to elicit a response that mimics the response to biotic stress [6, 7].

[1] A version of this chapter has been submitted for publication. Abbott, E., Hall, D., Hamberger, B. and Bohlmann, J. (2010) Isolation of individual tissues from white spruce stems using laser microdissection and extraction of high quality RNA, enzymatically active protein and metabolites for analysis of specialized metabolism.

A number of different methods have been developed to isolate and enrich individual cell- or tissue-types from plants. In conifers and in other tree species such as poplars, enriched cell populations from stem tissues can be obtained by separating bark from wood [6, 8], taking xylem scrapings [9, 10] and by tangential cryosectioning across the CZ [11-13]. Other methods that have been applied in herbaceous plant species include isolation of glandular trichomes or epidermal cells from plant surfaces by abrasion [14, 15] and generation of protoplasts for fluorescence activated cell sorting [16]. However, these latter methods would be difficult, if not impossible to apply for the isolation of specific cell- or tissue-types from the inner parts of woody stems of perennial species.

Laser microdissection (LMD) is a specific form of laser-assisted microdissection that uses a UV cutting laser to isolate tissues of interest from thin sections of biological samples, which are collected by gravity below the sample. LMD and other forms of laser-assisted microdissection are being applied widely in both animal and plant research [17, 18]. The most common application of laser-assisted microdissection is for RNA isolation and transcript analysis by qRT-PCR and more recently by sequencing using high-throughput technologies [19]. Protein, enzyme and metabolite analysis has been limited partly because amplification is not possible for these molecules. Microdissected tissues have been successfully analyzed using proteomics [20] and metabolomics techniques [21], but there are few reports (none in plants) of isolation of intact protein samples for enzyme assays [22, 23]. LMD has recently been applied successfully for microchemical analysis of stone cells from Norway spruce (*Picea abies*) stems [24], but laser-assisted microdissection has not been widely applied to woody plant tissues. There have been recent reports of a combined analysis of RNA, protein and metabolites from individual

cell-types isolated by other methods [25-27], but to our knowledge these approaches have not been applied to laser microdissected samples.

In woody perennials, laser-assisted microdissection has the potential to further improve the degree of spatial resolution of sample dissection for the study of dynamic, tissue-specific processes. For example, the CZ controls several processes of interest including the periodically alternating events of stem growth and dormancy, wood development and induced defense of traumatic resin duct formation. In CRDs, laser-assisted microdissection may be used to form a better understanding of tissue- and cell-type specific processes of constitutive and possibly induced defense.

This paper reports the successful use of LMD technology for the isolation of individual specialized tissues from white spruce (*Picea glauca*) stems suitable for subsequent combined analysis of RNA transcript abundance, enzyme activity and metabolite profiles. In validation of these combined methods, it is shown that genes involved in terpenoid biosynthesis and defense exhibit differential gene expression patterns between CRD and CZ tissues and in response to methyl jasmonate (MeJA) treatment. It is further demonstrated that active terpene synthase (TPS) enzyme and terpenoid metabolites can be detected and analyzed in laser microdissected CRD and CZ tissues. The methods for LMD combined with analysis of gene expression, enzyme activity and metabolite profiles in microdissected samples will enable a more comprehensive analysis of complex metabolic processes at multiple levels of regulation in individual tissues that are otherwise difficult to access in woody plants.

2.2 Results and discussion

2.2.1 Application of LMD technology to spruce stems

To maximize the number of cells harvested from a sample required the ability to cut large regions from relatively thick tissue sections, which is possible using the LMD system as collection is aided by gravity. Plant tissue is inherently resistant to laser cutting due to the presence of cell walls, which can be highly lignified in spruce stem tissues. LMD was not effective in cutting spruce stem tissue mounted on glass slides because the laser power required was sufficient to etch the surface of a glass polyethylene naphthalate (PEN)-membrane slide resulting in diffusion of the beam and decreased laser cutting efficacy. However, the gravity assisted collection method of LMD permits the use of glass-free, steel frame polyethylene terephthalate (PET)-membrane slides that eliminates the need for glass support, facilitates the use of increased laser power and also allows collection of microdissected tissues in an empty PCR tube cap, which is required for metabolite extractions. LMD was used successfully to cut large areas (>1 mm diameter) from thick sections (>30 μm) mounted on PET-membrane frame slides. For routine LMD applications, 25 μm sections were used. The LMD platform using PET-membrane frame slides was determined to be a highly suitable system for microdissection of spruce stem tissue.

2.2.2 Overview of LMD from spruce stem samples

Sample preparation protocols for LMD can vary substantially depending on the type of tissue and downstream analysis. A single method is described for microdissection of two different specialized tissues from white spruce stems that is suitable for analysis of RNA,

protein and metabolites (Figure 1). Briefly, frozen stem sections are taken from 2-year old white seedlings for cryosectioning in the tangential plane. Cryosections are mounted on a membrane support and specific tissues are isolated using LMD.

2.2.3 Preparing tangential cryosections for LMD

Prior to sectioning, specimens are often fixed and embedded to preserve delicate cell structures [17, 18, 28]. However, fixation can be time consuming, non-uniformly reduces the extractability of molecules from the tissue sample [17, 29] and can be a source of contamination [21]. Formalin fixed, paraffin embedded cross-sections of spruce stems were found to have good morphology, but the RNA was degraded compared to unfixed samples. Sucrose was tested as a cryoprotectant to preserve morphology but was found to interfere with laser cutting. Cryosections without cryoprotection or fixation had reduced morphology, but were still of high enough quality to identify specialized cell types and tissues and gave higher RNA yield and integrity. Therefore, cryosections were taken from unfixed, frozen stem pieces in either cross-section or tangential section orientation. Cryosections were then transferred onto a PET-membrane frame slide containing 100% ethanol (for RNA extraction) or DTT (for protein or metabolite extraction) and allowed to dry thoroughly.

While stem cross-sections allow for the identification of many different tissue types within a single section (Figure 2, top), there is often damage to the cortex, CRD epithelia and CZ tissue, and these cryosections are prone to curling as they dry on the slide (not shown). In contrast, tangential cryosections of woody stems were found to be intact (Figure 2A-D, left panel) and do not curl on the slide unless there is a substantial amount of xylem tissue present. Cell morphology was sufficient to identify major tissue

types from tangential cryosections including CRDs, phloem, CZ and xylem (Figure 2A-D, middle and right panel). By carefully adjusting the sectioning plane, axially oriented tissue can be observed to extend the entire length of a tangential section (up to 10 mm long) compared to cross-sections where these same tissues may occupy only a small percentage of the section area. Tangential cryosections treated with 10 mM DTT had similar morphology but retained pigments such as chlorophyll in the outer stem tissues that are removed by ethanol treatment (not shown). Delicate tissues such as CZ or CRD epithelia were intact even after treatment with 100% ethanol as demonstrated by subsequent staining for cellular contents in CRD epithelial cells (Figure 3A and B). Ethanol dried cryosections have similar morphology to cryosections mounted in 50% glycerol (Figure 3C).

A tangential sectioning orientation is well suited for LMD of spruce stems because morphology is well preserved without cryoprotection or fixation and larger regions of individual tissues are accessible compared to cross-sections, thus requiring fewer cryosections to obtain a large quantity of cells using LMD. Tangential cryosections with similarly intact morphology have also been successfully produced from white spruce needles (data not shown) suggesting that these techniques may be successfully applied to a broad range of cell- and tissue-types from large or small specimens.

2.2.4 LMD of CRD and CZ tissues from tangential cryosections of spruce stems

A series of tangential cryosections from the cortex to the xylem was prepared and mounted on a single LMD frame slide. CRD and CZ tissues were chosen to test LMD applications and subsequent RNA, protein and metabolite analysis because they consist

of metabolically active cells that represent a very small proportion of the spruce stem and are thus good candidates for high resolution enrichment using LMD. CRDs carry terpene-rich oleoresin and terpene synthase enzymes have been shown to be localized to CRD epithelial cells [30]. The CZ contains initial cells for differentiation of all secondary xylem and secondary phloem tissue and thus plays a vital role in stem growth and development as well as the formation of traumatic resin ducts.

Laser microdissected CRD and CZ tissues were collected separately into an empty PCR tube cap and tissues were checked for morphology after laser cutting (Figure 2A-D, middle and right panel). CRD tissue included the epithelial cells immediately lining the resin duct lumen as well as the second cell layer (Figure 2A, left panel and Figure 3). CZ tissue included all thin-walled, light colored cells that could be visually distinguished from fully differentiated xylem and phloem (Figure 2C-D, left panel). Laser settings were the same for cryosections treated with ethanol or DTT. Slides were mounted on the LMD system with tissue sections dried on the bottom surface to prevent microdissected cells from becoming trapped on top of the membrane after cutting. Laser cutting was very efficient with ~90% of microdissected regions being released from surrounding tissue and immediately falling into collection tubes. Regions that did not fall were dislodged using a laser pulse. To avoid cross contamination between different tissues cut from the same cryosections, each tissue type was harvested completely before selecting regions for the next tissue. Cryosections treated with DTT required a longer time to dry on the slide and sections that were not completely dried were difficult to cut with the laser.

The amount of tissue isolated using laser-assisted microdissection is often reported as the number of cells collected. However, cell size and metabolic state varies between different tissues and plant species and the total number of cells is difficult to estimate because the number of partially cut cells contained in thin cryosections depends on the section thickness. To facilitate better comparison of methods for laser-assisted microdissection the amount of microdissected tissue is reported as "µl LMD volume", which is calculated as the microdissected area multiplied by the section thickness. For two year old white spruce stems, the amount of tissue obtained using LMD was greater for CRD tissue with 2.9 ± 0.9 µl LMD volume/cm stem length (n=14) compared to CZ tissue with 1.1 ± 0.3 µl LMD volume/cm stem length (n=14).

2.2.5 RNA extraction from CRD and CZ tissues isolated by LMD

Total RNA was extracted from CRD and CZ tissue isolated by LMD from ethanol treated cryosections obtained from a 6 mm long half stem segment. Cryosections were dried in 100% ethanol and microdissected tissue was collected in RNA lysis solution for extraction. For each RNA extraction, the average LMD volume used was 0.72 ± 0.16 µl (n=3) for CRD and 0.37 ± 0.12 µl (n=3) for CZ, which was sufficient for use with a standard RNA isolation protocol rather than a modified protocol specifically designed for microdissected samples. RNA yield was normalized to the total LMD volume for each tissue type. CZ yielded 357 ± 58 ng RNA/µl LMD volume (n=3), which was more than twice the yield from CRD tissue at 150 ± 12 ng RNA/µl LMD volume (n=3). Incubation of samples at 42°C in lysis buffer prior to RNA extraction did not increase yields. RNA samples were DNase treated and concentrated by ethanol precipitation. There was no measurable loss of RNA during ethanol precipitation (not shown).

RNA integrity was assessed using the Bioanalyzer RNA Pico Assay and expressed as an RNA integrity number (RIN). RNA yield was quantified using the Ribogreen assay, which was found to be robust with no interference from buffer components. High integrity RNA suitable for qRT-PCR analysis and construction of cDNA libraries was obtained from microdissected tissues with CZ RNA being of slightly higher quality than CRD RNA (Figure 4). RNA samples with an RIN greater than 5.0 are suitable for qRT-PCR [31] and an RIN greater than 7.0 is the standard for construction of cDNA libraries [Personal communication, Yungjun Zhao, BC Cancer Agency Genome Sciences Centre, Vancouver, BC]. DNase treatment eliminated background genomic DNA contamination (Figure 4) without decreasing RNA integrity, whereas ethanol precipitation resulted in a decrease of 0.7 RIN units. RNA integrity is of particular concern during LMD because each slide may be at room temperature for over an hour while regions of interest are selected and cut. To assess RNA degradation during this time, whole cross-sections were dried in ethanol on a PET-membrane frame slide and collected in RNA lysis buffer immediately or after incubation at room temperature. There was no detectable decrease in RNA integrity for slides left at room temperature for up to four hours (data not shown). Slides were also stored at -80°C for eight days with no decrease in RNA integrity (data not shown). However, cryosections may fall off the slide during storage and slides must be stored in a dry, airtight container to prevent condensation when they are warmed to room temperature.

To test if RNA integrity can be affected by LMD extensive laser cutting was applied to whole cross-sections but only observed a slightly lower RNA integrity (<1.0 RIN units) compared to RNA extractions from intact whole cross-sections. It was found

that RNA integrity from CRD and CZ tissue after microdissection, DNase treatment and ethanol precipitation is of sufficient quality for downstream applications. It was not necessary to apply any specific treatments to remove polysaccharides or polyphenols from LMD tissue and the use of the Plant RNA Isolation Aid (Ambion, USA) to remove these compounds was found to substantially reduce RNA yields. These results suggest that polysaccharides or polyphenols may not be abundant in CRD and CZ tissue or that these compounds may be efficiently removed during sample preparation and RNA extraction.

2.2.6 Transcript analysis from CRD and CZ tissue isolated by LMD

qRT-PCR is the most common method of quantitative transcript analysis. When transcript abundance is reported as a normalized value relative to a reference gene it is critical to carefully evaluate the reference gene to ensure that it is expressed at constant levels under experimental conditions being tested. Three candidate reference genes were evaluated, including translation initiation factor (TIF), elongation factor (ELF) and tubulin α-subunit (TUB), for expression in spruce stem CRD, CZ and whole cross-sections (Figure 5A). TIF was the most appropriate reference gene because it had the lowest standard deviation across the tissue types tested. Primers specific to a white spruce TPS gene (β-pinene synthase) [GenBank:BT105745] were used to compare relative transcript abundance for a representative monoterpene synthase gene involved in terpenoid defense metabolism [32] between CRD, CZ and whole cross-section tissues by qRT-PCR. β-Pinene synthase transcripts were 12-fold more abundant in CRD tissue, the primary site for constitutive terpenoid accumulation, compared to CZ and whole cross-section tissues (Figure 5B). Similarly, transcripts for levopimaradiene/abietadiene

synthase (LAS) representing a major diterpene synthase for diterpene resin acid biosynthesis was more abundant in CRD than in CZ in untreated trees (Figure 6).

MeJA-inducible changes in transcript abundance were evaluated for four different genes involved in terpenoid biosynthesis, including two monoterpene synthases, a diterpene synthase, and one gene encoding 1-deoxyxylulose-5-phosphate synthase (DXPS) (Figure 6). CRD and CZ tissue were harvested eight days after MeJA treatment as this timepoint has been shown in Norway spruce to be the peak of TPS transcript abundance [32]. Specific TPS genes exhibit unique spatial expression patterns in response to MeJA treatment. In CZ tissue, expression of β-pinene synthase and LAS was up-regulated in response to MeJA treatment, but induction was not significant for 3-carene synthase. High TPS induction in CZ tissue is likely associated with localized traumatic resin duct formation. In CRD tissue, a slight up-regulation is observed for β-pinene synthase and LAS, but this induction is not significant due to high constitutive expression of these genes. However, a significant induction was observed for 3-carene synthase in CRD tissue where constitutive expression is low. Since CRDs are preformed prior to biotic stress they are sometimes referred to as constitutive resin ducts [6, 33, 34]. Since inducible changes in gene expression were observed in this tissue it is suggested that these structures be referred to simply as cortical resin ducts. CRDs are preformed, but can also be activated by MeJA.

DXPS transcript levels were also up-regulated in CRD tissue in response to MeJA treatment, but surprisingly this gene was down-regulated in CZ tissue in response to MeJA treatment. The particular DXPS gene tested here is 100% identical at the nucleotide level to a type II inducible DXPS from Norway spruce that was found to be

up-regulated in the outer stem tissue (including bark and CZ tissue) in response to mechanical wounding, MeJA treatment and inoculation with fungal elicitors [35]. DXPS up-regulation in CRD tissue is consistent with a role in terpenoid oleoresin production. Down-regulation of this same DXPS gene in CZ tissue suggests subfunctionalization of this enzyme between these tissues. Since tracheid formation is transiently arrested during traumatic resin duct formation it is possible that this DXPS gene is involved in wood formation during constitutive growth. White spruce homologues have been identified for each of the three Norway spruce DXPS genes [35] and it is likely that one of these genes plays a role in terpenoid oleoresin production in developing traumatic resin duct tissue.

In previous analysis of DXPS genes in Norway spruce [32, 35], individual tissues were not separated and any detectable down-regulation of DXPS in CZ tissue was likely masked by the up-regulation of DXPS in CRD and other more abundant tissues. It is only by LMD isolation of CZ tissue from other tissues that DXPS down-regulation could be observed in the present study. Thus, LMD is an effective tool for elucidating tissue-specific gene expression patterns that is not possible using more conventional techniques of tissue separation.

2.2.7 Protein extraction from CRD and CZ tissue isolated by LMD and detection of TPS enzyme activity in microdissected CZ tissue

Two-year old, MeJA treated white spruce trees used for protein extraction were harvested 8 days post-induction. This time point coincides with the peak of protein abundance for TPS enzymes in MeJA-induced Norway spruce [32]. For protein extractions, two stem pieces (13-15 mm total length) were taken from the apical end of the first interwhorl (previous year's growth) for LMD of CRD and CZ tissues from three independent

biological replicates. The average LMD volume used for protein extraction was 5.14 ± 1.44 μl (n=3) for CRD tissue and 1.44 ± 0.25 μl (n=3) for CZ tissue. The protein extraction protocol was optimized to isolate active enzymes instead of applying higher yield protocols that use solvent extractions optimized for general proteomics analysis where active enzymes are not required. CZ yielded 31 ± 5 μg protein/μl LMD volume (n=3), double the yield from CRD (16 ± 2 μg protein/μl LMD volume (n=3)). A higher protein yield from CZ compared to CRD tissue correlates well with RNA yield from these tissues.

Monoterpene synthase assays were performed using approximately 30 μg total protein for CRD tissue, 15 μg for CZ tissue and 10 μg for whole cross-sections. The amount of protein used for each assay varied based on the total protein yield from each tissue. Monoterpene synthase enzyme activity was detected in assays with protein extracts from CZ samples and from whole cross-sections. Due to high levels of endogenous monoterpenes that were co-purified during the protein extraction, monoterpene formation could not be detected above background in assays with protein from CRD samples. However, monoterpene synthase enzyme activity was detectable in microdissected CZ tissue. The monoterpene synthase specific activity in MeJA-induced CZ tissue was 1.92 ± 0.31 pkat/mg total protein (n=3), which was twice as large as in whole cross-sections (0.98 ± 0.29 pkat/mg total protein (n=3)). The products of monoterpene synthase activity detected in CZ and whole cross-section extracts are shown in Table 1. Monoterpene synthase activity in the MeJA-induced CZ may be associated with the onset of traumatic resin duct development [6] and the differences in the product

profiles of the monoterpene synthase activity in CZ tissue and whole cross-sections suggests differential expression of TPS gene family members in these tissues.

Treatment of cryosections with DTT was found to be critical to preserving enzyme activity. Using concentrations lower than 10 mM DTT or volumes lower than 2 µl resulted in browning of sections due to oxidation. Protein extractions from oxidized cryosections were degraded with smeared bands on silver stained SDS-PAGE gel (not shown) and no detectable monoterpene synthase enzyme activity. A decrease in the level of specific monoterpene synthase enzyme activity due to laser cutting was not observed.

2.2.8 Extraction and analysis of terpenoid metabolites in CRD and CZ tissue isolated by LMD

Sample preparation for metabolite extraction was similar to protein extraction. Tangential cryosections were taken from two-year old stems harvested 8 days after treatment with MeJA. Metabolite extractions were performed from a single stem piece (5-9 mm long) from the apical end of the first interwhorl of three independent biological replicates. The average LMD volume used for metabolite extraction was 2.68 ± 1.37 µl (n=3) for CRD and 0.70 ± 0.25 µl (n=3) for CZ. Microdissected tissues were transferred to a 2 ml glass gas chromatography vial using a pipette tip that had been dipped in water to reduce static. Metabolites were extracted in 500 µl methyl tert-butyl ether (MTBE) and split into two samples for independent analysis of mono- and diterpenes by gas chromatography-mass spectrometry (GC/MS).

The total monoterpene yield was higher from CRD tissue with 2.39 ± 0.42 µg monoterpenes/µl LMD volume (n=3) compared to 1.81 ± 0.41 µg monoterpenes/µl LMD volume (n=3) from CZ tissue. This trend supports the observation that CRDs are the

primary specialized tissues for oleoresin terpenoid accumulation in conifer stems [3]. The relative abundance of specific monoterpenes is similar between CRD, CZ and whole cross-section tissue, but the total monoterpene abundance is higher in the specialized tissues found in microdissected CRD and CZ samples (Figure 7). While this result is consistent with the general observation that terpenoids accumulate in CRDs and in the MeJA-induced traumatic resin ducts formed from initials in the CZ [3, 6, 7], to the best of our knowledge, these results are the first to specifically localize terpenoid profiles in these tissues. The most abundant monoterpenes detected from CZ tissue [(+)-α-pinene, (-)-α-pinene, (-)-β-pinene and (-)-β-phellandrene] correspond with the most abundant monoterpenes produced from GPP in cell free extracts isolated from this tissue (Table 1). However, the most abundant monoterpenes detected in whole cross-sections (including CRD tissue) do not correspond to the most abundant enzyme products. Monoterpenes are present in large quantities in the constitutive CRD oleoresin, so it is not surprising that newly induced TPS enzymes in this tissue may not have contributed substantially to the total monoterpene content at the time point measured.

Monoterpenes are volatile compounds and also may diffuse to some degree into the drop of 10 mM DTT during sample preparation. Therefore, it was necessary to evaluate the effect of sample processing on the terpenoid profile. Metabolite extractions were performed on cross sections immediately after sectioning or after drying on a drop of 10 mM DTT. As expected, the total monoterpene yield was lower in dried samples, which can be attributed to monoterpene volatility (Additional File Figure S1A), but the relative abundance of individual monoterpenes was not changed substantially (Additional File Figure S1B). The extent of monoterpene diffusion was evaluated by placing a series

of tangential cryosections on 4 μl drops of 10 mM DTT, pipetting up and down, and then collecting the aqueous phase and tangential sections for separate metabolite extractions. Approximately 2-3% of individual monoterpenes were found in the aqueous phase (Data not shown). This is an upper limit for monoterpene diffusion since cryosections are normally placed on a smaller drop of DTT and are not agitated by pipetting. Since volatility and diffusion do not substantially alter the relative monoterpene composition it is likely that the monoterpene profiles measured in microdissected tissues is representative of their relative abundance *in vivo*. However, due to the volatility of monoterpenes the measurement of absolute abundance of monoterpenes using these methods requires the additional use of internal standards.

The feasibility of a qualitative analysis of diterpenoids, the principal non-volatile component of conifer oleoresin [30], was also tested in CRD and CZ tissues isolated by LMD from stem cryosections. The diterpene compounds detected in laser microdissected CRD and CZ tissues were predominantly the diterpene resin acids abietic acid, dehydroabietic acid, isopimaric acid, levopimaric acid, neoabiatic acid, palustric acid, and sandaracopimaric acid, along with minor amounts of the corresponding diterpene aldehydes, alcohols and olefins.

2.3 Conclusions

Laser-assisted microdissection has previously been applied in several herbaceous plant species, mostly for the analysis of RNA transcripts [17, 18]. A method is described for the isolation of individual tissue-types from the stem of white spruce, a woody perennial species that is particularly recalcitrant to tissue- and cell-type specific analysis using

conventional techniques. Sample preparation is simple, robust and may be broadly applicable to RNA, protein and specialized metabolite analysis. The use of tangential cryosections was instrumental in obtaining sufficient quantities of microdissected tissues for protein and metabolite analysis, which is often challenging due to the inability to amplify these molecules. Microdissected tissue quantities were sufficient for RNA isolation using standard protocols designed for larger, non-microdissected samples and transcript analysis by qRT-PCR without RNA amplification. Terpenoid biosynthetic genes were shown to exhibit differential patterns of gene expression between CRD and CZ tissues and in response to MeJA treatment. The detection of TPS enzyme activity from laser microdissected tissues suggests that sample preparation and LMD do not interfere with sensitive downstream biochemical analyses. Successful extraction and quantification of volatile monoterpenes and detection of a range of diterpenoids suggests that metabolite analysis should be amenable to different classes of metabolites.

The combined analysis of RNA, protein, enzyme activity and metabolite profiles from individual conifer stem tissues will be extremely powerful when combined with the use of genomics resources [36, 37] and the application of transcriptomics [10, 38], proteomics [32, 39, 40] and metabolite profiling [32]. Tissue-specific analysis supported by LMD has the potential to substantially improve our understanding of complex processes including cell differentiation and specialization associated with stem growth, wood development and the inducible formation and activation of defense-related structures such as resin ducts. The methods described here should be broadly applicable to both woody and herbaceous species and will be of particular value for those perennial

woody systems where genomics resources are already available such as poplar [41, 42] and grapevines [43, 44].

2.4 Materials and methods

2.4.1 Plant material, methyl jasmonate (MeJA) treatment, and collection of stem samples

White spruce (*Picea glauca*) seedlings of the genotype PG653 were clonally propagated by somatic embryogenesis and provided by Dr. Krystyna Klimaszewska (Natural Resources Canada, Canadian Forest, Sainte-Foy, Québec). Two-year old trees were maintained outside at the University of British Columbia greenhouse and moved, prior to experiments, into controlled greenhouse environments as previously described [8]. Trees were used for experiments at the beginning of the third year growth season and were actively growing with flushing buds present. Untreated trees for RNA extractions were harvested on April 27, 2009. MeJA treated trees for RNA, protein and metabolite extraction were induced on June 1, 2009 by applying a fine spray of 100 ml of 0.1% MeJA (Aldrich, USA) in 0.1% Tween 20 (Fisher Scientific, USA) over the entire stem as described previously [8] and samples were harvested eight days post-induction. Trees were cut at the base and the lateral branches and basal-most 2 cm of the stem were removed and discarded. The remaining stem was divided into pieces of 6 to 8 mm length using a thin razor blade (Wilkinson Sword, Classic), immediately flash frozen in liquid nitrogen and individually stored in 1.5 ml microcentrifuge tubes at -80°C.

2.4.2 Cryosectioning

An individual stem piece was transferred to a Leica model CM3050 cryostat (Leica Microsystems, Germany) and allowed to equilibrate in the cryostat chamber (chamber temperature -15°C and object temperature -25°C) (Figure 2.1B). For subsequent LMD applications, the stem piece was divided in half along the longitudinal axis using a thin razor blade (Wilkinson Sword, Classic) (Figure 2.1C). Care was taken to ensure that any stem defects or buds were oriented away from the cryosectioning surface of the specimen. Each half stem segment was quickly and gently placed flat side down onto a small drop of Optimal Cutting Temperature embedding medium (Sakura Finetek USA, Inc., USA) (Figure 2.1D). Tangential cryosections of 25 μm thickness were taken with the stem axis oriented vertically during sectioning. Cryosections were immediately transferred to either a pool of cold 100% ethanol for RNA extractions or a 2 μl drop of 10 mM DTT for protein and metabolite extractions on a PET-membrane frame slide (#11505151, Leica Microsystems, Germany). Cryosections for morphological characterization were stained with 0.05% Safranin O in 50% glycerol. For other applications not involving LMD, the stem was mounted upright and four to eight cross sections, each of 25 μm thickness, were taken and transferred to a PET-membrane frame slide as for microdissected samples and collected using forceps.

2.4.3 Laser microdissection (LMD)

Cryosections on PET-membrane frame slides were allowed to dry at room temperature before microdissection using a Leica model LMD6000 Laser Microdissection Microscope (Leica Microsystems, Germany) with 5x magnification, laser intensity between 110-128 and speed of 2. Laser microdissected CRD or CZ tissues were

collected into the caps of nuclease free 0.5 ml PCR tubes (Axygen, USA) containing the appropriate buffer for RNA and protein extractions (described below) or into empty caps for metabolite extractions. Buffer crystallization on the LMD collection tube holder was reduced by adding buffer to the cap before mounting it on the holder. When microdissecting multiple tissues from a single cryosection, dissection of one tissue type was completed before starting to cut the next tissue type to avoid cross contamination of tissue samples. The LMD volume for a given sample collection was calculated by multiplying the section thickness by the total area of all microdissected regions for each tissue. Standard deviations are reported for LMD volume.

2.4.4 RNA extractions

RNA was extracted independently from three separate trees using the standard-volume protocol (non-LCM) for the RNAqueous-Micro RNA Isolation Kit (Ambion, USA). Briefly, laser microdissected tissues were collected in 30 µl of RNA lysis solution in a 0.5 ml PCR tube cap and stored at -80°C. PCR tubes were thawed upside down on ice and microdissected tissues were transferred with lysis solution to a nuclease free 1.5 ml microcentrifuge tube containing an additional 30 µl lysis buffer. The PCR cap was washed twice with 20 µl lysis solution to give a total volume of 100 µl lysate for a silica column-based purification according to the manufacturer's protocol with elution of total RNA using 3 x 20 µl elution buffer heated to 75°C. DNase treatment was performed with reagents provided as described in the manufacturer's protocol. RNA samples were concentrated by ethanol precipitation by adding 0.1 volumes of DEPC treated 3M sodium acetate and 2.5 volumes of cold 100% ethanol before freezing at -80°C for at least 30 minutes. RNA pellets were collected by centrifugation (18,000 x *g*, 30 min, 4°C),

supernatant was removed by pipetting and pellets were washed with 100 µl of 70% ethanol made with DEPC-treated water. Centrifugation (18,000 x *g*, 10 min, 4°C) and removal of supernatant was repeated and the pellet was dried at room temperature for 10 min before resuspension in 10 µl DEPC-treated water. RNA quality was assessed using the RNA Pico Assay for the 2100 Bioanalyzer (Agilent, USA). RNA yield was determined by two replicate measurements using the Ribogreen assay (Invitrogen, USA). Standard deviation is reported for RNA yield in order to represent the variation of the extraction protocol.

2.4.5 Quantitative real time PCR (qRT-PCR)

RNA from three independent trees was reverse transcribed in separate reactions using random hexamers and Superscript III reverse transcriptase (Invitrogen, USA). Gene targets were translation initiation factor (TIF), elongation factor (ELF), tubulin α-subunit (TUB), β-pinene synthase (PIN), 3-carene synthase (3CAR), levopimaradiene/abietadiene synthase (LAS) and 1-deoxyxyulose-5-phosphate synthase (DXPS) with the following sequences: ELF-f GTTGCTGTAACAAGATGGATGC; ELF-r CCCTCAAAACCAGAGATAGGC; TIF-f CATCCGCAAGAACGGCTACATC; TIF-r GTAACATGAGGGACATCGCAG; TUB-f TATGATGCCCAGTGATACGTCG; TUB-r ATGGAAGAGCTGCCGGTATGC; PIN-f CTACAAGGCGGACAGAGCC; PIN-r TGATCATGGCGTTGATATGGTC; 3CAR-f GGCTCTCCGTAGACCAACCTCAACTG; 3CAR-r GCACAAACAATATCTCTCCCAGGTCCAATG; LAS-f GGACGATCTCAAGTTGTTTTCCGATTC; LAS-r TGAGAACCACTGTTCCCAGCGC; DXPS-f

AGAAACTCCCTGTGAGATTTGCCCTT; DXPS-r CAACAGTAACTGATATGCCCTGCTGAG. qRT-PCR reactions were performed in 96-well plate (HSP9655, BioRad, USA) sealed with a plastic film (MSB1001, BioRad, USA) using a DNA Engine Opticon 2 (MJ Research, USA) as follows: *Reaction mix:* 0.1 µl cDNA (1.5-14 ng/µl), 3.65 µl DEPC-treated water, 3.75 µl pre-mixed primers (1.2 µM each), 0.03 µl HK-UNG thermolabile uracil N-glycosylase (Epicentre Biotechnologies, USA) and 7.5 µl DyNAmo HS SYBR Green qPCR 2x master mix (Finnzymes, Finland). *PCR program:* 30 minutes at 37°C, 15 minutes at 95°C followed by 45 cycles of (10 s at 94°C, 30 s at 56°C, 30 s at 72°C followed by measurement of reaction fluorescence) and a 10 minute final extension at 72°C. A melting curve was generated from 65°C to 95°C at 0.2°C intervals holding each temperature for 1 s before measuring reaction fluorescence. Data was analyzed using Real Time PCR Miner [45]. Relative transcript abundance was calculated using the equation $1/(1+E)^{CT}$ where the cycle threshold (CT) is the average of four technical PCR replicates and the efficiency (E) is the average of all reactions across all templates for each primer set. Relative transcript abundance was then normalized to TIF expression. Product identity and specificity were confirmed by sequencing amplicons from representative reactions. Reactions with non-discrete melting curves or other anomalies were excluded from analysis.

2.4.6 Protein extractions

Protein was extracted independently from three separate trees treated with MeJA. For each tree, microdissected tissue from two stem pieces (~16 mm combined length) was collected in four 0.5 ml PCR tube caps each containing 30 µl protein extraction buffer (50 mM HEPES pH 7.2, 5 mM DTT, 5 mM ascorbic acid, 5 mM sodium bisulfite, 10

mM $MgCl_2$, 10% glycerol, 1% polyvinylpyrrolidone (PVPP), 0.1% Tween 20) and stored at -80°C. Samples from each tree were thawed upside down on ice and contents were pooled into a 1.5 ml microfuge tube and the PCR tube caps were each washed with 20 µl protein extraction buffer for a total volume of 200 µl. This volume was then supplemented with PVPP (1% w/v) and the protease cocktail described in Lippert et al., (2009) [40] modified by adding 10 mM 1,10-phenanthroline (Sigma, USA), 0.5 mM PMSF (Sigma, USA) and by using 1 µM E64 and 0.5 mM AEBSF. The sample was then ground by hand with a microtube pellet pestle (Kontes, USA) for 1 min, gently vortexed intermittently for 1 min and sonicated for 10 min in a 4°C water bath sonicator (Model 1510, Branson, USA). Homogenized samples were centrifuged at 120 x *g* for 10 min at 4°C and 130 µl of supernatant was desalted using a PD25 spintrap desalting column (#28-9180-04, GE Healthcare, USA) equilibrated with metal ion free desalting buffer (25 mM HEPES pH 7.2, 100 mM KCl, 10% glycerol). Protein concentration was determined by Bradford assay. Standard deviation is reported for protein yield in order to represent the variation of the extraction protocol.

2.4.7 Monoterpene synthase enzyme assays

Enzyme assays were performed on protein samples from three separate MeJA-treated trees based on previously published methods developed for large volume biological samples [6, 46, 47] with minor modifications for laser microdissected tissue samples. Briefly, 40-55 µl cell free total protein extract with a protein content of 10-30 µg was combined with geranyl pyrophosphate (GPP, Echelon Biosciences Inc., USA) and monoterpene synthase buffer (25 mM HEPES pH 7.2, 100 mM KCl, 10 mM $MnCl_2$, 10% glycerol, 5 mM DTT) to a total volume of 500 µl and a final substrate concentration of 50

μM in a 2 ml GC vial. As a control, protein samples were boiled for 10 min prior to assay. The aqueous assay mixture was overlaid with 0.5 ml pentane containing 2.5 μM isobutyl benzene as an internal standard and incubated at 30°C for 100 minutes. Assays were vortexed for 20 s and immediately stored for at least 30 min at -80°C before centrifugation (1000 x *g*, 4°C, 30 min) and GC/MS analysis as described below. Standard error is reported for enzyme activity.

2.4.8 Metabolite extractions

Terpenoid metabolites were extracted from three separate MeJA-treated trees based on previously published methods [6, 48] with the following modifications for laser microdissected tissues. Microdissected tissues from a single stem piece (~8 mm length) were collected in an empty 0.5 ml PCR tube cap and transferred to a GC vial (#5183-2072, Agilent, USA) using a wet pipette tip (to reduce static) and stored at -80°C until extraction. Metabolites were extracted in 500 μl MTBE containing 3.0 μM isobutyl benzene as an internal standard and shaken vigorously overnight at room temperature. For monoterpene analysis, 400 μl of MTBE extract was combined with 150 μl of 0.1 M ammonium carbonate (pH 8) and vortexed for 20 s before carefully transferring the top ether layer to a new GC vial insert for GC/MS analysis. For diterpene analysis, 100 μl of MTBE extract was combined with 40 μl methanol and 40 μl TMS-diazomethane and incubated for 45-60 min at room temperature. Samples for diterpene analysis were dried under high purity grade nitrogen and resuspended in 100 μl diethyl ether (inhibitor free, HPLC grade) and transferred to a fresh GC vial insert for GC/MS analysis. Standard error

is reported for metabolite yields in order to represent the variation of the extraction protocol.

2.4.9 Metabolite analysis by gas chromatography-mass spectrometry (GC/MS)

The protocol for GC/MS analysis of mono- and diterpenoids was based on previously published methods [6, 48]. Metabolites in 1 or 2 μl pentane (extracted from enzyme assays) or MTBE (extracted directly from tissue) were identified using a GC (Agilent 6890A series) coupled with a mass spectrometer (5973N mass selective detector, quadropole analyzer, electron ionization, 70 eV). Metabolite identification was based on comparison of retention times to authentic standards as well as comparison to mass spectral libraries (Wiley7Nist05). Monoterpenes were separated using a DB-WAX capillary column (J&W 122-7032, 0.25 mm diameter, 30 m length, 0.25 μm film thickness) with the following program: 4 min at 40°C, increase by 3°C/min to 85°C, increase by 30°C/min to 250°C, hold for 2.5 min (Injector = 250°C, initial flow rate = 1.4 ml He/min, total run time 27.00 min). Stereochemistry of monoterpenes was determined for compounds where authentic standards were available by separation on a Cyclodex-B chiral capillary column (J&W 112-2532, 0.25 mm diameter, 30 m length, 0.25 μm film thickness) with the following program: 1 min at 55°C, increase by 1°C/min to 100°C, increase by 10°C/min to 230°C, hold for 10 min (Injector = 250°C, initial flow rate = 1.0 ml He/min, total run time 69.00 min). Diterpene compounds were separated using an AT-1000 capillary column (Alltech A-13783, 0.25 mm diameter, 30 m length, 0.25 μm film thickness) with the following program: 1 min at 150°C, increase by 1.5°C/min to 220°C,

increase by 20°C/min to 240°C, hold for 15 min (Injector = 250°C, initial flow rate = 1.0 ml He/min, total run time 63.67 min).

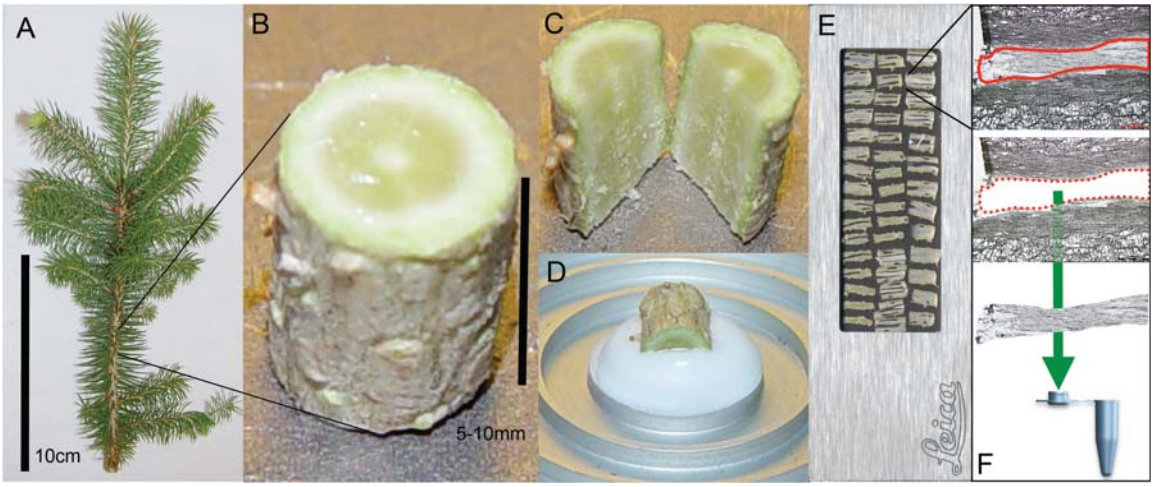

Figure 2.1 – Schematic overview of sample preparation for laser microdissection of spruce stems. (A) 2-year old white spruce harvested above the root. (B) Stems were divided into 5 – 10 mm long pieces that were flash frozen in liquid nitrogen. (C) Stem pieces were split in half longitudinally prior to cryosectioning. (D) Half stem segment mounted for tangential cryosectioning. (E) Tangential cryosections on a PET-membrane frame slide. (F) Laser microdissection: Regions of interest are selected, cut with a laser and selected cells fall by gravity into a collection tube.

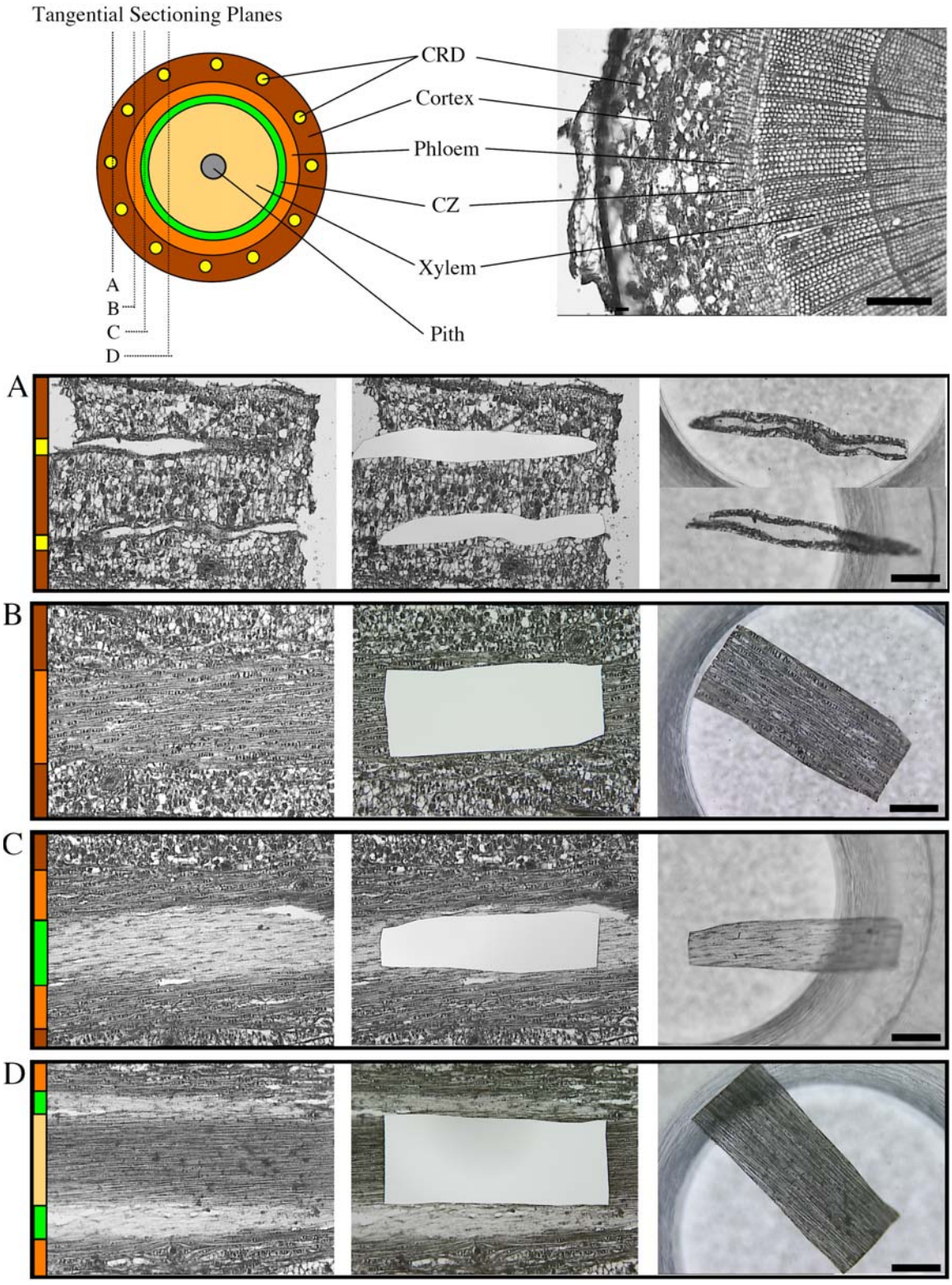

Figure 2.2 – Identification and microdissection of individual tissue types from tangential cryosections. (Top) Schematic diagram and image of a stem cross-section

showing an overview of tissue types. Letters A, B, C and D in the schematic indicate tangential cryosection planes corresponding to the figure panels (A), (B), (C) and (D) below. (A-D) Tissue images of tangential cryosections at different stem depths. For each horizontal figure panel (A) – (D), the left image shows tissue cryosection prior to laser cutting; the centre image shows the same tissue cryosection after laser cutting; and the right image shows the laser microdissected tissues in collection tube. Tissue types are indicated by the color bar on the left side of panels (A) – (D) where colors correspond to the colors in the schematic of the cross section (Top): Brown is cortex, yellow is cortical resin duct (CRD), orange is phloem, green is cambium zone (CZ), yellow is xylem, and grey is pith. (A) Tangential cryosection containing cortex and CRD tissues. (B) Tangential cryosection containing cortex and phloem tissues. (C) Tangential cryosection containing cortex, phloem, and cambium zone tissues. (D) Tangential cryosection containing phloem, cambium zone, and xylem tissues. CRD: Cortical resin duct; CZ: Cambium zone. Length of scale bar is 200 μm for the cross-section and 400 μm for tangential panels (A) – (D).

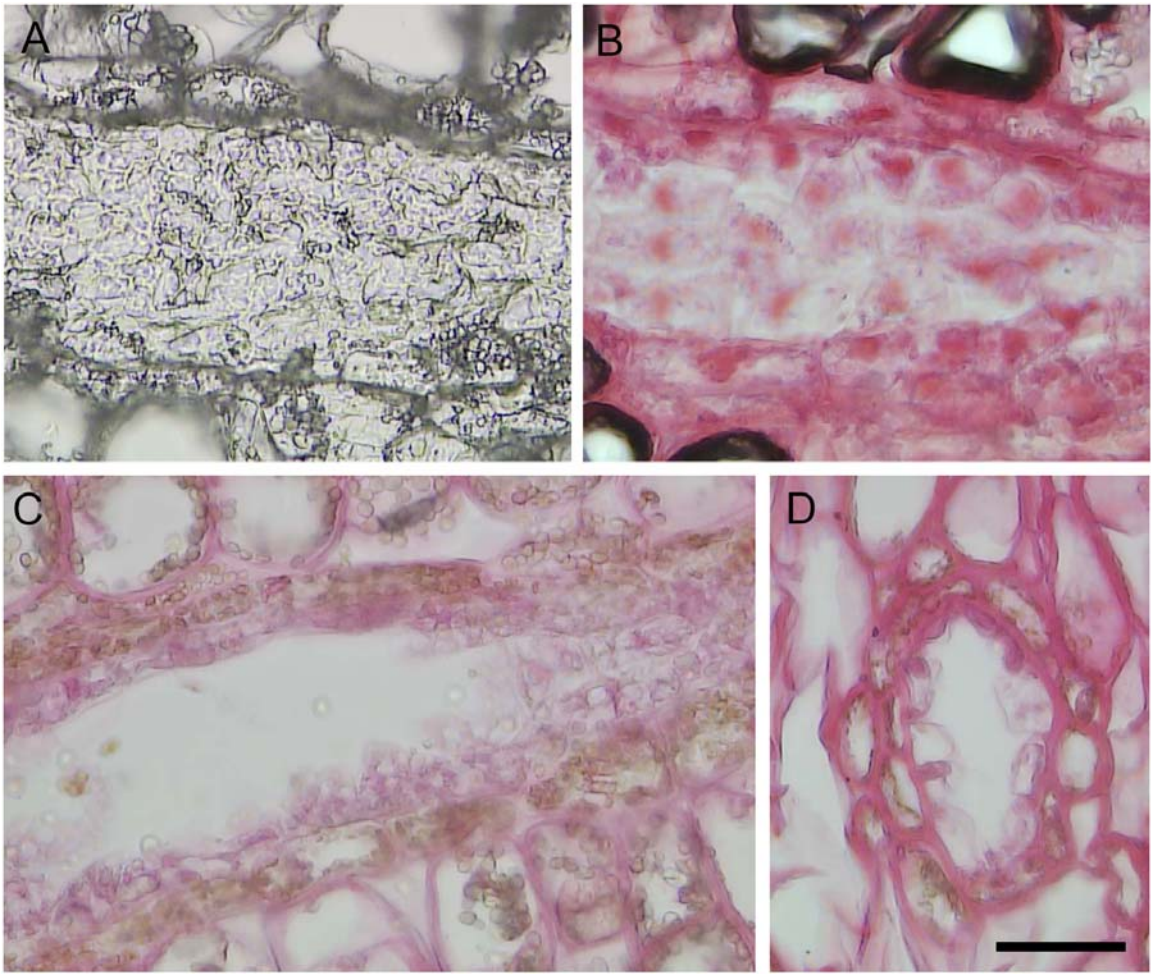

Figure 2.3 – Characterization of CRD morphology before and after treatment with 100% ethanol. (A) Cross-section of CRD and surrounding cortex tissue stained with Safranin O. (B) Tangential section of CRD stained with Safranin O. (C) Tangential section of CRD treated with 100% ethanol. (D) Tangential section of CRD treated with 100% ethanol and stained with Safranin O. Length of scale bar is 50 µm.

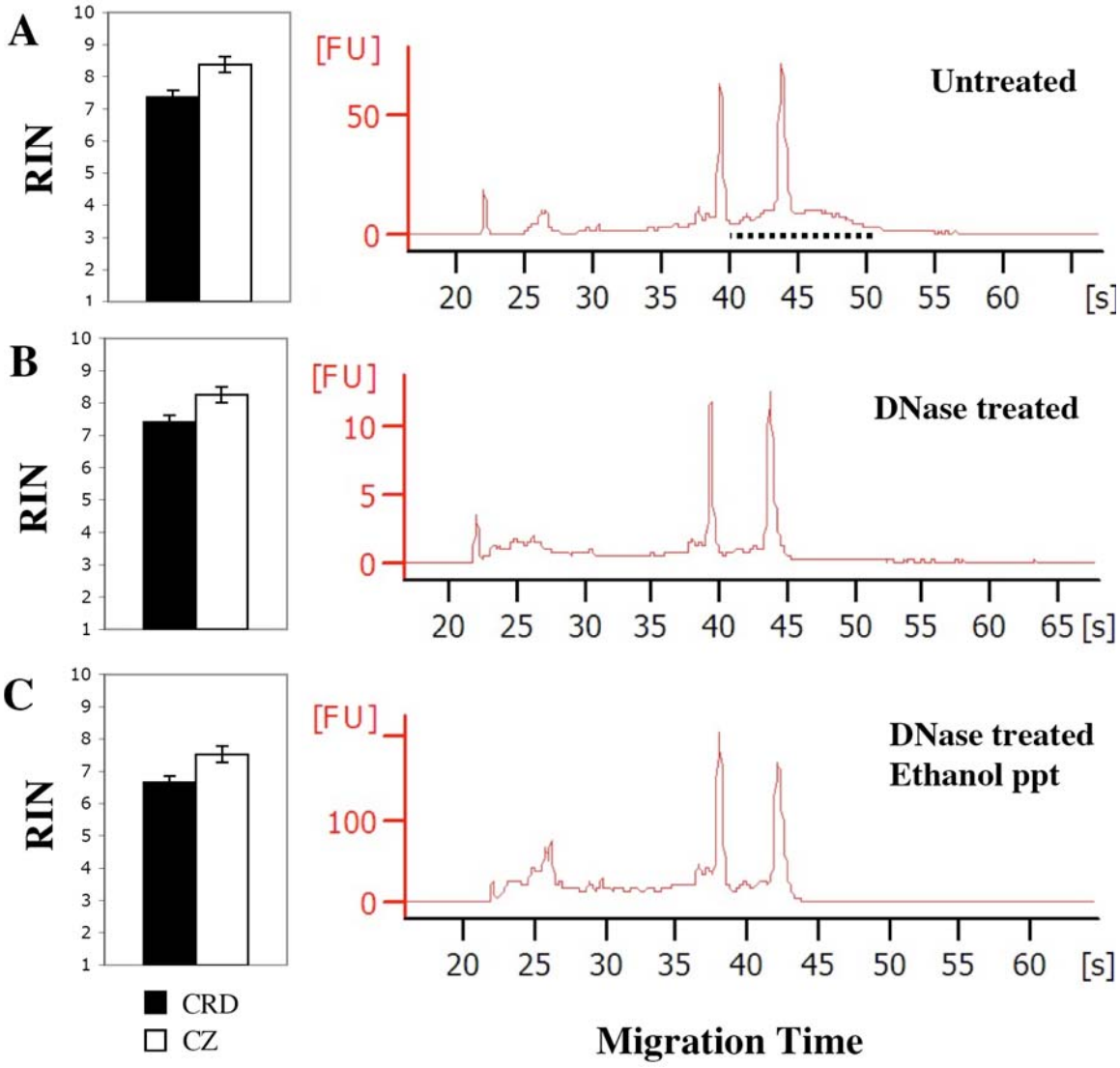

Figure 2.4 – RNA integrity of untreated, DNase treated and DNase/ethanol precipitated RNA samples from CRD and cambial zone tissue. RNA integrity number (left) and a representative Bioanalyzer electropherogram (right) is shown for each treatment. (A) Untreated, (B) DNase treated, (C) DNase treated and ethanol precipitated RNA samples. Error bars represent standard error of three biological replicates. The dotted line in untreated samples (A) represents a region of genomic DNA background contamination that is removed by DNase treatment (B and C). RIN: RNA integrity number; FU: Fluorescence units.

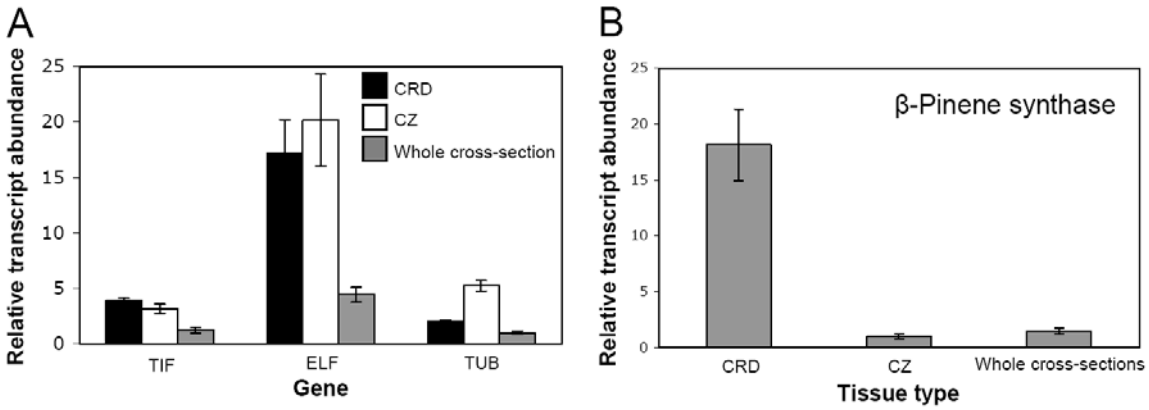

Figure 2.5 – Validation of reference genes for evaluation of monoterpene synthase transcript abundance between CRD, CZ and whole cross-section tissues. (A) Relative mRNA abundance of candidate reference genes normalized to RNA concentration. TIF: Translation initiation factor; ELF: Elongation factor; TUB: Tubulin α-subunit. (B) Relative transcript abundance of β-pinene synthase in different tissue types. Error bars represent the standard error of three biological replicates.

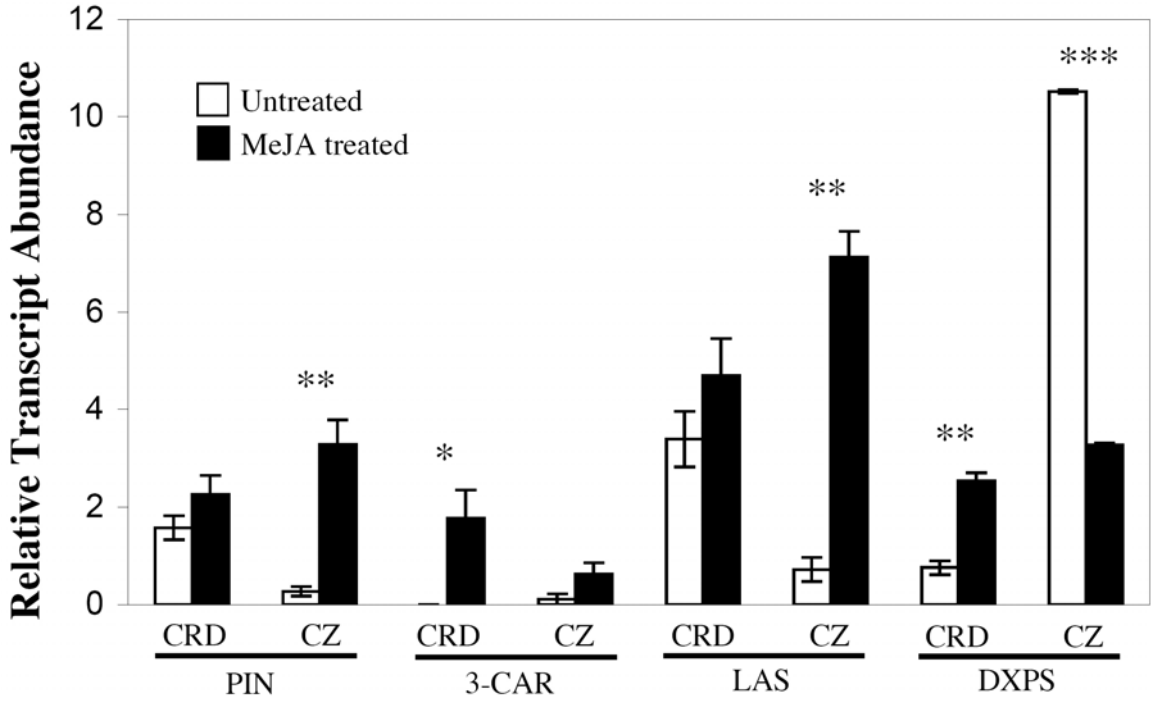

Figure 2.6 – Relative transcript abundance of selected terpenoid biosynthetic genes. PIN: β-pinene synthase; 3-CAR: 3-carene synthase; LAS: levopimaradiene/abietadiene synthase; DXPS: 1-deoxyxyulose-5-phosphate synthase. Error bars are the standard error of three biological replicates for all CRD and MeJA treated CZ but was restricted to two biological replicates for untreated CZ due poor template quality for one sample and limited quantity of biological material. Statistical significance represents a Student's T-test comparison of MeJA treated versus untreated samples with (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$.

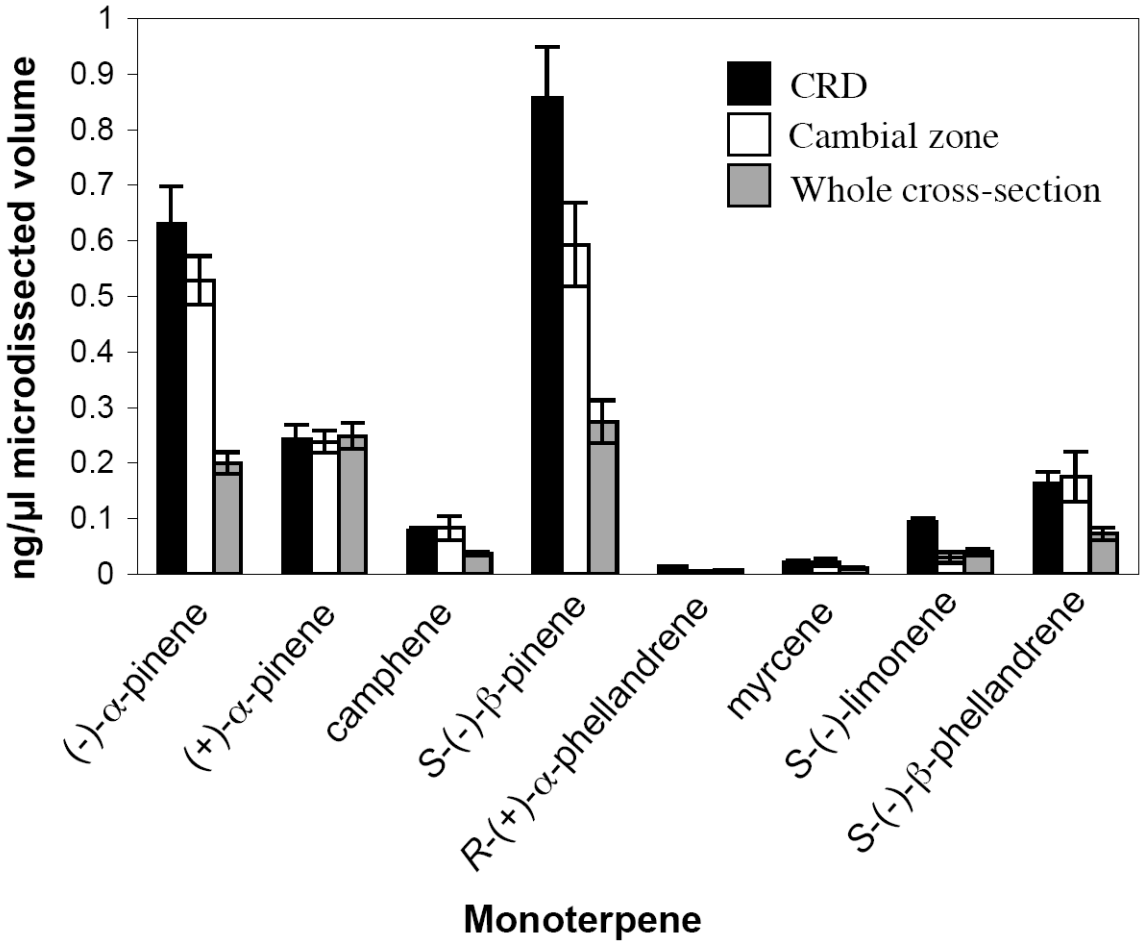

Figure 2.7 – Monoterpenes detected by GC/MS in metabolite extracts from CRD, CZ and whole cross-section tissue. Only monoterpenes representing >1% total monoterpene content are shown. Error bars are the standard error of three biological replicates.

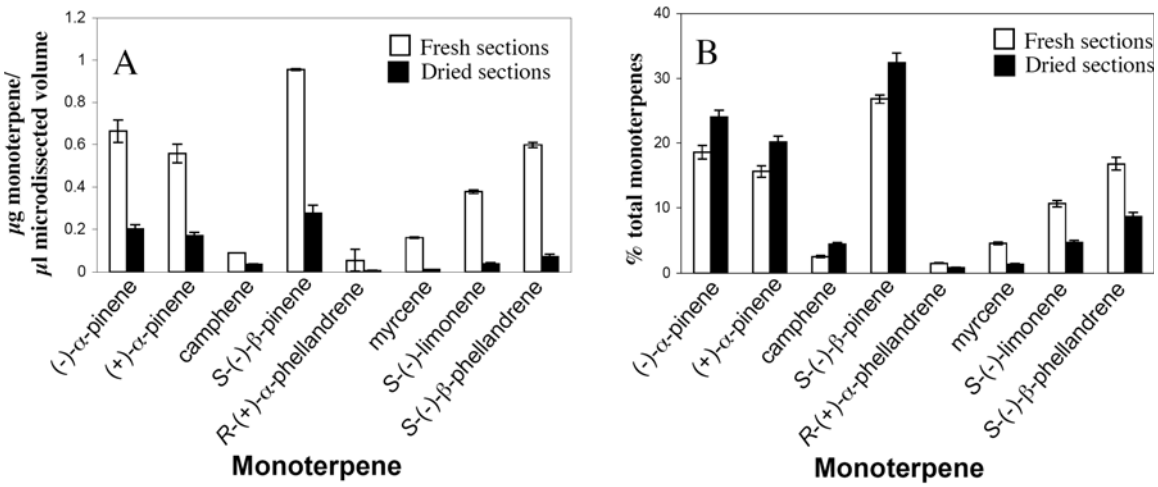

Figure 2.8 – Effects of drying whole cross-sections in a drop of 10 mM DTT on monoterpene yield and relative monoterpene profile. (A) Monoterpene yield; (B) Relative monoterpene profile. Only monoterpenes representing >1% total monoterpene yield are shown. Error bars represent the standard error of three biological replicates.

Table 2.1 – Monoterpenes formed from geranyl diphosphate in cell-free enzyme assays of total protein extracts from CZ laser microdissected tissue and whole cross sections.

	Cambial Zone		Whole Cross Sections	
Product	**% Total**	**St. Error**	**% Total**	**St. Error**
(-)-α-Pinene	22.2	1.0	3.9	0.8
(+)-α-Pinene	19.0	0.8	2.9	0.5
(-)-β-Phellandrene	28.4	0.7	12.3	3.7
(-)-β-Pinene	20.7	1.0	8.4	0.3
(+)-3-Carene	8.1	1.2	25.6	2.2
(-)-Limonene	1.7	0.9	15.6	2.1
α-Terpinolene	nd	nd	14.9	2.1
(+)-Sabinene	nd	nd	11.9	2.4
Myrcene	nd	nd	4.5	1.9

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3. CONCLUSION

3.1 Suitability of LMD system for application to woody plant stems

3.1.1 Comparison of different LAM systems

Laser-assisted microdissection has been utilized for a broad range of analyses in a variety of plant species. While RNA can be amplified from very small samples, protein and metabolite analysis require large quantities of microdissected samples. Large samples are most efficiently obtained by selecting large areas from relatively thick cryosections. Three separate laser-assisted microdissection platforms were considered for application to spruce stem tissue: laser capture microdissection (LCM; Pixcell IIe, Arcturus, USA), laser microdissection (LMD; LMD6000, Leica Microsystems, Germany) and laser microdissection pressure catapulting (LMPC; PALM MicroBeam, Carl Zeiss, Germany).

The LCM system uses a thermoplastic film that is activated by an infrared laser to expand and adhere to cells of interest. The LCM platform was is the oldest type of laser-assisted microdissection [1] and has been widely utilized for application to plants [2, 3]. The LCM method requires that the selected cells be torn away from the rest of the tissue, which requires a cryosection thickness of 5-10 μm. While the LCM method has been successfully applied to herbaceous plant species, it was expected that the dense, highly lignified tissues in the secondary phloem and secondary xylem would not tear easily from surrounding tissue using this system, especially with thicker cryosections. Also, LCM is time consuming for large areas because a relatively thin laser beam must trace across each point within the area. Thus, the LCM platform did not receive additional consideration.

The LMPC and LMD systems both use a UV laser to cut around the perimeter of a region of interest within a cryosection. Applications to herbaceous plants have been reported for both LMPC [4-10] and LMD [11-20], but only LMD has been reported to be used on woody spruce stem tissue [18]. Metabolite analysis has been reported using both LMPC [21] and LMD [18, 19], but only the LMPC system has been reported for application to protein analysis [4].

For LMD, cut regions fall by gravity into a collection tube below the sample, whereas for LMPC the cut regions are catapulted into a collection tube above the sample using a defocused laser pulse. Both systems are well suited to the isolation of larger areas since the laser path is shorter when tracing the perimeter instead of the area. However, during demonstrations spruce stem tissues could not be reliably cut using the LMPC system, even with very high laser power. Similar problems were encountered using the LMD system where the laser power required to cut spruce stem tissues were capable of etching the glass surface of the slide, resulting in diffusion of the laser beam. This problem was circumvented with the LMD system by using glass-free steel frame membrane slides. This type of slide is not compatible with the LMPC system because cut cells must be supported on a glass surface before they are catapulted into the collection tube. PALM states that regions up to 1000 μm could be obtained using the LMPC system (http://palm.dasat.ch/dasat/index.php?cid=100150&sid=dasat), but there is no such theoretical limit for the LMD system with collection aided by gravity. The LMD system is capable of collecting cut cells in an empty collection tube, whereas the LMPC system requires that cut cells be captured on the liquid surface within an inverted collection tube above the sample. The liquid surface may become saturated with large quantities of cut

cells, which imposes a limit to the number of cells that can be harvested into a single tube using the LMPC system.

The ability to use glass-free, steel frame membrane slides enables the use of high laser power necessary to cut through thick cryosections (>20 μm) of secondary xylem and secondary phloem tissue. The gravity collection method enables the isolation of very large regions of individual tissues. The LMD system was therefore determined to be best suited for isolation of individual tissues from spruce stems. It is also interesting to note that the same authors who used the LMPC system for gene expression analysis of nematode infected soybean roots [22] later switched to using the LMD system for a microarray study of the same biological material [17].

3.1.2 LMD of spruce stem tissue in the context of previous applications of laser-assisted microdissection in plants

While laser-assisted microdissection is not a new concept, it has been primarily used for biomedical applications or for the study of herbaceous plant species. The type of sample, tissue of interest and type of downstream analysis dictate how samples must be prepared for LMD. Tissue fixation and histological staining are often required to obtain cryosections of sufficient morphological quality to identify tissues of interest for laser-assisted microdissection. However, fixation and staining can result in degradation and reduced extractability of biological molecules [2, 4, 23]. Fortunately, due to the rigid nature of a spruce stem, it was possible to take cryosections from unfixed, frozen samples. Cryosections can be dehydrated by treatment with 100% ethanol without substantially compromising tissue morphology (Figure 2.3).

Small sample sizes are often a limiting factor for laser-assisted microdissection studies. RNA samples may be amplified, whereas extensive and laborious tissue harvesting may be required to obtain large enough samples for protein or metabolite analysis. By using tangential cryosections of spruce stems, it is possible to identify several individual tissues including the periderm, cortex, CRDs, secondary phloem, CZ, secondary xylem, pith and ray parenchyma. Cryosections may be taken from lengths of stem up to 10 mm in length, which allows for LMD of large quantities of tissue from relatively few sections, thus reducing harvesting time.

The LMD method was applied for the isolation of CRD, CZ and other stem tissues from tangential cryosections of white spruce stems. It is difficult to compare tissue quantities between different laser-assisted microdissection studies due to inconsistent estimations of cell number. I propose that "microdissected volume" is a more consistent measure for comparison of microdissected samples. For example, laser microdissection was performed on ~500 *Arabidopsis* cross-sections (30 µm thick) to obtain 5000 vascular bundle regions for proteomic analysis [4]. Using an estimate of 12,000 μm^2 for the cross sectional area of each vascular bundle this is equivalent to a microdissected volume of 1.80 µl. By comparison, 1.44 µl of CZ tissue was obtained from ~40 tangential sections of white spruce stems.

Thus, the LMD methods described here are well suited for isolation of individual tissues such as CRD and CZ from spruce stems. A tangential cryosectioning approach facilitates the isolation of large quantities of tissue from relatively few cryosections. Since fixation and staining are not required, sample processing time is reduced relative to other protocols and the integrity of biological molecules is preserved enabling a broad

range of downstream analyses. Initial experiments have shown that these methods can be applied for the isolation of individual tissues from tangential cryosections of white spruce needles using LMD. This demonstrates that these approaches can be applied to non-woody tissue and suggests that they may be applied to a range of herbaceous plant species.

3.1.3 Detailed protocols for application of LMD to spruce stems

A detailed protocol was developed for isolation of individual tissues from spruce stem samples using the LMD system. A tangential cryosectioning approach was critical for the efficient procurement of large quantities of CRD and CZ tissues, which are difficult to harvest from cross-sections. While the necessary protocol details are described in Chapter 2.4, an extended description of these protocols is given in the appendices, which includes a discussion of method development, trouble-shooting and optimization. Appendix A describes a protocol for harvesting spruce seedlings into frozen stem segments suitable for cryosectioning and LMD. Appendix B describes methods for cryosectioning these stem segments and isolation of individual tissues by LMD for subsequent extraction of RNA, protein or metabolites. These appendices are intended to act as standalone reference documents for future users of the LMD system for isolation of individual tissues from spruce stems.

3.2 RNA analysis

3.2.1 qRT-PCR analysis

RNA extracted from microdissected CRD and CZ tissue was of sufficient quality for gene expression analysis by qRT-PCR (Figure 2.4) [24]. A single reference gene (TIF)

was chosen from a panel of three candidates based on low deviation in expression between CRD, CZ and whole cross-section tissue (Figure 2.5A). While TIF is certainly a useful reference gene, it is important to note that a reference must always be evaluated for constant expression under the experimental conditions tested. Future analysis of different tissues and cell-types, or additional experimental treatments such as insect feeding and fungal inoculation will require validation of candidate reference genes under these conditions. Thus, it would be useful to evaluate additional candidate reference genes that could be used under these conditions. Alternatively, multiple reference genes may be combined to calculate a single normalization factor with lower variation than the expression of any single reference gene [25, 26].

Spatial and temporal patterns of gene expression were evaluated for a set of genes involved in terpenoid metabolism (Figure 2.6). Prior to MeJA treatment, expression of β-pinene synthase and LAS was higher in CRD tissue compared to CZ tissue, which is consistent with the constitutive accumulation of terpenoid-rich oleoresin in CRDs. Constitutive expression of 3-carene synthase was low in both CRD and CZ tissue. DXPS expression was highest in CZ relative to CRD tissue.

After MeJA treatment, β-pinene synthase and LAS expression increased in CZ tissue. At eight days post-MeJA treatment, developing TRDs are contained within the CZ tissue as normal tracheid production has not yet resumed to enclose TRDs within the secondary xylem [27-31]. Therefore, increased TPS expression in CZ tissue in response to MeJA treatment is likely associated with developing TRD cells. In CRD tissue, a slight increase in β-pinene synthase and LAS expression is observed, but is not statistically significant due to high constitutive expression of these genes. However, a significant up-

regulation is observed for 3-carene synthase in CRD tissue after MeJA treatment. CRDs have been previously referred to in the literature as “constitutive resin ducts” because they are pre-formed [32-34]. Since inducible gene expression changes were specifically localized to CRD tissue in response to MeJA treatment, it is suggested that CRDs be referred to simply as “cortical resin ducts”. This will facilitate discussion of the differences between constitutive CRD (cCRD) and induced CRD (iCRD) tissue.

DXPS exhibited differential induction patterns between CRD and CZ tissue. Expression of DXPS was up-regulated in CRD tissue in response to MeJA treatment, which is consistent with previously observed up-regulation in Norway spruce bark [35]. Surprisingly, DXPS expression was down-regulated in CZ tissue in response to MeJA treatment. The differential induction pattern observed here suggests subfunctionalization of this DXPS gene between CRD and CZ tissue. For example, DXPS may be involved in terpenoid oleoresin production in CRD tissue, whereas the same gene may contribute to wood development in CZ tissue. There have been three DXPS genes identified in Norway spruce [35] and it is likely that another DXPS gene is involved in terpenoid production in developing TRD tissue.

Analysis of whole bark tissue has shown maximum TPS expression [36] as well as increased DXPS expression at eight days post-MeJA treatment. Bark samples are comprised of many tissues including periderm, cortex, secondary phloem and CZ. The spatial and temporal differences in TPS and DXPS expression between CRD and CZ tissue described above are masked by analysis of whole bark samples. This serves to underscore the utility of an LMD-based approach for the analysis of tissue-specific gene expression patterns between individual tissues. This approach may be applied widely to

the study of terpenoid defenses by incorporating a larger set of TPS and DXPS genes, additional upstream genes such as prenyl transferases and terpenoid modifying enzymes such as cytochromes P450. Broader applications for conifer defense may include analysis of phenylpropanoid metabolism, chitinases and defense-related transcription factors and transporters. Applications also exist for the study of developmental processes controlled by CZ tissue such as wood development and seasonal dormancy patterns within the stem.

3.2.2 Transcriptomic analysis

Transcriptome analysis by cDNA library sequencing typically requires large amounts of RNA, usually greater than 1 µg of total RNA. To the best of my knowledge, all cDNA sequencing analyses from microdissected plant samples published to date have required amplification of RNA (or cDNA) samples and/or pooling of samples. The total RNA yield from microdissected samples was 419 ± 105 ng RNA/cm stem length for CZ tissue and 351 ± 69 ng RNA/cm stem length for CRD tissue. Thus, greater than 1 µg total RNA can be obtained from CRD or CZ tissues microdissected from less than 3 cm of stem length. Thus, using the protocols described here it is possible to obtain sufficient quantities of total RNA for construction and sequencing of a cDNA library without the need for amplification or pooling of biological replicates. However, protocols requiring larger quantities of RNA for cDNA library production or for microarray analysis would require an additional amplification step.

In our lab, cDNA libraries have recently been generated from microdissected CRD and CZ tissue for Sanger sequencing. The EST quality is comparable to other libraries generated from whole organs such as bark, wood and needles using similar

protocols. It is expected that these libraries will be useful resources for discovery of rare transcripts such as low expression enzymes, transcription factors and transporters that may be localized to these tissues. In addition to expanding our list of genes involved in terpenoid metabolism, it would be particularly interesting to mine this resource for possible terpenoid transporters. To date, no functionally characterized terpenoid transporter has been identified and the mechanism by which terpenoid oleoresin is synthesized and accumulates in the lumen of a resin duct is unknown. It would also be interesting to study the developmental transition that occurs in CZ tissue during TRD formation in response to MeJA treatment.

3.3 Protein analysis

3.3.1 Terpene synthase enzyme assays

Total protein was extracted from CRD and CZ tissue from MeJA treated stems for TPS enzyme assay. The protein yield was 53 ± 12 μg protein/cm stem length for CRD tissue and 30 ± 9 μg protein/cm stem length for CZ tissue. Total protein yields from 13-15 mm of stem were sufficient for three TPS enzyme assays. Terpene product formation was detected in protein extracts from CZ tissue. The relative abundance of terpenoid products produced from CZ assays was different from similar assays from whole cross-section tissue (Table 1). To the best of my knowledge, this is the first report of enzyme activity localized to individual plants tissues obtained by laser-assisted microdissection. In fact, only a single additional report was found describing enzyme activity of ornithine decarboxylase from laser microdissected rat lung alveolar and epithelial tissue [37].

The absence of enzyme analysis from laser microdissected cells in the literature can be attributed to the difficulty of obtaining large enough sample quantities to perform enzyme assays. The fact that TPS enzyme activity was not able to be detected from laser microdissected CZ tissue highlights the utility of this method for obtaining large quantities of individual tissues from spruce stems. TPS enzymes are prone to degradation during long term storage, which suggests that the methods described do not degrade sensitive protein samples. Therefore, these methods should be readily applicable to the study of other enzymes purified from laser microdissected tissues.

TPS enzyme activity was not detected above background in protein extracts from CRD tissue. Any activity in these samples was likely masked by high levels of endogenous terpenoids. The level of endogenous terpenoid background in CRD protein samples is comparable to protein extractions from whole organs such as bark and wood and is therefore unrelated to the cryosectioning and LMD methods. The sensitivity of TPS enzyme assays from cell free protein extracts may be increased by using radio-labeled substrates and detection using radio-gas chromatography.

3.3.2 Proteomic analysis

Proteomic analysis of laser microdissected samples has been applied in the biomedical field, but there have been few reports in plants [3]. Only a single report was found describing the analysis of vascular bundles from *Arabidopsis* using either two-dimensional gel electrophoresis (2D-GE) or liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) [4]. In this study, 5000 vascular bundle regions were isolated from 30 µm thick cross-sections using the LMPC method. Using an

estimate of 12,000 μm^2 for the cross sectional area of each vascular bundle this is equivalent to a microdissected volume of 1.80 µl. By comparison, the microdissected volume obtained from 13-15 mm of spruce stem was 5.14 ± 1.44 µl for CRD tissue and 1.44 ± 0.25 µl for CZ tissue. Since the tissue quantity is similar or greater, it should be possible to apply 2D-GE and LC-MS/MS techniques for proteomic analysis of CRD and CZ tissues.

These techniques have also been performed on poplar phloem exudates in response to wounding using 4 µg for 2D-GE and 10 µg for SDS-PAGE and LC-MS/MS [38]. Recently, individual TPS proteins were quantitatively analyzed in Norway spruce bark following treatment with MeJA using selective reaction monitoring (SRM) of 20 µg protein samples [36]. The average total protein yield was approximately 80 µg for CRD tissue and 45 µg for CZ tissue. Thus, the microdissected volumes and protein yields obtained from CRD and CZ tissue should be suitable for future proteomic analysis using techniques such as 2D-GE, LC-MS/MS and SRM. These techniques could be used to identify abundant proteins in CRD or CZ tissues, many of which may be important for oleoresin production and wood development. In addition, the observation of differential protein induction patterns in CZ tissue in response to MeJA treatment may provide insight into the regulation of the developmental transition between wood development and TRD formation. Similarly, analysis of differential protein induction patterns in CZ tissue during cold onset may provide insight into the regulation of seasonal dormancy. It should be noted that the methods described here were optimized for extraction of intact proteins for enzyme assays and that extraction using solvents such as acetone and

methanol [36, 38] may increase protein yields for proteomic analyses where intact protein is not required.

3.4 Metabolite analysis

Terpenoid metabolites were successfully extracted from CRD and CZ tissue and identified using GC/MS (Figure 2.7). There was minimal monoterpenoid solubilization into the aqueous solution suggesting that there is little movement of these compounds across the cryosections as they dry on the slide (Figure 2.8). Due to the volatile nature of monoterpenes, it was impossible to determine their absolute quantities in these tissues. However, the relative abundance of individual monoterpenoids did not change substantially as the cryosections dried. The relative abundance of individual monoterpenes was similar between both tissues and was consistent with enzyme activities and transcript abundance in these tissues. Diterpenoids were also detected in CRD and CZ tissues, which may be better suited to quantitative analysis in future studies since they are non-volatile and non-soluble in aqueous solution.

The methods developed here may be applied for the analysis of other types of metabolites. The phenolic contents of PP cells, ray cells and other parenchyma have been well characterized at a histological level [27, 29, 30, 34], but there is little information about specific phenolic compounds within these cells. An NMR-based approach was recently used to measure phenolic contents of laser microdissected stone cell from Norway spruce stems [18], which may be complemented by additional analysis using GC/MS or LC/MS. Similar approaches may be applied to PP cells, ray cells and other parenchyma.

3.5 General perspectives for tissue-specific analysis of spruce stems

The recently reported microchemical analysis of stone cells from Norway spruce marked the first application of laser-assisted microdissection to a woody plant species [18]. The methods described here extend this approach for application of LMD to a variety of tissues within the spruce stem that are amenable to a multi-leveled analysis of RNA, protein and metabolites. These methods should be directly applicable to the study of individual tissues from other woody plant species such as poplar, and may be more broadly applied to herbaceous plants.

This thesis describes the isolation of CRD and CZ tissues from MeJA-induced white spruce stems. Unique spatial and temporal expression patterns were observed for genes involved in terpenoid metabolism. TPS enzyme activity measured in CZ tissue was shown to be distinct compared to whole cross-section samples. Terpenoid profiles measured in CRD and CZ tissue were found to correlate well with TPS gene expression and enzyme activity. This result demonstrates the utility of the LMD method for application to terpenoid defense. These approaches should be extended to include additional genes involved in terpenoid biosynthesis such as prenyl transferases, a larger set of TPS genes and cytochromes P450. The temporal patterns can be further developed by including analysis of the induced defense response over a time course after treatment with MeJA. Experiments may eventually be performed with induction by insect feeding or fungal inoculations, but due to the structural damage associated with these treatments it will be first necessary to evaluate the suitability of these samples for cryosectioning and LMD.

Protein and metabolite analysis of laser microdissected samples has not been widely reported due to the inability to amplify these molecules and the extensive labor involved in collecting enough tissue for analysis. Using the tangential cryosectioning and LMD approach described here, sample sizes are greater than previously reported proteomic and metabolomic studies in laser microdissected plant tissues. Therefore, application of LMD to spruce stem tissues provides an opportunity to develop these types of analysis to a degree that is unprecedented in plant tissue. For a proteomics study of defense response in CRD and CZ tissue, probes exist for quantitative analysis of specific terpenoid metabolic genes using SRM [36] and a more general approach may be applied using 2D/GE or LC-MS/MS. This proteomic analysis could be complemented by GC/MS analysis of terpenoid profiles in CRD and CZ tissue.

It would be particularly interesting to study the developmental transition that occurs as TRDs are formed from initials in the CZ tissue during the induced defense response. This process has been well documented in histological studies but has not been characterized at the molecular level. Genes that are induced or repressed during this process may be identified from CZ-specific cDNA libraries or proteomic analysis. Target genes could then be further analyzed by qRT-PCR or SRM to assess their role in induced TRD formation or wood development.

Applications of LMD have been demonstrated for CRD and CZ tissue within white spruce stems. There is great potential for applying these methods to other tissues. Ray parenchyma cells are interesting targets for characterization due to their association with developing TRDs and their potential role in radial signaling of the defense response. Preliminary results from our lab show that monoterpene metabolites are detected in

cortical tissue surrounding CRDs. It would be interesting to investigate whether monoterpenes are exuded from CRDs into the surrounding tissue or produced in other parenchyma within the cortex. Needles also play a role in the emission of volatile monoterpenes and sesquiterpenes [39] and it would be interesting to determine the role of different needle tissues (i.e. central canal, resin ducts and mesophyll tissue) in the biosynthesis, storage and emission of terpenoids from spruce foliage.

In summary, the methods described here provide a solid foundation for cryosectioning and LMD of spruce stem tissue. A multi-leveled approach for analysis of gene expression, enzyme activity and metabolite profiling is demonstrated for the study of terpenoid defense response in white spruce. These methods are robust and will serve as a useful tool for the study of complex metabolic processes in individual tissues and cell-types from spruce and other plant species.

3.6 References

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APPENDICES

Appendix A: A detailed protocol for harvesting spruce seedlings for cryosectioning and LMD

Introduction:

A typical method for harvesting seedlings has been to separate secondary phloem from secondary xylem for storage in Falcon tubes. However, tissues harvested and stored in this manner are not easily cryosectioned. Furthermore, cutting small pieces from frozen tissue is difficult and may cause damage to the tissue. This protocol is used to produce small stem segments from 1-2 year old white spruce seedlings (PG-653) that are suitable for cryosectioning and laser microdissection (LMD). It can easily be modified for other tree species, lateral branches, etc.

Materials:

-Garden clippers	-Liquid N_2
-Camera	-Forceps (medium sized)
-Ruler	-Dry ice
-Styrofoam container (for liquid N_2)	-Microcentrifuge tubes (labeled – holes in lid)
-White paper	-Gloves
-Razor blades (fine and coarse)	

Protocol:

1. Cut the stem as close to the base as possible using garden clippers.

2. Take a picture of the tree on a white paper background with a ruler to measure height.

3. Remove and discard all lateral branches using garden clippers.

4. Remove and discard the basal 1-2 cm from the stem using a coarse razor blade.

5. Cut a stem segment about 8 mm in length from the base of the stem using a fine razor blade (a fine razor blade is required to prevent crushing the stem tissue when applying pressure).

6. Hold the stem segment using forceps and submerge it in liquid nitrogen for a few seconds.

7. Roll the frozen stem segment gently with your fingers to remove needles and buds.

8. Place each stem segment in a labeled microcentrifuge tube with a hole poked in the lid (to prevent exploding tubes).

9. Store samples on dry ice and transfer to -80°C for long term storage.

Notes:

1. Work quickly! Total harvest time for each tree is ~15 min.

2. Be careful not to drop stem segments into the liquid nitrogen.

3. Avoid freeze/thaw – This leads to separation of bark from the wood.

Appendix B: A detailed protocol for cryosectioning and LMD of spruce stem tissue

Introduction:

Laser microdissection (LMD) can be used to isolate inidividual tissues and cell-types from cryosections obtained from fresh frozen stem tissue. High quality RNA, active enzymes and metabolites have been extracted from specific tissues isolated from spruce stems. This protocol provides details for producing cryosections from flash frozen stem segments of spruce seedlings for isolation of cortical resin duct (CRD), cambial zone (CZ) and other tissues using LMD. Microdissected tissues are suitable for RNA, protein and metabolite extraction. Unless otherwise specified, the species used is white spruce seedlings (PG-653, 1-2 years old).

Equipment:

Leica CM5060S cryostat

Leica LMD6000 laser microdissection system

Materials:

-Spruce stem segments (see Appendix A)
-Specimen discs
-Tissue-Tek OCT mounting medium
-Fine-tipped brushes (x2)
-Forceps
-Kimwipes
-Fine razor blades
-Cryostat blades (Fisher 12-634-1C No. S35)
-Leica PET frame slides (#11505151)
-Ethanol (100%)
-10 mM DTT (in nuclease-free water)
-Pasteur pipette
-0.5 ml PCR tubes (Nuclease-free, flat cap)
-Lysis buffer from Ambion RNAqueous-Micro RNA Extraction Kit
-Protein extraction buffer (50mM HEPES pH 7.2, 5mM DTT, 5mM ascorbic acid, 5mM sodium bisulfite, 10mM $MgCl_2$, 10% glycerol, 1% polyvinylpyrrolidone (PVPP), 0.1% Tween 20)

Procedure:

1. Set cryostat to the following settings: *Object temperature (OT) = -25℃*

Chamber temperature (CT) = -15°C

Blade angle = 0°

Thickness = 10 μm (adjusting orientation)

= 20-25 μm (collecting sections)

2. Bring stem segment into the cryostat chamber to allow it to equilibrate temperature (~30 min)

3. Clean the cryostat pressure plate, blade and anti-roll plate using a Kimwipe and 100% ethanol.

4. Place stem segment on the pressure plate and divide it in half longitudinally using a fine razor blade. Ensure that any stem defects (buds, branches or physical damage) are at the edge of the cut stem segment so that they do not interfere with the sectioning surface.

5. Cover a small region of the specimen disk with OCT. Use forceps to place a half stem segment flat side down (bark side up) gently onto the surface of the OCT. The stem should be barely submerged. Avoid excessive use of OCT to speed up freezing time and reduce movement of the specimen before the OCT solidifies. Avoid contact of OCT with regions to be sectioned – OCT is suspected to inhibit laser cutting.

6. Mount the specimen disk on the specimen head with the stem segment in vertical orientation and tighten it in place. If there are defects in the stem segment make sure they are positioned at the top so that these regions are sectioned at the end of a cutting cycle.

7. Ensure that the section thickness to 10 μm and the anti-roll plate is up. Move the specimen closer to the blade gradually until the specimen barely grazes the blade. Use the positioning screws to achieve a tangential sectioning orientation. This is indicated by even contact of the blade at equal depth from the top to the bottom of the stem piece. With each adjustment, move the specimen back away from the blade slightly to avoid damaging the specimen and anti-roll plate. The section thickness is set to 10 μm to conserve the specimen during orientation adjustment. Fine adjustments to the sectioning plane can be made throughout sectioning, but it is a good idea to get as close as possible to a perfect tangential plane before starting to collect sections. With practice, this can be achieved within a few sections of the periderm tissue.

8. Set the section thickness to 20-25 μm. If necessary, clean the anti-roll plate, blade and pressure plate with a Kimwipe (dry or with a small amount of ethanol) and place the anti-roll plate down. Adjust the position of the anti-roll plate using the adjustment knob (clockwise = away from the blade, counter-clockwise = towards the blade). Always move the anti-roll plate a slight turn away from the blade before beginning sectioning. Move the anti-roll plate slowly forward (1/8 turn or less per cycle) until the best quality sections are obtained.

> *Take care not to hit the anti-roll plate with the specimen during sectioning! Specimen contact with the anti-roll plate is indicated by any movement, sound or vibration of the anti-roll plate during any part of the cutting cycle (up or down-stroke)*

Avoid sectioning too deep into the secondary xylem. Cryosections with a large proportion of secondary xylem tend to curl and adhere poorly to the slide. However, if additional cortex and CZ tissues are required they may be collected if the secondary xylem is manually removed first. To do this effectively, hold a fine-tipped brush with bristles flat on top of the cortex on one side of the section. Hold the brush in place and use the tip of a pair of forceps to press on the secondary xylem tissue and pull it away from the cam

9. Transfer each section immediately to an LMD steel frame PET membrane slide using a dry, fine-tipped paintbrush. Place sections as close as possible to each other to maximize the number of sections per slide.

RNA analysis – Keep slide inside the cryostat. Fill the space on top of the membrane with a thin layer of 100% ethanol. Transfer individual sections onto the slide

Protein and metabolite analysis – Keep slide outside the cryostat. Place a 2 μl drop of 10 mM DTT onto the membrane. Briefly hold the slide inside the cryostat as the section is transferred onto the drop. Gently touch the surface of the section on the slide to distribute the DTT to the edges of the section.

10. When a slide is filled with sections, allow it to dry completely at room temperature. Use the corner of a Kimwipe to wick away any excess liquid. Keep slide “section side up” while drying.

The slide should NOT be dried in the cryostat for four reasons: (1) Condensation will form on the slide when it is brought to room temperature and nucleases are active in the presence of water; (2) CZ can be more easily distinguished from secondary phloem when dried at room temperature; (3) Sections are more likely to fall of when dried within the cryostat; (4) It simply takes too long.

11. Remove any sections that have curled excessively and that are not adhering well to the membrane. This helps prevent whole sections from falling off the slide during LMD.

12. Place slide "section side down" on the LMD slide tray and load the tray on the LMD system. *Note: Slides may also be cut "section side up", but in this case they are more likely to become trapped above the membrane after cutting.*

13. Select all regions of interest for a single tissue-type. Do not select multiple tissue-types at one time to avoid cross-contamination. Check the "close shape" option in order to calculate the area of each shape. *Note: Selected regions disappear when you eject the slide holder, so be careful!*

14. Turn on the laser to allow it to warm up.

15. Add 30 µl of RNA lysis buffer or protein extraction buffer to the bottom of a 0.5 ml PCR tube cap. For metabolite extractions, leave the cap empty. Insert the PCR tube into the appropriate space in the collection tube holder and mount it on the LMD system. *Note: Adding*

buffer after the tube is in the collection tube holder often results in buffer crystallization on the edges of the holder.

16. Confirm laser settings and calibrate the laser (must be done separately for each objective). For most applications, power = 110 and speed = 2. If cutting is difficult, increase the power and/or decrease the speed. *Note: In practice, laser does not need to be calibrated each time.*

17. Press the "start cut" button. *Note: If the "erase shapes after cutting" option is selected (look under Options→Misc) then you will lose all shapes if you terminate cutting prematurely.*

18. After cutting has finished, check that all sections have been cut completely and are dislodged from the slide. Dislodge any incompletely cut regions by using the pen screen on "move and cut" mode.

19. Remove collection tube carefully and place cap-down in a -80˚C freezer. Tubes may be stored upright after it has frozen.

20. If required, proceed to select and cut the next tissue-type from the sections (Step 13-19). *Note: Cutting multiple tissues from a single section increases the risk that unselected regions may fall into the collection tube.*

21. Proceed to RNA, protein or metabolite extractions as described in Chapter 2.

Optimizing cutting:

Getting good sections is less of a science and more of an art. Pinpointing the cause of poor sections can be difficult and small adjustments can make a big difference. Below is a list of factors to consider when troubleshooting. The order is related to the order in which they should be considered, but it is only a rough guide (i.e. If the blade was just changed then it is likely not dull).

1. Clean the pressure plate and anti-roll plate with a Kimwipe (dry or with ethanol).
2. Move to a new part of the blade.
3. Adjust position of anti-roll plate.
4. The periderm on each side can catch on the knife or anti-roll plate and cause the section to bunch up or curl. This can be reduced by moving the anti-roll plate back, rubbing the periderm on each side of the sectioned surface with the blunt side of a pair of forceps, or by backing up slightly when bunching starts before continuing the cut. This problem may also be reduced by using a faster cutting speed.