CONIFERIN METABOLISM IN THE CAMBIAL REGION OF *PINUS STROBUS* L.

by

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DEDICATION

This dissertation is dedicated to my family.

To my parents, Peggy and Ronnie Steeves, whose love, support, guidance and encouragement have motivated me to get to this point.

To my brothers, Grant and Ryan Steeves, whose love and loyalty to their sister have always been much appreciated. I am so fortunate to have such amazing brothers.

To my husband, Malcolm Reeves, whose love, support and patience has seen me through some of the tougher times.

Much love to each of you, today and always.

ABSTRACT

E-Coniferin has only been detected in the cambial region of conifers during cambial activity, appearing prior to lignification and disappearing prior to cambial dormancy. Thus, its presence serves as an indicator that cambial growth is occurring. It follows then that the enzymes involved in coniferin metabolism, coniferin β-glucosidase (CBG) and uridine 5'-diphosphoglucose: coniferyl alcohol glucosyltransferase (UDPG: CAGT), may be linked to regulation of the seasonal cycle of cambial growth and dormancy in conifers. In addition, these enzymes may also play a regulatory role in lignification in conifers, where CAGT sequesters coniferyl alcohol (CA) and CBG releases it. Thus, it is critical that we understand both CBG and CAGT.

One of the roadblocks in CBG research is its cellular location. Reports conflict as to whether the enzyme is soluble or cell wall-bound. In this study, CBG activity was investigated in cambium and developing xylem of *Pinus strobus* L. by a novel experimental approach. CA accumulated up to 28-fold in developing xylem during secondary wall formation, and also in actively dividing cambium cells. The findings strongly indicate that this abnormal CA accumulation was a result of CBG hydrolysis of coniferin, and also that CBG is a soluble catalyst in cambium and developing xylem of

P. strobus. Dormant cambium was not competent for CA production, indicating a temporal association between CBG and cambial activity. This is the first report of CBG activity in cambium cells.

CAGT from *P. strobus* was found to have higher activities for sinapyl alcohol, sinapaldehyde and coniferaldehyde, compared to coniferyl alcohol. CAGT Michaelis constants, K_m, are reported for sinapaldehyde and coniferaldehyde for the first time. When dual substrates were administered to CAGT, the most pronounced product inhibition occurred when coniferyl alcohol and sinapaldehyde were administered together, with CAGT activity for sinapaldehyde being more strongly affected. This research also provides the first evidence for in vitro glucosylation of dihydroconiferyl alcohol by CAGT. Together, these findings, coupled with the discovery of seasonal patterns of CAGT activity toward coniferaldehyde and sinapaldehyde, indicate that CAGT has a prominent role in phenylpropanoid metabolism in relation to secondary growth.

PREFACE

Much of the research presented here has been published, and the format of the publications has been preserved here; therefore, the formatting varies slightly throughout this dissertation. Chapter 1 is a literature review and introduction to the dissertation and has not been previously published. Chapter 2 has not been published; however, it will be prepared for submission to *Phytochemistry*, which is the format used to present it here. The experimental design, procedure and analysis are my own, as is the writing. Chapter 3 has been published in *Phytochemistry*[a], where I am a joint first author. For this project, I was responsible for the design and performance of chemical analyses, particularly NMR work. I learned about the techniques used in this study and I was responsible for a majority of the writing. Chapter 4 is a chapter published in *Cell and Molecular Biology of Wood Formation*[b]. Again, I performed the NMR work and analysis, and wrote the chapter with H. Förster. Appendix A[c] is also a chapter published in *Cell and Molecular Biology of Wood Formation*. The experimental design, analysis and writing are my own, with R.A. Savidge's help in editing the manuscript and with initial project design.

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[a] **Steeves, V.**, Förster, H., Pommer, U., and Savidge, R. 2001. Coniferyl alcohol metabolism in conifers - I. Glucosidic turnover of cinnamyl aldehydes by UDPG: coniferyl alcohol glucosyltransferase from pine cambium. Phytochemistry 57, 1085 - 1093.

[b] Förster, H., **Steeves, V.**, Pommer, U., and Savidge, R.A. 2000. UDPG: coniferyl alcohol glucosyltransferase and coniferin biosynthesis - a regulatory link to seasonal cambial growth in conifers. In: Cell and Molecular Biology of Wood Formation. Savidge, R.A., Barnett, J.R., and Napier, R. (Eds). Oxford: BIOS Scientific Publishers Ltd. pp. 189 - 201.

[c] **Steeves, V.J.**, Savidge, R.A. 2000. Cambial coniferin content as an indicator of the health status of conifers. In: Cell and Molecular Biology of Wood Formation. Savidge, R.A., Barnett, J.R., and Napier, R. (Eds). Oxford: BIOS Scientific Publishers Ltd. pp. 57 - 65.

Please see the Curriculum Vitae presented at the end of this dissertation for a full list of recent publications and conference presentations.

ACKNOWLEDGMENTS

I owe many people sincere thanks for helping me along my studies. First of all, I would like to thank my supervisor, Dr. Rod Savidge, without whom this would not have been possible. He not only provided guidance in the lab, but also helped me to realize my potential and he was instrumental in my discovery for my own passion for teaching.

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Several people have helped me with my work over the years, mostly in the form of field work, and often simply as company out in the woods! Grant (my brother), Charlene (a good friend), Natascha (another good friend), Debbie and Jennifer.

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My family, to whom this dissertation is dedicated, deserves all of my thanks for their love and support.

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LIST OF ABBREVIATIONS

Abbreviation	
4CL	4-coumarate: CoA ligase
β-gluc	β-glucosidase
BSTFA	N,*O*-bis (trimethylsilyl) trifluoroacetamide
C3H	*p*-coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CA	coniferyl alcohol
CAD	cinnamyl alcohol dehydrogenase
CAGT	coniferyl alcohol glucosyltransferase
CAld5H	coniferyl aldehyde 5-hydroxylase
CBG	coniferin β-glucosidase
CCoA3H	coumaroyl CoA 3-hydroxylase
CCoAOMT	caffeoyl-CoA *O*-methyltransferase
CCR	cinnamoyl CoA reductase
CoA	coenzyme A
COMT	caffeic acid *O*-methyltransferase
COSY	correlation spectroscopy
CZ	cambium
DFRC	derivatization followed by reductive cleavage
DHCA	dihydroconiferyl alcohol
DX	differentiating xylem
EC	enzyme classification
EDAX	energy dispersive analysis of X rays
ER	electrical resistance
eV	electron-volt

F5H	ferulate 5-hydroxylase
g	gram
g_{fw}	gram fresh weight
G lignin	guaiacyl lignin
GC	gas chromatography
GL	D-glucono-1,5-lactone
h	hour
H lignin	*p*-hydroxycoumaryl lignin
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
Hz	hertz
i.d.	inner diameter
IEF	isoelectronic focussing
IR	infrared
kat	katal
LW	latewood
M	molar
MeOH	methanol
min	minute
mol	mole
MPA	methylphosphonic acid
MS	mass spectrometry
NAA-BAP	naphthalene acetic acid - benzylaminopurine
NB	New Brunswick
NMR	nuclear magnetic resonance
NT	new tracheids

PAL	phenylalanine ammonia-lyase
ppm	parts per million
RE	radially expanding cambial derivatives
s	second
S1	outer secondary wall layer
S2	middle secondary wall layer
S3	inner secondary wall layer
S lignin	syringyl lignin
SAD	sinapyl alcohol dehydrogenase
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SL	expanded derivatives undergoing lignification in their secondary walls
STM	scanning tunneling microscopy
TEM	transmission electron microscopy
UDPG	uridine 5'-diphosphoglucose
UV	ultra violet

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

"Because of its importance as the progenitor of wood, the cambium is worthy of a greatly intensified scientific study." (Stewart, 1957)

1. Introduction

To date, there exists a number of differing attitudes towards both forest and urban trees. For instance, those with a utilitarian perspective aim to produce trees for industry and profit, while those with an ecosystem perspective aspire to grow and maintain healthy trees and healthy, whole forest ecosystems. In either case, manipulations at both environmental and genetic levels are made in attempts to 'grow better trees' (Berlyn and Battey, 1985). The term 'better' here is rather subjective, again depending on the individual's perspective. For example, to those with the almighty dollar in mind, 'better' refers to growing trees with thicker, taller and straighter stems, and doing so in the shortest amount of time possible. The paradox is that genetic manipulations are performed without fundamental knowledge of the systems that are being altered.

Wareing (1958) acknowledged nearly 50 years ago the importance of understanding factors controlling behaviour of the cambium if we are to understand the

processes involved in wood formation. While Savidge (1996) has recently pointed out that "no plant meristem can be more abundantly obtained in pure form than the cambial zone from trees, and no developing tissue can be more readily had for molecular physiology studies than that comprising cambial derivatives on their way to becoming wood," he also recognizes that our understanding of seasonal cambial activity, especially in relation to wood biosynthesis, is still underdeveloped (Savidge, 2000), attributed to a lack of reported biochemistry data on the cambium and its derivatives. Recently, Savidge (2000) reviewed the importance of cambial activity and its products (i.e., wood) to humankind, claiming that fundamental research into the biochemistry of the cambium and its derivatives is required, and amply justified. A compilation of continued and novel research into the cambium, with regards to cell and molecular biology of wood formation has been published (Savidge et al., 2000). However, much more fundamental work on the cambium and its derivatives is necessary before complete comprehension of wood formation can be achieved.

Despite the importance of primary meristems for extension growth of all plants, secondary meristems, especially the vascular cambium which is responsible for diameter growth, are possibly even more essential on a global scale (Chaffey, 2002). Chaffey explains that wood is of primary importance to us, not only as building material, but as a long term carbon sink (atmospheric carbon), and renewable source of energy (relative to fossil fuels). He points out the need to understand the fundamental processes of wood formation before truly understanding genetic engineering of wood. Chaffey (2002) also indicates that funding for research into the cambium remains meager partially because

there remain so many obstacles, such as the lack of a model species, the research is too difficult, and lack of interest, for example. In an earlier article, Chaffey (1999) suggests reasons why using herbaceous plants, such as *Arabidopsis thaliana* (L.) Heynh. and *Zinnea elegans* Jacquin. may not be representative of the 'tree', and also why the use of *in vitro* systems (i.e., single cell system, single cell type produced, unnatural cell types, no cycle of activity/dormancy, restricted extension and radial growth) may not provide answers that are applicable to *in vivo* systems.

In addition to the limitations outlined by Chaffey (1999, 2002), researchers of the cambium must also take into consideration defence/wound responses that may occur when cambial tissue is extracted for analysis (Kahl, 1978). During phenological studies, continuously removing samples from the same individual tree poses concerns of defence/wound responses, as well as infection, which may alter cambium biochemistry, in turn potentially affecting the outcome of subsequent analyses. This challenge can be partially overcome by using different trees for each sampling date, and reinforcing it by studying the cellular anatomy of each sample and correlating anatomical data with any biochemical data. Sectioning the cambium for anatomical observation provides a static image of the cambium at time of harvest. This practice is readily employed in our research (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998; Förster et al., 2000; Steeves and Savidge, 2000; Steeves et al., 2001).

The focus of this chapter is to provide a general introduction into areas of research presented in this dissertation, as well as to complement information presented in subsequent chapter literature reviews. In each of my research projects, I investigated

coniferin metabolism in pine cambial tissue. Coniferin is one of many products of phenylpropanoid metabolism, which is of global importance to forestry, as well as agriculture and the environment (Humphreys and Chapple, 2002). In trees, other products of phenylpropanoid metabolism include: lignins, lignans, salicylates, coumarins, hydroxycinnamic amides, flavonoids, which function as phytoalexins, pigments, UV light protectants, and antioxidants, for example (Dixon and Paiva, 1995). Thus, in addition to offering structural support and defence (i.e., lignins), phenylpropanoids are also responsible for plant qualities such as colour, texture and flavour (Mayer et al., 2001). *E*-Coniferyl alcohol (Figure 1a), a cinnamyl alcohol product derived from phenylpropanoid metabolism, has long been established as a *primary* precursor for lignin biosynthesis, along with two other cinnamyl alcohols: *p*-coumaryl alcohol (Figure 1b) and sinapyl alcohol (Figure 1c; c.f., Freudenberg, 1962). The 4-*O*-β-D-glucopyranoside of *E*-coniferyl alcohol, coniferin (Figure 1d), is found in abundance in the cambium of conifers during the growing season (Kremers, 1957; Kratzl, 1960; Freudenberg and Harkin, 1963; Freudenberg and Neish, 1968; Marcinowski and Grisebach, 1977; Schmid and Grisebach, 1982; Terazawa and Miyake, 1984; Terazawa, et al., 1984a; Savidge, 1988, 1989, 1991; Savidge and Förster, 1998; Steeves and Savidge, 2000) and has recently been implicated in tree health assessment (Steeves and Savidge, 2000). The research presented here investigates on a broad basis the enzymes involved in the interconversion of coniferyl alcohol and coniferin, coniferin β-glucosidase (CBG) and uridine 5'-diphosphoglucose: coniferyl alcohol glucosyltransferase (UDPG: CAGT; Figure 2), as well as a potential role of coniferin in

tree health assessment.

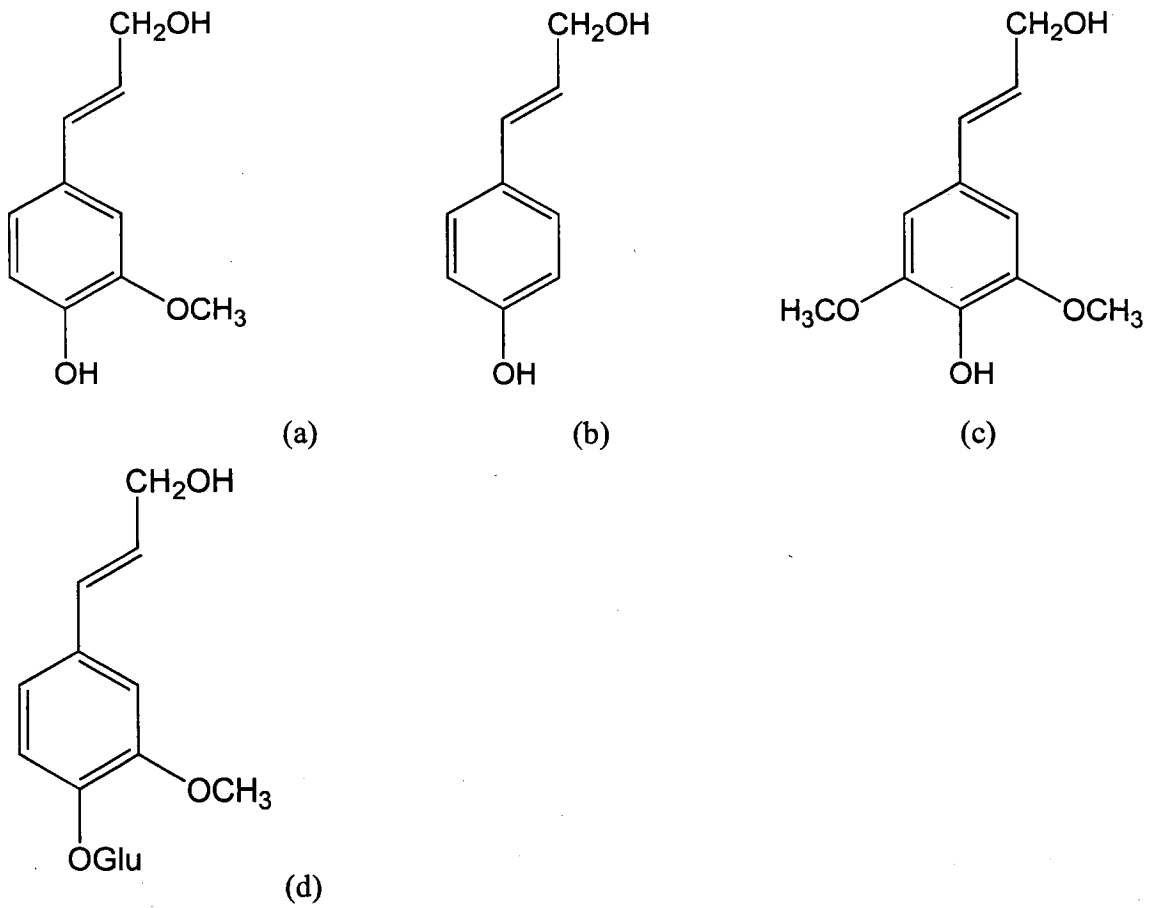


Figure 1. Structures of monolignols and coniferin. a) coniferyl alcohol; b) *p*-hydroxycoumaryl alcohol; c) sinapyl alcohol; and, d) coniferin.

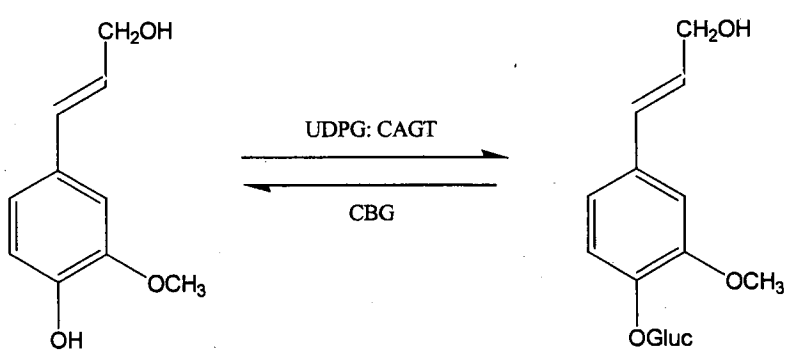


Figure 2. Interconversion of coniferyl alcohol and coniferin.

Since most of the research reported in this dissertation was carried out using cambial tissue (i.e., cambium and its derivatives) from eastern white pine (*Pinus strobus* L.), a brief discussion of white pine cambium, with special reference to pine cambium phenology is presented first. This is followed by a discussion of phenylpropanoid metabolism and more specifically coniferin metabolism.

2. Cambium of *Pinus strobus*

The vascular cambium is the secondary lateral meristem responsible for diameter growth in both shoots and roots of conifers, giving rise to both secondary phloem and secondary xylem (wood). In simplistic terms, the cambium lies between the bark and wood of the main stem, branches and roots of a tree, enveloping the entire circumference of each. As seen in cross sections, cambium cells along with adjacent xylem (centripetal) and phloem (centrifugal) cells are arranged in radial rows (or 'radial files'; see Figure 3) and lack intercellular spaces (Srivastava and O'Brien, 1966). Cambium cells are distinguished from other cells by their thin, primary walls and narrow radial diameters (Murmanis, 1970, 1971). Composed of both meristematic fusiform and ray cells, cambium in healthy white pine generally consists of 6 - 15 cells per radial file (Wilson, 1966), however, the number of cambial zone cells per radial file oscillates with species, time of year and tree vigour (Gregory, 1971). Fusiform cells are compressed radially (Priestley, 1930a), typically having radial dimensions 5 μm or less (Bailey, 1930; Bannan, 1955, 1962a; Murmanis, 1971; Savidge and Förster, 1998) and lengths up to several hundred times as long, from 200 - 9000 μm (Bailey, 1919, 1920; Bannan, 1967;

Catesson, 1994). Parenchymatous ray cells in the cambium are relatively small and isodiametric (Bailey, 1920; Lev Yadun and Sederoff, 2000). Xylem ray cells remain alive (i.e., not terminally differentiated) for many years and function in radial movement of water and various solutes, and in storage of organic material such as starch and lipids (Bailey, 1952). Cambial fusiform and ray initials are uninucleate in many species, including white pine (Bailey, 1919, 1920); however, multinucleate cambial cells have been reported, mostly in tropical species (Catesson, 1994).

Figure 3. Cross-section of *Pinus strobus* stem during tracheid differentiation. CZ = cambium; RE = radially expanding cambial derivatives; SL = expanded derivatives undergoing lignification in their secondary walls; NT = new tracheids; LW = previous year's latewood.

In a review on the structure and functions of the vascular cambium, Lachaud and

coworkers (1999) claim that "it is generally admitted that the cambium is uniseriate." That is to say that the *cambium* itself is a single layer of cells (termed 'initials'), while the *cambial zone* consists of cambial initials, along with xylem and phloem mother cells (mother cells arise from division of the initial, whereby one daughter cell retains status as the initial, and the other becomes either a xylem or phloem mother cell; Bannan, 1955; Wilson et al., 1966). Mother cells are then capable of further division, but do not necessarily divide, before maturation into secondary vascular elements (i.e., xylem tracheids and phloem sieve cells in pine). Each mother cell that divides is thought to give rise to groups of four cells in the xylem, derived from two successive divisions of the initial. These four cells, collectively known as Sanio's four, are the initial, the xylem mother cell and two daughter cells (Newman, 1956; Mahmood, 1968; Murmanis, 1970). Groups of four cells neighbouring Sanio's four are called the 'enlarging four' (Mahmood, 1968). These cells, derived from the daughter cells in Sanio's four, are no longer capable of cell division and will differentiate into mature tracheids (Mahmood, 1968; Murmanis, 1970). However, Sanio's four and the enlarging four are not always observed in the cambial zone (Savidge, 2000).

While many accept the idea of a uniseriate cambium, others believe the cambium to be multiseriate (c.f., Bannan, 1955; Wilson, 1964; Murmanis, 1970), whereby all cells in the cambium are equally competent for cell division and differentiation (i.e., evidence in support of this theory has been recently reviewed by Savidge, 2000). Murmanis (1970) points out, in support of Srivastava and O'Brien (1966), that it is impossible to differentiate between what may be the initial and other cells of the cambial zone, in that

all cambial zone cells have similar appearances, even at the ultrastructural level. Murmanis (1970) goes on to suggest that a difference, should one exist, between a supposed initial and other cambial cells could be biochemical. A consensus has not yet been reached as to whether the cambium is uniseriate or multiseriate (for example, see Bannan, 1962a; Larson, 1994; Savidge, 2000, for reviews). In this dissertation, cambial zone (CZ) refers to all cells capable of continuing cell division (terminology of Wilson, et al., 1966).

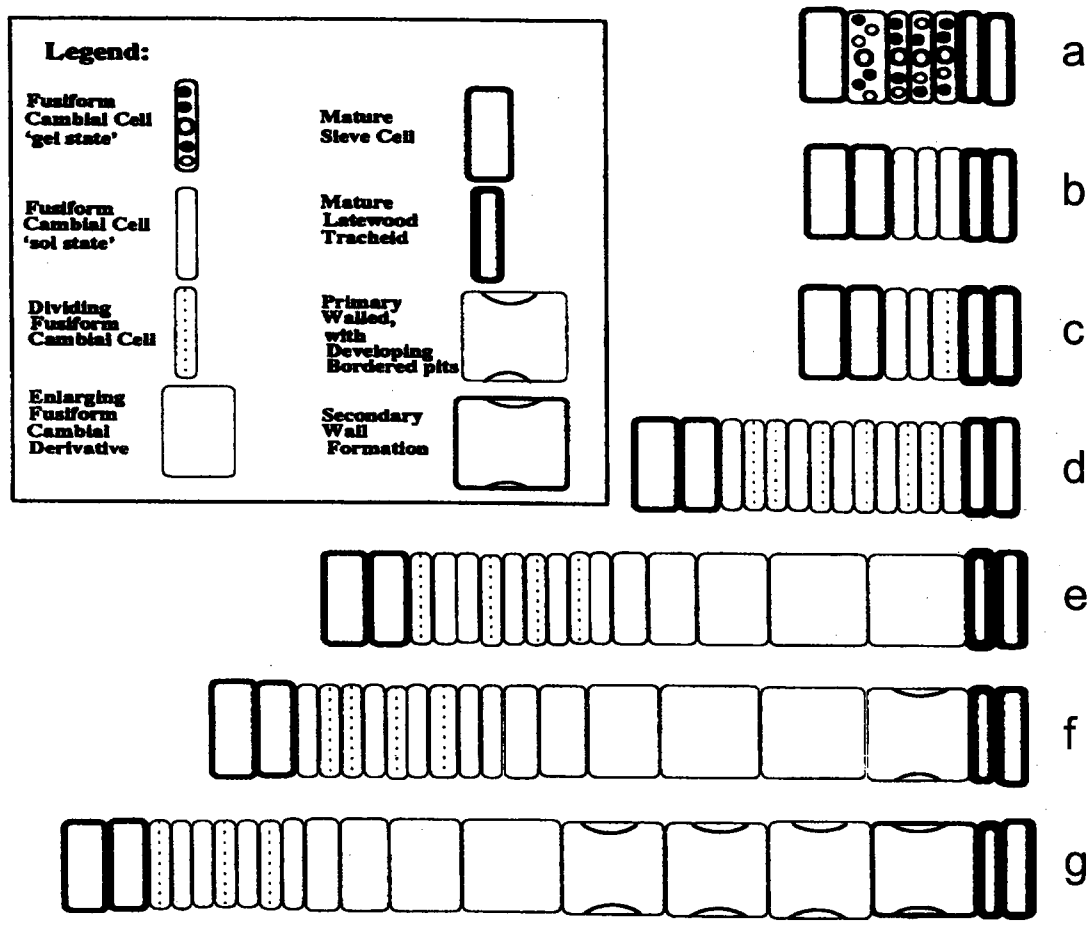
2.1 Phenology of *Pinus strobus* cambium

Predictable ultrastructural changes occur in cambium of temperate zone trees, such as white pine, during an annual cycle of meristematic activity (Bailey, 1919, 1920; Wilson, 1966; Murmanis and Sachs, 1969; Murmanis, 1971; Catesson, 1994). The cambium of eastern white pine in eastern Canada is dormant throughout the winter months. Dormant cambial cells (generally averaging 5 - 6 cells per radial file; Srivastava and O'Brien, 1966; Murmanis, 1971) contain numerous small vacuoles (Figure 4a), distinguishing them from active cells, which typically contain a single, large central vacuole (Bailey, 1930; Srivastava and O'Brien, 1966; Murmanis, 1970, 1971; Itoh, 1971). Dormant cambial cells also have thickened radial walls, dense protoplasm and contain relatively fewer organelles than do actively dividing cambial cells (Bannan, 1955, 1962a; Wareing, 1958; Murmanis, 1971). Nuclei also differ in dormant versus active cambial cells, being longer and narrower in the former (Bailey, 1920).

Cambial growth, to be considered herein as xylem or wood formation (Wareing,

1958; Kutscha et al., 1975), although it also produces new phloem tissue (this area of development is not considered) generally begins with rehydration and vacuolation of dormant fusiform cambial cells in white pine (Figure 4b) usually occurring in mid-spring (Srivastava and O'Brien, 1966; Murmanis, 1970, 1971; Reeves, personal observations). It should be noted that environment plays a role here, as open grown trees tend to reactivate earlier than those with slender boles in closed stands, as observed in *Thuja occidentalis* L. (Bannan, 1955). In general, vacuolation is closely accompanied by thinning of the radial walls (Wareing, 1958; Evert, 1963; Derr and Evert, 1967; Rao and Catesson, 1987). As rehydration occurs, cambial cells swell to as much as four times their initial widths (Brown, 1915; Bailey, 1930; Priestley, 1930a, 1930b; Fraser, 1952; Bannan, 1955; Wareing, 1958). Cell division activity in the cambium of conifers begins shortly following swelling (Brown, 1915; Wareing, 1958).

While in some conifers, cambial cell division activity appears to begin at the base of the live crown (Brown, 1912, 1915; Brown et al., 1953; Wilson, 1966; Savidge and Wareing, 1984; Savidge, 1985, 1993, 2000), conflicting reports exist (briefly reviewed in Stewart, 1957; Wareing, 1958). For example, Bailey (1920) concluded, based on numerous observations with many species, including white pine, that the initiation of cell division activity could occur in one or more portions of a stem at one time, while cells in adjacent portions remain inactive. Fraser (1952), studying white pine trees growing in different environments, determined that cambial activity initiated in the uppermost parts of the stem and moved downward; however, his study was limited not



only by a small sample size but also by a short sampling period with infrequent

(Savidge, 1993)

Figure 4. Representation of transition from dormancy to cell division activity and first earlywood formation for radial files of conifer cambium as seen in transverse sections. The stages from dormancy (a) to earlywood formation (g) are explained in the text (Section 2.1).

sampling. Rees (1929) observed initiation of cambial activity in the upper bole of *Picea rubra* Link. growing in different environments but found that reactivation in branches occurred later, at the same time as it was occurring in the lower bole. Brown (1912)

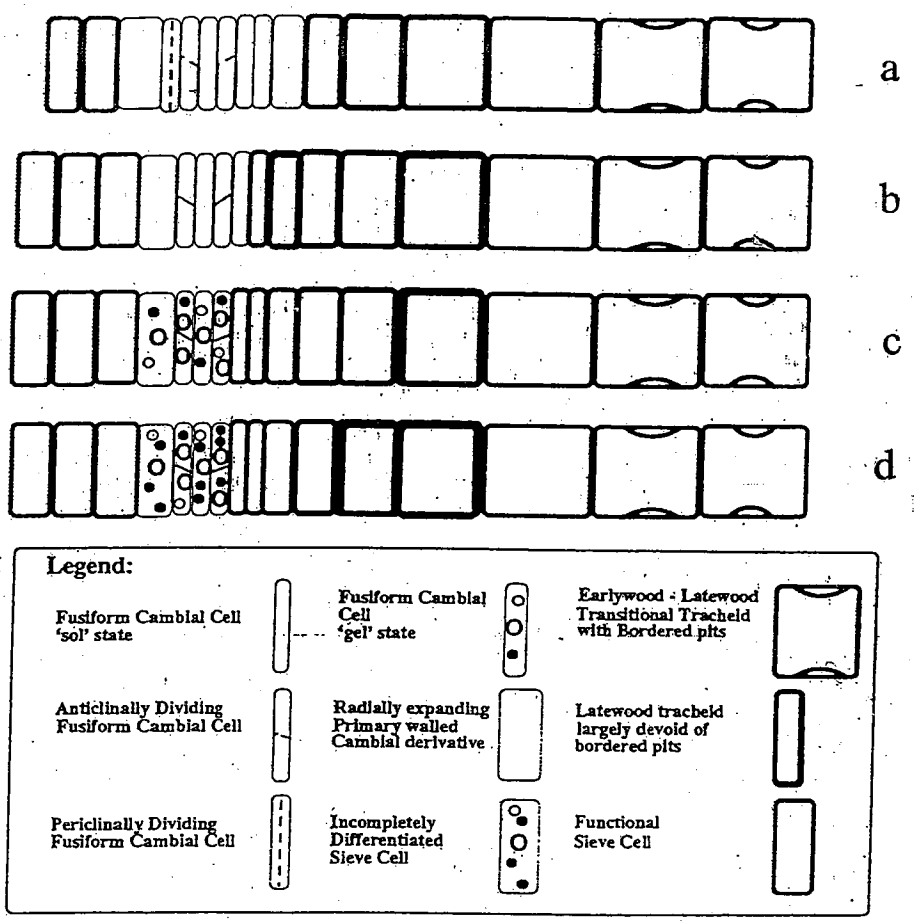
reported that cambial activity began in lower parts of the trunk in *Pinus rigida* Mill., and separately in branches, and spread both upward and downward from the initiation point. However, in a subsequent study on white pine, he determined that cambial activity commenced in the upper parts of the tree (Brown, 1915). Savidge and Wareing (1984) determined that cambial reactivation (i.e., resumption of cell-division activity) in *Pinus contorta* Dougl. began at the base of the live crown; however, no pattern of progression of reactivation was observed in either direction (i.e., basipetal or acropetal). Priestley's observations with different conifers led him to conclude that cambial activity was initiated at the base of buds in both branches and main stem and progressed downward through the stem (Priestley, 1930b).

Periclinal cell division activity (i.e., division in the tangential plane; Figure 4c) in the cambium results in an enlarged cambial zone (i.e., often triple the number of cells per radial file that are present during dormancy; Savidge, 1993; Figure 4d) in late spring followed shortly by maximal cambial cell division activity (Kienholz, 1934; Wilson, 1964, 1966; Murmanis, 1971). At this time, Murmanis (1970, 1971) counted 15 to 20 cambial cells per radial file in white pine. Faster growing trees have radially thicker CZ (usually 100 - 150 μm and up to 300 μm (Bannan, 1955, 1962a, as seen in *Thuja occidentalis*) than slower growing trees, as a result of an initial period of mitosis of cambial cells in the absence of differentiation (Bannan, 1955; Wilson, 1966; Gregory, 1971). Shortly after the onset of cambial cell division activity, primary wall radial enlargement (RE zone; Wilson et al., 1966) begins in cambial cells closest to the previous year's latewood that have ceased dividing (Figure 4e). At this time, radial walls

of white pine cambial derivatives expand from an average length of 10 μm in dividing cambial cells to roughly 30 μm in mature tracheids (Murmanis and Sachs, 1969). Following radial enlargement, initiation of bordered pit development in the absence of secondary wall deposition (Murmanis and Sachs, 1969) marks the beginning of secondary wall formation (SL zone; Wilson et al., 1966; Figure 4f) which is subsequently followed by deposition of polysaccharides, then lignification. Upon completion of secondary wall formation, cells autolyse, thereby terminally differentiating into new tracheids (NT; Figure 4g) and assuming their conductive function. First-formed tracheids of the growing season, designated as earlywood, have large radial diameters (average 20 - 30 μm; Murmanis and Sachs, 1969) and thin cell walls. Earlywood formation generally continues until termination of apical growth, after which time latewood formation begins (Wareing, 1958; Larson, 1960, 1962); however, the earlywood-latewood transition can be influenced by environmental factors such as drought. Latewood tracheids are characterized by reduced radial diameters (9 - 30 μm, progressively smaller in going from earlywood to cambium; Murmanis and Sachs, 1969) and thicker cell walls, as compared to earlywood tracheids (Bailey, 1952). Latewood formation is initiated at the base of the tree, and proceeds up the stem with time in *P. resinosa* (Gordon and Larson, 1968).

When latewood formation is underway the frequency of anticlinal divisions (i.e., division in the radial plane) increases (Brown, 1915; Bannan, 1967); in vigorous young stems, anticlinal divisions may occur throughout the growing season as their circumference increases more rapidly (Bannan, 1950), giving rise to new fusiform

cambial cells (Bannan, 1962b; Savidge, 1993; Figure 5a). It has been found that the rate of anticlinal divisions exceeds the required rate for sustainability of circumferential continuity; however, some of these newly formed fusiform cambial cells reduce to ray cells or disappear by becoming incorporated into the xylem (Bannan, 1950; Bannan and Bayly, 1956). For a few different conifer species, Bannan (1951, 1962b, 1965, 1967) and Bannan and Bayly (1956) determined that survival rate was highest for those cambial initials contacting the most ray cells.



(Savidge, 1993)

Figure 5. Representation of onset of dormancy in radial files of conifer cambium as seen in transverse sections. The stages from active cambial growth (a) into dormancy (d) are explained in the text (Section 2.1).

Near the completion of latewood formation, the RE zone disappears, cell division activity in the cambium ceases, and the cambium is back to roughly 6 cells per radial file (Figure 5b; Murmanis, 1971; Savidge, 1993; Savidge and Förster, 1998). The large central vacuole of the active fusiform cambial cell splits into numerous smaller vacuoles as found in the dormant cambium (Figure 5c; Murmanis, 1971; Rao and Catesson, 1987), while latewood cells continue to lignify and autolyse. By October, latewood formation is generally complete and cambial cells structurally resemble dormant cambial cells (Figure 5d; Murmanis, 1971; Savidge, 1993). Latewood cells that have not yet autolysed have been known to overwinter incompletely differentiated, continuing their differentiation upon springtime reactivation (Bannan, 1955; Murmanis and Sachs, 1969; Murmanis, 1971).

3. Phenylpropanoid metabolism

Over the past century, and mostly in the past 50 years, phenylpropanoid metabolism, especially in relation to lignin biogenesis, has been examined extensively. Thus, a brief discussion of lignin literature is fundamental to a review of coniferin biosynthesis (largely reviewed in Chapters 2 - 4), since much research on coniferin and coniferyl alcohol owes its existence to lignin (being the predominant research focus in many cases), and the *primary* fate of coniferyl alcohol is in lignin biogenesis.

3.1 Lignin - Our evolving interpretations

"*...it may be useful to remember that our belief is greater than our exact knowledge.*" A

quote from Erdtman (1957) when referring to lignin chemists who, in the 1950s, believed that the solution to the lignin puzzle was near.

Lignin, originating from the Latin word *lignum* meaning 'wood', evidently first appeared on Earth in the Upper Silurian, more than 400 million years ago (Harkin, 1967; Williams et al., 2000). Harkin (1967) speculated that lignin 'must be one of the oldest natural organic polymeric materials on the evolutionary scale.' It is thought that the appearance of lignin was important in terrestrial colonization of plants (Grisebach, 1981; Kubitzki, 1987; Raven et al., 1999), with the first conifers appearing in the Permian approximately 200 million years after the supposed emergence of lignin (Williams et al., 2000). Deposited in the secondary wall of developing xylem, lignin offers structural support, allowing for vertical growth in vascular plants, and is responsible for hydrophobicity in water-conducting tissue. It is thought to play a role in defence against pathogens, insects and other herbivores (Ye et al., 2001). Thus, lignin is *essential* to tree growth and development.

Lignin occurs in abundance in conifers, being the second most abundant biopolymer on Earth, next to cellulose (ratio of 60 to 100; Freudenberg, 1964), and accounts for 15 to 36 % of the dry weight of trees (Higuchi, 1985). Lignin also accounts for approximately 30 percent of all of the carbon (1.4×10^{12} kg) sequestered into plant material each year (Battle et al., 2000). It is thus extremely important to both global and individual plant carbon balances (Douglas, 1996). Boudet (2000) claims that "the chain of reactions leading to lignin is one of the most expensive biosynthetic processes in plants, in terms of energy demand." The heat of combustion of lignin has been measured

at 6.3 calories per gram, compared to 3.7 calories per gram of glucose (Kremers, 1957).

While it was early in the nineteenth century when lignin was first described (for historical reviews, see Brauns, 1952; Harkin, 1967), it was only in the late 1800s and early 1900s that Klason was able to gain insight into the structure of lignin. He was the first to suggest, based on work by Kübel (1866) and Tiemann and coworkers (Tiemann and Haarmann, 1874; Tiemann, 1875a,b; Tiemann and Mendelsohn, 1875; Tiemann and Nagai, 1875; Tiemann, 1878), that lignin must be a 'product of oxidation of coniferyl alcohol'. While many speculations about the chemical nature of lignin have gone without benefit of scientific evidence, Klason's theory that lignin was composed of coniferyl alcohol units still stands. He was the first to 'isolate' lignin for analytical purposes using 72 % sulfuric acid, now known as the Klason method. Brauns (1952), a well-known lignin chemist who wrote one of the first comprehensive treatises on lignin studies, declared Klason 'the father of lignin chemistry.'

Generally, although incompletely, defined as a complex phenylpropanoid polymer, lignin is *mainly* derived from dehydrogenative polymerization of three primary precursors, often referred to as monolignols: *p*-coumaryl (giving *p*-hydroxycoumaryl, or H lignin), coniferyl (giving guaiacyl or G lignin) and sinapyl (giving syringyl or S lignin) alcohols. Lignin in conifers is primarily composed of guaiacyl units with small amounts of *p*-hydroxycoumaryl units, while lignin in angiosperms is primarily composed of both guaiacyl and syringyl units, with small amounts of *p*-hydroxycoumaryl units (Whetten et al. 1998). However, lignin composition and distribution is more varied than any other natural polymer, thus using the term 'lignins' may be more appropriate. For instance,

lignin composition not only varies between species, but within a single tree, within a single cell, and across cell walls (Saka et al., 1982; Timell, 1982; reviewed in Saka and Goring, 1985; Campbell and Sederoff, 1996; Donalson, 2001; Anterola and Lewis, 2002). In addition, evidence suggests that a host of other molecules can be polymerized into the lignin matrix (c.f., Pillonel et al., 1991; Halpin et al., 1994; Doorsselaere et al., 1995; Hibino et al., 1995; Ralph, 1996; Ralph et al., 1997, 1998, 1999, 2001; Matsui et al., 2000; Higuchi, 2003; reviewed in Sederoff et al., 1999).

Lignin is deposited in the carbohydrate matrix of the primary and secondary cell wall of developing xylem (and phloem, although focus here is on xylem), marking one of the final steps in xylem cell differentiation (Donaldson, 2001; Section 2.1). The secondary wall of xylem tracheids (i.e., in conifers) is made up of three layers, S1 (outer), S2 (middle) and S3 (inner). Carbohydrate deposition (cellulose and hemicelluloses) occurs prior to lignin deposition in each of the layers. In conifers, lignification begins in the cell corners of the primary wall, while cellulose is being deposited in the S1 layer (Terashima et al., 1988; Higuchi, 1990, 2003). Lignin deposition proceeds through the primary wall and in the outer layer of the secondary wall (Donaldson, 2001) while lignification of the remainder of the secondary wall generally occurs only once the carbohydrate matrix of the S2 layer is well established (Hepler et al., 1970; Takabe et al., 1981; Boerjan et al., 2003). Lignin forms chemical bonds with the hemicellulose portion of the carbohydrate matrix in which it is embedded, forming what are known as lignin-carbohydrate complexes (LCC; Iversen, 1985; Higuchi, 1990; Donaldson, 2001), anchoring lignin within the wall.

While much work has been done in the last 50 years, a *complete and comprehensive* understanding of lignification and the heterogeneous lignin structure have yet to be achieved. For example, it is still not known whether lignin formation and deposition occur in a random or orderly fashion (Lewis, 1999).

Many reviews of lignin and its biosynthetic pathways (specifically the phenylpropanoid pathway) have been written (for example: Brauns, 1952; Freudenberg, 1959, 1962, 1964, 1965; Neish, 1960; Brown, 1961, 1966, 1969; Freudenberg and Neish, 1968; Adler, 1977; Grisebach, 1977, 1981; Hahlbrock and Grisebach, 1979; Gross, 1979, 1980, 1985; Grand et al., 1982; Higuchi, 1985, 1990; Monties, 1989; Yamamoto et al., 1989; Lewis and Yamamoto, 1990; Chen, 1991; Whetten and Sederoff, 1995; Boudet et al., 1995; Campbell and Sederoff, 1996; Douglas, 1996; Boudet and Grima-Pettenati, 1996; Whetten et al., 1998; Lewis and Sarkanen, 1998; Lewis et al., 1998; Jung and Ni, 1998; Boudet, 1998, 2000; Baucher et al., 1998; Lewis, 1999; Grima-Pettenati and Goffner, 1999; Donaldson, 2001; Dixon et al., 2001; Hatfield and Vermerris, 2001; Humphreys and Chapple, 2002; Anterola and Lewis, 2002; Boerjan et al., 2003; Ralph et al., 2004). From the foregoing and additional, uncited works, it is evident that there is strong interest in this challenging, unresolved aspect of wood formation. Focus of these papers is incongruous, and includes topics such as lignin isolation methods, phenylpropanoid pathway enzymes and their (plausible) functions, polymerization, and genetic engineering, for example. As new technologies have become available, new doors have been opened.

Slow and uncertain progress in lignin research was partially to blame for the

limited effort to understand lignin biosynthesis up to the mid-1900s, according to Stewart (1957). However, the 1950's and 1960's saw rapid advances toward understanding lignin biosynthesis (and, thus, coniferin and coniferyl alcohol biosynthesis). Many earlier studies on lignin biosynthesis were conducted by Freudenberg, Neish, Brown, Higuchi and their coworkers (c.f., Brown et al., 1953; Brown and Neish, 1955a,b, 1959; Freudenberg, 1959, 1962, 1964, 1965; Brown et al., 1959; Neish, 1960, 1961, 1964; Brown, 1961, 1966, 1969; Higuchi, 1962; Higuchi and Brown, 1963a,b,c; Freudenberg and Harkin, 1963), and of these, enzymatic studies using radio-labelled molecules (such as carbon dioxide, glucose, L-phenylalanine, cinnamic acids, and cinnamyl alcohols, for example) provided insight into identifying plausible lignin precursors (c.f., Brown et al., 1953; Brown and Neish, 1955a,b, 1959; Eberhardt and Schubert, 1956; Nord and Schubert, 1957; Wright et al., 1958; McCalla and Neish, 1959; Brown et al., 1959; Kosuge and Conn, 1959; Schubert and Acerbo, 1959; Hasegawa et al., 1960; Neish, 1961; Higuchi, 1962; Finkle and Nelson, 1963; Higuchi and Brown, 1963a,b,c; El-Basyouni et al., 1964; Bland and Logan, 1967; Fujita and Harada, 1979; Terashima et al., 1986, 1988; Terashima and Fukushima, 1988; Fukushima et al., 1996, 1997; Brunow et al., 1998a). For example, Brown and coworkers (1953) fed labelled carbon dioxide ($^{14}CO_2$) to wheat plants (*Triticum vulgare* Vill., var. Thatcher) for a mere twenty minutes and recovered radio-label in lignin only four hours following the feeding (cellulose acquired radio-label more rapidly than lignin). Tracer experiments revealed that all enzymes necessary to convert L-phenylalanine to lignin (Higuchi, 1962) and even glucose to lignin were present in the

cambial zone of higher plants (Schubert and Acerbo, 1959; Hasegawa et al., 1960; Brown, 1966; Brunow et al., 1998a), although it is still possible that lignin precursors are transported from other parts of the plant (Hasegawa et al., 1960).

Tracer experiments revealed that shikimic acid is the likely precursor of the aromatic ring in lignin (Brown and Neish, 1955b; Eberhardt and Schubert, 1956; McCalla and Neish, 1959). Labelled shikimic acid was fed to cut ends of stems of wheat (*Triticum vulgare* Vill., var. Thatcher) and ash-leaved maple (*Acer negundo* L., var. *interius*; Brown and Neish, 1955b), intact sugar cane plants (*Saccharum officinarum*; Eberhardt and Schubert, 1956; Nord and Schubert, 1957) and *Salvia splendens* (McCalla and Neish, 1959). In all cases, the label was recovered in lignin, leading Neish (1960) to conclude that "the shikimic acid pathway is probably an important route for formation of aromatic C_6-C_3 compounds in plants." Nord and Schubert (1957), using living sugar cane plants, were able to determine that the carbon atoms in the cyclohexene ring in shikimic acid were not rearranged upon aromatization and incorporation into lignin. They later described lignification as "but one illustration of the more general phenomenon of aromatization" (Nord and Schubert, 1962).

Brown (1966), in a review on lignin studies, pointed out limitations of *in vivo* tracer experiments, such as 'inability to establish obligate pathways' and 'possibility of enzyme induction by the administration of the tracer.' Thus, while tracer experiments revealed plausible intermediates in lignin biosynthesis, and gave researchers an idea as to what enzymes were functioning along the pathway to lignin formation, isolation of these enzymes provided the next step in understanding lignin biogenesis. Throughout the

latter part of the 1960's and into the 1970's and 1980's, phenylpropanoid pathway enzymes were isolated (and eventually purified and characterized) from a number of different plant species. The 1990's and into the new millennium have seen rapid advances in the broad field of molecular biology, with characterization of genes coding for specific phenylpropanoid enzymes. Genetic manipulation, such as down-regulation, has resulted in altered lignin compositions and characteristics in a variety of plant types (Anterola and Lewis, 2002). Rate-determining enzymes have also been identified. However, it is clear that lignification is highly complex and despite many advances, our understanding of phenylpropanoid metabolism and lignin biogenesis is still incomplete. Different species and even different cell types within a single organism may not follow the same sequence along these biosynthetic pathways (Whetten and Sederoff, 1995).

In 1962, Nord and Schubert remarked upon the complexity and immensity of lignin bio-formation from glucose: "the almost complete disparity of chemical nature between a carbohydrate on the one hand, and an aromatic polymer (i.e., lignin) on the other, clearly implies an extended series of far-reaching enzyme reactions in order to effect this profound transformation." Focus in lignin research has largely been on the phenylpropanoid pathway, which includes the conversion of L-phenylalanine to cinnamoyl-CoA thioesters, and the monolignol pathway, which involves the reduction of these esters to cinnamyl alcohols. Figure 6 represents my current understanding of phenylpropanoid metabolism, based on research by many groups over several years to date (most recent reviews include: Whetten and Sederoff, 1995; Boudet and Grima-Pettenati, 1996; Boudet, 2000; Dixon et al., 2001; Anterola and Lewis, 2002; Baucher et

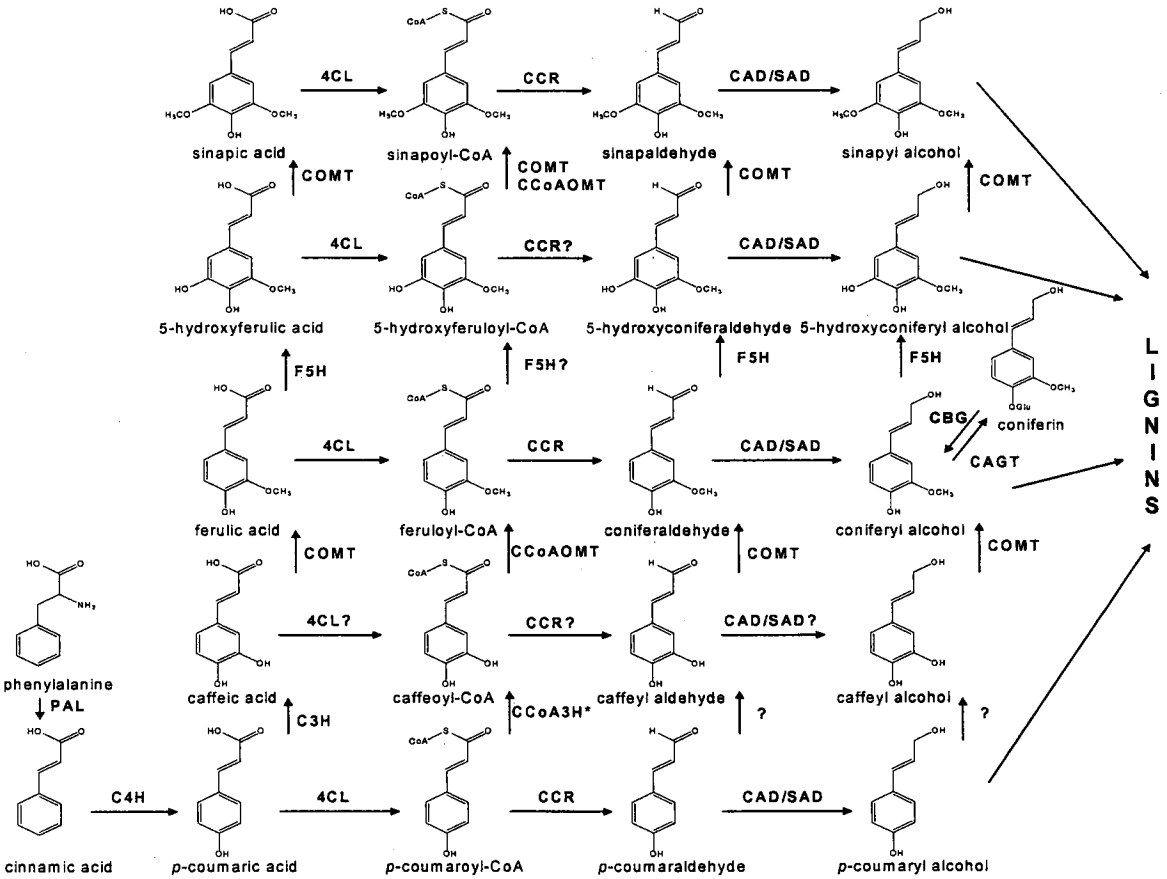


Figure 6. Current understanding of monolignol biosynthesis via phenylpropanoid metabolism. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; C3H, *p*-coumarate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyl-transferase; CBG, coniferin β-glucosidase; CAGT (also UDPG: CAGT), uridine 5'-diphosphoglucose: coniferyl alcohol glucosyltransferase; F5H, ferulate 5-hydroxylase. *CCoA3H, coumaroyl CoA 3-hydroxylase; an alternate route from p-coumaroyl-CoA to caffeoyl-CoA is through shikimic acid and quinic acid esters of these two compounds (Schoch et al., 2001; Nair et al., 2002). Not all conversions are known (those with ? remain unconfirmed *in vivo*).

al., 2003; Boerjan et al., 2003; Higuchi, 2003). The research presented in this dissertation focusses on coniferin metabolism, such as the interconversion of coniferyl

alcohol and coniferin, it's 4-*O*-β-D-glucoside, and thus the enzymes responsible, UDPG: coniferyl alcohol glucosyltransferase (CAGT) and coniferin β-glucosidase (CBG). These are reviewed more extensively in subsequent chapters. The discovery of the enzymes presented in Figure 6 is not discussed further here.

The sub-cellular location of phenylpropanoid enzymes has been investigated over the past four decades. Earlier autoradiographic investigations indicated that lignin precursors were associated with the Golgi apparatus and cell walls (Pickett-Heaps, 1968; Wooding, 1968; Fujita and Harada, 1979, Takabe et al., 1985). More recently, PAL, COMT, 4CL and CAD (Figure 6) were localized to the cytosol of developing xylem of *Populus kitankamiensis* (Takabe et al., 2001) which is in agreement with Kersey et al.'s (1999) findings with *Medicago sativa* L. (alfalfa). Hydroxylase enzymes C3H, C4H and F5H (Figure 6) are membrane-bound P450 enzymes, purportedly fulfilling their role of hydroxylating cinnamic acid derivatives on the surface of the endoplasmic reticulum (Chapple, 1998; Schoch et al., 2001). Peroxidases, one class of enzymes involved in dehydrogenative polymerization of monolignols (others enzymes thought to play a role in the final stage of lignin biosynthesis are laccases, polyphenol oxidases and coniferyl alcohol oxidase; Higuchi, 1985; O'Malley et al., 1993; Savidge et al., 1998; reviewed in Boerjan et al., 2003), have been localized to the cell wall (Hepler et al., 1972), and more specifically, the cell membrane in *P. kitankamiensis* (Takabe et al., 2001). Thus, monolignols formed in the cytoplasm must be transported to the cell membrane where they can be dehydrogenatively polymerized into lignin. As Boerjan et al. (2003) recognize, it is not yet known if transport of monolignols or their glucosides, such as

coniferin, occurs via Golgi-derived vesicles, although this is the preferred hypothesis (Dixon et al., 2001; Higuchi, 2003). For a visual representation, Takabe et al. (2001) and Dixon et al. (2001) both provide interesting schematic representations of monolignol synthesis within the cell.

3.2 Lignin isolation methods

While advances in molecular biology and genetic engineering have led to better understanding of wood formation processes, many lignin isolation and characterization methods (such as the Klason method, acetyl bromide determination, and thioacidolysis, for example; Morrison, 1972) were and still are lacking in molecular resolution. That is not to say that with better instrumentation and technique there have not been improvements in structural analysis. The fact is that many analyses still rely on earlier processes (such as nitrobenzene oxidation, for example) for both qualifying and quantifying lignin composition and content, especially after genetic alterations have been applied (Anterola and Lewis, 2002). Degradation products resulting from these isolation methods are elusive representatives of lignin monomers, although they are used to both qualify and quantify structures and linkages present in three-dimensional lignin polymers. In the introduction chapter to their book *Methods in Lignin Chemistry*, Lin and Dence (1992) reiterate: "In an ideal procedure, the lignin is recovered quantitatively, free of non-lignin contaminants, and chemically unmodified." Reviews of technical limitations in both past and present lignin analysis methods can be found in Lewis and Yamamoto (1990), Lin and Dence (1992), Lapierre (1993), Terashima et al. (1998),

Anterola and Lewis (2002) and in selected chapters of Lewis and Sarkanen (1998).

Estimates of lignin monomer composition (via nitrobenzene oxidation, Creighton et al., 1941; Chen, 1992; hydrogenolysis, Hwang and Sakakibara, 1981; Sakakibara, 1992; and more recently carbon-13 NMR, Manders, 1987, Terashima et al., 1995a; derivatization followed by reductive cleavage (DFRC), Lu and Ralph, 1998; permanganate oxidation, Lewis and Yamamoto, 1990; Gellerstedt, 1992; thioacidolysis, Lapierre et al., 1986, 1988; Rolando et al., 1992; Lapierre, 1993; and pyrolysis GC/MS, Ralph and Hatfield, 1991; Meier and Faix, 1992; Zhong, 1996, for example), often using methoxyl content of degradation products (benzaldehydes and thioesters) to differentiate between three main 'types' of lignin: *p*-hydroxycoumaryl (H-lignin), guaiacyl (G-lignin) and syringyl (S-lignin), suffer from severe limitations, as well. For example, ratios of the three lignin residues, H, G and S, obtained by these methods can not be taken as accurate, as H residues are more condensed than G residues, which in turn are more condensed than S residues and the more condensed the structures are, the more difficult they are to extract (Lapierre, 1993). Another problem is that internal standards are often not used in these analyses. Furthermore, ratios obtained by methods such as nitrobenzene oxidation and thioacidolysis usually represent only a small fraction (from 10 to 50 percent) of lignin present (Anterola and Lewis, 2002). In addition, non-lignin phenolic extractives, as well as carbohydrate degradation products, are sometimes falsely analysed as lignin, resulting in erroneous H/G/S ratios, and percent lignin (Terashima et al., 1998; Lu and Ralph, 1998; Gang et al., 1998). Other limitations of these methods are noted below.

The derivatization followed by reductive cleavage, or DFRC method, is more selective than thioacidolysis, requires milder conditions and does not cleave ester linkages. However, severe interference from carbohydrate degradation products affects analysis, and it is a multi-step process, with many structural changes and potential for artefact formation (Lu and Ralph, 1998). Pyrolysis offers high sensitivity in detecting lignin in tissues, whereby one run requires only a few micrograms of sample (Lapierre, 1993, Zhong, 1996). Lignin isolation is not a necessary pre-treatment of samples in pyrolysis techniques, as no interference from polysaccharides have been observed (Meier and Faix, 1992). In determining monomer composition, pyrolysis monomers represent less than 20 % of core lignins (Lapierre, 1993), which is slightly better than those obtained by thioacidolysis and nitrobenzene oxidation. In thioacidolysis, secondary reactions occur between degradation products, thereby resulting in artefact formation and decreased yields (Lapierre, 1993). Fourier-transform infrared spectroscopy has also been employed in lignin molecular characterization investigations (Sarkanen et al., 1967a,b; Faix, 1992; Zeier and Schreiber, 1999).

Chen (1992) implies that the accuracy of nitrobenzene oxidation (originally developed by Freudenberg, 1939; Freudenberg and Lautsch, 1939), as well as cupric oxide oxidation, is only 3 - 5 % in quantitative analyses. He goes on to say that these two methods "are effective in the characterization of lignins if they are applied imaginatively" (Chen, 1992)! Another method initially developed by Freudenberg is permanganate oxidation (detailed in Gellerstedt, 1992). Here, aliphatic side chains are degraded to carboxylic acids and subsequently methylated to esters. Methoxyl units

bound to the aromatic ring remain intact while functional groups on the phenolic rings are derivatized. Thus, differences in monomer composition are revealed by differences in methoxyl content; however, differences in aliphatic side chains, as well as linkage types, go undetected (Gellerstedt, 1992).

Partial lignin isolation can also be achieved through biotic action, in particular using brown-rot fungi. Brown-rot fungi, such as *Poria vaporaria*, *P. monticola*, *P. contigua*, *Lenzites abietina* and *L. trabea*, for example, consume both cellulose and hemicellulose from the cell wall, leaving lignin mostly intact, with some alteration, such as partial demethylation and degradation to smaller products (Kirk and Highley, 1973; Kirk, 1975; Haider and Trojanowski, 1980; Highley et al., 1985; Highley and Dashek, 1998). The remaining lignin residue has a brownish color, hence the name brown-rot fungi. However, brown-rot fungi have been shown to degrade cell wall lignin, even in cell corners and the middle lamella, which are rich in lignin (Highley et al., 1985), and to degrade lignin all the way to carbon dioxide in certain cultures (Haider and Trojanowski, 1980). Lignin degradation is achieved by white-rot fungi, such as *Coriolus versicolor*, *Phanerochaete chrysosporium* and *Phlebia radiata* (Higuchi, 1990). White-rot fungi degrade all wood cell wall components; however, they consume lignin at a faster rate than cellulose or hemicellulose (Highley and Dashek, 1998). While Kirk and coworkers are largely responsible for much of the earlier work on isolating white-rot fungi enzymes (Kirk and Moore, 1972; Kirk and Highley, 1973; Kirk and Chang, 1974, 1975; Kirk et al., 1975; Tien and Kirk, 1983), Higuchi and his coworkers have advanced our understanding of white-rot fungi by determining the probable mechanisms

responsible for lignin degradation by enzymes (peroxidases and laccases) from these fungi, using radio-labelled lignin substructure dimers and oxidizing reagents (Umezawa and Higuchi, 1985, 1989; Higuchi, 1993, 2004, 2006).

NMR spectroscopic methods have been used (examples of application include Nimz, 1974; Nimz et al., 1981; Leary et al., 1986; Sakakibara et al., 1987; Robert et al., 1989; Lundquist and Stern, 1989; Fukagawa et al., 1991; Landucci et al., 1992; Lundquist, 1992; Robert, 1992; Eberhardt et al., 1993; Lapierre, 1993; Terashima et al., 1995a; Ralph, 1996; Schroeder et al., 1997; Brunow et al., 1998b; Ralph et al., 1998, 1999, 2001; Chabannes et al., 2001), often in combination with degradation/isolation methods, to identify building blocks of lignin (Ralph et al., 1999), linkages among phenylpropane units (Ralph et al., 2001), and carbohydrate associations (Eglinton et al., 2000), as well as to determine monolignol ratios (Manders, 1987; Robert et al., 1989). Both proton and carbon-13 NMR have been used to identify differences, if any, in degradation products of tissues involved in feeding experiments (Eberhardt et al., 1993; Terashima et al., 1997), as well as in lignins from plants with down-regulated phenylpropanoid enzymes (Ralph et al., 1998). However, these methods also suffer serious limitations. When applied to lignin macromolecules (i.e., not chemically degraded), it becomes quite difficult to assign signals in the spectra, due to severe overlapping signals (for example, Xie and Terashima, 1991; Robert, 1992; Xie et al., 1994). Interference from cell wall-bound phenolics also reduces accuracy. Oftentimes, chemical shifts are compared to model compounds, usually dimers, for chemical characterization (Manders, 1987). Clearly, chemical shifts of carbons (i.e., in ^{13}C NMR)

in polymers may vary from similar carbon atoms present in larger polymers (Robert, 1992). Lignin degradation products are sometimes acetylated before they are analysed via NMR spectroscopy, to render them more soluble; however, Ralph (1996) isolated endogenously acetylated lignin from kenaf, indicating that previous NMR studies may have missed acetylated lignins in the past. Robert (1992) suggests that a major advantage of using ^{13}C NMR for lignin analysis is that it provides information for all types of carbon atoms present in the polymer.

Techniques for investigating lignin *in situ* have also been developed. Microauto-radiography is one example of a tool used to investigate intact lignins (Terashima et al., 1988), as are UV microscopy (Sarkanen et al., 1967a,b; Saka et al., 1982), scanning-electron microscopy (SEM; Westermark et al., 1988), scanning tunneling microscopy (STM; Radotic et al., 1994), transmission electron microscopy with $KMnO_4$ staining (TEM; Hepler and Newcomb, 1963; Hepler et al., 1970; Pickett-Heaps, 1968; Fujita and Harada, 1979), and bromination combined with energy dispersive analysis of X rays (EDAX; Saka et al., 1978; also called EDXA; Westermark et al., 1988).

Due to the variety of limitations plaguing lignin structural analysis methods, and more importantly, isolation methods, a shadow of doubt must be cast over results obtained from molecular biology and genetic engineering studies which have tended to rely on suspect chemical methods of identifying lignin monomeric composition following genetic manipulation (Anterola and Lewis, 2002). Failure to develop a reliable technique for both qualifying and quantifying lignin heterogeneous structures remains a major obstacle to progress and has serious implications on our understanding of the

phenylpropanoid pathway, as well as on the polymerization process in lignin biogenesis. Thus, Figure 6 represents only one interpretation of phenylpropanoid metabolism, but with emergence of new methods and techniques, perhaps even greater variety in lignin monomeric composition will be discovered, and the metabolic grid (i.e., of phenylpropanoid metabolism) proposed by Dixon and coworkers (2001) rendered ever more complex!

4. Coniferin

Much of the following information is again provided within Chapters 2 - 4 and Appendix A, but the following discussion of coniferin provides a more cohesive and comprehensive look at research involving this peculiar compound.

E-Coniferin (i.e., 4-*O*-β-D-glucoside of coniferyl alcohol; designated simply as coniferin) is the dominant aromatic glucoside in conifer cambial tissue (c.f., Freudenberg, 1959; Freudenberg and Harkin, 1963; Marcinowski and Grisebach, 1977; Schmid and Grisebach, 1982; Terazawa and Miyake, 1984; Terazawa et al., 1984a,b; Savidge, 1988, 1989, 1991; Savidge and Förster, 1998; Savidge et al., 1998). Nearly a century and a half ago, Kübel (1866) isolated coniferin from several conifers, and demonstrated that it was a glucoside. At the time, coniferin was called abietin; however, Kübel (1866) changed its name to coniferin, noting its wide-spread occurrence in conifers. Coniferin was first chemically characterized by Tiemann and coworkers (Tiemann and Haarmann, 1874), an achievement subsequently considered by Falshaw and coworkers (1969) to be 'one of *the* classical structural investigations' in terms of

chemical structure elucidation. Falshaw et al. (1969) were among the first to spectroscopically identify coniferin using infrared spectroscopy (IR; partially described previously by Hergert, 1960), nuclear magnetic resonance spectroscopy (NMR), ultraviolet spectroscopy (UV), and mass spectrometry (MS).

E-Coniferin can be readily obtained from cambium and developing xylem of conifers (c.f., Savidge, 1989), and straightforward methodology for synthesizing coniferin and related monolignol glucosides has been reported (Matsui et al., 1994; Terashima et al., 1995b; Daubresse et al., 1998; Beejmohun et al., 2006). *In vitro*, coniferin can be synthesized via transfer of glucose from uridine 5'-diphosphoglucose (UDPG) to coniferyl alcohol, as catalysed by UDPG: coniferyl alcohol glucosyltransferase (UDPG: CAGT; EC 2.4.1.111; Ibrahim and Grisebach, 1976).

E-Coniferin has been reported to accumulate in the cambial region of conifers, such as Norway spruce (*Picea abies* (L.) Karst.; Freudenberg and Harkin, 1963; Marcinowski and Grisebach, 1977; Schmid and Grisebach, 1982), western hemlock (*Tsuga heterophylla* (Raf.) Sarg; Krahmer et al., 1970), Japanese larch (*Larix leptolepis* Gord.; Terazawa and Miyake, 1984; Terazawa et al., 1984b), lodgepole pine (*Pinus contorta* Dougl. var. *latifolia*; Savidge, 1988), white spruce (*Picea glauca* (Moench) Voss), jack pine (*Pinus banksiana* Lamb.; Leinhos and Savidge, 1993; Leinhos et al., 1994; Förster and Savidge, 1995), white pine (*Pinus strobus* L.) and tamarack (*Larix laricina* (Du Roi) K. Koch; Savidge, 1989, 1991), to name a few earlier examples. Coniferin has been isolated from differentiating xylem of ginkgo (*Ginkgo biloba* L.; Tsuji et al., 2005). Coniferin has also been isolated from angiosperm cambial tissue,

such as members of the *Magnoliaceae* and *Oleaceae* families (Ibrahim, 1977; Terazawa et al., 1984a,b; Fukushima et al., 1996) as well as *Dendrobium* orchid (Nan et al., 1997). While earlier investigations report an absence of coniferin in leaves (Freudenberg and Torres-Serres, 1967; Marcinowski and Grisebach, 1977; Savidge, 1989), coniferin has been reported in needles of Douglas fir (*Pseudotsuga menziesii* (Mirb.); Morris and Morris, 1990) and Norway spruce (Slimestad and Hostettmann, 1996). Coniferin has also been isolated from cell suspensions of *Picea abies* L. (Kärkönen et al., 2002), *Linum flavum* L. (Berlin et al., 1986; van Uden et al., 1990; Hagendoorn et al., 1994; Oostdam and van der Plas, 1996), *Linum album* Kotschy (Smollny et al., 1998), *Paulownia tomentosa* Sieb. & Zucc. bark (Sticher and Lahloub, 1982), galls of *Picea glauca* (Moench) Voss (Kraus and Spiteller, 1997), transformed root cultures of *Campanula glomerata* L. var. dahurica Fish. (Tanaka et al., 1999), aerial parts of *Artemisia stolonifera* (Max.) Kom. (Lee et al., 1996), flue-cured tobacco (*Nicotiana tabacum* L.) leaves (Ito et al., 2000), *Daphne oleoides* Schreb stems (Ullah et al., 1999), leaves and stems of *Viscum album* ssp. (Deliorman et al., 1999), leaf/stem and root extracts of *Linum flavum*, *L. flavum compactum*, and *L. capitatum* (Broomhead and Dewick, 1990), *Osmanthus asiaticus* Nakai bark, which likely includes cambial tissue, although it isn't clear (Sugiyama et al., 1993), light-grown *Arabidopsis thaliana* L. Heynh. roots (Hemm et al., 2004), hairy root cultures of *L. flavum* (Lin et al., 2003), methanolic root extracts of Valerianaceae (*Centranthus longiflorus* Stev. ssp. *longiflorus*; Kuruüzüm-Uz et al., 2002), citrus peel molasses (*Citrus sinensis* (L.) Osbeck cv. Valencia, *C. paradisi* Macf. cv. Duncan and *C. paradisi* x *C. reticulata*

Blanco. cv. Murcott; Tatum and Berry, 1987; Manthey and Grohmann, 2001; *C. limon* (L.) Burm.; Manthey and Grohmann, 2001), and water-acetonitrile extracts of ground tea leaves (Japanese green tea, Chinese green tea, Chinese oolong tea and Darjeeling black tea; Nishitani and Sagesaka, 2004). Coniferin has not yet been detected in seeds (Marcinowski and Grisebach, 1977; Marcinowski et al., 1979). Its presence in such a wide variety of species led Paris (1963) to suggest that coniferin is of little taxonomic value.

Z-coniferin (also known as faguside; Morelli et al., 1986) has been isolated (Dübeler et al., 1997), along with *Z*-syringin, *Z*-coniferyl alcohol and *Z*-sinapyl alcohol, from bark of American beech (*Fagus grandifolia* Ehrh.) where significant accumulation is reported (Morelli et al., 1986; Lewis et al., 1988; Yamamoto et al., 1990). Corresponding *E*-isomers were not detected. *Z*-Isoconiferin and *Z*-syringin were also isolated from bark of *F. sylvatica* L. (Chapela et al., 1991; Dübeler et al., 1997). UDPG: CAGT from beech bark showed pronounced substrate specificity for *Z*-coniferyl alcohol, producing *Z*-coniferin (Yamamoto et al., 1990). Radiolabelling studies performed under conditions preventing photoisomerisation indicated that *E*-coniferyl alcohol was converted to *Z*-coniferyl alcohol in bark of *F. grandifolia* (Lewis et al., 1987).

An important physiological aspect of endogenous cambial coniferin is that its content oscillates in relation to the annual cycle of growth and dormancy (Section 2.1; Kremers, 1957; Kratzl, 1960; Freudenberg and Harkin, 1963; Freudenberg and Neish, 1968; Terazawa and Miyake, 1984; Fukushima et al., 1997; Savidge and Förster, 1998). It has not been detected in the cambium during winter dormancy, nor in the early spring,

prior to cambial reactivation (Freudenberg and Harkin, 1963; Terazawa and Miyake, 1984; Savidge, 1989, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998, 2001; Savidge et al., 1998). Following the dormancy period, coniferin appears in the cambial zone immediately prior to resumption of cell-division activity, well before the initiation of lignification (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Fukushima et al., 1997; Savidge and Förster, 1998; Savidge et al., 1998) and thereafter is present throughout the entire period of cambial growth. Coniferin in the cambial zone becomes non-detectable at the onset of cambial dormancy in early autumn, before the last produced cambial derivatives have concluded their differentiation into latewood tracheids (Savidge, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998; Savidge et al., 1998). Because coniferin content oscillates with cambial cell division activity over an annual cycle, and disappears during dormancy, its use as an indicator of cambial activity, or wood biogenesis, and thus, tree health, was investigated. The results are presented in Appendix A.

During peak cambial activity, endogenous coniferin levels have been detected as high as 10 mM in cambial tissues of conifers (Schmid and Grisebach, 1982; Savidge and Förster, 1998). The concentration of coniferin in isolated pine cambial protoplasts has been detected as high as 1.6 mM, accounting entirely for coniferin content in tissue extracts at the stage of development investigated (Leinhos and Savidge, 1993; Leinhos et al., 1994), indicating that very little coniferin, if any, is present in the walls of primary-walled radially-expanded cambial derivatives from which the protoplasts were collected. In order for endogenous coniferin to accumulate to high levels, it must either be

compartmentalized or biosynthesized at a greater rate than it turns over (Marcinowski and Grisebach, 1977, reported a half-life of 60 - 120 h for coniferin) or is utilised in support of lignification. Coniferin in cell suspensions of *Linum flavum* accumulated up to 12.4 % on a dry-weight basis, while callus material contained 5.6 % (van Uden et al., 1990).

Coniferin has long been considered a participant in lignification (Tiemann and Haarmann, 1874; Freudenberg and Niedercorn, 1958; Härtel et al., 1958; Kratzl and Faigle, 1958; Freudenberg, 1959; Goldschmid and Hergert, 1961; Freudenberg and Harkin, 1963; Freudenberg and Torres-Serres, 1967; Freudenberg and Neish, 1968; Terashima and Fukushima, 1988; Xie et al., 1994; Matsui et al., 1994, 1996; Fukushima et al., 1996, 1997; Förster et al., 1999, 2000) either as a reserve pool for coniferyl alcohol, the primary guaiacyl monolignol (Freudenberg and Niedercorn, 1958; Freudenberg and Harkin, 1963; Freudenberg, 1964; Freudenberg and Torres-Serres, 1967; Freudenberg and Neish, 1968; Marcinowski and Grisebach, 1977, 1978; Ibrahim, 1977; Schmid et al., 1982; Terazawa and Miyake, 1984; Gross, 1985; Terashima and Fukushima, 1988), or a transport form of coniferyl alcohol, rendering the toxic, insoluble aglycone non-toxic, more soluble, and less reactive (c.f., Pridham, 1960; Freudenberg and Torres-Serres, 1967). In earlier investigations, radioactivity from radiolabelled coniferin (only the aglycone moiety was radiolabelled) was readily incorporated into *Picea abies* (L.) Karst lignin (Freudenberg, 1964). Incorporation of isotopically labelled monolignol glucosides into lignin has been further investigated in autoradiographic studies by Terashima and coworkers (Terashima et al., 1986, 1993,

1995b, 1997; Terashima and Fukushima, 1988; Xie and Terashima, 1991). Monolignol glucosides enriched with carbon-13 at specific side chain carbons were administered to growing tree stems resulting in newly formed lignin shown to be carbon-13 enriched at the side chain carbons corresponding to those of the glucosides (Xie and Terashima, 1991; Xie et al., 1994; Matsui et al., 1994; Terashima et al., 1995a, 1997). Precocious accumulation of coniferin prior to lignification indicates that the presence of enzymes involved in phenylpropanoid metabolism (Figure 6) in the cambial region should not be considered as strong evidence that lignification is occurring.

While the primary function of coniferin seems to be in support of lignin biosynthesis in conifers, coniferin has also been purported in lignan biosynthesis (Kremers, 1957; Goldschmid and Hergert, 1961; Gross, 1985; Sakakibara et al., 1987); is known to inhibit coniferyl alcohol dehydrogenase (CAD; O'Malley et al., 1992) and 4-coumarate-coenzyme-A ligase (Voo et al., 1995) in loblolly pine, suggesting a possible role in regulating lignin biogenesis; has been shown to induce *vir*-gene activity in *Agrobacterium tumefaciens*, causing crown gall disease (Morris and Morris, 1990; Castle et al., 1992; Delay et al., 1994), suggesting a plausible role in microbial resistance in cambial regions; and, has been shown to stimulate podophyllotoxin (a cytotoxic lignan with anticancer properties; Lin et al., 2003) synthesis in cell suspension cultures of various *Linum* ssp. (van Uden et al., 1990; Woerdenbag et al., 1990; Smollny et al., 1998). A brief look at physiological roles of other cinnamyl alcohol glucosides is provided in Chapter 4.

Manskaya (1948) reportedly demonstrated that the amount of coniferin produced

during an annual cycle of growth in conifers was inversely related to the amount of peroxidase (i.e., an enzyme implicated in monolignol polymerization yielding lignin) present; however, Savidge's group (1998) reported continual presence of peroxidase throughout an annual cycle of growth in cambium of several conifer species, completely uncorrelated with coniferin content. They also found no evidence in support of coniferin being polymerized directly into lignin, using peroxidase preparations (Savidge et al., 1998); however, it is possible that other enzyme(s) may fulfill this role. van Uden and coworkers (1990) were also unable to demonstrate a relationship between coniferin and peroxidase activity in cell suspension cultures of *Linum flavum*. There are a large number of distinct peroxidases in vascular plants, and it is possible that these researchers were investigating different peroxidases.

Based on its phenological pattern of appearance and disappearance, its response to stress, as well as its precocious accumulation in relation to lignification in conifers, coniferin is an interesting, if not exceptional secondary metabolite. In studying coniferin, one must consider the two major enzymes that take part in coniferin metabolism, coniferin β-glucosidase (CBG) and UDPG: coniferyl alcohol glucosyltransferase (CAGT). Chapter 2 provides a more detailed examination of literature pertaining to CBG, while both Chapters 3 and 4 provide a look at literature involving CAGT activity.

5. Outlook of dissertation

Induced CBG activity was investigated in cambium and developing xylem tissue cultures of *P. strobus* and the results are reported in Chapter 2. Substrate specificity of

CAGT extracted from *P. strobus* was investigated, and novel substrates, both in terms of aglycones and sugar moieties, are reported in Chapters 3 and 4. A phenological pattern similar to that of coniferin content in cambial tissues of conifers was observed for CAGT activity in the cambium and its derivatives of *P. strobus* (results reported in Chapter 4). Finally, a potential application of coniferin use in tree health assessment is provided in Appendix A.

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CHAPTER 2

NOVEL *IN VITRO* INDUCTION OF CONIFERIN β-GLUCOSIDASE ACTIVITY IN CAMBIUM OF *PINUS STROBUS* L.

Abstract

Coniferyl alcohol accumulation in cambium and developing xylem tissues of *Pinus strobus* L. was investigated via HPLC and GC/MS using labelled $^{13}C_6$-coniferyl alcohol as an internal standard. Coniferyl alcohol accumulated 28-fold in developing xylem tissue incubated at 25 °C for 4 hours. Evidence indicates that coniferin β-glucosidase is the enzyme responsible for the observed accumulation of coniferyl alcohol. Coniferyl alcohol also accumulated in cambium and cytosolic fractions of developing xylem, indicating that coniferin β-glucosidase is a soluble enzyme in the cambial region of *P. strobus*.

Keywords: *Pinus strobus*; Coniferophyta; Pinaceae; eastern white pine; coniferyl alcohol; coniferin; coniferin β-glucosidase; lignification; cambium.

1. Introduction

Despite increased research interest over the past two decades, the structure of lignin and its mode of assembly have yet to be completely elucidated, and the process of lignification could be considered nearly as mystifying now as when it was first described roughly two centuries ago (Lewis, 1999; Anterola and Lewis, 2002). Deposited in both the primary and secondary wall of developing xylem, lignin offers structural support allowing for vertical growth in vascular plants. It is also responsible for hydrophobicity in water-conducting tissue and has been implicated in playing a role in defence against pathogens, insects and other herbivores (Ye et al., 2001). Thus, lignin is *essential* to tree growth and development. Given lignin's ecological and economic importance, it follows that every effort should be made to understand lignin structure and biosynthesis prior to undertaking the profound task of genetically altering lignin chemistry or content, especially without an awareness of potentially negative pleiotropic effects (Chaffey, 2002).

Lignin is a complex, heterogeneous polymer mainly composed of three primary monolignols: *p*-coumaryl alcohol (giving *p*-hydroxycoumaryl, or H lignin), coniferyl alcohol (giving guaiacyl, or G lignin), and sinapyl alcohol (giving syringyl, or S lignin). However, lignin content and composition varies with species (Campbell and Sederoff, 1996). Lignin in conifers, such as *Pinus strobus* L., is predominantly derived from dehydrogenative polymerization of coniferyl alcohol, giving G lignin, with small amounts of H lignin and no observed S lignin. Coniferyl alcohol that becomes

incorporated into lignin is thought to be formed directly by reduction of coniferylaldehyde by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195; Grisebach, 1981). However, endogenous coniferyl alcohol has not been isolated in abundance from lignifying tissue (i.e., developing xylem; Savidge and Förster, 2001). In contrast, its 4-*O*-β-D-glucoside, coniferin, accumulates to levels as high as 10 $\mu mol/g_{fw}$ in cambium and developing xylem of conifers during the growth season (Förster et al., 2000). The primary role of coniferin is thought to be in support of lignification, conceivably as a storage reserve for coniferyl alcohol (c.f., Freudenberg and Harkin, 1963; Marcinowski and Grisebach, 1977, 1978; Ibrahim, 1977; Schmid et al., 1982; Terazawa and Miyake, 1984). Another possibility is that coniferin is a transport form of coniferyl alcohol, rendering the toxic, insoluble aglycone non-toxic, more soluble, and less reactive (c.f., Pridham, 1960; Freudenberg and Torres-Serres, 1967). In either case, accumulated coniferin must be hydrolysed by a β-glucosidase in order to liberate coniferyl alcohol for polymerization into lignin.

In earlier investigations, Freudenberg (1964) postulated that coniferin is synthesized 'somewhere between the green shoots and the cambium, or in the cambium' and that upon diffusion into cells differentiating into developing tracheids, coniferin comes in contact with a β-glucosidase, releasing coniferyl alcohol for polymerization into lignin (Freudenberg and Harkin, 1963; Freudenberg, 1965). To verify this, indican (indoxyl β-D-glucoside) was applied to transverse 1-year-old stem sections of *Araucaria excelsa* and a resulting blue color (the aglycone of indican, indoxyl, is oxidised by air into

indigo) appeared where β-glucosidase was active, 'concentrated in the proximity of the cambium' (Freudenberg, 1964). Using light microscopy, the blue oxidation product was observed in radially expanding cambial derivatives not yet lignifying, as well as in lignifying derivatives, but not in fully lignified tracheids (Freudenberg et al., 1955; Freudenberg, 1964). Freudenberg was unable to demonstrate specificity toward coniferin, however. Savidge re-examined *A. excelsa* stem sections with indican and found β-glucosidase restricted to cell walls of fully lignified tracheids, with no response in the cambium or differentiating xylem elements (unpublished observations). Marcinowski and coworkers (1979) were unable to repeat Freudenberg's observations with *A. excelsa* using indican and *Picea abies* L. Karst. seedlings. A number of glucosidases and enzymes with glucosidase activity capable of hydrolysing indican have been discovered in mature xylem which are not necessarily capable of hydrolysing coniferin (for example Hösel et. al., 1978; Marcinowski and Grisebach, 1978; Marcinowski et al., 1979; Leinhos et al., 1994, Dharmawardhana et al., 1995).

However, several β-glucosidases capable of hydrolysing coniferin have been reported. Leinhos et al. (1994) isolated an acidic coniferin β-glucosidase (CBG) from the cytosolic fraction of pulverized developing xylem of *Pinus banksiana* Lamb., which had negligible homology to an acidic CBG isolated from the cell wall fraction of pulverized developing xylem of *Pinus contorta* var *latifolia* Engelm. (Dharmawardhana et al., 1995, 1999). Dharmawardhana's group (1999) were able to isolate the full length cDNA sequence of this CBG. Marcinowski and his group (1978, 1979) isolated a CBG to the

inner secondary wall of young *Picea abies* L. Karst. seedlings via immunohistochemical methods, while Burmeister and Hösel (1981) immunolocalized a CBG to epi-, endo- and exodermis of xylem elements in stems and roots of *Cicer arietinum* L. seedlings. Tsuji and coworkers (2005) isolated a CBG from the developing xylem of *Ginkgo biloba* L. capable of deglucosylating both coniferin and coniferyl aldehyde glucoside, but with much higher activity toward coniferyl aldehyde glucoside. While it remains unclear whether these are all distinct enzymes, these researchers and others agree that a coniferin-specific β-glucosidase is involved in lignification due to the spatial and temporal association of CBG with lignifying tissue (Marcinowski and Grisebach, 1978; Marcinowski et al., 1979; Burmeister and Hösel, 1981; Leinhos et al., 1994; Dharmawardhana et al., 1995, 1999; Boudet and Grima-Pettenati, 1996; Grima-Pettenati and Goffner, 1999). This lends support to Freudenberg's original hypothesis that a β-glucosidase capable of hydrolysing coniferin, and the other cinnamyl alcohol glucosides, is critical to the lignification process (c.f. Freudenberg, 1964).

Tsuji's group has investigated possible CBGs in a variety of species recently (Tsuji and Fukushima, 2004; Tsuji et al., 2004, 2005). Tsuji and coworkers (2004) administered labelled coniferin and coniferyl alcohol to excised shoots of magnolia and eucalyptus and recovered the label in lignin in both cases, providing evidence that a CBG was likely responsible for liberating coniferyl alcohol from coniferin before polymerization into lignin. The label was more pronounced in syringyl lignin for trees fed coniferin rather than those fed coniferyl alcohol, indicating a potential role for CBG in regulating

monolignol composition (Tsuji et al., 2004). In another study, Tsuji and Fukushima (2004) administered labelled coniferin, syringin, coniferaldehyde glucoside, and sinapaldehyde glucoside to cut stem segments of magnolia and oleander. Their results suggested that coniferaldehyde and/or coniferaldehyde glucoside are important in biosynthesis of syringyl lignin in these species, as the efficiency of incorporation of coniferaldehyde glucoside was greater than that of sinapaldehyde glucoside (Tsuji and Fukushima, 2004). Their results also suggested that coniferin must be converted to coniferaldehyde via coniferaldehyde glucoside before being incorporated into lignin, which was also observed in ginko (Tsuji et al., 2005). In the ginkgo study, Tsuji and coworkers (2005) extracted CBG from developing xylem and assayed the protein extract with coniferin and coniferaldehyde glucoside and found that the enzyme had a greater substrate specificity for coniferaldehyde glucoside than for coniferin. This led them to hypothesize that endogenous coniferaldehyde may not accumulate in developing xylem of conifers because CBG is more active for it than for coniferin. They found that coniferaldehyde glucoside and coniferin were incorporated similarly both spatially and temporally into lignin (Tsuji et al., 2005). Taken together with the findings that cinnamyl aldehydes are good substrates for UDPG: CAGT (uridine 5'-diphosphoglucose: coniferyl alcohol glucosyl transferase; Ibrahim and Grisebach, 1976; Schmid and Grisebach, 1982; Steeves et al., 2001) and the seasonal pattern of CAGT activity is the same for coniferaldehyde as it is for coniferin in *P. strobus* (Steeves et al., 2001), it is plausible that the route from coniferin to coniferyl alcohol does go through the aldehyde glucoside in this system, although the authors are careful to caution that this was observed in

wounded tissue and may not occur *in planta*. It is unclear, at this time, whether the glucosidase activity observed by Tsuji's group is the result of CBGs mentioned above.

Coniferin-specific β-glucosidase (CBG) isolated from lignifying tissues of *Pinus banksiana* was found only in low abundance (Leinhos et al., 1994). Its catalytic activity evidently was not competitive with that of UDPG: CAGT (i.e., the enzyme responsible for catalysing glucosylation of coniferyl alcohol to give coniferin) found in the same tissues, at the same developmental stage (Savidge and Förster, 1998). CAGT activity was readily detectable in the cambial zone (CZ) of *P. banksiana* and *P. strobus* before cambial derivatives began differentiating into tracheids, and remained detectable in differentiating xylem elements until cambial dormancy (Savidge et al., 1998; Förster et al., 2000). Thus, it appears that the catalytic environment of the cambium and its derivatives, at least in conifers, favours coniferin accumulation. In addition, elevated CAGT activity in the active cambium provides a mechanism of continuous removal of coniferyl alcohol (i.e., in support of coniferin biosynthesis; up to 10 mM when it begins to inhibit CAGT activity; Savidge and Förster, 1998), thus possibly forcing CAD equilibrium between coniferyl alcohol and coniferylaldehyde to favour coniferyl alcohol production, according to Le Châtelier's Principle (Savidge, 1989; Förster et al., 1999; Förster et al., 2000). Recall that Le Châtelier's Principle states that if a system at equilibrium is disturbed, it will react so as to minimize the disturbance and re-establish equilibrium. For example, the removal of coniferyl alcohol shifts the equilibrium in favour of minimizing the loss of coniferyl alcohol by producing more coniferyl alcohol.

Seasonal patterns of β-glucosidase activity in relation to the annual cycle of cambial growth have been investigated with *Pinus sylvestris* L., *Picea abies* (L.) Karst. and *Betula pendula* Roth. (Marjamaa et al., 2003). CBG activity was not detectable in protein extracts (cell wall material remaining after extraction steps was discarded) of mature xylem of dormant stems, and became detectable in developing and mature xylem prior to lignin deposition in the secondary wall and remained detectable throughout the period of cambial growth. These results agree with observations by Savidge and Leinhos (unpublished data) that indicate that β-glucosidase activity was weak, possibly absent, from dormant cambium, and peaked in developing xylem during cambial activity, further supporting its role in lignification. Coniferin has not been isolated from dormant cambium during winter months (Freudenberg and Harkin, 1963; Terazawa and Miyake, 1984; Savidge, 1989, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998, 2001; Savidge et al., 1998), which is likely tightly linked to the absence of detectable CBG activity in dormant tissue. Hösel et al. (1982) reported CBG activity in parsley (*Petroselinum hortense*) cell suspension cultures that was closely associated with lignification. CBG activity became detectable with the onset of activity of other lignin biosynthesis enzymes, and this was followed closely by lignin deposition. Hosokawa and coworkers (2001) determined, using HPLC and GC-MS, that coniferyl alcohol concentration in cultured *Zinnia elegans* L. mesophyll cells peaked at initiation of secondary wall thickening, then decreased rapidly during secondary wall thickening, and finally increased again. This may also be evidence for seasonal fluctuation in CBG activity, with higher CBG activity at the start of secondary wall deposition in order to

release CA for lignin biosynthesis.

One roadblock in our path to understanding CBG activity is that its cellular location remains unclear, making it difficult to determine if monolignols are transported to the cell wall as free aglycones or as glucosides. For example, Freudenberg's work (1964) with *A. excelsa* suggests that it is cytosolic (i.e., soluble). In induced *Escherichia coli* cells, the soluble protein fraction displayed coniferin hydrolysing activity, while the insoluble did not (Dharmawardhana et al., 1999). Wardrop (1976) histochemically localized a β-glucosidase capable of hydrolysing both indican and L-naphthyl-β-D-glycopyranoside to the cytoplasm of differentiating xylem cells of *Eucalyptus goniocalyx* F. Muell. and *Pinus radiata* D. Don.; however, he did not use coniferin as a substrate. van Uden et al. (1990) reported low CBG activity in the soluble fraction of *Linum flavum* L. cell suspension cultures. In microsomal preparations of developing xylem of *Pinus strobus* L., Savidge and Förster (2001) report little-to-no CBG activity, providing further support for cytosolic residence. Kärkönen and coworkers (2002) found much higher glucosidase activity toward both coniferin and 4-nitrophenyl β-glucopyranoside in soluble versus cell wall bound protein extracts of *Picea abies* L. suspension cultures.

Other reports indicate that the enzyme is wall-bound. For instance, Castle and coworkers (1992) isolated a CBG gene, *cbg1*, from *Agrobacterium tumefaciens* that, when expressed in *E. coli*, demonstrated activity toward coniferin in cultures with intact or disrupted cells, and no activity in cultures containing no cell wall material. Marcinowski

and Grisebach (1978) characterized a CBG in the cell wall fraction of hypocotyls and roots of *Picea abies* seedlings undergoing lignification. Interestingly, they also found β-glucosidase activity in the cambium itself, but it was not active for coniferin. They indicated that strong peroxidase activity might be masking the glucosidase's activity toward coniferin. Inhibition of peroxidase to 90 % activity still revealed no activity of the cambial β-glucosidase for coniferin (Marcinowski and Grisebach, 1978). Burmeister and Hösel (1981) isolated CBG activity to cell walls of *Cicer arietinum* L. (chickpea) seedlings by immunofluorometrical methods. Marcinowski and coworkers (1979) observed, using immunofluorescent techniques with an antiserum against CBG, that the enzyme is active at the 'inner layer of the secondary wall.' As noted in Steeves et al. (2001), antibodies that are assumed specific have been found to bind non-specifically to secondary cell walls (R.A. Savidge, unpublished data). Using enzyme-gold probes and a polyclonal antibody against CGB isolated from *Pinus contorta*, Samuels et al. (2002) demonstrated CBG activity only in the secondary cell wall in developing xylem. Again, use of antibodies specific for CBG is in question and Samuels et al. (2002) acknowledge the high phenolic levels in the tissue they analysed, which may provide non-specific binding sites.

Much evidence supports CBG being wall-bound, and yet evidence also suggests that it is soluble. Hösel and coworkers (1982) reported different findings altogether. They found that CBG activity became increasingly associated with the cell wall fraction of *C. arietinum* cells cultured in NAA-BAP medium following the onset of lignification, with

nearly half of total coniferin-hydrolysing activity isolated to the soluble fraction only two days after the cells were placed in the medium, and before the onset of lignification. Forty days after cultivation in NAA-BAP medium, nearly 90 % of CBG activity was isolated to the cell wall fraction (Hösel et al., 1982). As Samuels et al. (2002) point out "identification of the sub-cellular location of the coniferin β-glucosidase would help determine whether lignin precursors are exported as glucosides or as aglycones, an important distinction for predicting the gene products and mechanisms involved in monolignol export and incorporation into lignin." For instance, if CBG is wall-bound, it is likely that monolignols are transported as their glucosides to the wall, where they are then deglucosylated for polymerization into lignin; however, if CBG is cytosolic, it is conceivable that monolignol glucosides are cleaved within the cytoplasm, and then transported to the cell wall for polymerization as the aglycones.

In this study, induced CBG activity in the cambium and developing xylem of *P. strobus* was investigated. This is the first report, to my knowledge, of CBG activity for coniferin in isolated cambium cells. Results indicate that CBG is active in the cytosol and possibly at the cell wall of developing xylem, indicating that the enzyme is soluble in both the cambium and developing xylem of *P. strobus*.

2. Results and Discussion

2.1 Profiling work

HPLC profiles (λ280 nm; Savidge 1988) of the methanolic extracts of cambium and

developing xylem at different stages of development are depicted in Figure 1 (profiles at λ254 nm, λ310 nm are not presented), along with anatomical data. Coniferin content increased in synchrony with cambial activity, as evidenced by HPLC profiles, and as expected (Savidge and Förster, 1998). A peak eluting at 11 minutes appeared in conjunction with increased lignification in the May 24th extract. At this time, a well-established zone of radially expanded cells undergoing lignification in their secondary cell walls (SL) was present, which was not present on earlier sampling dates. The peak was collected and analysed by UV and GC/MS to be *E*-coniferyl alcohol. HPLC chromatography of a standard sample of coniferyl alcohol confirmed the elution time. The appearance of such a large peak corresponding to coniferyl alcohol in the HPLC chromatogram prompted a more detailed look at coniferyl alcohol content in cambial tissue of *P. strobus*, as coniferyl alcohol is not known to accumulate to high concentrations in the cambium or its derivatives, although its concentration does increase over the period of cambial growth (Savidge and Förster, 2001).

2.2 Incubation studies

Limited $^{13}C_6$-coniferyl alcohol was available for quantitative analysis, thus numerous qualitative investigations were performed initially, without labelled coniferyl alcohol (CA). These results are summarized in Table 1, which also provides details of each incubation.

Table 1. Qualitative HPLC investigations of endogenous coniferyl alcohol accumulation in incubated cambium and developing xylem tissue cultures of *Pinus strobus*.

Tissue/Stem[a]	Stage of development[b]	Time stopped with HOAc (hr)	Coniferyl alcohol peak? (yes/no)	Number of replicates[c]
DX	RE + SL	0	no	3
DX	RE + SL	4	yes	3
DX	RE + SL	0 (but incubated for 4 hours after)	no	3
DX	RE + SL	4 (boiled for 15 minutes at t=0)	no	3
Cytosol fraction of DX	RE + SL	4	yes	2
Cell wall fraction of DX	RE + SL	4	yes (although much smaller peak than cytosol fraction)	2
Compression wood, DX + C, pooled	RE + SL	4	yes	1
Intact stem, DX	RE	4	no	2
Intact stem, C	RE	4	no	2
DX	RE	0	no	2
DX	RE	4	yes	2
C	RE	4	yes	2
C	RE + SL	0	no	3
C	RE + SL	4	yes	3
C + DX, pooled	dormant	0	no	2
C + DX, pooled	dormant	4	no	2
DX in 10 mM D-glucono-1,5-lactone	RE + SL	4	no	3
C in 10 mM D-glucono-1,5-lactone	RE + SL	4	no	3
DX, prepared and incubated without light	RE + SL	4	yes	3

[a] DX = differentiating xylem; C = cambium

[b] RE = zone of primary-wall radial expansion; SL = zone of differentiation where lignification of secondary cell wall is occurring.

[c] Unless otherwise indicated, each replicate was performed with tissue from a different tree.

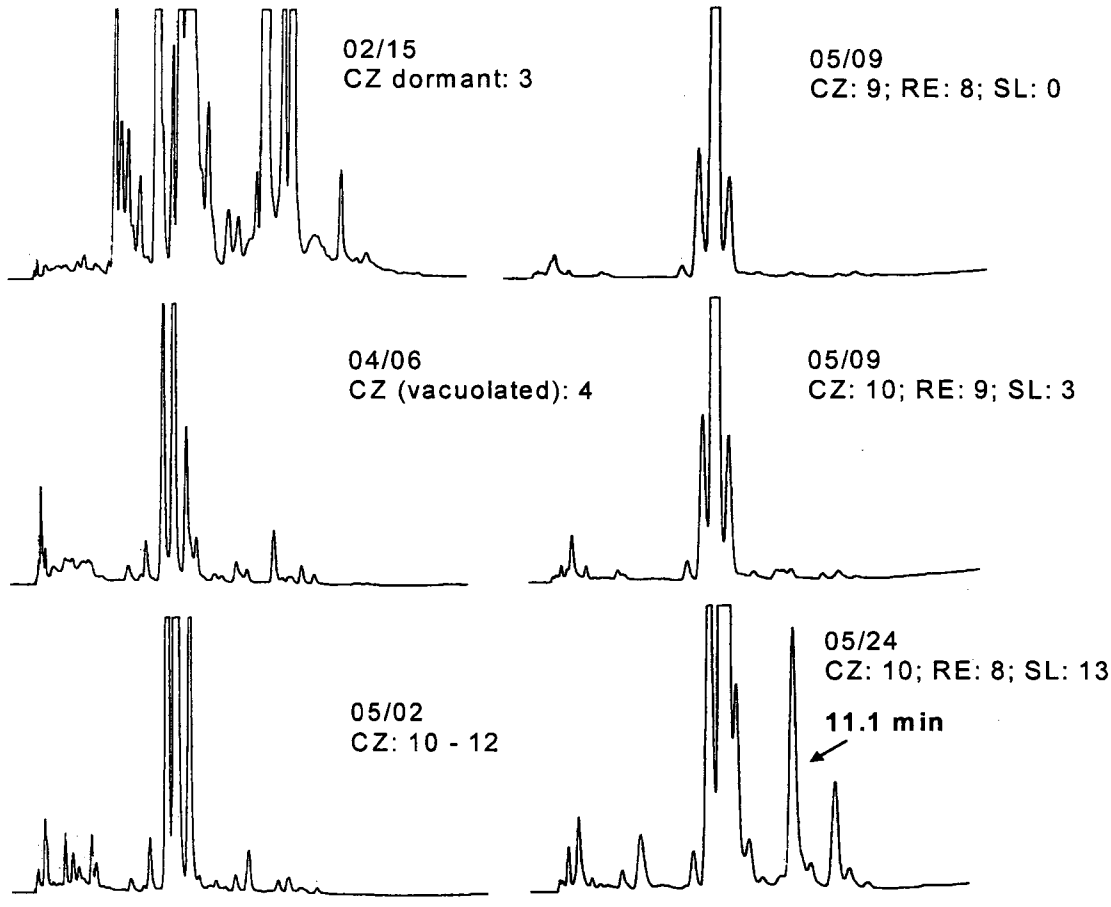

Figure 1. HPLC profiles of methanolic extracts from developing xylem of *Pinus strobus* at λ280 nm. Dates are given (month/day) and numbers of cells per radial file are also provided. CZ = fusiform cambial cells; RE = radially expanding cambial derivatives not yet lignifying; SL = radially expanded cambial derivatives undergoing bordered pit formation and lignification in their secondary walls. Note 05/09 has two samples, one with SL, and one without. Tissue from three trees was pooled for each profile.

For trees at the RE stage of development (radially expanding cambial derivatives not yet lignifying; Steeves and Savidge, 2000), two phenotypes were used. For trees at the RE + SL stage (where radially expanded cambial derivatives are undergoing both bordered pit development and lignification in their secondary wall; Steeves and Savidge, 2000), three phenotypes were used. Dormant tissue used in these investigations was harvested in

early February and stored in liquid nitrogen. CA is recorded (Table 1) as either present or absent, based on the appearance of the 11-minute peak in HPLC profiles, which was collected and confirmed to be CA via GC/MS. Figure 2a is an example of an HPLC chromatogram where no CA was detected while Figure 2b represents one where CA was detected, collected and confirmed.

According to HPLC analysis, endogenous CA accumulated in differentiating xylem tissue (i.e., both with (RE + SL) and without (RE) a zone of secondary wall deposition/lignification), both cytosol and cell wall fractions of differentiating xylem separated from one another via centrifugation, and cambial tissue after 4 hours of incubation at 25 °C. To determine if CA accumulation was enzymatic in nature, samples were boiled for 15 minutes immediately upon pulverization, and then incubated. No CA was detected in these samples after 4 hours of incubation at 25 °C, providing evidence that CA accumulation was a result of enzymatic activity, probably CBG.

To be sure that CA was not an artefact of acidic cleavage of the glucose moiety from coniferin (which was abundantly present in all but the dormant tissue) in acetic acid, acetic acid was added immediately upon pulverization and the mixture was then incubated for 4 hours at 25 °C. Again, no CA was detected, indicating that the accumulation of CA was not the result of non-enzymatic cleavage of the β-*O*-4 linkage between the coniferyl alcohol aglycone and the glucose moiety; however, enzymatic cleavage remained to be investigated.

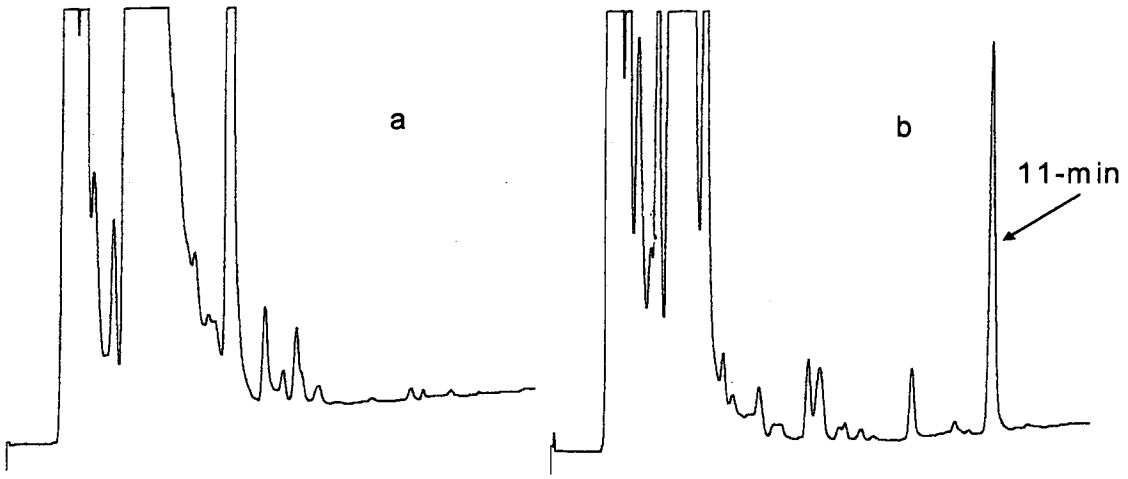

Figure 2. HPLC profiles of extracts of incubated developing xylem. a) No coniferyl alcohol detected; b) coniferyl alcohol detected at 11 minutes.

No CA was detected by HPLC in samples that were analysed at time zero (i.e., freshly harvested tissue). Acetic acid was added immediately following pulverization of these samples in liquid nitrogen. Neither CA nor coniferin were detected by HPLC in dormant tissue at time zero, or after 4 hours of incubation. This provides further support for the hypothesis that the accumulating CA in developing xylem and cambium incubated for 4 hours could have arisen through release of CA from endogenous coniferin, as coniferin has not been isolated from dormant cambial tissue (Savidge 1989, 1991; Förster and Savidge 1995; Savidge et al., 1998). To investigate whether wounding (scraping and pulverizing the tissue) was responsible for the observed increase in CA over 4 hours, intact stem segments (i.e., 15 - 20 cm in length) were incubated. CA was not detected by HPLC in either the CZ or RE + SL tissue of these samples following 4 hours at 25 °C. Care was taken to leave 2.5 cm buffer zones that were not scraped from either end of the

stem segments to negate effects of wounding at the ends of the stem segments. These results provide strong evidence that wounding did play a key role in CA accumulation.

To determine whether CA was being released from coniferin via β-glucosidase activity, a known coniferin β-glucosidase inhibitor, D-glucono-1,5-lactone (Hösel and Todenhagen, 1980; Surholt and Hösel, 1981; Dharmawardhana et al., 1995) was added to both cambium and developing xylem tissue prior to incubation. No CA was detected in either the cambium or developing xylem incubated with D-glucono-1,5-lactone after 4 hours, indicating that the observed accumulation of CA was due to coniferin β-glucosidase (CBG) activity.

Compression wood was available in one of the trees investigated, and was thus analysed separately. CA accumulated in compression wood and normal wood from the same tree. Removing all sources of light did not appear to have an effect on coniferin hydrolysis activity, as developing xylem (RE + SL) prepared and incubated in the absence of light accumulated CA similar to developing xylem prepared and incubated in daylight.

Taken altogether, the results obtained through numerous HPLC analyses (including numerous preliminary analyses not reported here) indicated that CBG activity toward coniferin was responsible for the observed accumulation of CA in incubated samples of actively-dividing cambium and developing xylem. This prompted a closer examination of these tissues using labelled CA as an internal standard. Questions that needed to be

answered included: 1) Was CA really absent in samples whose HPLC profiles revealed no CA peak?; 2) Did β-glucono-1,5-lactone really inhibit CA production?; 3) Which fraction accumulated more CA - the cytosolic or cell wall fraction?; 4) Is CBG activity found in the cytosol, cell wall, or both?; and 5) In general, how much CA accumulated in each sample?

Quantitative investigations were conducted using $^{13}C_6$-coniferyl alcohol as an internal standard (Savidge and Förster, 2001). Since results obtained with the internal standard are more rigorous than the HPLC investigations mentioned above, the remaining discussion will focus on the quantitative data. The limited amount of labelled CA allowed for only 1 replicate of each sample, and for only a small number of samples. The protocol for stopping reactions was altered from using acetic acid to using methanol, for increased CA extractability. Results are summarized in Figure 3. While HPLC analyses did not reveal the presence of CA in non-incubated tissue (Figure 3), CA was quantified from all samples investigated. All tissue at the RE/SL stage was obtained from one phenotype, and all tissue at the RE stage was obtained from a different phenotype.

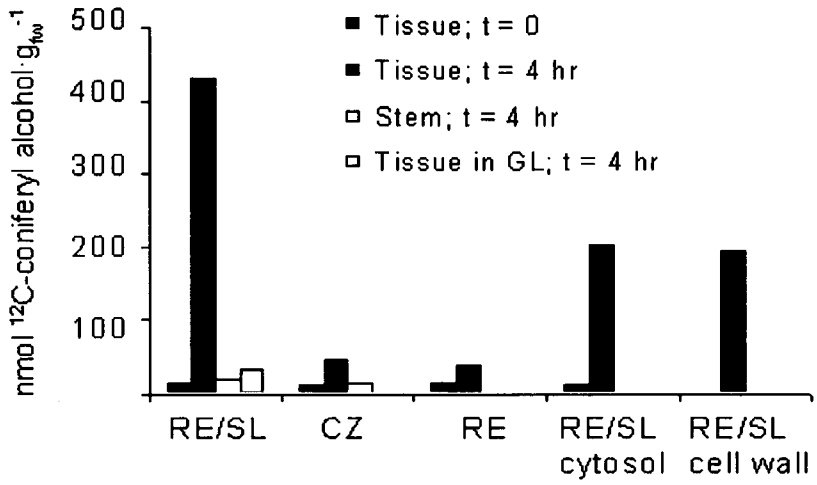

Figure 3. Endogenous coniferyl alcohol content in incubated (0 or 4 hr; 25 °C) *Pinus strobus* cambium and developing xylem, as determined by GC/MS (*m/z* 309, 315, 324, 330) with $^{13}C_6$-coniferyl alcohol as an internal standard. 'Tissue' = pulverized tissue; 'stem' = whole stem was incubated and tissue removed and pulverized at 4 hr; GL = tissue incubated in D-glucono-1,5-lactone.

Good separation was achieved during GC analysis and baseline resolution between MS peaks made it feasible to obtain integrated peak areas (Figure 4) for analysis. For discussion purposes, 'tissue' will refer to the scraped tissue that was incubated, while 'stem segment' will be used for intact stem segments that were incubated.

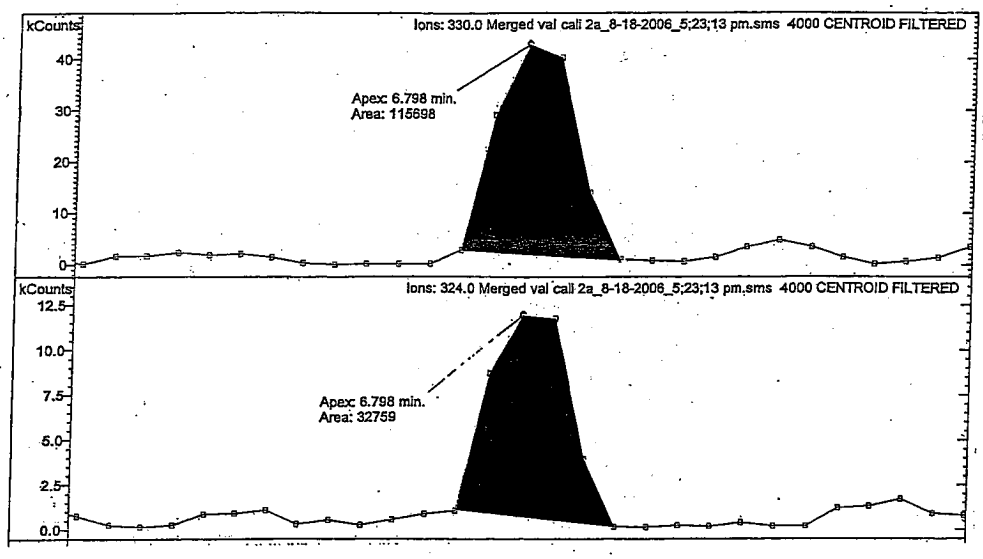

Figure 4. MS peaks with integrated peak area (*m/z* 330, 324)

Intact stem segments that were incubated for 4 hours did not accumulate CA the way that scraped tissue samples did, with CA content in stem segments after 4 hours being very similar to CA content in scraped tissue at time zero (taken as representative of the amount of CA in the tissue at the time of harvest from the tree). For example, differentiating xylem from stem segments had a CA concentration of 18.9 nmol · g_{fw}^{-1} after 4 hours compared to differentiating xylem analysed at time zero, which contained 15.3 nmol · g_{fw}^{-1} CA. For cambial tissue, compare 13.4 nmol · g_{fw}^{-1} CA in cambial tissue at time zero to cambium from stem segments with 14.4 nmol · g_{fw}^{-1} CA after 4 hours. Because CA content in stem segments was not significantly greater than that extracted from tissue at time zero, it is possible that the small difference is due to a natural variation in CA content within the plant material rather than increased CBG activity in the stem segments. Thus, the data strongly suggest that wounding via pulverization was responsible for increased CBG activity.

Developing xylem tissue undergoing lignification in the secondary wall (RE + SL) had the most pronounced accumulation of CA, with endogenous CA content increasing by a factor of 28 in 4 hours as compared to developing xylem tissue extracted with methanol immediately upon pulverization in liquid nitrogen (t = 0). This represents a rate of CA biosynthesis, or CBG activity, of 28.9 pmol $\cdot$ g_{fw}^{-1} $\cdot$ s^{-1}. Hösel et al. (1982) reported rates of coniferin hydrolysis between 3 and 18 nmol $\cdot$ g_{fw}^{-1} $\cdot$ min^{-1} (or 50 - 300 pmol $\cdot$ g_{fw}^{-1} $\cdot$ s^{-1}) for tissue cultures growing on NAA-BAP medium for 24 days, for a number of dicot plants; however, no trees were investigated in their experiments and exogenously applied coniferin served as a substrate. No GC/MS data were obtained. Also, NAA-BAP was not added to *P. strobus* tissue cultures. All of these differences combined make it difficult to compare results with *P. strobus* to those of Hösel et al. (1982). Kärkönen and coworkers (2002) determined CBG activity in soluble protein extracts of *Picea abies* suspension cultures to be 164 pmol $\cdot$ g_{fw}^{-1} $\cdot$ s^{-1}, similar to rates observed by Hösel et al. (1982). CBG activity in the cell wall bound protein fraction was only 6.7 pmol $\cdot$ g_{fw}^{-1} $\cdot$ s^{-1}. The higher CBG activity toward coniferin observed in protein extracts (Hösel et al., 1982; Kärkönen et al., 2002) compared to rates reported here for CBG activity in cambial tissue could be due the extraction of the enzyme from the tissue.

Marjamaa and coworkers (2003) investigated CBG activity toward exogenously applied coniferin in xylem protein extracts of *Pinus sylvestris* L., *Picea abies* (L.) Karst., and *Betula pendula* Roth. CBG activity for coniferin in *P. sylvestris* and *P. abies* was not detectable during dormancy and in *P. sylvestris* became detectable prior to radial

expansion of cambial derivatives, and only when radially expanded cambial derivatives were differentiating (RE zone) in *P. abies*. Their results agree closely with those reported herein for *P. strobus*, with no CBG activity detected in dormant tissue and detectable CBG activity in tissue at the RE stage of development. Cambial derivatives not yet undergoing radial expansion were not investigated in *P. strobus*. Activity reached a maximum of roughly 18 fkat (i.e, fmol $\cdot$ g_{fw}^{-1} $\cdot$ s^{-1}) for *P. sylvestris* and roughly 14 fkat for *P. abies* during secondary wall deposition (Marjamaa et al., 2003). CBG activity in *B. pendula* remained below 5 fkat throughout the period of investigation (January through September). Because CBG activity was detected well before the onset of lignification and decreased during peak lignification, they concluded that CBG activity should not be considered as being tightly linked to lignification (Marjamaa et al., 2003). They suggested that the observed decrease in CBG activity toward coniferin during the height of secondary wall formation might be due to covalent binding of CBG to the cell walls, which were discarded following extensive extraction in their preparations (Marjamaa et al., 2003). The results for *P. strobus* support their hypothesis, as CBG activity was detected in both the cytosol and cell wall fractions, although activity was higher in the cytosol (Figure 3). It is possible that CBG was contained within vesicles and that these vesicles are associated with the cell wall via fusion with the plasma membrane (Pickett-Heaps, 1968; Northcote, 1989; Takabe et al., 2001).

To determine if any of the CA accumulating in the wall fraction after 4 hours was due to CA, coniferin or even CBG from the cytosol, labelled CA was administered to whole,

pulverized differentiating xylem tissue, which was subsequently centrifuged according to section 3.3.2. The resulting pellet and supernatant were then incubated separately for 4 hours. Labelled CA was recovered from both fractions, with a smaller *m/z* 324:330 ratio in the cytosol compared to the cell wall (compare 42.8 to 50.1). Recovery of the label from the wall fraction suggests that a portion of endogenous CA associated with the pellet may also have its origins within the cytosol, since labelled CA (and it is assumed that endogenous CA would exhibit the same behaviour) became associated with the wall fraction, perhaps via diffusion into the pulverized tissue during sample preparation. The results were not quantifiable since there was no way to determine how labelled CA was partitioned between the cytosol and cell wall fractions during separation; however, it does indicate that 195 nmol · g_{fw}^{-1} CA in the cell wall fraction after 4 hours is possibly artificially high and CBG activity is not as pronounced in this tissue compared to the cytosol. Unfortunately, there was not enough labelled CA to measure the amount of CA in the cell wall fraction at time zero, although it could be considered as being close to negligible, given that 15.3 nmol · g_{fw}^{-1} CA were detected in whole differentiating xylem at time zero, and 13.3 nmol · g_{fw}^{-1} CA in the cytosolic fraction of the same tissue, at time zero. It is also possible, as previously mentioned, that CBG is located within vesicles associated with the cell wall and the conditions used here (pulverizing and centrifuging) were unable to release these from the plasma membrane, leaving CBG associated with the cell wall fraction. This is being further investigated and should not be considered as evidence that CBG is firmly embedded within the cell wall matrix.

CBG activity in the incubations reported herein was much higher than that observed by Marjamaa et al. (2003) for *P. sylvestris* and *P. abies*, on the order of 1000 times higher in some instances, possibly because of the increased incubation time (i.e., their incubations were for 30 minutes at 30 °C); however, the rate of hydrolysis may not change over time, depending on the precursor supply. The tissue investigated by Marjamaa et al. (2003) was heavily extracted prior to incubation and incubation material contained a number of exogenous reagents in addition to differentiating xylem. The systems investigated herein contained only crude tissue and added internal standard (labelled CA), with the exception of the incubations performed in the presence of β-glucono-1,5-lactone. In both studies, tissue was pulverized and thus not representative of *in planta* situations. It is possible that the higher CBG activity in *P. strobus* reported here resulted because all cell components were included, even if they were pulverized, compared to the extracted material in Marjamaa et al.'s (2003) study. It is also unclear what effect endogenous coniferin had on their investigations, if any, as they measured CBG activity toward exogenously applied coniferin, as endogenous coniferin should have been removed during the extraction process (Marjamaa et al., 2003). No GC/MS data was reported.

CBG extracts from ginkgo preferred labelled coniferaldehyde glucoside to coniferin in feeding experiments (Tsuji et al., 2005). This was not investigated here with *P. strobus*. It is possible that they are not the same glucosidase. Heavy extraction methods combined with buffers and exogenously applied precursors make their experimental set-up (Tsuji

et al., 2005) rather different from that for *P. strobus* reported here.

Tissue incubated in 1 mL of 10 mM D-glucono-1,5-lactone (GL) accumulated CA, but to a much lessened extent than tissue incubated alone (31.9 vs 431 nmol · g_{fw}^{-1} for tissue incubated in GL versus the same tissue incubated without GL, respectively; Figure 3). GL has been reported as an effective inhibitor against CBG (Hösel and Todenhagen, 1980; Surholt and Hösel, 1981; Dharmawardhana et al., 1995, 1999). It is reported to have varying inhibitory effects on other β-glucosidases. For example, 10 mM GL inhibited a β-glucosidase isolated from *Alternaria alternata* (Fr.) Keissler by 45 % (Martínez et al., 1988), while 10 mM GL inhibited a β-glucosidase isolated from *Aspergillus ornatus* by 93 % (Yeoh et al., 1986). The inhibitory effect of GL seen here, along with documented cases of GL's inhibitory effect on both CBG and other β-glucosidases, support CBG's role in CA accumulation in *P. strobus* tissue cultures.

To my knowledge, this is the first report of CBG activity for coniferin in cambium cells, isolated from stems with actively differentiating xylem. CA content increased more than three-fold in cambium incubated for 4 hours at 25 °C, compared to tissue analysed at time zero. As previously mentioned, it is well established that coniferin accumulates in both the cambium and differentiating xylem; however, coniferin content is higher in differentiating xylem than in the cambium itself (c.f., Savidge, 1989). It is not surprising, then, that CBG activity was higher in differentiating xylem than in the cambium. Marcinowski and Grisebach (1978) reported glucosidase activity in the cambium of

spruce seedlings, but it had no activity for coniferin.

Data with cambial tissue and with developing xylem at the RE stage, which each contain no secondary cell wall material, along with the fifteen-fold increase in CA content in the cytosolic fraction of developing xylem after 4 hours compared to the same tissue at time zero, provide overwhelming evidence that CBG, at least in *P. strobus*, is a soluble enzyme. Because intact stem segments did not accumulate CA, the act of scraping and pulverizing the tissue prior to incubation appears to be responsible for increased CBG activity. The evidence suggests that CBG is compartmentalized within the cytosol of these tissues, and upon scraping and pulverizing, the compartments are broken open, releasing CBG into the cytosol where it comes in contact with endogenous coniferin. Dharmawardhana and coworkers (1999) suggested, following studies with CBG extracted from developing xylem of *P. contorta*, that CBG may be presented to its substrates in membrane-bound compartments through vesicle trafficking mechanisms. The data presented here for *P. strobus* support this. The concentration of coniferin in isolated pine cambial protoplasts has been detected as high as 1.6 mM, accounting entirely for coniferin content in tissue extracts (Leinhos and Savidge, 1993; Leinhos et al., 1994), indicating that very little coniferin, if any, is present in the walls of primary-walled radially-expanded cambial derivatives from which the protoplasts were collected. The accumulation of coniferin in protoplasts could indicate that coniferin itself is either compartmentalized within the protoplast or produced at a greater rate than it is metabolised. Marcinowski and Grisebach (1977) reported a half-life of 60 - 120 hours

for coniferin.

Samuels et al. (2002) observed vesicles fusing with the plasma membrane in developing xylem of *P. contorta*, as well as unattached vesicles in the cytoplasm. They reported that "in the developing tracheids where the secondary wall was labelled [with antibodies for CBG], no labelling of CBG epitopes could be detected in the cytoplasm and very little or no antibody binding was observed in the Golgi and associated vesicles." As mentioned in the introduction, antibodies that are assumed specific have been shown to bind non-specifically to secondary walls (Savidge, unpublished). CBG could be present in the vesicles observed by Samuels et al. (2002) and be presented to the developing matrix of the secondary wall via fusion of the vesicle with the plasma membrane. This could explain the accumulation of CA observed in the cell wall fraction of *P. strobus* developing xylem reported here. Samuels et al. (2002) reported that CBG was "found only in the secondary wall." This is subject to further investigation, as the data presented here with *P. strobus* are in contradiction with data for *P. banksiana* reported by Samuels et a. (2002). CBG is associated with cell wall material in *P. strobus*; however, CBG activity from the cytosolic fraction is greater. It is possible that CBG is produced in the protoplast and is then transported to the cell wall in vesicles that are initially associated with the Golgi system (Samuels et al., 2002) where it becomes associated with the cell wall. Northcote (1989) suggested that phenylpropionic acids could be converted to their corresponding alcohols in vesicles that fuse with the plasma membrane, subsequently delivering lignin precursors to the wall. In wheat xylem cells that were fed labelled

lignin precursors, such as phenylalanine and cinnamic acid, and label that was observed at the endoplasmic reticulum and in Golgi apparatus was also associated with the cell wall (Pickett-Heaps, 1968). Radioactive lignin precursors have been located in the Golgi apparatus and lignifying cell walls in *Cryptomeria japonica* D. Don (Fujita and Harada, 1979; Takabe et al., 1985). Based on these reports, it is possible that CBG is itself synthesized within the endoplasmic reticulum (Takabe et al., 1985) then transported to the cell wall through Golgi-derived vesicles. It could be presented to coniferin either in the vesicles or at the cell wall (Dharmawardhana et al., 1999). This needs to be further investigated.

Additional experiments were performed where labelled CA was added at the end of the 4-hour incubation time, in order to determine how much CA actually remained in incubated tissue after 4 hours. The results indicated that CA was readily metabolized over the 4-hour incubation period, possibly for formation of defence polyphenolics in response to wounding (Daurade-Le Vagueresse and Bounia, 1992; Kraus and Spiteller, 1997). The integrated peak area (*m/z* 330) for the sample where labelled CA was added initially was compared to that where the label was added at the end of the incubation period in order to determine how much labelled CA was metabolized over 4 hours. It was assumed that the rate of metabolism of labelled CA was the same as the rate of metabolism of endogenous CA. The results are plotted in Figure 5. It is unclear at this point how CA was metabolised or what the metabolic product(s) were. Also, tissue incubated in GL had much lower CA metabolism than tissue incubated alone. These

findings should be investigated in future experiments.

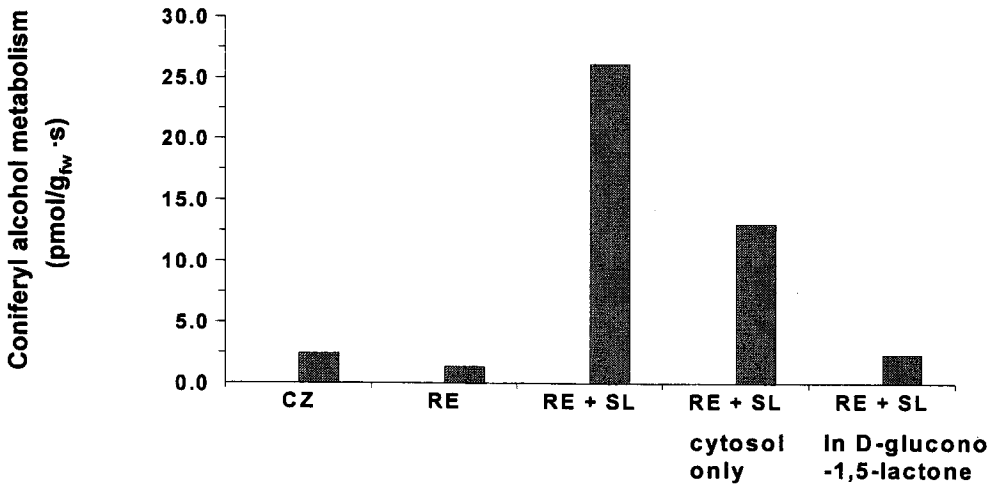

Figure 5. Coniferyl alcohol metabolism in cambium and developing xylem incubated for 4 hours at 25 °C.

Coniferin hydrolysis as a result of stress or wounding has been investigated in a few species. In investigations of *Pinus sylvestris* inoculated with *Ceratocysis*, coniferin content was found to decrease, potentially to release CA for polymerization into defence polyphenolic molecules and glucose for an energy source (Daurade - Le Vagueresse and Bounias, 1992). No observed increase in CA was reported in this study. *Z*-coniferin levels reportedly decreased in beech (i.e., a species that accumulates *Z*-coniferin, similar to accumulation of coniferin in conifers) following combined attack by beech scale (*Cryptococcus fagisuga*) and a bark canker fungus (Dübeler et al., 1997). The observed decrease is possibly a result of a lignification response to wounding; however, this was

not investigated. Coniferin has been described as an *Agrobacterium tumefaciens* (i.e., the causal agent of crown gall disease) *vir*-gene inducer (Morris and Morris, 1990; Castle et al., 1992; Delay et al., 1994; Nan et al., 1997). *A. tumefaciens* strains with high β-glucosidase activity responded to coniferin and subsequently infected Douglas fir seedlings, while strains with low β-glucosidase activity were largely unable to respond to coniferin and were considered avirulent on Douglas fir (Morris and Morris, 1990). Thus, as Morris and Morris (1990) have stated, "hydrolysis of coniferin to coniferyl alcohol clearly plays an important role in presenting the VirA protein or phenolic binding receptor with an active virulence region inducer from Douglas fir during infection." Melchers and coworkers (1989) studied a number of phenolic compounds for inducing behaviour and found that the basic guaiacol structure was required for activity.

Savidge and Förster (2001) reported a ten-fold increase in CA levels in dormant cambium above and below a ring of removed bark vs. non-girdled dormant cambium. Because CA accumulated both above and below the removed ring, its accumulation evidently was not due to translocation from other parts of the crown, and was likely a response induced by wounding. In these investigations, it seems unlikely that CA production was a result of coniferin cleavage by CBG, given that coniferin is not present in dormant cambia of conifers.

While many researchers report that CAD catalyses the *last* step in providing coniferyl alcohol to the cell wall for oxidative polymerization into lignin (c.f., Walter et al., 1988;

Boudet and Grima-Pettenati, 1996; Damiani et al., 2005; Sibout et al., 2005), it should be considered that in conifers, at least, coniferin β-glucosidase could be partially responsible for catalysing the last step by releasing coniferyl alcohol from accumulated coniferin for polymerization into the cell wall matrix. Thus, understanding this enzyme is critical to developing a full understanding of the lignification process. Data presented herein for *P. strobus* provides strong evidence that CBG is a soluble enzyme and a facile, straight-forward method for achieving elevated CBG activity has been demonstrated. Future experiments should focus on determining the nature of the association of CBG with the cell wall, extracting the enzyme and investigating its role in lignin biosynthesis. As Dharmawardhana and coworkers (1999) state: "Manipulation of the CBG activity in transgenic plants will not only allow a genetic test of the centrality of the monolignol-glucoside/glucosidase cycle for lignin biosynthesis in conifers, but could offer the potential to modify the overall rate of lignin deposition, which is one of the current goals in forest tree biotechnology." In addition, investigating the balance between CAGT and CBG in the same tissue, over the course of a season, will provide a better understanding of how these two enzymes function together in producing coniferin and then hydrolysing it for lignin biosynthesis in developing xylem of conifers.

3. Experimental

3.1 HPLC profiling work

3.1.1 Material

On each date, reported in Figure 1, three 15 to 20-year-old eastern white pine (*Pinus*

strobus L.) trees were obtained from the University of New Brunswick woodlot, Fredericton, NB (totalling 12 trees). Branches were removed and stems brought directly to the laboratory for processing. The bark was peeled from the wood and the exposed inner bark surface (i.e., cambial zone) and wood surface (i.e., developing xylem) were scraped individually into cryogenic vials (Savidge et al., 1982). Tissue was extracted from the portion of stem segment supporting live branches and foliage. The tissue was then stored in liquid nitrogen until analysis. Microscopy was performed to determine stage of development (Figure 1; Steeves and Savidge, 2000).

3.1.2 Tissue extraction

Prior to analysis, cambium and developing xylem were pulverized together in liquid nitrogen. Tissue from all three trees harvested on the same date was pooled and weighed (all three trees were confirmed to be at the same developmental stage). Methanol was added in a 10 ml/g tissue fresh weight ratio. Methanol-soluble compounds were extracted for 24 hours at 4 °C. The tissue was then filtered off and the filtrate collected in a round-bottom flask (rbf) and dried under vacuum. In a ratio of ml solvent per mg tissue (fresh weight), 5 % methanol in pH 3 water (pH adjusted using acetic acid) was added to the rbf. One-ml aliquots were removed and centrifuged for 20 min at 12 000 rpm and 4 °C and the supernatant was analysed by HPLC.

3.1.3 HPLC analysis

Reversed-phase HPLC of 100 µl aliquots (i.e., 100 µl ≡ 100 mg tissue, fresh weight) of supernatant was performed according to Savidge (1989) on a Milton Roy system with a semi-preparative C18 ODS Ultrasphere column (Beckman, 5 µm spheres, 150 ×10 mm

i.d.). A linear gradient over 30 min (5 - 100 % methanol, adjusted to pH 3.0 with acetic acid) with a flow rate of 5 ml/min and UV detection at 254, 280 and 325 nm, respectively, were applied (according to Savidge 1988, 1989). The large 11-minute peak that appeared on May 24 was collected and analysed by UV and GC/MS.

3.1.4 Gas chromatography/ mass spectrometry

Ten-µl aliquots of the HPLC-collected 11-minute peak were dried down under a stream of nitrogen gas, and taken up in 10 µl BSTFA for 1 h derivatisation at 75 °C. Following derivatising, 1 µl was analysed with a 59970C Chem Station (Hewlett Packard) and a 5890A gas chromatograph (GC) coupled to a 5970B mass selective detector (Hewlett Packard). Ionization potential was 70 eV. Separation was accomplished using a DB-1 (methyl silicone) 0.18 mm (i.d.) × 16 m fused silica column with a He flow of 0.6 ml/ min (40 cm/ s). Injection temperature was 200 °C and oven temperature was set at 40 °C, holding at 40 °C for 1 min and purging the septum after 1 min. The transfer line was set at 220 °C, and temperature programming was from 40 °C to 280 °C at 20 °C/ min, then isothermal at 280 °C.

3.2 Incubation studies I. Qualitative investigations

3.2.1 Material

Five open-grown 15- to 20-year-old eastern white pine (*Pinus strobus* L.) trees were obtained from the backyard of a domestic residence in Fredericton, NB, at different times during May. Branches were removed and stems brought directly to the laboratory for processing, which was performed according to Section 3.1.1. Small stem pieces were left intact for incubation of whole-stem sections. Microscopy was performed to

determine stage of development.

3.2.2 Tissue incubation

Prior to incubation, developing xylem and cambium were pulverized separately in liquid nitrogen. The frozen powder was weighed out in 500 ± 3 mg quantities, into 2-ml capped vials. For whole-stems being incubated, the ends were trimmed and stems were placed upright in a beaker. Each sample was incubated in a shaking water bath at 25 °C, according to Table 1. In qualitative investigations using equivalent masses of tissue (not reported here), the largest CA peak was obtained at 25 °C (-18, 4, 15, 20, 25, 30, 35 and 40 °C were investigated), thus all subsequent incubations were performed at this temperature. Similarly, a time study was performed to determine an optimal incubation time, and 4 hours was long enough to obtain an appreciable-sized peak and short enough to perform incubations and analysis in one day. CA was detectable by HPLC after only 30 minutes of incubation, which was the shortest incubation time investigated, other than tissue analysed at time zero. Incubations of tissue cultures were arrested by adding 500 µl of 50 % acetic acid and mixing on a vortex mixer. Incubation of stem segments was stopped by peeling the bark from the wood, leaving 2.5 cm at each end; the exposed inner bark surface (i.e., cambial zone) and wood surface (i.e., developing xylem) were scraped individually into 50 % acetic acid immediately. All samples were then centrifuged at

12 000 rpm for 20 minutes at 4 °C. The supernatant was collected for HPLC analysis.

3.2.3 Analysis

Reversed-phase HPLC of 100 µl aliquots (i.e., 100 µl ≡ 100 mg tissue, fresh weight) of

supernatant was performed on the same system as Section 3.1.2 with UV detection at 262 nm: R_t coniferyl alcohol 11.1 min.

Gas chromatography/ mass spectrometry were performed as above, to confirm the identity of the coniferyl alcohol peak.

3.3 Incubation studies I. Quantitative investigations

3.3.1 Material and chemicals

Three open-grown 15- to 20-year-old eastern white pine (*Pinus strobus* L.) trees were obtained from the backyard of a domestic residence and three from the University of New Brunswick woodlot, both in Fredericton, NB. Branches were removed and stems brought directly to the laboratory for processing as per Section 3.1.1. Small stem pieces were left intact for incubation of whole-stem sections. Microscopy was performed to determine stage of development. $^{13}C_6$-Coniferyl alcohol from Savidge and Förster (2001) was used.

3.3.2. Tissue incubation

Prior to incubation, developing xylem and cambium were pulverized separately in liquid nitrogen. The frozen powder was weighed out in 100 ± 2 mg quantities, into 2-ml capped vials. For whole-stems being incubated, the ends were trimmed and stems were placed upright in a beaker. Each sample was incubated in a shaking water bath at 25 °C, according to Figure 3. $^{13}C_6$-Coniferyl alcohol was added to samples (approximately 800 ng $^{13}C_6$- CA / g_{fw}) prior to incubation, except with intact stems, where it was added to tissue after incubation. Incubations were arrested by adding 300 µl of methanol and

mixing on a vortex mixer. Stem segments were processed according to section *3.2.2.* but scraped into methanol (for increased solubility of CA) instead of acetic acid. Samples were then centrifuged at 12 000 rpm for 20 minutes at 4 °C. Supernatant was removed to fresh 2-ml vials and dried down under a stream of nitrogen gas with low heat. Remaining residue was taken up in 500 ul of 20 % methanol in pH 3 water. Samples were centrifuged at 12 000 rpm for 15 minutes at 4 °C and the supernatant collected for HPLC analysis.

3.3.3. Semi-preparative HPLC analysis

Reversed-phase HPLC of all of the collected supernatant was performed on the same system (Section 3.1.3). A linear gradient over 30 min (20 - 100 % methanol, adjusted to pH 3.0 with acetic acid) with a flow rate of 5 ml/min and UV detection at 262 nm were applied: R_t coniferyl alcohol 6.2 min (note change in gradient, accounting for change in retention time). The coniferyl alcohol peak was collected into a round-bottom flask and dried under vacuum. Methanol was added to the rbf until further preparation for GC/MS analysis.

3.3.4 Gas chromatography/ mass spectrometry

Methanol extract from the rbf was pipetted into GC/MS tapered vials and dried down under a stream of nitrogen gas with heat. After drying, 20 µl BSTFA was added to each vial for 30 minutes of derivatisation at 75 °C. Following derivatising, 1 µl was analysed with a Varian 3800 gas chromatograph coupled to a Varian 4000 mass spectrometer, using a Varian 60 m cross-linked methyl silicon (3 %) 0.53 internal diameter megabore GC column with temperature programming from 40 - 300 °C at a rate of 28 °C/min,

holding for a half minute. Injection temperature was 180 °C and He carrier gas flow rate was 5 mL/min. Ionization was off for the first 4.5 min, then on from 4.5 to 9.79 min, scanning *m/z* 70 to 350. The integrated peak area ratio (*m/z* 324:330) was used to construct a calibration curve (Figure 6), while the integrated peak area ratio for *m/z* 309:315 served as a check (Savidge and Förster, 2001). Based on the calibration function $y = 1.03\ x$ ($R^2 = 0.999$) the mass ratio of ^{12}C:^{13}C coniferyl alcohol in each sample was obtained. The mass of endogenous CA could be thus be determined.

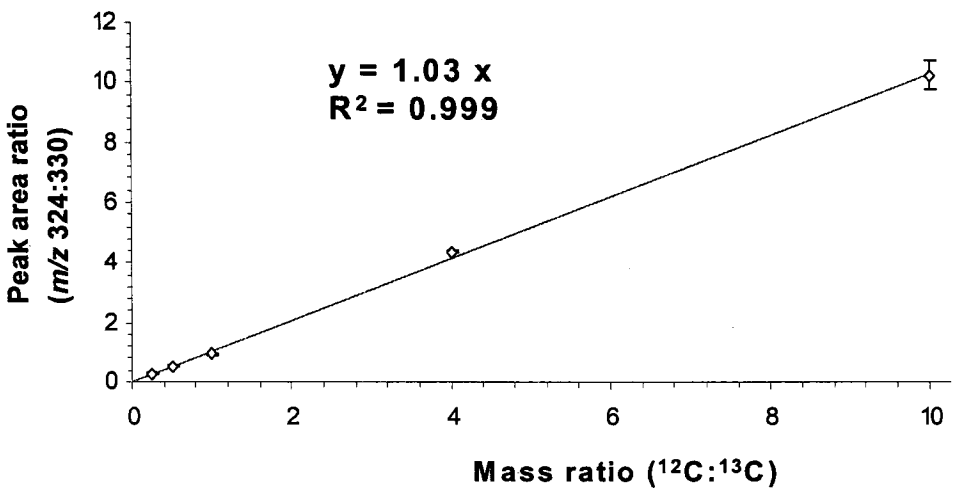

Figure 6. Integrated peak area ratio (*m/z* 324: 330) as a function of mass ratio of coniferyl alcohol. Error bars represent standard errors (n = 3).

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CHAPTER 3

CONIFERYL ALCOHOL METABOLISM IN CONIFERS - I. GLUCOSIDIC TURNOVER OF CINNAMYL ALDEHYDES BY UDPG: CONIFERYL ALCOHOL GLUCOSYLTRANSFERASE FROM PINE CAMBIUM

Abstract

UDPG: coniferyl alcohol glucosyltransferase (CAGT; EC 2.4.1.111) isolated from cambial tissues of *Pinus strobus* was able to convert cinnamyl aldehydes as well as dihydroconiferyl alcohol into their corresponding 4-*O*-β-D-glucosides in vitro. Cinnamyl aldehydes were glucosylated with comparable efficiency to coniferyl alcohol, the physiological substrate for CAGT. Seasonal patterns of CAGT activity for aldehydes were similar to those of coniferyl alcohol. Formation of cinnamyl aldehyde and additional monolignol glucosides indicates that precursor flux and availability for lignification is likely greater than previously recognized.

Key words: *Pinus strobus*; Coniferophyta; Pinaceae; Eastern white pine; Cinnamyl aldehyde; Cinnamyl alcohol; Dihydroconiferyl alcohol; Glucoside; Glucosyltransferase; Seasonal cambial growth

1. Introduction

The 4-O-β-D-glucopyranosides of trans-cinnamyl alcohols, i.e., *E*-coniferin and *E*-syringin, frequently accumulate in the cambial region of woody plants, and they have long been considered as participants in lignification, conceivably as either storage reserves (see, for example, Terazawa and Miyake, 1984) or transported forms of monolignols (Freudenberg and Torres-Serres, 1967). While coniferin is known to accumulate in conifer cambial tissues (earlier examples include: Freudenberg and Harkin, 1963; Marcinowski and Grisebach, 1977; Schmid and Grisebach, 1982; Terazawa and Miyake, 1984; Terazawa et al., 1984a,b; Savidge, 1988, 1989, 1991), it has also been isolated from cambial tissues of various angiosperms (Terazawa et al., 1984a, b), needles of Douglas fir (Morris and Morris, 1990) and Norway spruce (Slimestad and Hostettmann, 1996), cell suspensions of *Linum flavum* (van Uden et al., 1990), *Paulownia tomentosa* bark (Sticher and Lahloub, 1982), flue-cured tobacco leaves (Ito et al., 2000), *Daphne oleides* stems (Ullah et al., 1999) and leaves and stems of *Viscum album* ssp. (Deliorman et al., 1999). However, its accumulation evidently is greatest in conifers, such as *Pinus banksiana* (Leinhos and Savidge, 1993; Leinhos et al., 1994; Förster and Savidge, 1995), *Larix laricina* (Savidge, 1991) and *Picea abies* (Schmid and Grisebach, 1982), where the contents of endogenous coniferin were reported to be as high as 10 $\mu mol/g_{fw}$. In isolated cambial protoplasts the concentration of coniferin reached 1.6 mM and accounted fully for the content found in tissue extracts (Leinhos and Savidge, 1993; Leinhos et al., 1994), indicating that very little if any was present in walls of the primary-walled radially expanded cambial derivatives from which

the protoplasts were obtained.

In springtime, the cinnamyl alcohol glucosides accumulate in cambial derivatives of conifers in the complete absence of lignification (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Fukushima et al., 1997; Rowland and Arora, 1997; Savidge et al., 1998); however, there is ample evidence that once lignification is underway they can contribute to that process (Förster et al., 1999, 2000), as earlier surmised by Freudenberg and Harkin (1963). In order for coniferin to accumulate to high levels, it must either be compartmentally shielded or produced many times more rapidly than it turns over (Marcinowski & Grisebach (1977) report a half-life of 60 – 120 hours for coniferin) or is utilised in support of lignification. Freudenberg (1964) reported that when the coniferyl alcohol moiety of coniferin was radiolabelled, the radioactivity was readily incorporated into spruce lignin. The incorporation of isotopically labelled monolignol glucosides into lignin has been further investigated in autoradiographic studies by Terashima et al. (1986, 1993, 1995, 1997, Terashima and Fukushima, 1988; Eglinton et al., 2000). Monolignol glucosides enriched with carbon-13 at specific side chain carbons were administered to growing tree stems resulting in newly formed lignin shown to be carbon-13 enriched at the side chain carbons corresponding to those of the glucosides (Xie and Terashima, 1991; Xie et al., 1994; Terashima et al., 1995).

In agreement with the concept that the activity of coniferin-hydrolysing β-glucosidase may influence the rate of lignification (Grima-Pettenati and Goffner, 1999), a β-glucosidase specific to coniferin and of much lower abundance and/or activity than numerous other β-glucosidases of wood and cambial tissues was isolated, primarily on

the basis of its unique ability to hydrolyse authentic coniferin to *E*-coniferyl alcohol, as confirmed by GC/MS (Leinhos et al., 1994). It remains unclear whether that coniferin-specific β-glucosidase is identical to the enzyme investigated by Dharmawardhana et al. (1995, 1999), the N-terminal sequences of the two being notably distinct although both evidently have similar p*I* values, or to the enzyme isolated from inner secondary cell walls in young spruce seedlings by Marcinowski et al. (1979), or that localized to xylem epi-, endo- and exodermis in stems and roots of chick pea seedlings by Burmeister & Hösel (1981). A problem may exist, however, in using antibodies for localisation; supposedly specific antibodies have been repeatedly observed to bind non-specifically with lignified secondary cell walls (R.A. Savidge, unpublished data).

Coniferin has been found present in the cambial zone and its derivatives throughout the period of cambial growth, but it becomes undetectable at the onset of cambial dormancy in autumn, before the last cambial derivatives have completed their maturation into latewood (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge et al., 1998). Coniferin has never been detected in stems during cambial dormancy (Freudenberg and Harkin, 1963; Terazawa and Miyake, 1984; Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge et al., 1998). Quantitative investigations of endogenous indol-3-ylacetic acid, sucrose and coniferin in *Larix laricina* cambium revealed that, of the three, only coniferin content varied parallel to seasonal cambial activity (Savidge, 1991), and biochemical investigations revealed that the presence or absence of UDPG: coniferyl alcohol glucosyltransferase (CAGT; EC 2.4.1.111) activity is the explanation for the qualitative seasonal variation of coniferin,

indicating that seasonal variation in coniferin has its explanation in seasonal change in gene expression (Förster and Savidge, 1995; Savidge and Förster, 1998; Förster et al., 2000).

Ibrahim & Grisebach (1976) were first to report purification and characterization of a CAGT, isolated from suspensions of Paul's scarlet rose. The enzyme catalysed the transfer of glucose from UDPG to coniferyl alcohol and had the highest substrate specificity reported to date for a CAGT (K_m = 3.3 x 10^{-9} M for coniferyl alcohol; K_m = 2 x 10^{-5} M for UDPG). In 1977, Ibrahim confirmed the substrate specificity using CAGT isolated from young lignifying stems of *Forsythia ovata*, demonstrating it to be distinct from other glucosyltransferases, which are capable of nonspecifically glucosylating hydroxy phenols, flavonols, anthocyanidins and phenolic acids.

Schmid & Grisebach (1982) purified and characterized CAGT from *Picea abies*, and found coniferin to inhibit CAGT in a noncompetitive manner, with product inhibition starting at approximately 10 mmol/g. Schmid et al. (1982) also immunolocalized CAGT to the epidermis, subepidermis and vascular bundles of young *P. abies* seedlings. Intracellularly, it was located in the parietal cytoplasmic layer, indicating that CAGT is a soluble enzyme of the cytoplasm, in agreement with Ibrahim (1977). Förster & Savidge (1995) reported that coniferin below 10 $\mu mol/g_{tissue}$ showed no inhibitory effect on activity of CAGT of *Pinus banksiana*, but it did inhibit CAGT above 10 μmol/g. The catalytic activity of the coniferin-specific β-glucosidase isolated from lignifying tissues was not found to reside in CAGT obtained from the same tissues, at the same developmental stage (Savidge and Förster, 1998). Recently, Förster

et al. (1999, 2000) confirmed the pronounced specificity of CAGT for UDPG; however, they unexpectedly found that CAGT from *P. strobus* also expressed high activity when UDP-galactose was supplied to the enzyme.

E-Coniferaldehyde has been detected in the cambial sap of conifers (Freudenberg and Neish, 1968), bamboo (Tanaka et al., 1998) and oak (Fernàndez de Simòn et al., 1996). Despite the generally low endogenous coniferylaldehyde levels in plants and the efficient reduction of cinnamyl aldehydes to cinnamyl alcohols, coniferyl aldehyde glucosides have nevertheless been isolated from bark of *Syringa velutina* (Park et al., 1999) and *Kolopanax pictus* (Sano et al., 1991).

Cinnamyl aldehydes have also been reported as suitable substrates for CAGT (Ibrahim and Grisebach, 1976; Schmid and Grisebach, 1982; Förster et al., 1999, 2000). Preliminary investigations indicated that CAGT catalysed the conversion of cinnamyl aldehydes to their corresponding 4-O-β-D-glucosides in vitro (Förster et al., 1999, 2000). CAGT from *Pinus strobus* appeared to produce higher amounts of cinnamyl aldehyde glucosides than monolignol glucosides; however, the specific turnover rate (i.e., ratio between administered substrate and obtained product) was approximately 8 times lower (Förster et al., 2000). In seasonal investigations of CAGT activity, Förster et al. (2000) presented evidence for in vitro generation of coniferyl aldehyde glucoside to parallel similar seasonal patterns to that observed for coniferin. In the present study, we confirm, for the first time, by proton NMR spectroscopy, the enzymatic glucosidic turnover of cinnamyl aldehydes catalysed by CAGT isolated from pine cambial tissue. Seasonal patterns of CAGT activity for monolignols and cinnamyl aldehydes are

provided and their K_m values reported for the first time. This is the first report of in vitro biosynthesis of dihydroconiferyl alcohol glucoside.

2. Results and discussion

Molecular structures of the predicted enzymatic products were confirmed by ^{1}H NMR spectroscopic analyses. Two-dimensional COSY and HMQC NMR spectra were obtained to aid in structure elucidation. For both semi-preparative and analytical HPLC, an assay was developed to effectively separate the cofactor uridine 5'-diphosphoglucose (UDPG), the monolignol substrates and the corresponding product glucosides, allowing exact quantitation of the catalysed reaction. Based on this assay, enzymatic products were separated using semi-preparative HPLC techniques, collected and lyophilized, resulting in mg quantities of the desired compounds.

The monolignol-specific UDPG was extracted and partially purified (Savidge and Förster, 1998) from approximately 400 g of differentiating xylem from *Pinus strobus* harvested in late May. IEF and native PAGE indicated purification to homogeneity, however, SDS-PAGE and N-terminal sequencing of the resulting major band revealed the presence of two proteins, one without any evident homology to known proteins and the other yielding perfect homology to the internal sequence of a glucosyltransferase precursor. Because of the almost identical physico-chemical features (p*I* 5.0 and molecular weight 43 kDa) of both proteins, complete purification could not yet be achieved (Förster et al., 2000).

All substrates tested in the assay were successfully converted to their 4-*O*-β-D-

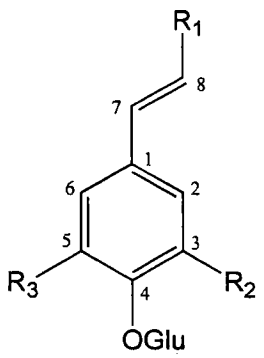
glucosides, as evidenced by the chemical shifts of the glucosyl moieties (not reported) in the ^{1}H NMR spectra. The glucosyl moiety did not bond to the alcoholic hydroxyl group of the phenylpropane side chain of the monolignol substrates (i.e., as in *iso*-coniferin), which is in agreement with Schmid and Grisebach's (1982) findings for *Picea abies*. Although Ibrahim (1977) obtained small amounts of *iso*-coniferin (i.e., *trans*-coniferyl alcohol-1-*O*-β-D-glucoside) from *Forsythia ovata*, this isomer of coniferin to our knowledge has not yet been reported in conifers.

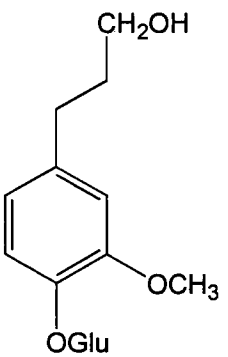
^{1}H NMR data for the enzymatic products, along with that of coniferyl alcohol, are presented in Table 1, while corresponding molecular structures are found in Figure 1. Carbon 9 (Table 1) refers to either the primary alcoholic carbon atom (i.e., as in coniferin (**1**), syringin (**2**), dihydroconiferyl alcohol glucoside (**5**) and coniferyl alcohol) or the aldehydic carbon atom (i.e., as in coniferyl aldehyde glucoside (**3**) and sinapyl aldehyde glucoside (**4**)). As evidenced by large coupling constants for the 7-H/8-H of the double bond (i.e., J = 15.8, 15.8, 16.6, and 14.8 Hz for **1** - **4**, respectively), products **1** - **4** maintained a *trans* orientation. Chemical shifts for glucosyl hydrogens are not reported, but were observed ranging from δ = 3.1 - 3. 8 ppm.

Chemical shifts of 9.61 and 9.63 ppm, corresponding to the aldehyde hydrogens for **3** and **4**, respectively, clearly demonstrate that the glucosyl moiety must have attached at the 4'-position of the aromatic ring, as opposed to the primary alcohol of the phenylpropane side chain.

When comparing the ^{1}H NMR spectra of coniferin and coniferyl alcohol, a larger up-field shift (i.e., up to 0.38 ppm) is observed for the aromatic hydrogens compared to

the aliphatic ones. This suggests the glucose attached to the phenol oxygen, and not that of the primary alcohol. Since the structures and chemical shifts for **2** and **5** are similar to **1**, the same conclusions were obtained.





1. Coniferin: $R_1 = CH_2OH$, $R_2 = OCH_3$, $R_3 = H$
2. Syringin: $R_1 = CH_2OH$, $R_2 = OCH_3$, $R_3 = OCH_3$
3. Coniferylaldehyde glucoside: $R_1 = CHO$, $R_2 = OCH_3$, $R_3 = H$
4. Sinapaldehyde glucoside: $R_1 = CHO$, $R_2 = OCH_3$, $R_3 = OCH_3$
5. Dihydroconiferyl alcohol glucoside

Figure 1. Structure of monolignol glucosides.

As previously mentioned, *E*-coniferin formation has been reported in various plants throughout the plant kingdom; however, by way of contrast, *Fagus grandifolia* Ehrh. (American beech) exceptionally accumulates *cis*-monolignols. Yamamoto et al. (1990) reported a glucosyltransferase in bark of *F. grandifolia*, which showed an unusual preference for *Z*-coniferyl alcohol catalysing the formation of *Z*-coniferin. The glucosyltransferase isolated from cambial tissues of *Pinus strobus* did not accept *cis*-coniferyl alcohol and a mixture of *cis* and *trans* substrates always resulted in formation of *E*-coniferin, as evidenced by HPLC separation (results not shown). Plausibly, the maintenance of a *trans* orientation during catalysis is an essential requirement for

Table 1

^{1}H NMR spectral data for the monolignol glucosides and coniferyl alcohol

	δ ^{1}H (ppm) [int, mult, *J* (Hz)]					
Carbon	Coniferyl aldehyde glucoside	Sinapyl aldehyde glucoside	Dihydroconiferyl alcohol glucoside	Coniferin	Coniferyl alcohol	Syringin
2'	7.32 [1H, *d*, 1.6]	7.03 [1 of 2H, *s*]	6.86 [1H, *d*, 2.2]	7.06 [1H, *d*, 1.9]	7.00 [1H, *d*, 1.9]	6.74 [1 of 2H, *s*]
5'	7.21 [1H, *d*, 8.3]	-----[a]	7.07 [1H, *d*, 8.2]	7.10 [1H, *d*, 8.3]	6.72 [1H, *d*, 8.0]	-----[a]
6'	7.25 [1H, *d*, 8.3]	7.03 [1 of 2H, *s*]	6.74 [1H, *dd*, 8.2, 2.1]	6.94 [1H, *dd*, 8.3, 1.9]	6.84 [1H, *dd*, 8.1, 1.6]	6.74 [1 of 2H, *s*]
7	7.61 [1H, *d*, 16.0]	7.61 [1H, *d*, 16.0]	2.63 [2H, *t*, 7.6]	6.54 [1H, *br d*, 15.8]	6.50 [1H, *br d*, 15.9]	6.54 [1H, *br d*, 15.9]
8	6.71 [1H, *dd*, 16.6, 7.7]	6.78 [1H, *dd*, 14.8 ,7.0]	1.82 [2H, *tt*, 7.7, 6.3]	6.27 [1H, *dt*, 15.8, 5.6]	6.19 [1H, *dt*, 15.6, 5.9]	6.32 [1H, *dt*, 15.8, 5.6]
9	9.61 [1H, *d*, 8.0]	9.63 [1H, *d*, 7.7]	3.55 [2H, *t*, 6.5]	4.20 [2H, *dd*, 5.8, 1.4]	4.19 [2H, *dd*, 6.0, 1.1]	4.21 [2H, *dd*, 5.6, 1.0]
OCH_3	3.91 [3H, *s*]	3.86 [6H, *s*]	3.85 [3H, *s*]	3.87 [3H, *s*]	3.85 [3H, *s*]	3.85 [6H, *s*]

[a] Both C3' and C5' methoxyls yield equivalent proton shifts.

converting suitable substrates to 4-*O*-β-D-glucosides in the cambium of *Pinus strobus*.

The catalytic activity of UDPG: CAGT in the formation of monolignol glucosides increased more than 3-fold in early June compared to CAGT isolated from cambial tissue of *Pinus banksiana* (Savidge and Förster, 1998); however, considerable differences in CAGT activity observed between cambium and developing xylem of *P. banksiana* did not appear in corresponding tissues of *Pinus strobus* (data not shown). Seasonal fluctuations in CAGT activity and levels of monolignol glucosides in cambial tissue parallel each other (Fig. 2; Savidge et al., 1998) with comparable efficiencies in the formation of aldehydic and alcoholic glucosides.

In addition, dihydroconiferyl alcohol (DHCA) was accepted as a suitable substrate for the pine CAGT and converted into its corresponding 4-*O*-β-D-glucoside (Fig. 1, Table 1). The 4-*O*-β-D-glucoside has been reported in needles of *Pinus contorta* (Higuchi et al., 1977). Ralph et al. (1997) recently reported dihydroconiferyl alcohol as a main constituent of lignin in a mutant loblolly pine. The involvement of dihydrophenylpropanoids in the biosynthesis of plant pigments (Schmitt and Schneider, 1999) suggests, in general, that dihydro-compounds play a role in secondary metabolism. The effective turnover of both cinnamyl alcohols and cinnamyl aldehydes by CAGT during the growing season was confirmed by determination of the apparent K_m values of the various substrates (Table 2). The apparent K_m values were obtained from Lineweaver-Burk plots at saturating concentrations of UDPG. For coniferyl alcohol, when assayed in phosphate buffer at pH 7.6, a K_m of 120 μM was obtained. Sinapyl alcohol, although not synthesized in pines, also served as a substrate with an apparent

K_m of 154 µM. The data confirm former results for *Pinus banksiana*, where sinapyl alcohol reached 96 % of the relative activity of coniferyl alcohol (Savidge and Förster, 1998). A comparable K_m value (250 µM) was reported for spruce (Schmid and Grisebach, 1982).

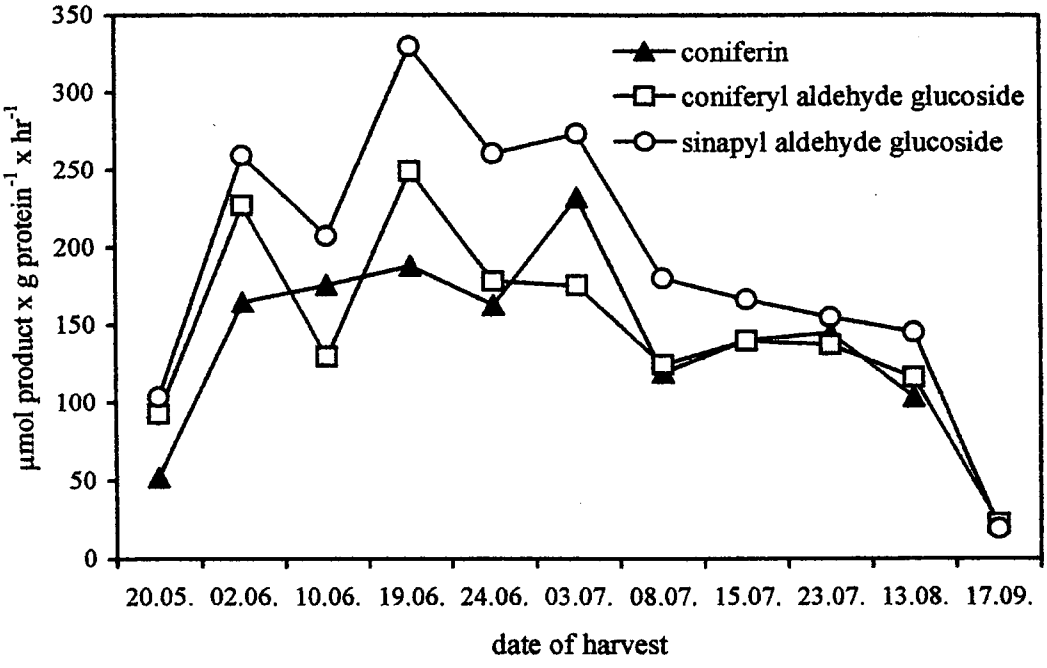


Figure 2. Seasonal activity pattern of UDPG:CAGT in developing xylem of *Pinus strobus* as determined by in vitro experiments.

Coniferyl aldehyde and sinapyl aldehyde glucosylation resulted in K_m values of 317 and 242 µM, respectively, thus working in close proximity to the order of cinnamyl alcohols. Nevertheless, existing differences in the enzymatic turnover of cinnamyl alcohols and aldehydes, evident by the reported K_m values, underline that the hydroxyl

group of the phenylpropane side-chain constitutes a more efficient substrate configuration. As reported before (Förster et al., 2000), cinnamyl alcohols and cinnamyl aldehydes compete with each other for CAGT when provided as dual substrates, with alcohols being more efficient in inhibiting the formation of cinnamyl aldehyde glucosides.

Table 2. Enzymatic parameters of the partially purified UDPG:CAGT from cambium of *Pinus strobus*

Substrate	Enzymatic product	K_m (μM)	V_{max} (nM × $mg_{protein}^{-1}$ × min^{-1})
Coniferyl alcohol	Coniferin	119.6	1.17
Sinapyl alcohol	Syringin	153.5	1.54
Coniferylaldehyde	Coniferylaldehyde-4-*O*-β-D-glucopyranoside	317.4	1.85
Sinapaldehyde	Sinapaldehyde-4-*O*-β-D-glucopyranoside	241.7	1.38

Cinnamyl aldehydes have been previously reported as appropriate substrates; however, structure and physiological relevance was not investigated. The amount of aldehydic glucosides obtained in investigations of the substrate specificity of CAGT in

Picea abies (Schmid and Grisebach, 1982) and suspension cultures of Paul's scarlet rose (Ibrahim and Grisebach, 1976) was expressed as relative activity in comparison to coniferyl alcohol. Because of the lack of authentic compounds for co-chromatography, the structure of the presumed glucosidic products could not be unequivocally elucidated. Hence, the data in this report based on HPLC separation and NMR investigations (Table 2) confirm, to our knowledge for the first time the real structure of the enzymatic products (Fig. 1). In contrast to the glucosyltransferase purified from *Forsythia ovata* (Ibrahim, 1977) and in agreement with the glucosyltransferase purified from *Picea abies* (Schmid and Grisebach, 1982) the CAGT from *Pinus strobus* expressed almost no activity with cinnamic acids (Förster et al., 2000). The glucosylation of cinnamic acids (Nurmann and Strack, 1980; Mock and Strack, 1993; Lorenzen et al., 1996; Wang and Ellis, 1998; Fons et al., 1998; Lim et al., 2001) has been demonstrated previously, with the glucosyl moiety attached to the carboxyl group of the side-chain and to the 4'-hydroxy group of *cis-p*-coumaric acid and *cis*-caffeic acid, whereby the *trans*- isomer was not a suitable substrate (Rasmussen and Rudolph, 1997).

The observed seasonal fluctuations of both cinnamyl alcohol and aldehyde glucoside formation (Fig. 2) coincide with events of wood formation, i.e., formation of primary wallled radially enlarged cells and the first appearance of differentiated tracheids undergoing secondary wall formation and lignification (Savidge and Förster, 1998). Preliminary examination revealed both high activities of the glucosyltransferase and developmental processes during June and July (Fig. 2) in the cambial zone, thus concentrating the characterization and purification of the CAGT to this time of the

season. Lignification is not developmentally correlated to the formation of monolignol glucosides as shown previously (Leinhos and Savidge, 1994; Savidge and Förster, 1998). However, some observed metabolic connections to wood formation and lignification once the glucosides are produced in abundance needs further clarification.

CAGT, in catalyzing the formation of cinnamyl alcohol glucosides, evidently competes with coniferyl alcohol dehydrogenase (CAD) and monolignol oxidising enzymes for coniferyl alcohol. Our data (Figure 2, Table 2) indicate that the glucosyltransferase must also compete with CAD for available cinnamyl aldehydes by forming the corresponding aldehydic glucosides (Figure 1).

Results obtained in the past few years have prompted a reconsideration of the established lignin biosynthetic pathway. It is clear now that inter-conversions of lignin precursors may occur at varied points along the lignification pathway, and not necessarily solely at the phenylpropanoid acid stage. In addition, the ratio of guaiacyl to syringyl units can be modified at the level of CoA esters (Grimmig et al., 1999), cinnamyl aldehydes (Osakabe et al., 1999), cinnamyl alcohols (Chen et al., 1999a, b) and monolignol glucosides (Matsui et al., 1996, 2000).

Taken together, the questions of if and how CAGT (i.e., alone or in a regulatory manner with the coniferin-specific β-glucosidase) may participate in controlling availability of cinnamyl alcohols and/or cinnamyl aldehydes for lignin formation deserves further efforts.

3. Experimental

3.1 General experimental

Coniferyl alcohol, sinapyl alcohol, coniferyl aldehyde and sinapyl aldehyde were purchased from Aldrich. *E*-coniferin was isolated from *P. strobus* (Savidge, 1989) and dihydroconiferyl alcohol was obtained as previously described by Savidge (1987). Before processing and HPLC analysis, handcut sections of cambium and developing xylem were examined by microscopy as previously described by Savidge (1988).

3.2 Trees

Healthy *Pinus strobus* between 15 and 18 years old growing in the University of New Brunswick woodlot, Fredericton, NB, Canada, were selected. Sufficient amounts of protein for this study were isolated for purification from a number of trees harvested during the growing season (April - September) for investigations into seasonal CAGT activity patterns. On each date, stem segments from the main axis were removed with a handsaw and brought directly to the laboratory for processing. The bark was peeled from the wood and the exposed inner bark surface (i.e., cambial zone) and wood surface (i.e., developing xylem) were scraped individually into cryogenic vials (Savidge et al., 1982). The tissue was then stored in liquid nitrogen until preparation for CAGT analysis.

3.3 Enzyme isolation

Buffer systems used include: extraction buffer (0.2 M phosphate (Na_2HPO_4, NaH_2PO_4), 10 % polyvinylpyrrolidone, 7.5 % glycerol, 15 mM aminocaproic acid, 10 mM

dithiothreitol, 14 mM β-mercaptoethanol, pH 7.6), equilibration buffer (0.1 M phosphate (Na_2HPO_4, NaH_2PO_4), 14 mM β-mercaptoethanol, pH 7.6), sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4, 100 mM, pH 7.6). Cambial tissue was pulverized to a homogeneous powder and extracted for 1 h in extraction buffer. The suspension was filtered and the filtrate centrifuged for 20 min (4 °C) at 15 500 × g. The supernatant was centrifuged (1 h, 4 °C) at 100 000 × g to remove microsomes. Protein in the supernatant was precipitated with $(NH_4)_2SO_4$. Protein precipitated between 40 and 85 % $(NH_4)_2SO_4$ was collected by centrifugation (23 300 × g, 20 min, 4 °C). The resulting pellet was resuspended in phosphate buffer (2.5 ml), then applied to Sephadex G-25 (20 mm i.d. × 100 mm, previously equilibrated in equilibration buffer). One ml fractions of soluble protein were collected. Fractions showing transferase activity were pooled and concentrated by centrifugation at 2455 × g using a Centricon YM-10 concentrator. All subsequent isolation and purification of UDPG: coniferyl alcohol glucosyltransferase (CAGT) was carried out according to Förster and Savidge (1995) and Savidge and Förster (1998).

3.4 Protein determination

Protein concentrations were determined using the method developed by Lowry et al. (1951), modifying final volumes to 40 % of the original assay. The UDPG: CAGT was then applied in a 40-fold dilution, using bovine serum albumin as a standard, and measured at 578 nm.

3.5 Semi-preparative HPLC and product separation

Reversed-phase HPLC of the enzyme assay aliquots was performed according to Savidge (1989) on a Milton Roy system with a semi-preparative C18 ODS Ultrasphere column (Beckman, 5 μm spheres, 150 × 10 mm i.d.). Assays were carried out on a larger scale (i.e., total volume 2 ml) in order to obtain sufficient product for analytical investigations. The enzyme incubation mixture consisted of 100 mM phosphate buffer (pH 7.6), 2 mM UDPG, 1 mM of the substrates in question and approximately 800 μg protein. Enzyme assay incubation time was 60 min at 36 °C. Product separation was achieved with a linear gradient over 30 min (5 - 100 % MeOH, adjusted to pH 3.0 with acetic acid) with a flow rate of 5 ml/min and UV detection at 260 nm: R_t coniferin 8.0 min, 266 nm: R_t syringin 8.7 min, 330 nm: R_t coniferyl aldehyde glucoside 9.0 min, 320 nm: R_t sinapyl aldehyde glucoside 9.8 min, 262 nm: R_t *p*-coumaryl alcohol glucoside 8.7 min, 276 nm: R_t dihydroconiferyl alcohol glucoside 8.3 min, 266 nm. Enzymatic products were collected from semi-preparative HPLC eluant and freeze dried. The compounds were characterised by GC-MS and ^{1}H NMR and used as standards in analytical HPLC.

3.6 Ultraviolet-visible spectra

Ultraviolet-visible spectra of the purified enzymatic products were obtained using a computer-controlled UV/vis spectrophotometer (Shimazu, UV-2102-PC). Spectra were recorded from 195 to 450 nm and compared to spectra obtained in analytical HPLC work-up.

Coniferin λ_{max} (MeOH) nm (log $\in$): 260 (1, 3) 214, λ_{min} (MeOH) nm: 238, $\lambda_{shoulder}$ (MeOH) nm: 296; syringin λ_{max} (MeOH) nm (log $\in$): 266, (1, 23), 221, λ_{min} (MeOH) nm: 240; coniferyl aldehyde glucoside λ_{max} (MeOH) nm (log $\in$): 330 (1, 16) 236, λ_{min} (MeOH) nm: 266, $\lambda_{shoulder}$ (MeOH) nm: 306; sinapyl aldehyde glucoside λ_{max} (MeOH) nm (log $\in$): 320 (0, 55), 236, λ_{min} (MeOH) nm: 268; dihydroconiferyl alcohol glucoside λ_{max} (MeOH) nm (log $\in$): 276, 224, λ_{min} (MeOH) nm: 248.

3.7 Nuclear magnetic resonance

Approximately 1-2 mg of coniferin, coniferyl aldehyde glucoside, syringin and sinapyl aldehyde glucoside were each dissolved in 600 - 700 µl deuterated methanol and placed in individual 5 mm NMR tubes. Only microgram quantities of dihydroconiferyl alcohol glucoside were available, which was subsequently dissolved in approximately 600 µl deuterated methanol, and placed in a 5 mm NMR tube. ^1H spectra were recorded on a 400 MHz, Unity 400 NMR spectrometer, at 25 °C. The residual solvent peak, CHD_2OD (3.30 ppm) was used as an internal standard. Two-dimensional COSY spectra and one-dimensional spectra were obtained.

3.8 Analytical HPLC and glucosyltransferase assay

Analytical HPLC was performed on a computer-controlled Jasco system consisting of an autosampler (AS 950-10), multiwavelength detector (MD 910), ternary gradient unit (LG 980-02) and a 3-line-degasser (DG 980-50) on a Peltier-thermostated Sepsil C18 column (Jasco, 5 µm spheres, 125 × 3 mm i.d.). Cinnamyl alcohol glucosides were

eluted in low pressure gradient mode in H_2O: MeOH (containing 1 % HCO_2H) over 55 min (2 - 39 % MeOH for 35 min, 39 - 100 % MeOH for 15 min), and cinnamyl aldehyde glucosides over 70 min (2 - 60 % MeOH for 50 min, 60 - 100 % MeOH for 20 min) with a flow rate of 0.5 ml/min. Column temperature was adjusted to 26 °C. Data analysis was accomplished using Borwin PDA (version 1.0) and chomatography (version 1.22.03) software.

The enzyme incubation mixture (i.e., total volume 200 µl) contained 200 nM UDPG in 10 µl H_2O, various amounts of substrate in 10 µl MeOH, 80 µl sodium phosphate buffer and approximately 100 µg protein dissolved in sodium phosphate buffer. The various substrates were incubated for 10 min at 36 °C, then terminated using 20 µl HOAc (50 %). K_m values were calculated at fixed concentration of UDPG (1 mM) by linear regression of 1/v vs. 1/s (Lineweaver-Burk plot) with Grafit (version 4.0.1). All experiments were performed in triplicate and compared to a control containing all constituents but terminated prior to catalytic activity by HOAc precipitation of the enzyme.

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CHAPTER 4

UDPG:CONIFERYL ALCOHOL GLUCOSYLTRANSFERASE AND CONIFERIN BIOSYNTHESIS - A REGULATORY LINK TO SEASONAL CAMBIAL GROWTH IN CONIFERS

1. Introduction

E-coniferin, the 'coniferin' present in the cambium of conifers, is the 4-*O*-β-D-glucopyranoside of *trans*-coniferyl alcohol. Coniferin has long been known to be a major cambial metabolite in the Coniferophyta, and it has also been reported in woody angiosperms (e.g., Terazawa *et al.*, 1984) including Liliopsida (i.e., a monocot) species (Nan *et al.*, 1997). Early research in Germany demonstrated that when the coniferyl alcohol moiety of coniferin was radiolabelled, the radioactivity was readily incorporated into lignin of spruce (Freudenberg, 1964). Freudenberg (1964) considered that coniferin served as a 'reservoir' of lignin precursor (i.e., of coniferyl alcohol) because, unlike coniferyl alcohol, the glucoside was found not to be susceptible to oxidation by enzymes of lignification. In a similar vein, Neish (1964) speculated that 'if for some reason there is an overproduction such as may occur in spruce cambium, I would imagine that coniferyl alcohol is stabilized as a glucoside, perhaps by reaction with UDP-glucose.'

In his initial investigations into the biosynthesis of coniferin, Ibrahim (1977)

again emphasized that during coniferin formation 'lignin precursors' were being glucosylated. There is no question today that coniferyl alcohol as the aglycone can be utilized as a lignin precursor (Lewis and Sarkanen, 1998), and when coniferin is hydrolysed by a coniferin-specific β-glucosidase, the resulting aglycone has been shown to be coniferyl alcohol (Leinhos *et al.*, 1994). Thus, it is not surprising that the physiological role of coniferin has been considered almost exclusively in relation to the known role of coniferyl alcohol in lignification. There have been many reviews which address that concept, including those by Grima-Pettenati and Goffner (1999), Baucher *et al.* (1998), Whetten *et al.* (1998).

Acknowledging that coniferin does have a role in supplying coniferyl alcohol for lignification, the primary focus of this chapter nevertheless is on what coniferin biosynthesis can tell us about the regulation of the seasonal cycle of growth and dormancy in conifers, of which lignification is only one aspect.

2. Coniferin biosynthesis and metabolism

2.1 *Background on CAGT*

Coniferin is only one of a great many glucosides which are produced in plants as both aliphatic and aromatic compounds (Harborne, 1964; Hopkinson, 1969). Plant glucosides arise through the activities of various glucosyltransferases (E.C. 2.4.1), enzymes which catalyse transfer of glucose from a sugar nucleoside diphosphate to the corresponding aglycone. The only enzyme known to catalyse *E*-coniferin biosynthesis is uridine 5'-diphosphoglucose:coniferyl alcohol glucosyl transferase (CAGT). Only very modest

CAGT activities have been detected in plants low on the evolutionary scale (e.g., Bryophyta and Polypodiophyta), and much higher activities have been found in most Pinophyta and a few woody Magnoliophyta (Ibrahim, 1977).

Ibrahim and Grisebach (1976) published the first report on purification and characterization of a CAGT. Despite the fact that CAGT is most readily available from conifers, the enzyme was first isolated from suspension cultures of Paul's scarlet rose and found to catalyse the transfer of glucose from UDP glucose (UDPG) to coniferyl alcohol, yielding the highest substrate specificity reported to date for a CAGT (K_m = 3.3 x 10^{-9} M for coniferyl alcohol; K_m = 2 x 10^{-5} M for UDPG).

Schmid and Grisebach (1982) subsequently purified and characterized CAGT from *Picea abies*. Immunofluorescence detection in young *P. abies* seedlings showed the enzyme being localized to, predominantly, the epidermis, subepidermis, and vascular bundles. Intracellularly, the enzyme was found in the parietal cytoplasmic layer (Schmid *et al.*, 1982). CAGT was subsequently investigated in several conifer species in greater detail (Förster and Savidge, 1995; Savidge and Förster, 1998). Despite the investigations of Ibrahim and subsequent reports, CAGT evidently has yet to be assigned a formal E.C. number.

As indicated in Figure 1, a coniferin-specific β-glucosidase exists in the cambial region, delivering oxidizable monolignols by hydrolysing cinnamyl glucosides (Marcinowski and Grisebach, 1978; Burmeister and Hösel, 1981; Leinhos *et al.*, 1994; Dharmawardhana *et al.*, 1995, 1999). This coniferin-specific glucosidase isolated from *Pinus banksiana* (Leinhos *et al.*, 1994) and *P. contorta* (Dharmawardhana *et al.*, 1995)

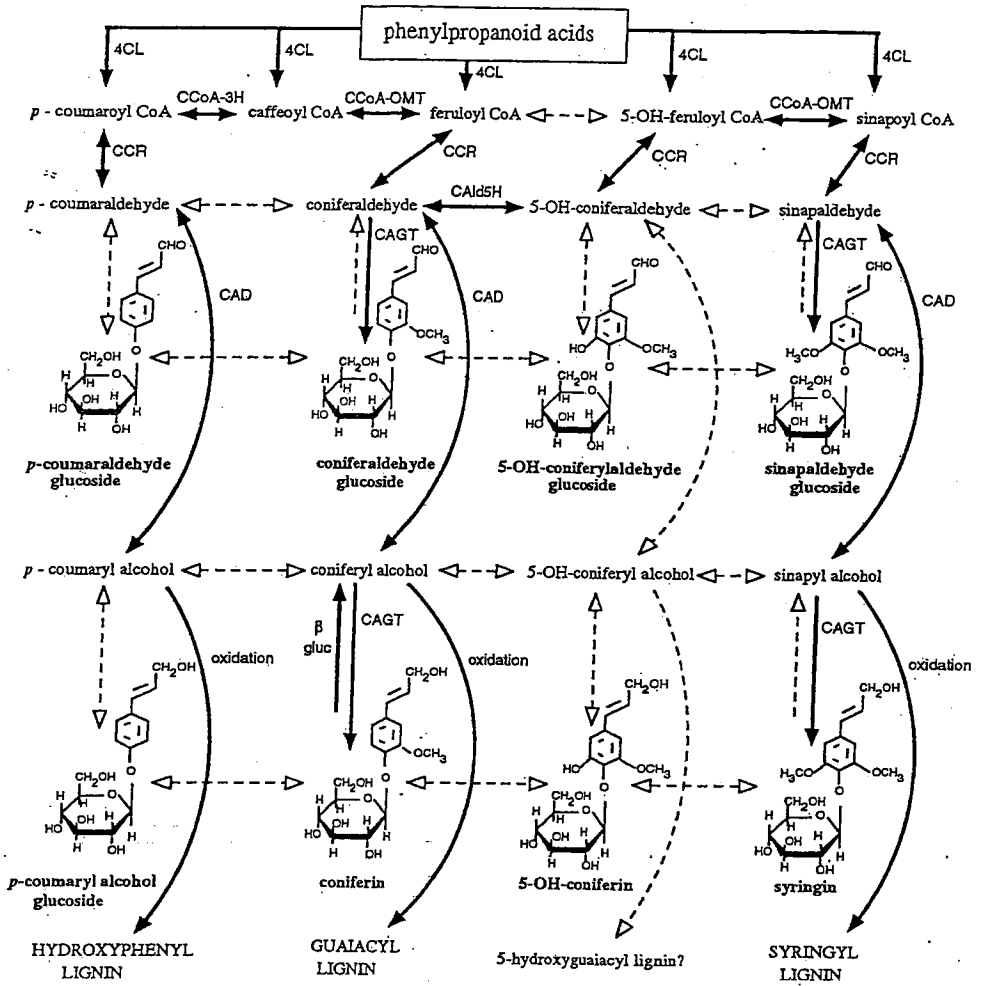

Figure 1. Pre-lignification metabolism of monolignols and monolignol glucosides (indicated by bold-faced labels). Solid arrows: catalysis has been demonstrated *in vitro*. Dashed arrows: circumstantial evidence exists for the indicated metabolic step but catalysis remains to be demonstrated *in vitro*. 4CL = 4-coumarate CoA ligase; CCoA-3H = coumaroyl CoA 3-hydroxylase; CCoA-OMT = coumaroyl CoA *O*-methyltransferase; CCR = cinnamoyl CoA reductase; CAld5H = coniferyl aldehyde 5-hydroxylase; CAD = coniferyl alcohol dehydrogenase; CAGT = uridine 5'-diphosphoglucose: coniferyl alcohol glucosyltransferase; β-gluc = β-glucosidase; oxidation = oxidative enzymes, for example, peroxidase, laccase, coniferyl alcohol oxidase.

was localized to developing xylem. Nevertheless, coniferin-specific β-glucosidase isolated from lignifying tissues was found only in low abundance (Leinhos *et al.*, 1994) and its catalytic activity evidently is not competitive with that of CAGT found in the same tissues, at the same developmental stage (Savidge and Förster, 1998). A cDNA encoding a glucosidase of *Pinus contorta* has recently been isolated and proposed to be a target gene for overall reduction in lignin deposition (Dharmawardhana *et al.*, 1999).

2.2 *Seasonal variation in endogenous coniferin*

In conifer species, formation of the active cambial zone (CZ) followed by a zone interior to the CZ of radially expanded primary-walled cambial derivatives (RE zone) requires several weeks, and only after both active CZ and RE zones have been generated does the initiation of wood formation and attending onset of lignification occur (Savidge and Wareing, 1981, 1984; Savidge *et al.*, 1998).

In a phenological study of cambial reactivation, Savidge (1988) found HPLC evidence for a biochemical indicator of impending tracheid differentiation in cambial extracts of *Pinus contorta*. The indicator metabolite was isolated and characterized as *E*-coniferin (Savidge, 1989). Quantitative investigation of endogenous indol-3-ylacetic acid, sucrose and coniferin in the cambium of *Larix laricina* throughout the course of a year revealed that, of the three, only coniferin content varied parallel to seasonal cambial activity (Savidge, 1991). In both CZ and developing xylem of *Pinus banksiana*, endogenous coniferin content oscillated in relation to the annual cycle of growth and dormancy (Savidge and Förster, 1998; Savidge *et al.*, 1998).

In springtime, coniferin first became detectable in the CZ in association with resumption of cell-division, indicating that coniferin biosynthesis occurred much earlier than the onset of lignification (Savidge, 1988, 1989, 1991; Fukushima *et al.*, 1997). Coniferin in the CZ (as isolated by bark peeling) remained present only during the period of active cambial growth, disappearing from the CZ in autumn prior to completion of lignification in the latewood of the annual ring (Savidge, 1988, 1989, 1991). In *Larix*, *Picea* and *Pinus* spp., coniferin was not detectable in dormant cambium by HPLC, gas chromatography, or selected-ion-monitoring gas chromatography-mass spectroscopy (Savidge, 1988, 1989, 1991).

2.3 *Seasonal variation in CAGT activity*

Seasonal changes in metabolic processes are frequently observed (e.g., Terazawa and Miyake, 1984; Savidge, 1991), but poorly understood. Our springtime investigations into *Pinus banksiana* indicated that both CAGT activity and the accumulation within the CZ of a detectable amount of endogenous coniferin are associated - both temporally and spatially - with resumption of cell-division, rather than with lignification (Förster and Savidge, 1995; Savidge and Förster, 1998; Savidge *et al.*, 1998). Similarly, late summer and autumn investigations indicated that CAGT activity in the CZ disappeared with the onset of dormancy despite ongoing lignification and maturation of the latewood elements (Förster and Savidge, 1995; Savidge and Förster, 1998). Thus, the annual cycle of cambial growth and dormancy in conifers appears to be linked to the production of the metabolite coniferin, and the evidence indicates that this qualitative relationship is

due to the expression of the corresponding glucosyltransferase. To our knowledge, CAGT is the first enzyme to be qualitatively linked to the annual cycle of growth and dormancy in perennial woody plants (Savidge and Förster, 1998). The fact that both coniferin and CAGT activity are present in the CZ only when the cambium is active points to a probable control mechanism for seasonal cambial activity at the level of gene expression (Savidge, 1996).

2.4 *Physiological roles of cinnamyl alcohol glucosides*

Cinnamyl alcohol glucosides (*p*-coumaryl alcohol glucoside, coniferin and syringin) have frequently been isolated from different plants (for a survey, see Förster *et al.*, 1999). Despite the fact that lignin is the second most abundant organic substance on earth, after cellulose, monolignols and their corresponding glucosides have only been found in small quantities in the Magnoliophyta. Cell suspensions of *Linum flavum* are a noteworthy exception, yielding coniferin at 5.5 mM (van Uden *et al.*, 1990). In conifers, concentrations of endogenous coniferin as high as 10 mM and 9 mM were reported in *P. banksiana* (Savidge and Förster, 1998) and *Larix laricina* saplings (Savidge, 1991), respectively. Large quantities (3.7 mM) were also obtained from cambial sap of spruce (Schmid and Grisebach, 1982). Coniferin content in the cambium of conifers varies depending on when during the growing season the cambium is harvested (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998).

Compounds in addition to lignin can be synthesized from monolignol glucosides and may have biological activity in plants. Addition of coniferin stimulated the

production of podophyllotoxin, a cytotoxic lignan (Woerdenbag *et al.*, 1990) and coniferin served as a precursor to lignans in the cambial region of *Tsuga heterophylla* (Krahmer *et al.*, 1970). A correlative behaviour between the levels of lignans and cinnamyl alcohol glucosides was observed before and after hatching of gall larvae in *Picea glauca* (Kraus and Spiteller, 1997). The promotion of cell division by dihydroconiferyl alcohol glucosides to initiate rapidly-growing crown gall tumours in *Vinca rosea* (Lynn *et al.*, 1987; Binns *et al.*, 1987) is especially remarkable, since the stimulation of cell growth and differentiation is usually attributed to phytohormones. A putative defence reaction is illustrated by the adaptive oscillation of *Z*-coniferin in *Fagus sylvatica* in response to attack by beech scale (Dübeler *et al.*, 1997). *Z*-Isoconiferin and *Z*-syringin were reported to induce wall shedding in ascospores of xylariaceae (Chapela *et al.*, 1991). Coniferin was also described as an inducer of *vir*-gene activities in *Agrobacterium tumefaciens* causing crown gall disease (Morris and Morris, 1990; Delay *et al.*, 1994; Nan *et al.*, 1997).

CAGT, in catalysing formation of *E*-coniferin and other cinnamyl alcohol glucosides (see below) evidently competes with coniferyl alcohol dehydrogenase and monolignol oxidizing enzymes for coniferyl alcohol (Figure 1). Coniferin is effective in inhibiting coumarate-CoA-ligase (Voo *et al.*, 1995) and cinnamyl alcohol dehydrogenase (CAD; O'Malley *et al.*, 1992), the latter being considered a core enzyme of lignification. Our data (see below) indicate that the glucosyltransferase must also compete with CAD for available cinnamyl aldehydes by forming the corresponding aldehydic glucosides (Savidge and Förster, 1998; Förster *et al.*, 1999). As illustrated in Figure 1, the substrate

competition possible between CAGT and other enzymes in the lignification pathway very probably diminishes precursor flow into lignification. Thus, it is apparent that monolignol glucosides could fulfil a role in regulating lignin biosynthesis.

Despite the focus on lignification in regard to coniferin, CAGT has not been considered as an enzyme capable of influencing either content or composition of lignin. There is evidence that syringyl lignin can arise from coniferyl aldehyde (Osakabe *et al.*, 1999); however, Matsui *et al.* (1996) also reported interconversion of labelled coniferin and syringin (the 4-*O*-β-D-glucoside of sinapyl alcohol) in *Magnolia kobus* by transformation of the corresponding aglycones. As Figure 1 indicates, the guaiacyl:syringyl ratio of lignin can be modified through the activities of enzymes forming and hydrolysing coniferin and syringin (Piumi *et al.*, 1998; Förster *et al.*, 1999). Repeating the ideas of Freudenberg (1964), Grima-Pettenati and Goffner (1999) suggested that coordinated transport, storage and mobilization of the glucosidic precursors may be rate limiting steps in lignification. CAGT activity may also be part of the explanation for the aldehyde:alcohol ratio in guaiacyl lignin, because CAGT competes with CAD for both coniferyl aldehyde and coniferyl alcohol (Savidge and Förster, 1998; see also Figures 2 and 3).

3. UDPG:coniferyl alcohol glucosyltransferase activity in *Pinus strobus*

3.1 *Material and methods*

Enzyme. P. strobus trees were harvested on dates throughout the growth season, and CAGT was extracted from tissues (cambial zone, CZ; zone of radial expansion, RE; and

zone of secondary wall formation and lignification, SL) separated by bark peeling. The CZ and xylogenic zones (RE, RE+SL, or SL) were scraped individually into liquid nitrogen. Enzyme isolation, purification and enzymatic assays were carried out according to Savidge and Förster (1998).

Product analysis. Aliquots of the enzyme assay were chromatographed by HPLC on a C18 ODS Ultrasphere column (Savidge, 1988; Förster and Savidge, 1995; Savidge and Förster, 1998). Coniferin peaks were detected at 262 nm and confirmed by co-chromatography using authentic *E*-coniferin crystallized from *P. strobus* (Savidge, 1989). Coniferyl aldehyde glucoside and sinapyl aldehyde glucoside peaks were quantified at 332 nm and 320 nm, respectively, and co-chromatographed with authentic references isolated and confirmed by ^{1}H NMR, as described by Förster *et al.* (1999). Coniferin and syringin were collected from the HPLC, lyophilized, derivatized with Sylon TP and analysed in scanning mode by GC-MS, using conditions as previously described by Savidge (1989).

Microscopy. Before extraction and HPLC analysis, handcut sections of cambium and developing xylem were examined by microscopy to ascertain the phenological stage of the trees being investigated.

3.2 *Results*

Seasonal coniferin content and CAGT activity. In agreement with the previous finding of

a direct correlation between seasonal CAGT activity and cambial growth in *P. banksiana* (Savidge and Förster, 1998), a similar relationship was found in *P. strobus* (Figure 2a). CAGT activity appeared in the CZ at the time when cell division activity was commencing for another growing season, about three weeks before the first produced cambial derivatives began developing into earlywood xylem. The major differences in coniferin content between developing xylem and CZ observed in *P. banksiana* (Savidge and Förster, 1998) were not found in *P. strobus*. Whereas more than 15 times higher CAGT activity was present in developing xylem compared to CZ of *P. banksiana*, the same two zones extracted from *P. strobus* exhibited more or less comparable catalytic rates of CAGT over the growing period (Figure 2a).

Specificity of CAGT. The CAGT isolated from *P. strobus* exhibited a pronounced specificity for uridine 5'-diphosphate glucose (Table 1). None of the other glucose nucloetides tested could be efficiently substituted for UDPG, although trace activities were detected with GDPG and CDPG in the case of coniferin formation, and with TDPG for coniferyl aldehyde glucoside formation. In agreement with results reported previously (Ibrahim and Grisebach, 1976; Ibrahim, 1977; Schmid and Grisebach, 1982), UDP-glucose was the preferred sugar donor for the glucosylation of cinnamyl alcohols. It should be mentioned, however, that in cambial sap of spruce TDP-glucose exhibited 17 % activity in comparison to UDPG (Schmid and Grisebach, 1982).

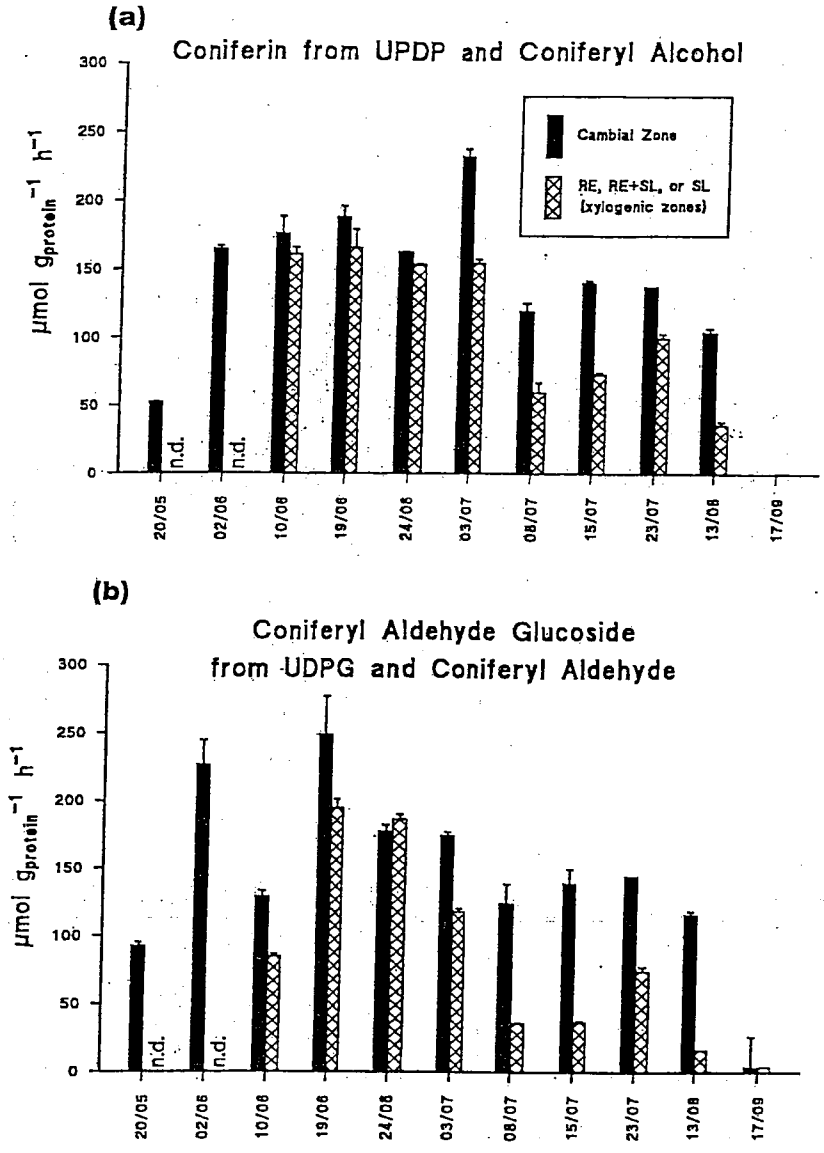

Figure 2. *In vitro* production (1 h, 36 °C) of coniferin (a) and the glucoside of coniferyl aldehyde (b) by partially purified enzyme preparations obtained from the cambial zone and zones of xylogenesis. The 13-14-year-old main stem region of 20-year-old Pinus strobus was assayed on the several successive dates (day/month) indicated under the x-axis. Product quantification was by reversed-phase HPLC (Savidge, 1988). n.d. = activity was not determined. Error bars are standard errors of mean (n = 3).

Table 1. Relative contributions of sugar nucleotides to coniferin biosynthesis *in vitro*.

Relative activity[a] (%)	Coniferin	Syringin	Coniferyl aldehyde glucoside	Sinapyl aldehyde glucoside
UDP-glucose	100.00	100.00	100.00	100.00
GDP-glucose	1.83	0.00	0.00	0.00
TDP-glucose	0.00	0.00	2.57	0.00
CDP-glucose	1.81	0.00	0.00	0.00
UDP-galactose	90.00	89.00	48.00	48.00

[a] Activities were relative to 100 % for UDP-glucose.

Unexpectedly, CAGT purified from *P. strobus* expressed high activity when UDP-galactose was provided to the enzyme (Table 1). Cinnamyl alcohols showed better turnover rates than cinnamyl aldehydes. It remains to be clarified whether the sugar moiety was inserted as glucose or galactose; UDP-glucose-4-epimerase activity in the enzyme preparation cannot be excluded (Dalessandro and Northcote, 1977). This possibility may be resolvable using UDP-xylose, a highly efficient inhibitor of the epimerase (Paczkowski and Wojciechowski, 1994).

Cinnamyl aldehydes were reported as suitable substrates for CAGT (Ibrahim and Grisebach, 1976; Schmid and Grisebach, 1982) and *in vitro* led to the formation of the corresponding 4-*O*-β-D-glucosides (Table 2, see also Förster *et al.*, 1999). Unexpectedly, CAGT from *P. strobus* produced higher amounts of cinnamyl aldehyde glucosides than monolignol glucosides although the specific turnover rate (i.e., ratio between administered substrate and obtained product) was about 8 times lower (Figure 2b). Nevertheless, the *in vitro* generation of coniferyl aldehyde glucoside revealed a

similar seasonal pattern to that observed for coniferin (compare Figure 2a and 2b).

Table 2. CAGT activity *in vitro* with a selection of aglycones.

Substrate	Activity[a] (%) relative to coniferyl alcohol				
	Pinus strobus	*Pinus banksiana*[b]	*Forsythia ovata*[c]	Paul's scarlet rose[d]	*Picea abies*[e]
Cinnamyl alcohols and aldehydes					
coniferyl alcohol	100.00	100.00	100.00	100.00	100.00
sinapyl alcohol	139.43	96.00	82.00	74.00	14.00
p-coumaryl alcohol	n.d	0.00	0.00	< 1	7.00
coniferyl aldehyde	106.26	n.d.	n.d.	46.00	63.00
sinapyl aldehyde	138.02	n.d.	n.d.	n.d.	48.00
Phenolics					
luteolin	3.14	n.d.	n.d.	< 1	2.00
apigenin	2.05	n.d.	n.d.	n.d.	4.00
naringenin	1.08	n.d.	n.d.	n.d.	8.00
trans-cinnamic acid	1.41	0.00	n.d.	n.d.	n.d.
p-coumaric acid	0.27	0.00	0.00	0.00	3.00
caffeic acid	0.49	0.00	n.d.	18.00	2.00
ferulic acid	2.38	0.00	18.00	20.00	4.00
sinapic acid	1.35	0.00	14.00	21.00	3.00
2-OH-benzoic acid	5.08	n.d.	0.00	< 1	n.d.
syringaldehyde	11.41	n.d.	n.d.	n.d.	n.d.
mandelic acid	0.00	0.00	n.d.	n.d.	n.d.
vanillin	2.76	0.00	n.d.	n.d.	n.d.
vanillic acid	0.00	0.00	0.00	< 1	14.00

[a]Activities were determined by calculation of the amount of UDPG incorporation into each substrate.
[b]Savidge and Förster (1998). [c]Ibrahim (1977). [d]Ibrahim and Grisebach (1976). [e]Schmid and Grisebach (1982). n.d. = not determined.

The substrate specificity of CAGT is summarized in Table 2. The observed substrate specificities of these partially purified enzymes generally agree, but there are also some exceptional differences. Notably, there are remarkably higher rates of glucosylation of cinnamic acids in angiosperms (*Forsythia* and *Rosa* spp.) compared to conifers. In general, aldehydes are suitable substrates for CAGT, evidently even in the case of syringaldehyde (however, the structure of the glucosidic product has not yet been investigated). Sinapyl alcohol was utilized by CAGT in *P. strobus* cambium to an even higher extent than coniferyl alcohol, but it was used much less effectively in *P. abies* (Table 2).

Inhibition of CAGT activity. Cinnamyl alcohols and cinnamyl aldehydes were in competition with each other for CAGT from the cambial zone (Figure 3). All of the substrates depicted in Figure 3 were applied as 1 mmol g^{-1} solutions. Coniferyl alcohol and sinapyl alcohol administered together diminished the formation of coniferin and syringin to 41 % and 43 %, respectively. Only 22 % of the original turnover was reached when the cinnamyl aldehydes were incubated together. In a mixture of cinnamyl alcohols and cinnamyl aldehydes, formation of cinnamyl aldehyde glucosides was more strongly affected (between 12 % and 16 % of the control) than that of cinnamyl alcohol glucosides (between 37 % and 41 %). However, it made almost no difference when coniferyl aldehyde or sinapyl aldehyde were incubated together with coniferyl or sinapyl alcohol (Figure 3).

Coniferin itself was reported to inhibit CAGT in a noncompetitive manner with product inhibition starting at about 10 mmol g^{-1} (Schmid and Grisebach, 1982).

However, coniferin added to CAGT of *P. banksiana* showed no inhibitory effect up to 10 mmol g^{-1} (Förster and Savidge, 1995). Thus strong inhibition of CAGT by the monolignols and their corresponding aldehydes at substrate concentrations ten times lower indicates efficient interference. Our *in vitro* results clearly demonstrate the possibility for *P. strobus* CAGT to catalyse 'sidestep' reactions along the lignification pathway.

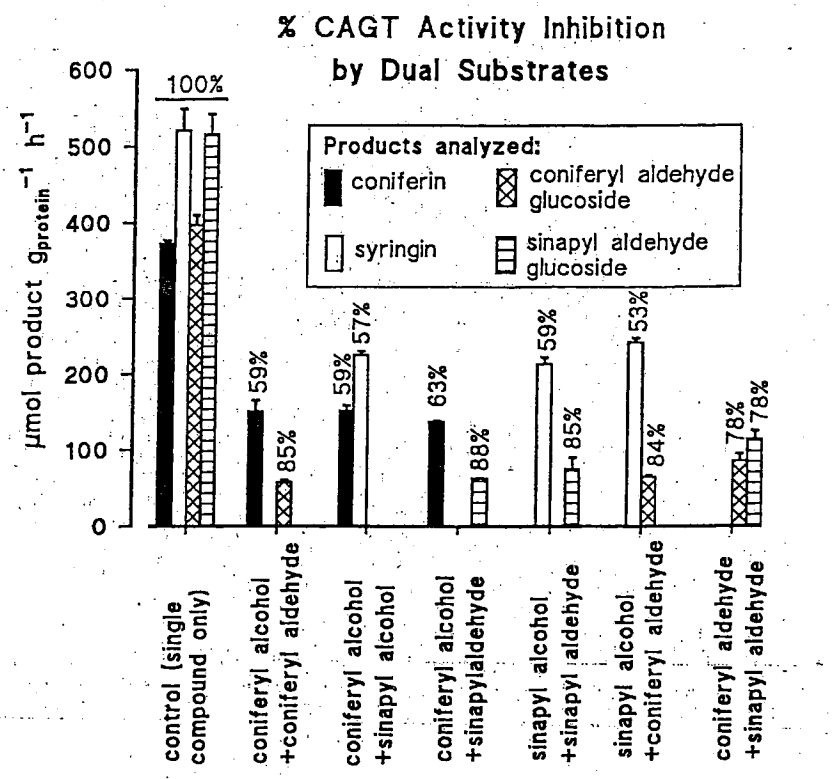

Figure 3. Percent reduction of in vitro CAGT activity by dual substrates, relative to controls receiving only a single substrate. All of the substrates were provided at a concentration of 1 mmolg^{-1} protein. Incubations (1h, 36 °C) were done using partially purified CAGT from the 14-year-old cambial zone of a 20-year-old Pinus strobus harvested on June 19th. Error bars are standard errors of mean (n = 3).

4. Conclusions and outlook

CAGT, catalysing formation of glucosides of both cinnamyl alcohols and aldehydes, has previously been considered merely as an enzyme catalysing production of a storage reserve of monolignols in support of lignification. To date, there is no evidence that glucosides such as coniferin are susceptible to oxidative polymerization or other kinds of metabolism, excepting hydrolysis; thus, if coniferin is only a storage reserve, there is no obvious reason for it not continuing to reside in the stem during dormancy. However, both coniferin and the activity of CAGT vanish from the cambial zone upon its entry into dormancy, which occurs well before the completion of lignification in the finally maturing latewood. Both coniferin accumulation and CAGT activity reappear in the cambial zone in springtime well before either lignification or any other aspect of earlywood formation are initiated. Thus, a growing body of evidence indicates that coniferin and the gene expression underlying CAGT activity, although capable of contributing to lignification, must be more fundamentally linked to the overall control of seasonal cambial growth in conifers. The physiological regulation of seasonal cambial growth is a longstanding, unanswered question and, to our knowledge, no other metabolites nor products of gene expression have been observed to have the qualitatively perfect spatio-temporal relation to the annual cycle of growth and dormancy that are displayed by coniferin and CAGT, respectively. Thus, continuing research into coniferin and CAGT would appear to be justified as a means of providing insight into the control of seasonal cambial growth.

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CHAPTER 5

CONCLUSIONS AND OUTLOOK

The research presented herein provides new insights into coniferin metabolism, with particular emphasis on the two key enzymes involved in coniferin metabolism, coniferin β-glucosidase (CBG) and UDPG: coniferyl alcohol glucosyltransferase (CAGT). CAGT catalyses the formation of *E*-coniferin from its aglycone, *E*-coniferyl alcohol with UDP-glucose, while CBG, in turn, catalyses the release of *E*-coniferyl alcohol from *E*-coniferin.

Key findings reported herein include:

Chapter 2: A novel method of obtaining elevated CBG activity toward coniferin in excised cambial tissue of *Pinus strobus* L. was developed. The enzyme was shown to be soluble in both the cambium and developing xylem. This is the first report of CBG activity in isolated cambium cells. A temporal association between CBG and cambial activity was observed.

Chapter 3: CAGT isolated from cambial tissue of *P. strobus* catalysed the glucosylation of sinapyl and coniferyl aldehyde to their 4-*O*-β-D-glucosides with comparable

efficiency to its activity for sinapyl and coniferyl alcohol. CAGT activity for the aldehydes followed a seasonal pattern similar to that observed for the alcohols. Michaelis-Menten constants, K_m, are reported for coniferyl and sinapyl aldehyde for the first time. This is the first report of CAGT activity toward dihydroconiferyl alcohol.

Chapter 4: CAGT product inhibition was investigated using various combinations of cinnamyl alcohols and aldehydes, with two substrates administered in each example. The most pronounced inhibition occurred when coniferyl alcohol and sinapyl aldehyde were administered together, with sinapyl aldehyde glucoside formation being more strongly inhibited.

Implications of the results reported in this dissertation are discussed in each of the chapters, as well as summarized here.

As noted throughout this dissertation, coniferin is known to accumulate in the cambium and developing xylem of conifers throughout the spring and summer months of cambial activity, appearing before cambial cell-division activity commences (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Fukushima et al., 1997; Savidge and Förster, 1998; Savidge et al., 1998) and disappearing at the onset of cambial dormancy in early autumn, before latewood formation is complete (Savidge, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998; Savidge et al., 1998). Coniferin has yet to be detected in dormant tissue during winter (Freudenberg and Harkin, 1963; Terazawa and

Miyake, 1984; Savidge, 1989, 1991; Förster and Savidge, 1995; Savidge and Förster, 1998, 2001; Savidge et al., 1998). Thus, while the presence of coniferin in the cambium and its derivatives is tightly linked to the season of cambial activity in conifers, coniferin's temporal association with lignification is less closely connected (i.e., it appears well in advance of lignification in the spring). This, along with the fact that coniferin has not been shown to accumulate in most angiosperm species to the same extent that it does in conifers (reviewed in section 4 of Chapter 1), has led some to argue that coniferin may not play a critical role in lignification (c.f., Brown and Neish, 1955; Wardrop, 1976).

In addition, to my knowledge, CBG itself has also not been isolated from angiosperm species; however, Tsuji and coworkers (2004) attribute this to an apparent lack of research with angiosperm trees rather than an absence of CBG activity. A few studies support CBG activity in angiosperms. For instance, Fukushima and Terashima (1990) demonstrated incorporation of the coniferyl alcohol moiety of labelled coniferin into lignin of various angiosperm species, such as magnolia, lilac, beech and poplar, indicating that a CBG must be active in these tissues. Tsuji and coworkers (2004) administered labelled coniferin and coniferyl alcohol to excised shoots of magnolia and eucalyptus and recovered the label in lignin in both cases, providing further evidence that a CBG is likely responsible for liberating coniferyl alcohol from coniferin before it is polymerized into lignin. In these feeding experiments, labelled precursors were fed to excised tissue, which is not necessarily representative of conditions *in planta*; however, the results do demonstrate that the tissue was competent for glucosidase activity toward

coniferin. In another study, Tsuji and Fukushima (2004) administered labelled coniferin, syringin, coniferaldehyde glucoside, and sinapaldehyde glucoside to cut stem segments of magnolia and oleander, and the label was recovered in lignin in all cases. Their results suggested that coniferaldehyde and/or coniferaldehyde glucoside are important in biosynthesis of syringyl lignin in these species, as the efficiency of incorporation of coniferaldehyde glucoside was greater than that of sinapaldehyde glucoside (Tsuji and Fukushima, 2004), although these experiments were performed with protein extracts of differentiating xylem rather than *in planta*.

Research presented here (Chapters 3 and 4) for *Pinus strobus*, and by Savidge and Förster (1998) for *P. banksiana*, demonstrates a seasonal pattern of CAGT activity toward coniferyl alcohol in cambium and developing xylem of these species, similar to the seasonal pattern of coniferin accumulation in the same tissues. Seasonal patterns of CBG activity seem to be emerging, as well. Dormant cambium of *P. strobus* was not competent for CBG activity toward coniferin; however, cambium and radially expanding derivatives (with an abundance of coniferin) did demonstrate CBG activity upon wounding, which was enhanced in wounded differentiating xylem undergoing lignification in the secondary wall (Chapter 2). These results agree with those of Marjamaa et al. (2003), who reported seasonal fluctuations in CBG activity in developing xylem of *P. sylvestris* and *Picea abies*. In conifers, it appears that the balance between CAGT activity for coniferyl alcohol and CBG activity for coniferin favours coniferin biosynthesis. The recent discovery of CBG activity in angiosperms, as well as the discovery of CAGT activity in suspension cultures of Paul's scarlet rose (Ibrahim

and Grisebach, 1976) suggest that these two enzymes may co-exist in angiosperm cambial tissue, as well. Certainly, if they do, the balance does not favour glucoside accumulation, as it has been shown to accumulate in relatively few angiosperm species (Ibrahim, 1977; Terazawa et al., 1984a,b; Fukushima et al., 1996; Nan et al., 1997). Tsuji's group has provided evidence that CBG may be more active toward coniferaldehyde glucoside rather than coniferin in angiosperms, and that it is through the aldehyde glucoside that guaiacyl units could be converted into syringyl units (Tsuji and Fukushima, 2004; this type of conversion was also noted in ginkgo in Tsuji et al., 2005). Clearly, more research is needed to fully establish the balance between CBG and CAGT activity over the period of cambial activity within the same tissue.

Both CAGT and CBG are soluble (or at least partially soluble; see Chapter 2; Schmid et al., 1982; Savidge and Förster, 1998) enzymes in conifer cambial tissue. This contradicts Samuels et al.'s (2002) report that CBG is found only in the secondary wall of developing xylem in *P. contorta*. As discussed in Chapter 2, evidence reported here suggests CBG is compartmentalized within the cytoplasm of cambium and differentiating xylem. It is unclear whether CAGT resides free in the cytoplasm or is compartmentalized. Since cinnamyl alcohol dehydrogenase (CAD; the enzyme responsible for reducing cinnamyl aldehydes, such as coniferaldehyde, to cinnamyl alcohols, such as coniferyl alcohol; see Chapter 1, Figure 6) has been located in the cytoplasm of differentiating xylem (Takabe et al., 2001), it is possible that coniferyl alcohol, upon being reduced from coniferyl aldehyde through CAD activity, comes in contact with CAGT in the cytoplasm of cambium and differentiating xylem. Coniferin

thus accumulates in these cells and only comes in contact with CBG when CBG is released from its compartments, via wounding, for instance (see Chapter 2), or perhaps at the plasma membrane (Samuels et al., 2002), or when CBG is presented to coniferin within compartments themselves (Dharmawardhana et al., 1999). This remains to be, and should be, investigated further.

There is no question that coniferyl alcohol derived from coniferin is incorporated into lignin in many systems. In addition to the examples already noted above, other examples are provided here. Radiolabelled coniferin was incorporated into lignin of: spruce through feeding experiments with young, intact spruce twigs (Freudenberg et al., 1955); *Pinus thunbergii* Parl. via feeding experiments with cut shoot segments (Terashima et al., 1988); *Ginkgo biloba* L. through uptake of labelled coniferin by excised stem segments (Xie and Terashima, 1991; Xie et al., 1994); and, *Triticum aestivum* L. cv. Marshall, MN (dwarf wheat) via injecting labelled coniferin into the second internode cavity from the top of the intact plant (Terashima et al., 1997). Also, coniferin has been shown to both enhance and inhibit CAGT activity, depending on its concentration (Savidge and Förster, 1998), and to inhibit both 4-coumarate-coenzyme-A ligase (O'Malley et al., 1992) and coniferyl alcohol dehydrogenase (Voo et al., 1995), two enzymes involved in the biosynthesis of lignin precursors (see Chapter 1, Figure 6).

Altogether, evidence in support of coniferin playing a role in lignin biosynthesis is very strong, either in providing coniferyl alcohol for polymerization through CBG activity, or in regulating enzymes involved in monolignol biosynthesis. Thus, both CAGT and CBG must also be considered as enzymes involved in lignin formation, in

conifers, at least (and perhaps in angiosperms, as more research is performed). Many researchers indicate that the last enzyme involved in monolignol formation, responsible for providing coniferyl alcohol to the cell wall for polymerization into lignin, is CAD (c.f., Walter et al., 1988; Boudet and Grima-Pettenati, 1996; Damiani et al., 2005; Sibout et al., 2005). The role of CBG in lignin formation in conifers has been hugely understated. CBG should be considered as the last enzyme involved in providing at least a portion of the coniferyl alcohol used in lignin formation to the cell wall in conifers. Hösel et al.'s (1982) work with parsley indicated a close temporal association of CBG activity with enzymes involved in monolignol biosynthesis, with CBG activity appearing in conjunction with phenylpropanoid enzymes and immediately prior to lignin deposition in the wall. Given its importance in catalysing such a critical step in lignin formation, future studies should focus on gaining further understanding of this critical, yet underappreciated enzyme. My research in Chapter 2 provides the stepping stone for future research on CBG. This work will hopefully be considered by others, as it is by me, to be my greatest contribution to the field of lignin biosynthesis, particularly in conifers. The next step should be to extract and isolate CBG from developing xylem of *P. strobus*. Once this has been achieved, genetic manipulations may then provide insights into CBG's exact role in lignin formation in conifers.

Another research project that can stem from the research presented here involves a closer look at specificities of CAGT and CBG *in planta*. For instance, CAGT extracted from developing xylem of *P. strobus* was found to glucosylate coniferaldehyde with similar efficiency to coniferyl alcohol (Chapter 2 and 3), and Tsuji et al.'s (2005) work

with feeding experiments with CBG extracted from ginkgo suggests that coniferaldehyde glucoside was a preferred substrate for CBG compared to coniferin. Also, CAGT used UDP-galactose with 90 % activity relative to UDP-glucose in *P. strobus*. These findings cannot necessarily be extrapolated to *in vivo* systems, and future experiments should focus on how to achieve this. As mentioned in Chapter 1, defence and wound responses may occur when cambial material is extracted, which should be taken into consideration when reporting results from experiments that rely on such destructive techniques (Kahl, 1978; Chaffey, 1999). That is why the observations with CBG in Chapter 2 are so critical. Evidence supports the presence of CBG within developing xylem prior to harvesting the trees and wounding, and the act of wounding simply released the enzyme into the cytoplasm where it could act upon coniferin.

It is my hope that the research presented here leads others to take an interest in the complex dynamic that exists between CAGT and CBG. Evidence is overwhelmingly in support of coniferin playing a role in providing coniferyl alcohol, the primary monolignol in conifer lignin, to the cell wall for polymerization into the lignin matrix. Thus, it follows that every effort should be made to understand coniferin metabolism in conifers. Best wishes!

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APPENDIX A

CAMBIAL CONIFERIN CONTENT AS A PLAUSIBLE INDICATOR OF TREE HEALTH IN CONIFERS

1. Introduction

Much controversy surrounds the harvesting of old-growth forests around the world. Scientists, industry and environmental groups have yet to arrive at reliable criteria for determining the health status of trees, and hence stands and forests. Most methods for predicting forest health rely on macro features, such as the amount and efficiency of foliage (i.e., Waring, 1983; Blanche *et al.*, 1985), growth abnormalities such as departures from typical bole taper (i.e., Waring, 1985), radial growth and internode elongation (i.e., Kostka and Sherald, 1982; Lewis, 1995; Ducrey *et al.*, 1996), and foliar discoloration (i.e., Bernier *et al.*, 1989; Allen *et al.*, 1992; Kolb and McCormick, 1993; Wilmot *et al.*, 1995).

However, as Ducrey *et al.* (1996) state, 'measurement of loss of vigor is difficult because directly observable symptoms often do not develop.' Thus, micro features such as the chemistry and physiology governing wood formation (i.e., a healthy tree is one that is producing wood) in the cambium must also be considered as tools for predicting

tree health.

As an example, cambial electrical resistance (ER) has been extensively studied as a possible indicator of tree health. Skutt *et al.* (1972) described a non-destructive method using resistance to pulsed electric current to detect discoloration and decay in living trees. Also in 1972, Tattar *et al.* determined that ER is a function of relative changes in concentrations of mobile ions, such as potassium. In 1974, Shigo and Shigo demonstrated application of an electrical meter, the Shigometer, for these measurements.

Cambial zones of healthy trees were shown to have lower resistance to pulsed electric current than stressed trees (Wargo and Skutt, 1975; Smith *et al.*, 1976), and Wargo and Skutt (1975) suggested that '...resistance is an indicator of tree vigor.' Resistance to pulsed electric current in the cambial zone was found to be inversely proportional to rate of tree growth (Shortle *et al.*, 1977) and this was subsequently used as a guide to thinning sugar maples (Shortle *et al.*, 1979). Measurements of ER were used successfully to predict Dutch elm disease in elm trees before visible symptoms were observed (Blanchard and Carter, 1980). Davis *et al.* (1980) found that balsam fir stands with increased levels of spruce budworm infestation had increased mean ER values. Kostka and Sherald (1982) described some limitations of ER values as indicators of tree health: 'Measurements of ER with the Shigometer were easily and quickly performed; however, ER readings did not reflect tree vigor as accurately as growth measurements.' Blanchard *et al.* (1983) found that lower ER values were related to higher growth rates in balsam fir, and Lindberg and Johansson (1989) corrected cambial ER of *Picea abies* to standard temperatures and found good correlation between growth

rate and ER for both dominant and suppressed trees.

This chapter focuses on another possible indicator of tree health, with emphasis on conifers. Coniferin is a secondary metabolite found in abundance in active conifer cambia. An important physiological aspect of coniferin is that its presence varies seasonally. It has not been detected in the cambium during winter dormancy, nor in the early spring, prior to cambial reactivation. Following the dormancy period, coniferin appears in the cambial zone immediately before the resumption of cell-division activity (Savidge, 1988, 1989, 1991; Förster and Savidge, 1995; Fukushima *et al.*, 1997; Rowland and Arora, 1997; Savidge *et al.*, 1998) and thereafter is present throughout the entire period of cambial growth. Coniferin in the cambial zone becomes undetectable at the onset of cambial dormancy in autumn, before the last cambial derivatives have completed their maturation into latewood (Savidge, 1991; Förster and Savidge, 1995). Coniferin has never been detected in stems during cambial dormancy (Freudenberg and Harkin, 1963; Terazawa and Miyake, 1984; Savidge, 1989, 1991; Förster and Savidge, 1995; Savidge *et al.*, 1998), and biochemical investigations have indicated that the presence or absence of UDPG:coniferyl alcohol glucosyltransferase activity is the explanation for the qualitative seasonal variation (Savidge and Förster, 1998).

Since coniferin is only present in the cambium of conifers during cambial activity, its presence serves as an indicator that cambial growth is occurring. This may be merely a coincidence of metabolism without physiological significance. On the other hand, it could be an indication that the gene expression underlying coniferin biosynthesis is linked to that controlling growth within the tree. If coniferin biosynthesis is obligately

linked to the primary metabolism underlying growth, as suggested by Savidge and Förster (1998), then coniferin content in the cambium should be affected when growth metabolism is experimentally altered. For example, Savidge (1989) found that girdling of tree stems, a treatment which accelerates cambial growth above and inhibits growth below the ring, had corresponding effects on cambial coniferin content.

To manipulate the vigour (i.e., growth metabolism) of conifers, different treatments can be used, including girdling, defoliation, debudding and derooting. The coniferin concentration in the cambium of these trees can be readily monitored by quantitative nuclear magnetic resonance (NMR) spectroscopy, concomitantly observing the extent of cambial cell-division activity by microscopy (Savidge *et al.*, 1998).

2. Materials and methods

2.1 *Trees*

Twenty-four, 1 to 3 m tall, 8-year-old plantation *Pinus resinosa* Ait. were selected from the University of New Brunswick woodlot for treatments and subsequent coniferin investigations in this study. Only those trees that appeared healthy, with no *visible* signs of decline or damage were chosen.

Fifteen, 4 to 6 m tall, 12-year-old plantation *Pinus strobus* L. were selected in late July from The University of New Brunswick woodlot for investigations into their coniferin content and cambial electrical resistance. The trees were deliberately selected to provide a range of health phenotypes, varying from completely dead snags to individuals having three ages (i.e., years) of dark green needles. The majority of the trees

presented only first and second year foliage, with the latter more or less yellowed.

2.2 *Treatments*

Seven treatments to 'stress' the trees were performed: (1) debudding; (2) defoliating; (3) debudding and defoliating; (4) derooting; (5) single ring in the 3-year-old main stem region (see Savidge, 1989); (6) double ring in the 3-year-old main stem region, removing the needles from the stem between the two rings; and (7) double ring in the 3-year-old main stem region, with needles between the rings intact. An eighth set of untreated trees was used as the control. Trees chosen for treatments 1, 2 and 3 were only 1 m tall, while the rest of the trees, including controls, were 2 to 3 m tall. Treatments were performed in early-mid June, with each treatment being replicated three times.

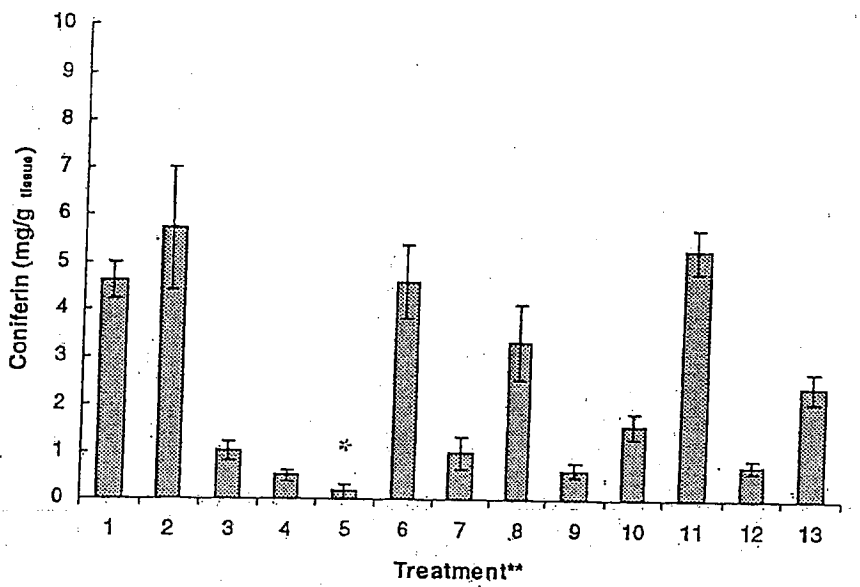
Six weeks after the treatments were performed (late July), the 3-year-old stem region of each treated tree and control was cut from the tree, and brought back to the laboratory for analysis.

2.3 *Microscopy*

Small (1.0 to 1.5 cm × 1.0 cm × 0.5 cm) specimens containing cambium sandwiched between the bark and the mature wood were removed 5 mm above and below rings, and midway between the rings in double-ringed specimen. For all others, including controls, specimens were removed from the midpoint of the 3-year-old main stem region. A cross-section from each specimen was mounted in water and observed under a light microscope.

In the microscopy analysis, the number per radial file of cells in the cambial zone (CZ), the zone where cambial derivatives were primary walled and undergoing radial

expansion (RE), the zone where secondary wall formation and lignification were occurring (SL), and the zone where the cells had completed protoplasmic autolysis to become new tracheids (NT) were all counted and recorded. The averages for the three replicates for each treatment and the controls were obtained and these results are depicted in Figure 1.



Tissue	Cell number per radial file												
CZ	9	5	0	6	14	10	0	8	11	0	6	0	0
RE	8	7	0	5	3	10	0	3	6	0	9	0	0
SL	10	7	4	5	0	11	4	5	3	8	11	6	6
NT	100+	60+	45	30	55	80+	70+	100+	80+	80+	65+	100	60+

Figure 1. NMR and anatomy data for treated and control *P. resinosa* trees. * Coniferin was not detected in one of three replicates. ** 1, control; 2, disbudded; 3, defoliated; 4, debudded + defoliated; 5, derooted; 6, above single ring; 7, below single ring; 8, above double ring (8 - 10 needles removed between rings); 9, between double ring; 10, below double ring; 11, above double ring (11 - 13 needles intact between rings); 12, between double ring; 13, below double ring.

2.4 *Coniferin Content by Physicochemical Methods*

The bark was peeled from the stem, and the exposed inner bark face and xylem surface were scraped together into a 2 ml cryogenic vial, which was then stored in liquid nitrogen. In the case of *P. resinosa*, the 3-year-old stem area was used for non-ringed trees, including controls. For double-ringed trees, 10 cm of stem, beginning 5 mm above the top ring or below the bottom ring were used. All of the area between rings was scraped, leaving a 5 mm buffer adjacent to each ring. For single-ringed trees, 10 cm of stem area were scraped both above and below the ring, beginning 5 mm from the ring. For *P. strobus*, the 8-year-old stem area was used following electrical resistance measurements (see below) in the same area.

Each of the samples was pulverized in liquid nitrogen followed by weighing of 100 mg of frozen powder. The powder was treated with 1.0 ml of extraction buffer containing 1.0 mg of methylphosphonic acid as an internal standard (Savidge *et al.*, 1998) and extracted for 15 minutes at 100 °C. The suspension was then centrifuged (12 000 x *g*, 4 °C) for 15 minutes and the supernatant pipetted into a 5 mm nuclear magnetic resonance (NMR) spectroscopy tube. Proton (^{1}H) NMR spectra were obtained at 400 MHz with a Unity 400 NMR spectrometer (see Figure 2).

2.5 *Electrical resistance measurements*

Cambial electrical resistance (ER) was measured in the eight-year-old stem area of standing trees using a Keithley 614 electrometer. As electrodes, two sharp sterile needles (Becton-Dickinson 27G ½) were pushed through the bark to the point where resistance at the wood surface was encountered. The needles were spaced 1.00 cm apart, one

axially above the other. Alligator clips were attached to the protruding stainless steel shafts and resistance values read immediately, taking each resistance measurement three times. The resistance over 1 cm between two points along the steel shaft was determined to be 0 KΩ, that is, lower than the sensitivity of the electrometer.

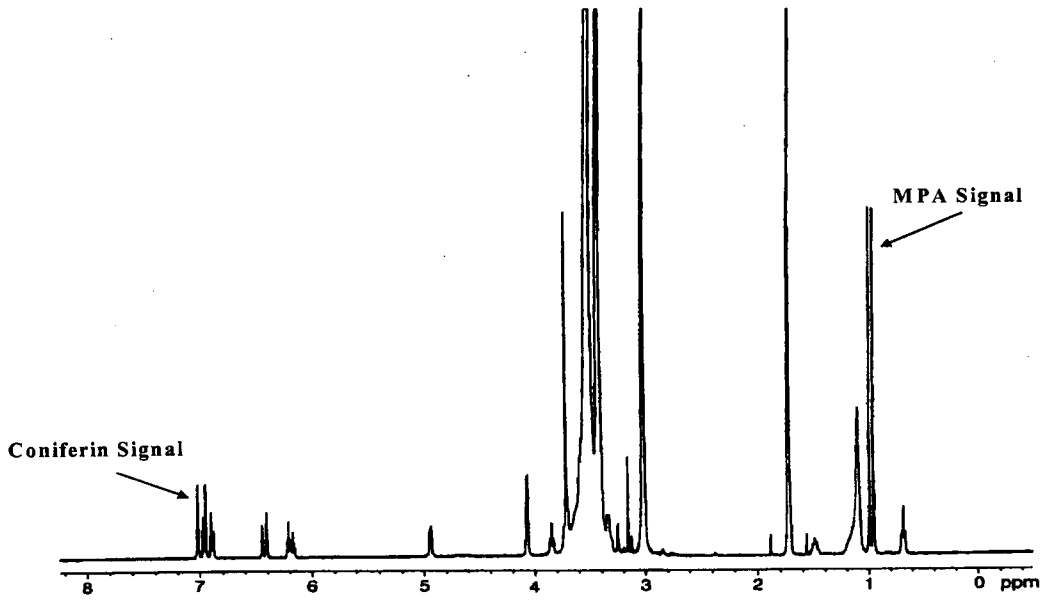


Figure 2. NMR spectrum of coniferin with methylphosphonic acid (MPA) as an internal standard.

3. Results and discussion

3.1 *Results*

The combined integration of the aromatic signals for coniferin, δ 6.8 - 7.1 ppm, and the integration of the methylphosphonic acid (MPA) signal, δ 1.0 ppm, were obtained from the ^{1}H NMR spectra (Figure 2). The integrated area for the coniferin signal was standardized with respect to the known mass of MPA. The concentration of coniferin in

each sample ($mg_{coniferin}$ /ml_{buffer}) was determined by substituting the integrated coniferin signal into the calibration function (R^2 = 0.99993). Since each sample was extracted in 1.0 ml buffer, the amount of coniferin, in mg, is equal to the concentration ($mg_{coniferin}$ /ml_{buffer}). The calculated mass of coniferin was then divided by the mass of tissue extracted, giving coniferin content in $mg_{coniferin}$ / g_{tissue}. The mean values for three replicates, with their standard errors, are depicted in Figure 1, with their standard errors . the minimum detection limit for coniferin was 12 μg.

Debudding had little effect on coniferin content in the cambial region of *P. resinosa*; however, defoliating resulted in a considerable decrease. A combination of debudding and defoliating resulted in an even greater decrease in coniferin content. Derooting had the greatest effect, with one of the three replicates having undetectable coniferin (Figure 1).

The amount of coniferin detected above single rings was not much different from controls, but the amount below rings was considerably reduced. Similar effects were seen in double-ringed specimens, with lower levels of coniferin below the bottom rings, and even lower levels between the rings, especially in the case where the needles were removed from the area between the rings (Figure 1).

The cell numbers per radial file found in the four anatomical zones of *P. resinosa* are shown in Figure 1. Microscopy indicated that the cambia of the controls were still active in July, and new tracheids were still forming in both RE and SL zones. The cambia in debudded trees, from above single rings, and from above double rings, were all still active and producing new tracheids. The cambia of defoliated trees, those from

below all rings, and those between the rings where needles were left intact, had all de-differentiated such that the cambial zone consisted only of axial parenchyma cells (hence, CZ = 0 in Figure 1). At these locations there was a considerable decrease in the number of new tracheids formed, particularly in defoliated samples. In derooted trees, all cambial activity and radial expansion activity ceased when the roots were cut from the trees; however, SL-zone cells that had already begun differentiation into new tracheids matured into new tracheids.

In *P. strobus*, coniferin was present in the cambium when it was active and could not be detected when the cambium was inactive or dead. Electrometer values for electrical resistance, as measured immediately after inserting the electrodes, varied from 1.84 to 6.83 Ω. Resistance values were unstable with time, increasing to a more stable value near 14 Ω in all but one tree (8 Ω) after elapse of several minutes. Correlation analysis (linear regression) of electrical resistance with coniferin yielded $r^2 = -0.00127$, indicating no relationship (Figure 3).

3.2 *Discussion*

Sink strength of the cambium is weak, relative to shoot and root apices (Eis *et al.*, 1965; Gordon and Larson, 1968; Rangnekar and Forward, 1973). For example, more than 60% of photosynthate produced in the shoots can travel through the phloem to the roots, without being unloaded to the stem's adjoining cambium (Bowen, 1985). The totality of factors regulating sink strength in general remain poorly understood, but trees presumably have feedback mechanisms for determining if there is sufficient nutriment

available to permit allocation to the cambium. Within this logic, if strong cambial growth is occurring, it is likely that the whole tree is healthy.

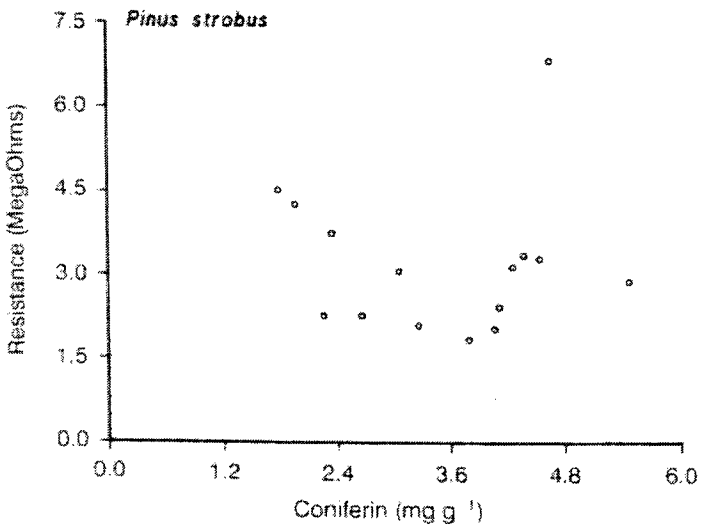


Figure 3. Electrical resistance versus coniferin content in *P. strobus* stems.

Our data with *P. resinosa* indicate that the cambium's content of coniferin varies with changing physiological state, supporting the hypothesis that coniferin biosynthesis is obligately linked to cambial growth (Savidge, 1989; Savidge and Förster, 1998). The glucosyltransferase required for coniferin biosynthesis is unquestionably present in the cambium during active growth (Savidge and Förster, 1998); however, coniferin content of the cambium drops noticeably in areas below rings and is also greatly reduced in defoliated specimens. Thus, our data indicate that living foliage fulfills a crucial role in maintaining the coniferin content of the cambium. The physiological explanation for this

is not clear at this time, although the absolute requirement of the crown in wood formation is well established. Coniferin content in the cambium was not found to be correlated with photosynthate nor auxin contents (Savidge, 1991), and an auxin-cytokinin combination was found to promote hydrolysis rather than biosynthesis of coniferin (Savidge, 1989). It appears, therefore, that coniferin biosynthesis is linked to an unknown factor(s) travelling to, or from, the needles. There is also evidence for coniferin biosynthesis in conifer needles (Slimestad and Hostettmann, 1996), raising the possibility that some coniferin may be exported from the needles to the cambium.

The lack of any correlation between ER and coniferin was not unexpected. Electrolyte concentration in solution is a function of the water potential of the tissue, and the electrical resistance of protoplasma is inversely related to its electrolyte concentration. Activity of the cambium in temperate-zone trees is invariably preceded by cambial rehydration and associated production of immense vacuoles in the fusiform cells. Moreover, many studies have shown that reduced water availability during summer can reduce or block the cambium's activity. It seems reasonable, therefore, to find relatively low ER values in association with active cambia and higher values with slow growing or inactive cambia. On the other hand, with current knowledge it would be difficult to argue that either the water content or the ER of the cambium was necessarily indicative of cambial activity. In spring, the living cambium rehydrates, but it does not necessarily reactivate and produce a new annual ring. In conifers, for example, diameter growth at the base of old branches and, in aged trees, at the base of the bole may not occur at all over many years, despite the continuing presence of the cambium at those

positions (R.A. Savidge, unpublished observations). Evidently, conduction of water through xylem and sap translocation through phloem can continue without need for annual increments of new vascular tissue in those locations. Moreover, electrical assessment of tree health is complicated by the fact that insertion of electrodes into a tree stem does not yield measurements only as they exist in the cambium, rather the net effect generated by all tissues (rhytidome, phloem, CZ, RE, SL, mature wood) contacting the electrodes. The presence of coniferin in the cambium does appear to be specifically indicative of metabolism in support of cambial activity. The coniferin method is more invasive than the electrical resistance method; however, it need not be destructive. Current research is focussing on development of a facile non-destructive field method to diagnose cambial coniferin content.

Based on our observations to date, it appears that cambial coniferin content can serve as a diagnostically useful biochemical indicator of tree health status. We consider that it should be possible to develop a useful quantitative index of tree health for various applications, for example to decide whether an old-growth tree is ready for harvest, based on the content of coniferin in the cambium during the season of growth. If unhealthy trees (or parts thereof) could be detected and harvested before signs of decline occurred, secondary problems in declining forest stands such as insect infestations and decay could be reduced. Conversely, aged trees having continuing competence to be productive and contribute to overall ecosystem health would not have to be harvested prematurely.

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CURRICULUM VITAE

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1. Publications:

a) Articles in Refereed Journals

Doerksen, R.J., **Steeves, V.J.**, and Thakkar, A.J. 2004. Are polarizabilities useful as aromaticity indices? Tests on azines, azoles, oxazoles and thiazoles. Journal of Computational Methods in Sciences and Engineering 4, 427-438.

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c) Other Refereed Papers

Bishop, M., Carr, M., Clarke, G., Colford, J., Fong, K., Foster, J., Hutchins, R., Joyce, V.M., Long, N., Lynch, E., Mengel, T., Pazienza, J., **Reeves, V.**, Roderick, C., Sharp, A., Spacek, R., Valk, J., and Zundel, P. 2006. Renaissance College: Learning Outcomes At The Program Level. Alan Blizzard Award Winning Project. Society for Teaching and Learning in Higher Education (STLHE). McGraw-Hill Ryerson, Toronto.

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2. Conference Presentations

Foster, J., Lynch, E., **Reeves, V.**, Roderick, C., Spacek, R., Valk, J., and Zundel, P. 2006. Outcomes-based learning at the whole program level. Society for Teaching and Learning in Higher Education (STLHE) annual conference: Knowledge and Its Communities. Blizzard Award Plenary Session. June 13 - 17, 2006. University of Toronto. Toronto, ON.

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