Biosynthesis of polyphenols in Norway spruce as a defense strategy against attack by the bark beetle associated fungus *Ceratocystis polonica*

Dissertation

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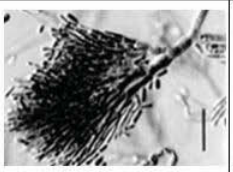
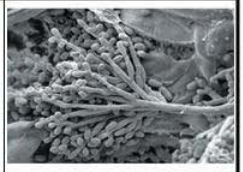
1. GENERAL INTRODUCTION

1.1 Trees from the family Pinaceae are attacked by bark beetles and their associated blue stain fungi

The family Pinaceae (order Coniferales) includes many environmentally and economically important softwood tree genera such as *Pinus* (pine), *Picea* (spruce), *Larix* (larch), *Abies* (fir) and *Tsuga* (hemlock). Pinaceae cover an estimated 1000 million hectare land area consisting of natural and planted forests (Global Forest Resources Assessment, FAO 2010), and are of great economic importance for the production of timber and fiber (Fenning and Gershenzon, 2002). The family Pinaceae also plays essential environmental roles by providing niches for smaller organisms (Dicki and Moyersoen, 2008), acting as a carbon dioxide buffer (Mitchell et al., 2009), preventing soil erosion (Huang et al., 2010) as well as reducing air and water pollution (Chiwa et al., 2004; Guerrieri et al., 2011).

Bark beetles (family Curculionidae, subfamily Scolitinae) are the greatest cause of tree mortality in natural and plantation softwood forests (Benz et al., 2010; Table 1.1). In order to overcome host defenses beetles initiating an attack emit aggregation pheromones which attract thousands of conspecifics to the same tree. Following successful colonization adult beetles mate and lay eggs under the bark of host trees. After hatching, bark beetle larvae feed on the phloem and dying cambium in the tree trunk until pupation.

Table 1.1: Economically important bark beetles and their associated blue stain fungi.

Common name	**European bark beetle**	**Mountain pine bark beetle**	**Southern pine beetle**	**Spruce bark beetle**
Species	*Ips typographus*	*Dendroctonus ponderosae*	*Dendroctonus frontalis*	*Dendroctonus rufipennis*
				
Associated blue stain fungus	*Ceratocystis polonica*	*Grosmannia clavigera*	*Ophiostoma minus*	*Leptographium abietinum*
				
Geographic range	Central Europe Scandinavia	USA Canada	SE USA	NW USA Canada Alaska

To complete their life cycle, the adults emerge and search for another host. As adult beetles bore into bark, they transmit very specific phytopathogenic fungi (Table 1.1) to the host tree which cause occlusion of xylem vessels and blue discoloration of sapwood (Figure 1.1). Although the interaction of these 'blue stain fungi' with their bark beetle vector is largely unknown, the association is believed to be mutualistic with the fungi assisting in weakening or killing trees (reviewed by Paine et al., 1997).

Figure 1.1: Cross section of Norway spruce sapwood infected by the blue stain fungus *C. polonica*. Photo: E. Christiansen.

Bark beetles usually occur in low densities and colonize trees recently felled by wind damage or kill stressed and dying trees. However, during certain environmental conditions, bark beetle populations increase rapidly in size (Raffa and Berryman, 1987). During such eruptive outbreaks, beetles can attack healthy trees and cause great damage to natural and plantation forests. During the last 50 years eruptive bark beetle outbreaks have increased in prevalence and severity (Raffa et al., 2008). The largest bark beetle outbreak recorded in history is presently occurring in Western Canada, where the mountain pine beetle (*Dendroctonus ponderosae*) has killed an estimated 500 million m^3 lodgepole pine (*Pinus contorta*) during the last 20 years (Kaervemo and Schroeder, 2010).

The increasing severity of bark beetle attacks on the Pinaceae correlates with warmer temperatures as well as with lower precipitation (Benz et al., 2010; Williams et al., 2010). Due to higher temperatures, bark beetles are expanding their range into niches in northern latitudes and higher elevations which were traditionally too cold for their survival (Cudmore et al., 2010). Higher temperatures and lower rainfall also contribute to shorter life cycles and increase the reproductive success of bark beetle populations (reviewed by Choi, 2011). The Pinaceae, on the other hand, are

poorly adapted to shifts in climate due to predetermined annual growth cycles, winter-chilling requirements (Aitken et al., 2008; Holliday et al., 2010) and sensitivity to drought stress (Breshears et al., 2009; Williams et al., 2010).

Boreal and temperate Pinaceae forests contain more than 30% of the earth's biomass (Global Forest Resources Assessment, FAO 2010). Therefore the health of these forests has a significant effect on the atmosphere. High tree mortality in northern forest ecosystems due to bark beetle attack could cause substantial increases in carbon dioxide levels (Pfeiffer et al., 2011), with consequent far reaching effects on global climate. Research on tree defense mechanisms against bark beetle attacks is thus important to produce Pinaceae accessions appropriate for sustainable biomass production during the 21st century.

1.2 Pinaceae can defend themselves against attack by bark beetles and their associated blue stain fungi

Bark beetles have co-evolved with their Pinaceae hosts since the late Mesozoic era (65 million years ago; Labandeira et al., 2001). It is therefore not surprising that these trees developed some structural and chemical strategies to defend against beetles and their fungal associates (reviewed by Franceschi et al., 2005). Structural defenses mainly include lignified cells with thickened secondary cell walls (Wainhouse et al., 1997) and cork tissue encrusted with calcium oxalate crystals (Hudgins et al., 2003), which may serve as a barrier against invasive insects. The Pinaceae also synthesize high amounts of natural products throughout their lifespan which are stored in specialized cellular structures in the bark and wood. During biotic or abiotic stress, this chemical defense system is increased through the *de novo* synthesis of inducible defense compounds (Franceschi et al., 2005).

The best known defense compound in the Pinaceae is oleoresin (reviewed by Zulak and Bohlmann, 2010). This viscous mixture of mono-, sesqui-, and diterpenoids is stored in specialized ducts lined with plastid-enriched epithelial cells. Terpenoid resins are synthesized by these cells and secreted into an extracellular lumen where their accumulation leads to increased pressure (Rosner and Hanrup, 2004). The biosynthesis of oleoresin is induced by the application of the defence-related phytohormone jasmonate and its volatile derivative methyl jasmonate (Franceschi et al., 2002), or by sub-lethal fungal inoculations. The *de novo* synthesized terpene mixture, which has been shown to increase in quantity and change in composition (Byun-McKay et al., 2006; Zhao et al., 2010), can protect trees against low density bark beetle attacks for at least one year (Christiansen et al., 1999; Krokene et al., 2003).

An equally important but less well-studied defense mechanism in the Pinaceae is the production and storage of phenolic compounds in specialized cells in the phloem parenchyma, the so-

called polyphenolic parenchyma cells or PP-cells (Franceschi et al., 1998). During a defense response, induced by wounding, methyl jasmonate or inoculation with the European blue stain fungus, *Ceratocystis polonica* (Table 1.1), PP-cells can expand and their inclusion bodies and develop a higher autofluorescent density (Figure 1.2; Hudgins et al., 2004; Nagy et al., 2004). Hence it has been thought that they contain phenolic compounds. Although it is likely that PP-cells form a protective barrier against pest species invading the tree's cambial zone (Christiansen et al., 1999; Krokene et al., 2003), very little is known about the biosynthesis and accumulation of polyphenols in these specialized cells.

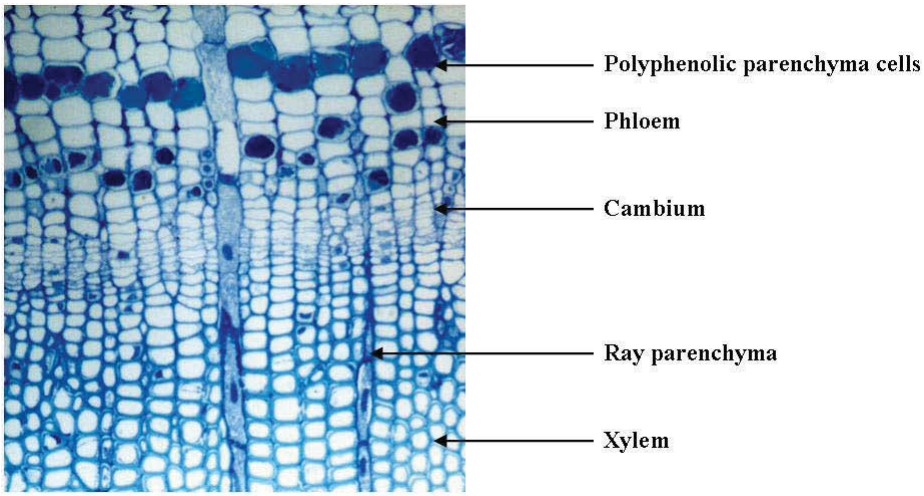


Figure 1.2: Typical cross section of the inner bark and sapwood region of the Pinaceae stained with Stevenel's blue. Cells staining dark blue are polyphenolic parenchyma cells (PP cells). Photo: V. Franceschi.

1.3 Phenylpropanoid function, structure and biosynthesis

Polyphenols or phenylpropanoids are abundant, structurally diverse natural compounds produced by almost all plant genera in response to environmental stimuli. The function of polyphenols includes structural support for upright growth on land, provided by polymeric phenylpropanoids such as lignin (Davin et al., 2008) and suberin (Franke and Schreiber, 2007). They are also responsible for protecting plants from harmful solar radiation and from reactive oxygen species produced during environmental stress (Bashandy et al., 2009). Phenylpropanoids also play important roles in plant interactions with other biota. They function as attractants for symbiotic microorganisms (Cesco et al., 2010), pollinators (Saito and Harbourne, 1992) and herbivores which disperse seeds. Compounds derived from the phenylpropanoid pathway (Figure 1.3) have also been

shown to negatively affect herbivore feeding behavior (Feeny, 1970) or hinder infection by phytopathogens (Franceschi et al., 2005).

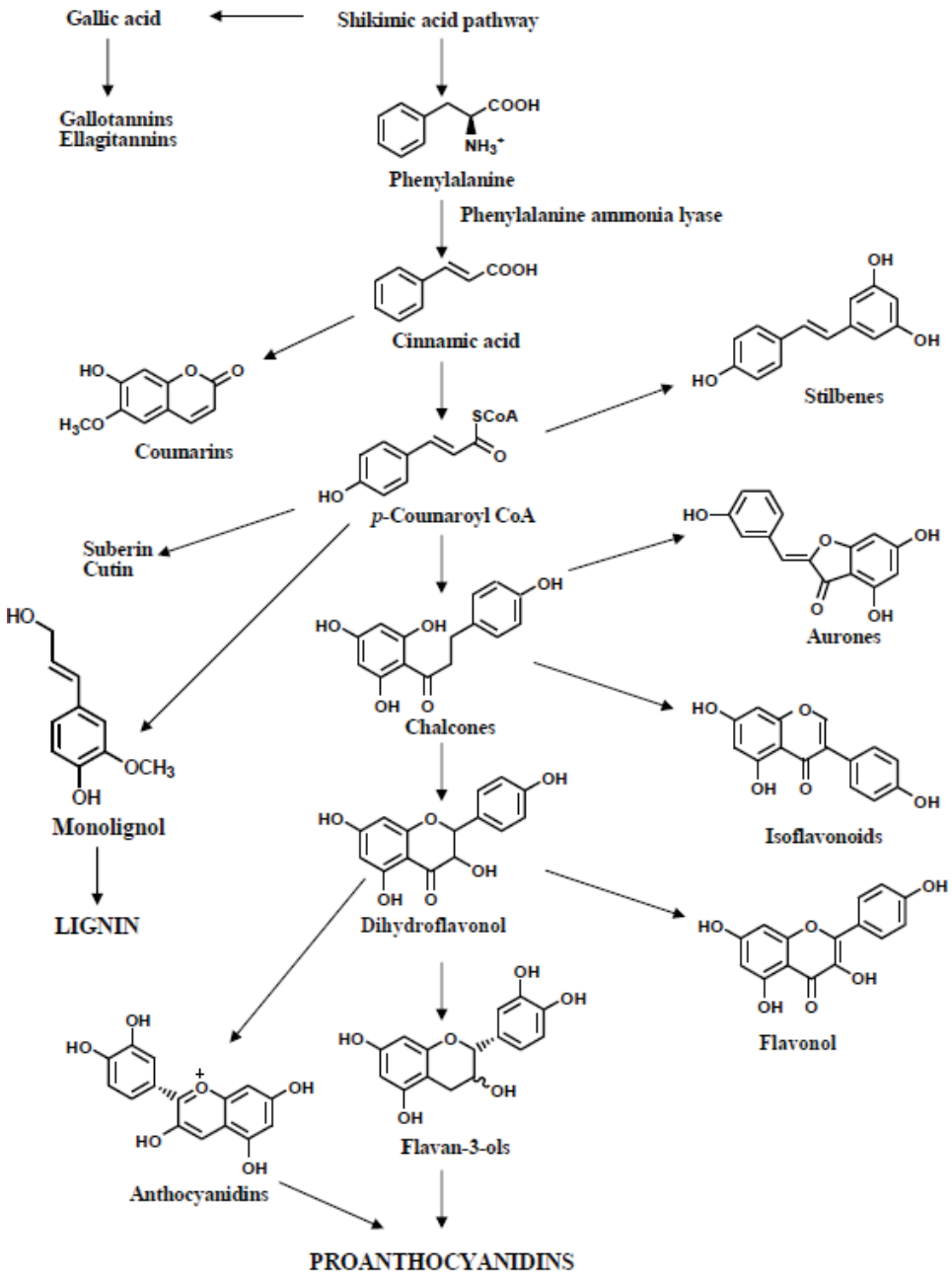


Figure 1.3: Diversity of phenylproanoids which are derived from phenylalanine. Phenylalanine is one of the products of the shikimic acid pathway.

Plant polyphenols are aromatic compounds derived from phenylalanine or tyrosine. These amino acids are synthesized by the shikimic acid pathway (Vogt, 2010). The committed step in polyphenol biosynthesis of most plant species is the deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) to form cinnamic acid (Figure 1.3). Cinnamic acid is then hydroxylated by a cytochrome P450 monooxygenase to form *p*-coumaric acid. Coenzyme A (CoA) activated *p*-coumaric acid in turn is the precursor for the two major downstream pathways in the plant phenylpropanoid pathway leading to monolignols and flavonoids.

Lignin, which is the principle metabolite for structural rigidity in plants, is derived from monolignol units which are phenylpropanoid alcohols. Monolignols decorated with various substitutions such as O-methylations or 3,5-hydroxylations are formed in an intersecting metabolic grid from the universal precursor *p*-coumaroyl CoA (Figure 1.3). Specific ratios of differently substituted monolignols are oxidatively coupled by laccase or peroxidase enzymes to form complex lignin polymers.

The other major downstream pathway in plant polyphenol biosynthesis leads to the production of polyketide-derived compounds. Chalcone synthase and stilbene synthase enzymes both catalyze the coupling of three acetate-derived malonyl-CoA molecules with *p*-coumaroyl CoA to form linear tetraketides. These enzymes then cyclize the tetraketide using different chemical mechanisms to form chalcones or stilbenoid phytoalexins (Figure 1.3).

Chalcones are the precursors of structurally diverse flavonoids, such as aurones responsible for yellow flower colour, isoflavonoids which are important phytoalexins in legumes and dihydroflavonols which are precursors for thousands of other flavonoid-like compounds (Figure 1.3). Dihydroflavonols can be converted to flavonols by an α-ketoglutarate dependent dioxygenase or can be reduced by a NADPH-dependent reductase to flavan-3,4-diols (leucoanthocyanidins). In subsequent steps, flavan-3,4-diols can be converted to anthocyanidins by α-ketoglutarate dependent dioxygenases or reduced by NADPH dependent reductases to form flavan-3-ols. These compounds are the precursors for the production of the anti-microbial and anti-oxidant proanthocyanidin polymers. Biosynthesis of chalcone-derived natural products occurs in a complex metabolic grid in which intermediates of the pathway are decorated by glycosylation, methylation, hydroxylation or acylation at specific control points unique to different plant species (Ferrer et al., 2008; Vogt, 2010).

1.4 Questions addressed in this thesis

Tree species of the Pinaceae contain autofluorescent rings of cells in the phloem parenchyma (PP-cells) which show an increase of fluorescent contents after fungal infection. Although it is believed that PP-cells synthesize polyphenolic compounds which are important in tree defense (Franceschi et al., 2005), very little is known about the biosynthesis, the structural diversity and biological roles of these compounds in the Pinaceae.

The objective of this research was to study phenylpropanoid biosynthesis in a representative species of the Pinaceae (*Picea abies*-Norway spruce) and to investigate the roles of these compounds in the interaction with a bark beetle-vectored blue stain fungus (*Ceratocystis polonica*). The first part of this thesis (Chapter 2) covers the biosynthesis of stilbene glycosides in *Picea* species. In this section it was shown that fungal inoculation increases the production of stilbene synthase-derived tetrahydroxystilbenes which originate from monohydroxylated *p*-coumaroyl CoA. The core theme of the second part of this thesis (Chapter 3) is the defense strategies of different isolates of *C. polonica* against stilbenes produced by *P. abies*. Feeding studies showed that *C. polonica* could detoxify tetrahydroxystilbene glycosides using an overlapping array of enzymatic reactions. Furthermore, it was revealed that virulent *C. polonica* had greater detoxification capabilities than avirulent *C. polonica*. In the last part of this work (Chapter 4) the biosynthesis and structural diversity of flavan-3-ols and proanthocyanidins was studied in *P. abies*. It could be shown that these defense compounds have a deleterious effect on fungal growth and undergo both quantitative as well as qualitative changes in spruce bark upon fungal infection.

2. Research Chapter 1

Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection

2.1 ABSTRACT

Stilbenes are dibenzyl polyphenolic compounds produced in several unrelated plant families that appear to protect against various biotic and abiotic stresses. Stilbene biosynthesis has been well described in economically important plants, such as grape, peanut and pine. However, very little is known about the biosynthesis and ecological role of stilbenes in spruce (*Picea*), an important gymnosperm tree genus in temperate and boreal forests. To investigate the biosynthesis of stilbenes in spruce, we identified two highly similar stilbene synthase (*STS*) genes in Norway spruce, *P. abies*, *PaSTS*1 and *PaSTS*2, which had orthologues with high sequence identity in Sitka (*P. sitchensis*) and white (*P. glauca*) spruce. Despite the conservation of STS sequences in these three spruce species, they differed substantially from angiosperm STSs. Inoculating spruce with fungal mycelium increased *STS* transcript abundance and tetrahydroxystilbene glycoside production, suggesting that one of the roles of stilbenes in spruce is anti-fungal defense. Several types of i*n vitro* and *in vivo* assays revealed that the *P. abies* STSs catalyze the condensation of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to yield the trihydroxystilbene resveratrol, but do not directly form the dominant spruce stilbenes, which are tetrahydoxylated. However, in transgenic Norway spruce over-expressing *PaSTS*1, significantly higher amounts of the tetrahydroxystilbene glycosides, astringin and isorhapontin, were produced. This result suggests that the first step of stilbene biosynthesis in spruce is the formation of resveratrol which is further modified by hydroxylation, O-methylation and O-glucosylation to yield astringin and isorhapontin.

2.2 INTRODUCTION

Stilbenes are plant secondary metabolites consisting of two phenol moieties linked by a C_2 bridge (Figure 2.1). These compounds are produced by species from a number of unrelated gymnosperm and angiosperm plant families including the Pinaceae, Cyperaceae, Vitaceae, Polygonaceae, Betulaceae, Fabaceae and Poaceae (Chong et al., 2009). Although the basic stilbene structure is widespread in plants, diverse species-specific substitution patterns exist. For example, pine (*Pinus*) species produce 3,5-dihydroxystilbene (pinosylvin, **5** in Figure 2.1) as a constituent of heartwood and roots (Chiron et al., 2000). Species from the Vitaceae, Fabaceae and Poaceae form mainly 3,5,4'-trihydroxystilbene (resveratrol, **6**) (Sparvoli et al., 1994; Shomura et al., 2005; Sobolev et al., 2007). Spruce (*Picea*) species synthesize the more complex 3,5,3',4'-tetrahydroxystilbene 3-glucopyranoside (astringin **10**) and 3,5,4'-trihydroxy-3'-methoxystilbene 3-glucopyranoside (isorhapontin **11**) (Toscano-Underwood and Pearce, 1991; Lieutier et al., 2003).

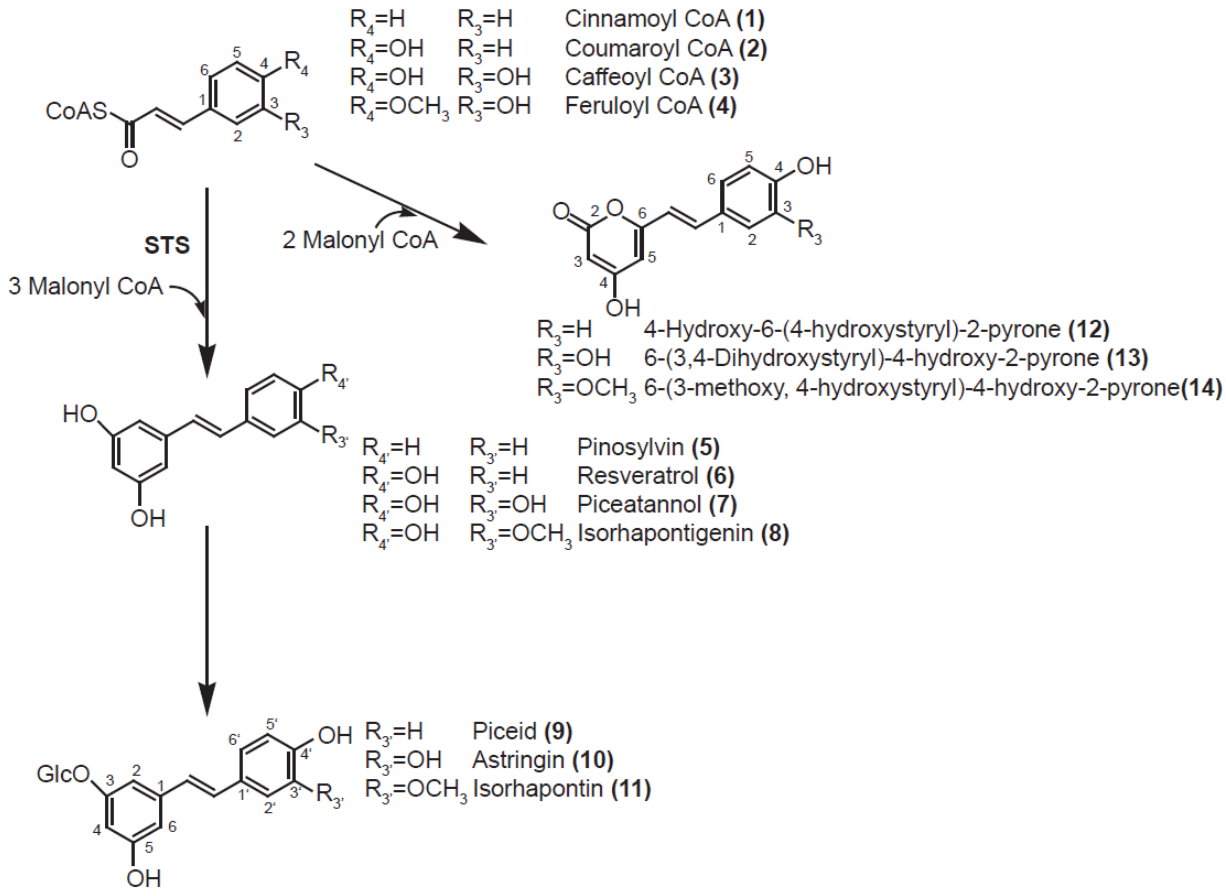


Figure 2.1: Potential intermediates, products and by-products of stilbene biosynthesis in spruce.

Although much has been learned about stilbene biosynthesis in recent years (Chong et al., 2009; Jeandet et al., 2010) the formation of tetrahydroxy-substituted stilbenes (such as **7**, **8**, **10** and **11** in Figure 2.1) is still unresolved. The stilbene skeleton is synthesized via condensation of three acetate units from malonyl-coenzyme A (malonyl-CoA) to a CoA-activated phenolic acid by an enzyme known as stilbene synthase (STS), which results in formation of a linear tetraketide intermediate. Ring closure to form the stilbene product is achieved via an intramolecular aldol condensation coupled to the loss of CO_2 (Austin et al., 2004). Stilbene synthases belong to the large polyketide synthase enzyme family whose best known representative in plants is chalcone synthase, catalyst of the first step in flavonoid biosynthesis. For the synthesis of simple di **(5)**- and trihydroxystilbenes **(6)** the CoA-activated phenolic acid which is incorporated into the tetraketide determines the substitution pattern of the stilbene formed (Austin and Noel, 2003). However, it is not known if complex tetrahydroxystilbenes, such as those produced by spruce, originate from the incorporation of more highly substituted CoA activated phenolic acids into the initial tetraketide or from initial formation of the basic stilbene ring system and subsequent oxidation. Greater knowledge of stilbene biosynthesis is of interest in plant biochemistry, but also in biomedical research. Stilbenes have been studied for many years for their roles in the prevention and cure of cancer (Athar et al., 2009) and heart disease (Pace-Asciak et al., 1995), and in extending life span. If the availability of these substances in human diets or as dietary supplements could be increased by molecular engineering of plants or microorganisms, there could be substantial medical benefits.

Within the plant, stilbenes appear to function as protectants against various biotic and abiotic stresses. For example their roles as constitutive or inducible anti-fungal defenses have been demonstrated in plants such as grape (Melchior and Kindl, 1990; Melchior and Kindl, 1991; Wiese et al., 1994), sorghum (Yu et al., 2005) and peanut (Sobolev, 2008). Stilbenes have also been shown to exhibit anti-fungal activity in non-stilbene producing species such as poplar (Seppaenen et al., 2004), wheat (Serazetdinova et al., 2005) and alfalfa (Hipskind and Paiva, 2000), when they were introduced by genetic engineering. These compounds inhibit fungal growth by interfering with microtubule assembly (Woods et al., 1995), disrupting plasma membranes and uncoupling electron transport in fungal spores and germ tubes (Pezet and Pont, 1990). Stilbenes have also been shown to protect plants against oxidative stress (He et al., 2008, Adrian et al., 1996, Rosemann et al., 1991), to deter herbivores (Torres et al., 2003) and to inhibit the growth of competing plants (Fiorentino et al., 2008). However, their functions in conifers are poorly studied despite their abundance in widespread genera, such as spruce and pine. In spruce, it has been suggested that phenolic compounds such as stilbenes, may play a pivotal role in defense against herbivores and pathogens due to the appearance of fluorescent inclusion bodies in the phloem parenchyma cells of fungal-treated bark (Franceschi et al.,

2005). The objective of this study was to learn more about the pathway of tetrahydroxystilbene formation in spruce as well as their role in tree defense. We isolated and sequenced two genes encoding stilbene synthase enzymes (STS1 and STS2) from *P. abies*, *P. glauca* and *P. sitchensis*. *In vitro* enzyme assays revealed that these STSs synthesize the trihydroxystilbene resveratrol **(6)**, which is not commonly observed in high concentrations in spruce tissue. But, by overexpressing *PaSTS*1 in *P. abies*, we could demonstrate that resveratrol is an intermediate in tetrahydroxystilbene glycoside biosynthesis in spruce. By measuring transcript accumulation and stilbene content after fungal inoculation of bark tissue, we could also show that STS enzymes in spruce are involved in fungus-induced defense responses.

2.3 RESULTS

2.3.1 Identification of spruce stilbene synthase genes and their phylogenetic relationships

In order to study the stilbene biosynthetic pathway in spruce (*Picea*), stilbene synthase (*STS*) gene candidates plus putative sequences for the very similar chalcone synthase (*CHS*) genes were identified using BLAST searches of spruce transcriptome resources with known sequences of the polyketide synthase (*PKS*) family. Search of more than 180,000 expressed sequence tags (ESTs) from *P. sitchensis* and 250,000 from *P. glauca* in the Treenomix database (Ralph et al., 2008) revealed two distinct contigs from each spruce species with high similarity to *STS* from *Pinus densiflora* (Kodan et al., 2002) (Figure S2.1). Additionally, more than eight distinct contigs were discovered for each spruce species which had high similarity to *CHS* from *Pinus sylvestris* (Fliegmann et al., 1992).

By using EST sequences from *Picea glauca* and *Picea sitchensis* as templates for primer design, two full-length *STS* candidates and eight *CHS* candidates were amplified from *Picea abies* cDNA by PCR. All of these sequences showed features similar to those of known plant *PKS* genes, including a malonyl-CoA binding site, conserved amino acid residues in the dimer interface and a conserved product binding site (Tropf et al., 1995; Austin and Noel, 2003). Predictive algorithms suggested that N-terminal signal sequences were absent.

Comparisons of these STS and CHS sequences among spruce species demonstrated high conservation within the genus. Deduced amino acid sequences for STS1 and STS2 were 99% identical within species and 98% identical between species (Figure S2.1). Outside of the genus, spruce STSs showed between 81-84% sequence identities to previously characterized pine STS encoding pinosylvin synthases (PSSs) (Raiber et al., 1995; Kodan et al., 2002).

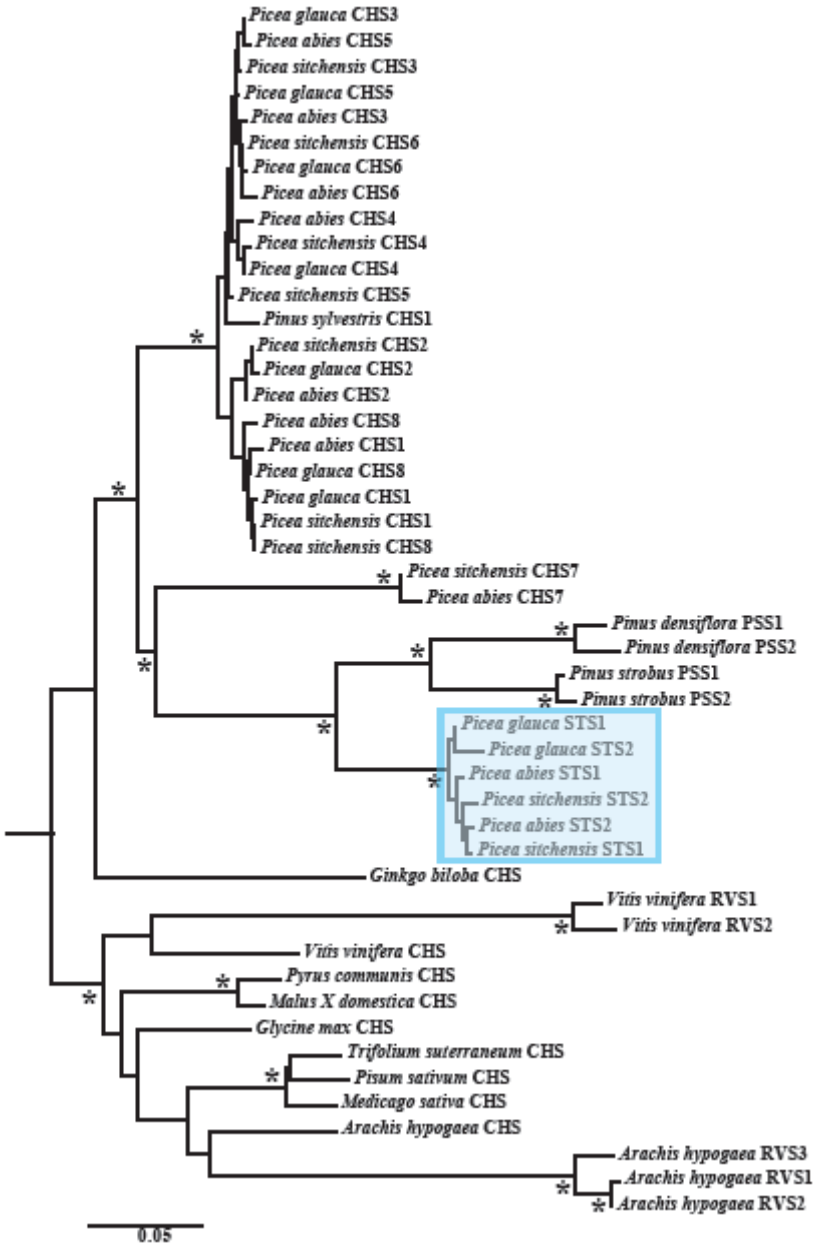


Figure 2.2: Phylogenetic relationships of stilbene synthase (STS) amino acid sequences. Neighbor joining tree of the *Pa*STS and representative STS and chalcone synthase (CHS) sequences from angiosperm and gymnosperm species. Branches supported with >95% confidence are indicated by*. Abbreviations for some stilbene synthases whose products have been previously characterized: RVS, resveratrol synthase; PSS, pinosylvin synthase. Genbank accession numbers are in supplementary table S2.2.

Amino acid sequences for CHS were 96-99% identical within and 95-100% identical between spruce species, with the exception of CHS7, which showed only 81-84% sequence identity to other

CHS within the genus. Outside the genus, spruce CHS showed 95-97% sequence identity to biochemically characterized CHS from *Pinus sylvestris* (Fliegmann et al., 1992).

Phylogenetic analysis revealed a clear evolutionary divergence between PKS from angiosperms and gymnosperms (Figure 2.2). In addition, the application of neighbor-joining algorithms resulted in separate clades for CHS and STS in gymnosperms ($p = 0.02$). Within the gymnosperm STS clade, enzymes from spruce grouped with high bootstrap support into a separate sub-clade from *Pinus* STS enzymes ($p < 0.001$), but without resolution of orthologs. Angiosperm PKS separated into clades according to the taxonomic affinities of their species of origin.

2.3.2 *In vitro* characterization of recombinant spruce stilbene synthases expressed in bacteria

Functional characterization of the two putative STS from *P. abies* (*Pa*STS1 and *Pa*STS2) was accomplished via heterologous expression in a bacterial system. SDS-PAGE as well as Western blotting revealed low level expression of these 45 kDa proteins which form homodimeric enzymes (data not shown). Catalytic activity was determined by incubation with malonyl-CoA and a phenylpropanoid-CoA ester (Figure 2.3). Reaction of the two *Pa*STS with *p*-coumaroyl-CoA **(2)** yielded the stilbenes (*E*)- and (*Z*)-resveratrol **(6)**, but neither enzyme converted cinnamoyl-CoA **(1)**, caffeoyl-CoA **(3)** and feruloyl-CoA **(4)** to stilbene products (Table 2.1).

Table 2.1: Products formed by *Pa*STS1 and *Pa*STS2 from various substrates under *in vitro* and *in vivo* conditions. Structures of numbered compounds are given in Figure 2.1. Products considered to be artifacts are listed in parentheses.

	Products		
Substrate	*In vitro* assay with phenylpropanoid-CoA esters (protein expressed in *E. coli*)	*In vivo* formation from phenylpropanoid acids (protein expressed in *E. coli* with 4CL)	*In vitro* assay with phenylpropanoid-CoA esters (protein expressed in *P. abies*)
1	none	none	none
2	**6**, (**12**)	**6**	**6**, (**12**)
3	(**13**)	(**13**)	(**13**)
4	(**14**)	not tested	(**14**)

Instead, caffeoyl-CoA and feruloyl-CoA afforded (*E*)- and (*Z*)-6-(3, 4-dihydroxystyryl)-4-hydroxy-2-pyrone **(13)** or 6-(3 methoxy, 4-hydroxystyryl)-4-hydroxy-2-pyrone **(14)**, respectively (Table 2.1). Incubation with *p*-coumaroyl-CoA yielded the corresponding (*E*)- and (*Z*)-4-hydroxy-6-(4-hydroxystyryl)-2-pyrone **(12)**. These 'derailment products' arise from two consecutive condensation reactions with malonyl-CoA and spontaneous cyclization after release from the active site (Austin and Noel, 2003). To form stilbenes, STS enzymes normally catalyze three consecutive

condensations of the phenylpropanoid-CoA ester with malonyl-CoA followed by ring closure and CO_2 loss. The formation of derailment products in variable amounts prevented the measurement of relevant kinetic parameters.

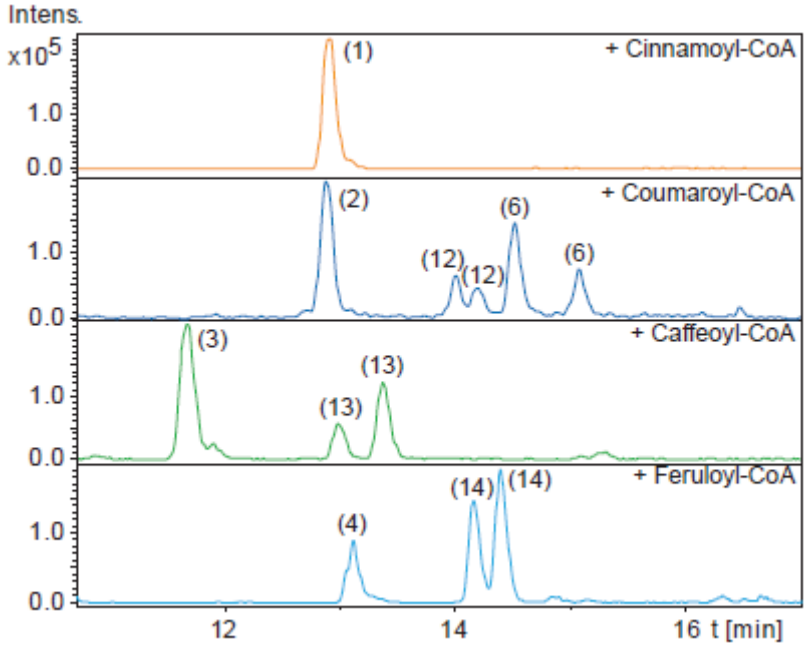


Figure 2.3: *In vitro* assay of *E. coli*-expressed STS. Representative liquid chromatography-mass spectrometry with electrospray ionization (LC-ESI-MS) traces for reaction products of *Pa*STS supplied with malonyl-CoA and a phenylpropanoid-CoA as substrates. With the cinnamoyl-CoA **(1)** no products were detected (upper panel). The substrate *p*-coumaroyl-CoA **(2)** yielded the derailment products (*E*)- and (*Z*)-6-(4-hydroxystyryl)-4-hydroxy-2-pyrone **(12)**, as well as (*E*)- and (*Z*)-resveratrol **(6)** (second panel). Caffeoyl-CoA **(3)** yielded (*E*)- and (*Z*)-6-(3,4-Dihydroxystyryl)-4-hydroxy-2-pyrone **(13)** (third panel). Feruloyl-CoA **(4)** yielded (*E*)- and (*Z*)-6-(3 methoxy, 4-hydroxystyryl)-4-hydroxy-2-pyrone **(14)** (bottom panel). Compounds were identified by mass spectrometry and comparisons with standards.

2.3.3 *In vivo* characterization of spruce stilbene synthases by co-expression with 4-coumaroyl-CoA ligase in *E. coli*

Enzyme activities of *Pa*STS1 and *Pa*STS2 were assayed *in vivo* in *E. coli* engineered to produce potential substrates. This was achieved by addition of phenolic acid starter units to the medium of *E. coli* co-expressing *PaSTS* along with 4-coumaroyl-CoA ligase from *Arachis hypogaea* (Watts et al., 2006) which produced CoA-esters in the bacterium. Addition of *p*-coumaric acid to the growth medium of such cultures resulted in production of resveratrol **(6)** which was released back into the medium without the formation of detectable derailment products. However, addition of caffeic acid yielded the derailment products (*E*)- and (*Z*)-6-(3, 4-dihydroxystyryl)-4-hydroxy-2-

pyrone **(13)**, but no stilbenes (Table 2.1). Ferulic acid could not be tested as substrate in this system due to the substrate specificity of the 4-coumaroyl-CoA ligase.

2.3.4 *In vivo* characterization of spruce stilbene synthase function by over-expression of *PaSTS*1 in *P. abies*

To determine the enzymatic activity of spruce STS *in planta*, embryogenic tissue from *P. abies* was transformed with *PaSTS*1 under the control of the inducible promoter *ubi*1 using a disarmed *Agrobacterium tumefaciens* strain. Two transgenic *PaSTS1* callus lines (line 5 and line 11) and one empty-vector control line were obtained (Figure 2.4 A), from which somatic embryos were produced by abscisic acid application under low light conditions (Figure 2.4 B).

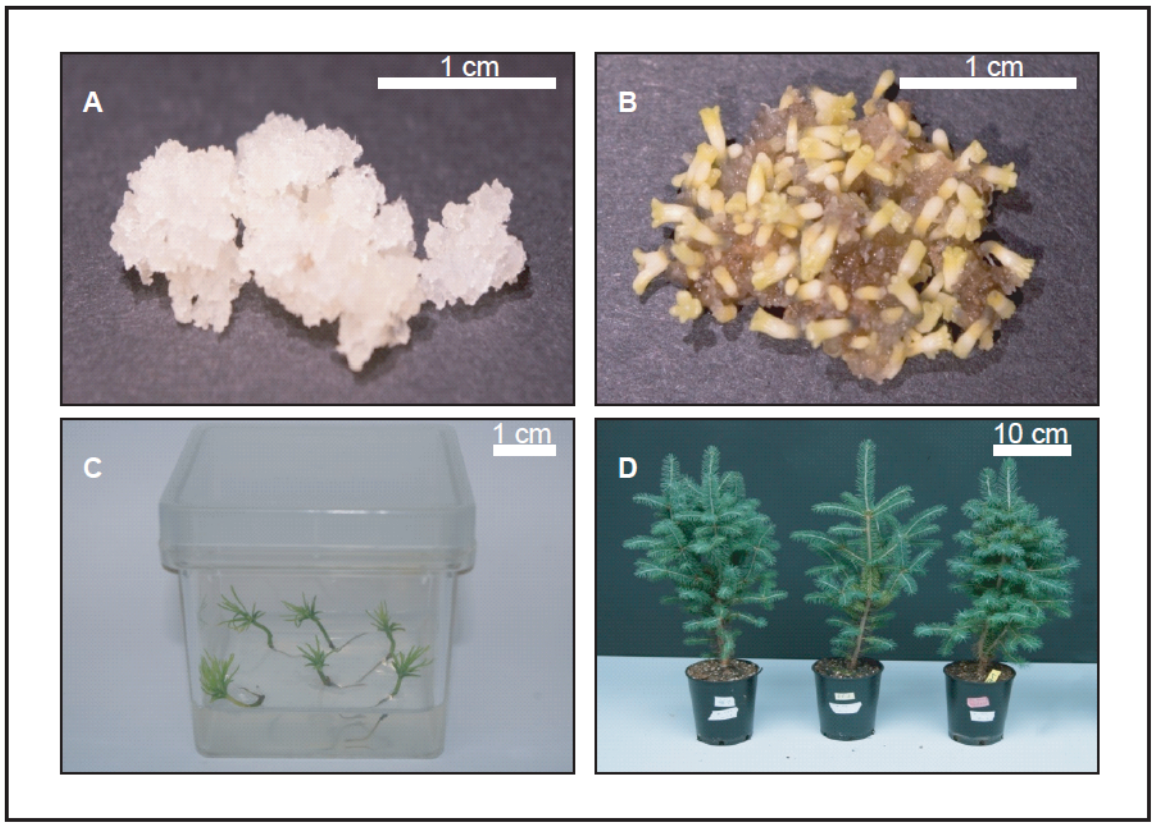


Figure 2.4: Transgenic *P. abies* lines were developed to overexpress STS. Depicted are callus **(A)**, somatic embryos **(B)**, seedlings **(C)** and young saplings **(D)**.

Shoots and roots were regenerated under moderate light conditions in the absence of plant growth regulators (Figure 2.4 C). One-year-old transformed saplings grown in potting soil were used for transcript and metabolite analysis (Figure 2.4 D).

Relative transcript accumulation of *PaSTS* (*PaSTS*1 and *PaSTS*2 were measured together by quantitative real-time PCR) was significantly higher in both *STS*1 over-expression lines than in the vector control line ($p < 0.001$). *PaSTS* transcript abundance in bark tissue was on average 30-fold higher in line 5 and 6-fold higher in line 11 than in the vector control line (Figure 2.5 A). Relative *PaSTS* transcript abundance was also measured in needles, stem and roots of line 5 and the vector control line. *PaSTS* transcript abundance was significantly higher in stems ($p < 0.01$) than in roots in both lines. Needles contained transcript levels intermediate between stems and roots (Figure 2.6 A).

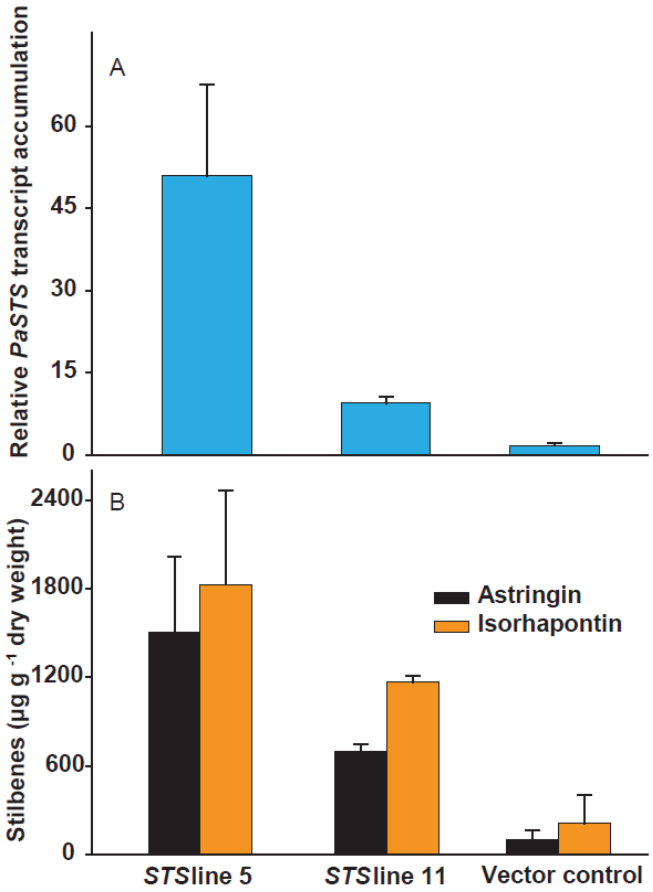


Figure 2.5: Transformation with *PaSTS* increased stilbene biosynthesis: relative transcript accumulation of *PaSTS*1 and *PaSTS*2 **(A)** and mean accumulation of the two major stilbenes, astringin and isorhapontin **(B)**, in two lines of *P. abies* transformed with *PaSTS*1 and a vector control line. Depicted are means ± SE of at least 4 replicates.

Stilbene glycoside concentrations in both *PaSTS*1 lines were significantly higher than in the vector control line ($p < 0.01$), providing further evidence that the *PaSTS*1 gene encodes a functional stilbene synthase (Figure 2.5 B). Concentrations of astringin **(10)** (3,4,3',5'-tetrahydroxystilbene 3-glucopyranoside) and isorhapontin **(11)** (3,4',5-trihydroxy-3'-methoxystilbene 3-glucopyranoside) in

bark tissue were more than nine-fold higher in line 5 and more than five-fold higher in line 11 than in the vector control. Stem and root tissue from *PaSTS*1 line 5 and the vector control contained significantly higher concentrations of stilbene glycosides (Figure 2.6 B) than the needle tissue ($p < 0.0001$). Other stilbenes, including resveratrol **(6)**, piceid (resveratrol 3- glucopyranoside **9**) as well as the aglycones of astringin and isorhapontin (piceatannol **(7)** and isorhapontigenin **(8)**) could be detected in bark and root tissue of the *PaSTS*1 lines and the vector control line using liquid chromatography-mass spectrometry (LC-MS), but could not be accurately quantified due to their low concentrations (Table 2.1).

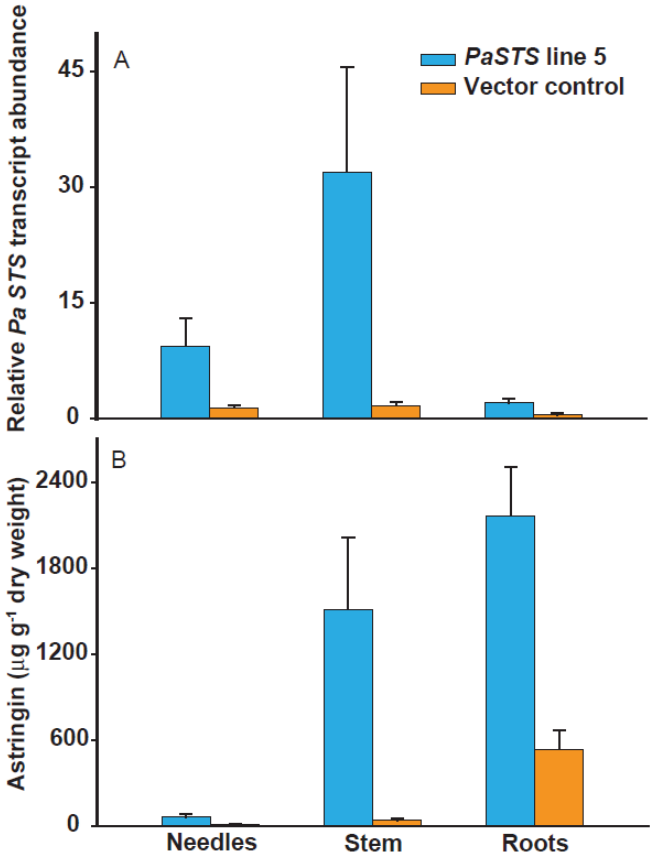


Figure 2.6: Organ-specific effects of *PaSTS* transformation: relative transcript accumulation of *PaSTS*1 and *PaSTS*2 **(A)** and accumulation of the stilbene astringin **(B)** in various organs of *PaSTS* transgenic line 5 relative to the vector control line. Depicted are means ± SE of at least 4 replicates.

To detect STS activities in transgenic spruce bark, crude extracts were enriched through reactive-red affinity chromatography. As in the previously described *in vitro* assays with *E. coli* expressing *Pa*STS1 and *Pa*STS2, the partially purified STS fraction from *P. abies* overexpressing *PaSTS*1 produced resveratrol **(6)** with the substrate *p*-coumaroyl-CoA **(2)**, but only 'derailment products' with the substrates caffeoyl-CoA **(3)** and feruloyl-CoA **(4)**.

2.3.5 Effect of transformation with *PaSTS*1 on other pathways of phenolic metabolism in spruce

To investigate whether *PaSTS*1 over-expression influenced the biosynthesis of other *P. abies* phenolics, we measured the accumulation of flavan-3-ols, the other major group of phenolics in this species besides stilbenes, and quantified gene transcripts encoding early and late biosynthetic enzymes in flavan-3-ol formation.

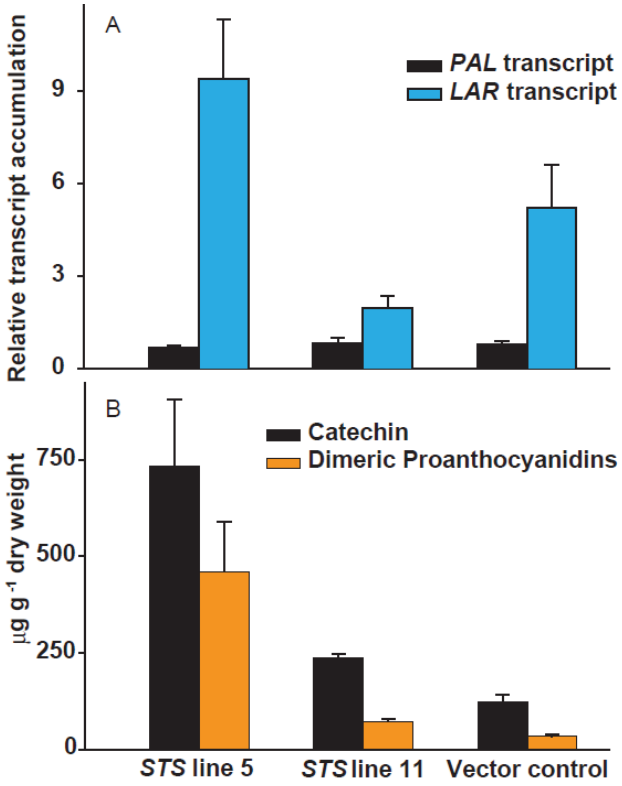


Figure 2.7: Effect of *PaSTS* transformation: relative transcript accumulation of *P. abies* genes encoding biochemically uncharacterized phenylalanine ammonia-lyase (*PAL*) and leucoanthocyanidin reductase (*LAR*) genes **(A)** and mean catechin and proanthocyanidin dimer accumulation **(B)** in a vector control line and two lines of *P. abies* transformed with *PaSTS*1. Depicted are means ± SE of at least 4 replicates.

We measured transcript abundance of a *P. abies* gene encoding phenylalanine ammonia lyase (PAL), the first step in the phenylpropanoid pathway (Noel et al., 2005), but transcript levels did not differ between *PaSTS*1 over-expressing lines and the vector control line (Figure 2.7 A). However, for leucoanthocyanidin reductase (LAR), which catalyzes the last step in flavan-3-ol biosynthesis (Tanner et al., 2003), relative transcript abundance of one of three putative *PaLAR* genes was significantly higher in the vector control line and in line 5 ($p < 0.0001$) than in line 11. No significant differences were observed for transcript levels of the other two *PaLAR* genes between the two transgenic *PaSTS*1 over-expressing lines and the vector control. Quantification of the products of

LAR enzymes, catechin and proanthocyanidin dimers, revealed that concentrations of these metabolites were significantly higher in *PaSTS*1 line 5 ($p < 0.001$) than in line 11 and the vector control (Figure 2.7 B).

2.3.6 Effect of fungal inoculation on stilbene synthase transcription and stilbene glycoside biosynthesis in *P. glauca* saplings

To determine if fungal inoculation leads to the activation of stilbene biosynthesis in spruce, two-year-old *P. glauca* (white spruce) saplings were wounded and inoculated with an avirulent strain of the bark beetle (*Ips typographus*)-associated fungus *Ceratocystis polonica*. Controls included unwounded saplings and those subjected to wounding without fungal inoculation. Given the ability of *C. polonica* to metabolize the stilbenes of its host, *P. abies* (unpublished data), the non-host *P. glauca* was used in this experiment to test if fungal inoculation leads to elevated stilbene levels. *P. glauca* is not a natural host of *C. polonica* due to non-overlapping geographic distribution.

Bark tissue was harvested for analysis 5, 15 or 25 days after treatment. While no lesions or typical 'reaction zones' (Krokene et al., 2001) could be observed in the inner bark of the inoculated saplings 25 days after the onset of the experiment, the level of *STS* transcript in inoculated *P. glauca* bark increased significantly between days 5 and 15 ($p < 0.001$). Compared to the non-wounded control, transcript levels in inoculated saplings were on average 3-fold higher 5 days post-inoculation and 8-fold higher 15 days post-inoculation. At 25 days post-inoculation, transcript levels were only 1.5-fold higher than in the non-wounded control. Saplings that were wounded without inoculation exhibited similar, although less pronounced changes in STS transcript accumulation. Compared to the non-wounded control, relative transcript levels only reached a maximum 3-fold increase 15 days post-wounding (Figure 2.8 B).

Fungal inoculation resulted in a significant increase in stilbene concentration in bark ($p < 0.001$) 15 to 25 days post-inoculation. Compared to non-wounded controls, total stilbene levels were 3-fold higher in inoculated bark (Figure 2.8 A). In wounded but not fungal-inoculated saplings, only a small, statistically insignificant, increase in total stilbene content could be detected. The increase in stilbenes was principally due to increases in the glycoside astringin, not in isorhapontin, which remained nearly constant over the time course of the experiment (data not shown).

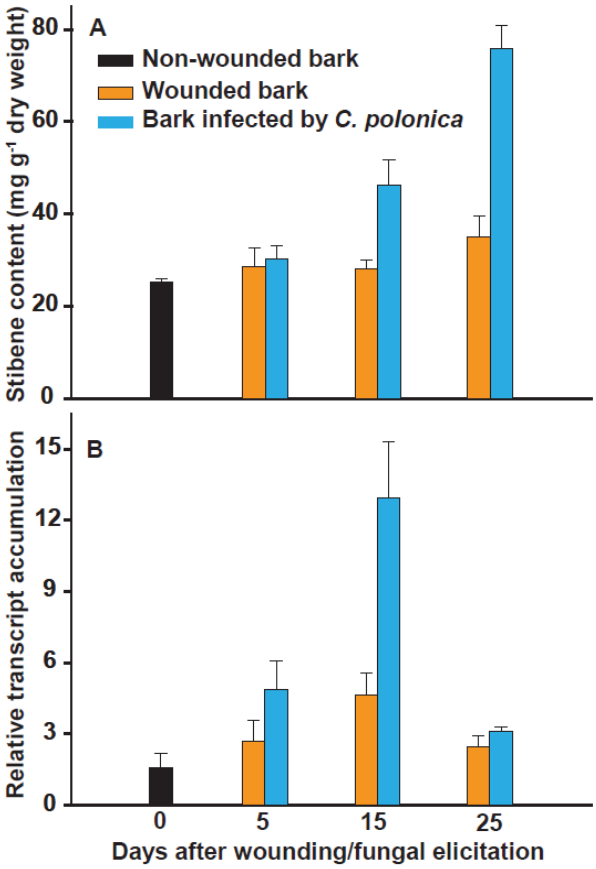


Figure 2.8: Fungal induction of stilbene biosynthesis: total stilbene accumulation **(A)** and *PgSTS*1 and *PgSTS*2 transcript accumulation **(B)** in non-wounded, wounded and *C. polonica*-inoculated bark tissue of *P. glauca* saplings. Depicted are means ± SE of at least 4 replicates.

2.4 DISCUSSION

2.4.1 Coding regions of stilbene synthases in the genus *Picea* are highly conserved

To learn more about the biosynthesis of stilbenes in the genus *Picea*, we investigated the genes encoding stilbene synthases (*STS*) which form the basic stilbene skeleton by condensing three molecules of malonyl-CoA with one phenylpropanoid-CoA molecule. Two genes were identified, *STS*1 and *STS*2, that had high sequence similarity within the three species studied, *P. abies*, *P. glauca* and *P. sitchensis*.

Fossil records and phylogenetic analysis reveal that the genus *Picea* originated in western North America in the Paleocene era about 62 million years ago (LePage, 2003), with *P. sitchensis* as the ancestral species (Ran et al., 2006). Spruce appears to have radiated from North America

westward to Asia and Europe with *P. abies* originating from a recent speciation event in the Pliocene era about 5 million years ago (LePage, 2001; Ran et al., 2006). At the amino acid level, STS sequences from *P. glauca*, *P. sitchensis* and *P. abies* were highly conserved with 99% similarity within taxa and 98% sequence similarity between taxa (Fig. S2.1). The high similarity of STS from the ancestral *P. sitchensis* to that of the more recently evolved *P. abies* and *P. glauca* indicates that genes for stilbene biosynthesis originated prior to the diversification of *Picea* and that these genes most likely fulfill the same function in all three spruce species studied.

The genes most closely related to *STS* in plants are the chalcone synthases (*CHS*), also members of the polyketide synthase gene family. CHS enzymes employ the same substrates as STS, but produce the C_{15} flavonoid skeleton instead of the C_{14} stilbene skeleton. Phylogenetic analysis of the known gymnosperm STS (from the genera *Picea* and *Pinus*) together with CHS revealed that gymnosperm enzymes form separate clades from angiosperm enzymes with the same function. This separation implies independent origins of STS after divergence of the angiosperm and gymnosperm lineages. In accordance with the phylogeny of the plant polyketide synthase family as reconstructed by Tropf et al. (1994), our analysis shows that polyketide synthases in general separate into clades that are based less on catalytic function and more on the taxonomic affinities of the species from which the genes were isolated. Our results provide further evidence that STS likely evolved from CHS on multiple occasions (Tropf et al., 1995) within stilbene-producing lineages, rather than originating from a single common ancestor.

The presence of multiple STS genes in individual species of the genera pine and spruce suggests several levels of control over stilbene formation. For example in *Pinus sylvestris* four *STS* genes which are almost identical have been described which are under the control of distinct promoters that activate transcription in response to different environmental cues and at different time intervals after a single stimulus (Preisig-Mueller et al., 1999). This level of complexity is further enhanced by the existence of multiple copies of the same *STS* gene in the genome with multiple 3'-untranslated regions. For example, *Pinus densiflora* contains three conserved *STS* genes with 12 different 3'-untranslated regions (Kodan et al., 2002). Attempts to determine gene copy numbers in *P. abies* using quantitative real-time PCR suggested the presence of numerous copies of both *STS*1 and *STS*2 genes in the spruce genome (Fig. S2.2). It is therefore conceivable that, similar to pine, spruce may also possess multiple *STS* genes that modulate stilbene biosynthesis differently in response to a range of internal and external factors.

2.4.2 Stilbene biosynthesis in spruce is a defense mechanism against fungal pathogens

One external factor that is known to influence stilbene biosynthesis is fungal challenge. In previous work, STS activity was found to increase substantially in the conifer *P. sylvestris*, when challenged by the fungal pathogens, *Botrytis cinerea* (Gehlert et al., 1990), *Leptographium wingfieldii* (Chiron et al., 2000) or *Lophodermium seditiosum* (Lange et al., 1994). Stilbene biosynthesis is also known to be induced in angiosperms by fungal pathogens (see Jeandet et al., 2010 for review). In the present study, inoculation of white spruce *P. glauca* saplings with an avirulent *C. polonica* isolate led to elevated transcript levels of *STS* followed by significant increases in tetrahydroxystilbene glycoside concentrations in the bark. Curiously induction of stilbene biosynthesis in spruce after fungal infection could not be clearly demonstrated in earlier studies (Brignolas et al., 1995; Virii et al., 2001). This may be due to variation in compatibility among the particular combinations of pathogens and hosts studied, a common occurrence in many plant-pathogen interactions (Vanetten et al., 1989). Nevertheless, our results provide evidence that stilbenes play a similar defensive role against pathogens in spruce as in pine and angiosperms.

2.4.3 *Picea abies* STS form a trihydroxylated product, resveratrol, which is an intermediate in the biosynthesis of the tetrahydroxy stilbenes, astringin and isorhapontin

The most abundant stilbenes in spruce are the 3,5,3',4'-tetrahydroxystilbene glycosides, astringin (**10**) and isorhapontin (**11**). STS condense phenylpropanoid-CoA esters with three molecules of malonyl-CoA to form the stilbene ring system (Austin and Noel, 2003). Several possible biosynthetic pathways can be envisioned that differ according to the substrate specificity of the STS reaction and the timing of oxidation (Figure 2.9): (I) The first possibility involves STS catalyzed formation of the 3,5-dihydroxystilbene, pinosylvin, from cinnamoyl-CoA. This 3,5-dihydroxystilbene product could then be further modified by 3'- and 4'-hydroxylation and glucosylation to give astringin (**10**), while additional 3'-O-methylation is required to yield isorhapontion (**11**). (II) Another possibility involves STS formation of a 3,5,4'-trihydroxystilbene, resveratrol (**6**), from *p*-coumaroyl-CoA, which could be further modified by 3'-hydroxylation and glucosylation (and 3'-O-methylation) to give astringin (and isorhapontin). (III) A third possibility is STS formation of a 3,5,3',4'-tetrahydroxystilbene, piceatannol (**7**), from caffeoyl-CoA (Chong et al., 2009), which could be further transformed by glucosylation (and 3'-O-methylation). Feruloyl-CoA could also serve as a STS substrate to give a product that does not require 3'-O-methylation.

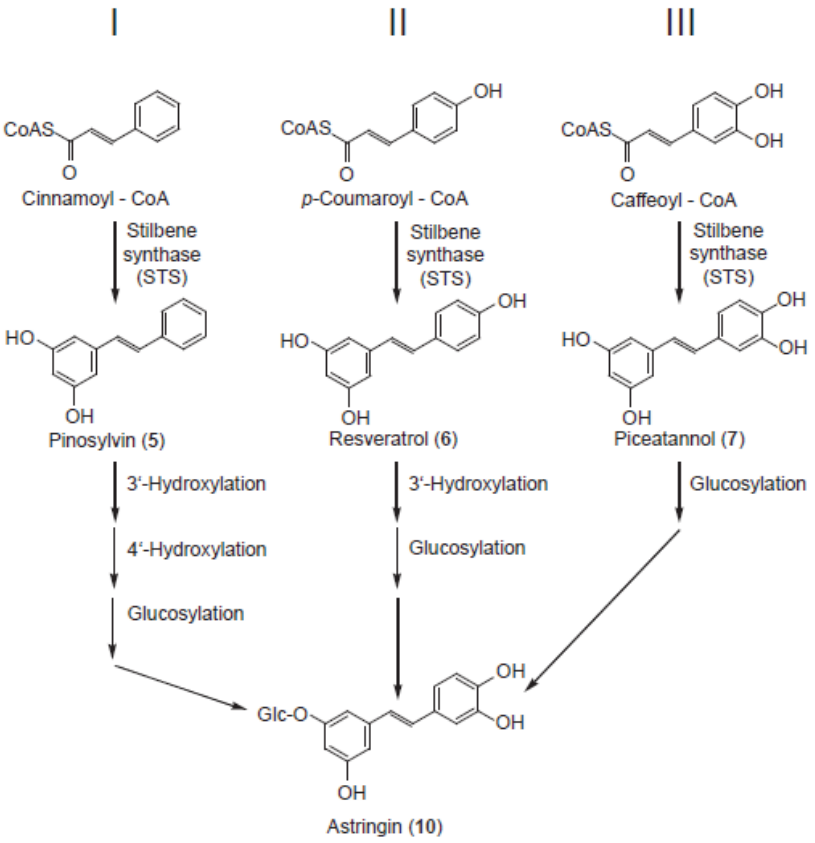


Figure 2.9: Biosynthetic pathway for astringin formation in *P. abies* depends on the substrate specificity of STS. Both STS1 and STS2 were found to utilize only *p*-coumaroyl-CoA as substrate, indicating the most likely pathway is **II**.

Of the phenylpropanoid-CoA substrates offered, the recombinant spruce STSs, *Pa*STS1 and *Pa*STS2, accepted only *p*-coumaroyl-CoA, producing the 3,5,4'-trihydroxy product, resveratrol. Neither cinnamoyl-CoA, caffeoyl-CoA, nor feruloyl-CoA were converted to stilbene products. However, both caffeoyl-CoA and feruloyl-CoA, but not cinnamoyl-CoA, were converted to styrylpyrones. These compounds are considered to arise from the condensation of the phenylpropanoid-CoA ester with two malonyl-CoA units followed by premature release from the active site and cyclization before the third condensation can occur. Formation of such 'derailment products' indicates that the STS cannot convert the respective phenylpropanoid-CoA substrate to a stilbene, probably due to limitations in the size of the active site cavity (Jez et al., 2002). Styrylpyrones can also arise from STSs due to sub-optimal *in vitro* conditions that lead to distortions in the active site cavity (Jez et al., 2002). In our work, styrylpyrone products were also formed in assays with *p*-coumaroyl-CoA, along with stilbenes. However, the substrates caffeoyl-CoA and feruloyl-CoA gave only styrylpyrone products and no stilbenes, and thus are unlikely to be native

substrates of spruce STSs. Styrylpyrones have never been observed *in planta*. Moreover, the *Pa*STS enzymes in genetically engineered *E. coli* expressing a 4-coumaroyl-CoA ligase assayed *in vivo* and the PaSTS enzymes in the bark of *P. abies* over-expressing *Pa*STS1 assayed *in vitro* both converted *p*-coumaroyl-CoA only to the stilbene resveratrol without any styrylpyrone formation, while caffeoyl-CoA and feruloyl-CoA were only converted to styrylpyrones.

In a previous characterization of spruce STS, it was demonstrated that resveratrol as well as small amounts of piceatannol (**7**) and isorhapontigenin (**8**) were formed by partially purified enzyme from cell culture extracts of *Picea excelsa* (Rolfs and Kindl, 1984). Our results confirm that resveratrol is the major product formed *in vitro* by STS from spruce. However, we could not detect piceatannol or isorhapontigenin.

Although *P. abies* STS1 and STS2 produced the trihydroxystilbene aglycone resveratrol, *in vitro* and *in vivo*, the major stilbenes accumulated in most spruce accessions are the tetrahydroxystilbene glycosides astringin and isorhapontin (Toscano-Underwood and Pearce, 1991; Lieutier et al., 2003). To determine if resveratrol is the precursor of the major stilbenes, *P. abies* was genetically engineered to overexpress *Pa*STS1. Overexpression lines had higher *PaSTS* transcript levels and produced significantly more astringin and isorhapontin than a control line transformed with an empty vector. Resveratrol levels, on the other hand, remained consistently low in both overexpression and vector control lines. Thus, *Pa*STS1 and *Pa*STS2 are involved in the biosynthesis of the major *P. abies* stilbenes, astringin and isorhapontin. Our *in vitro* characterization of *Pa*STS indicates that the first step of the pathway is the formation of resveratrol. This intermediate is then modified by 3'-hydroxylation, 3'-O-methylation and 3-O-glycosylation to yield the major stilbene glycosides accumulated in spruce.

In rhubarb (*Rheum rhaponticum*), a similar pathway for the production of the major stilbene, 3,5,3'-trihydroxy-3'-methoxystilbene-3-O-glucoside, from resveratrol was proposed after *in vivo* substrate feeding experiments (Rupprich et al., 1980). However, in this species resveratrol and another likely intermediate, resveratrol-3-O-glucoside, appear in high concentrations in the rhizome (Puessa et al., 2008). In contrast, in both wild-type *P. abies* as well as transgenic lines overexpressing the *Pa*STS1 resveratrol synthase, only minute amounts of resveratrol and its 3-O-glucoside were detected. This could indicate that metabolite channeling (Winkel-Shirley, 1999) of resveratrol to the next pathway enzyme, a stilbene 3'-hydroxylase, occurs in *P. abies* as has been shown for flavonoid biosynthesis in *Arabidopsis thaliana* (Burbulis and Winkel-Shirley, 1999).

STS enzymes evolved from CHS by gene duplication and neofunctionalization. It is therefore not surprising that both enzymes share the same substrate, *p*-coumaroyl-CoA. Thus, in both stilbene and flavonoid biosynthesis further oxidation, O-methylation or other modifications at positions on the

aromatic ring derived from the phenylpropanoid-CoA substrate must occur in later steps of the pathway.

2.4.4 Genetic transformation of spruce is a useful tool to study metabolic pathways in conifers

The use of stably transformed spruce in this study was critical to demonstrating that the STS enzymes, although only making resveratrol as their intermediate product, still contribute to the formation of the major stilbenes in the plant. Previous studies involving expression of genes involved in terpenoid resin production in *P. abies* and *P. glauca*, also showed the value of stable transformation of conifer genes in studying natural product biosynthesis (Godard et al., 2007; Schmidt et al., 2010). However, genetic transformation of woody plants is not without its difficulties, including the long time required for regeneration of intact plants.

Genetic transformation also produces unintended pleiotropic effects on occasion (Yabor et al., 2006; Abdeen and Miki, 2009) that negatively affect plant phenotype. In tobacco, for example, stilbene overexpression led to male sterility (Fischer et al., 1997; Hoefig et al., 2006) probably due to shortages of substrates for flavonoid and sporopollenin production. However, in the *Pa*STS overexpression lines described here, despite increased stilbene formation, we detected no negative impact on flavonoid biosynthesis. In fact, in one transgenic line (line 5) the transcript levels of putative leucoanthocyanidin reductase (LAR) enzymes (Tanner et al., 2003) responsible for the catalysis of late steps in the flavonoid pathway were elevated as compared to the vector control. The levels of the flavan-3-ols, catechin and proanthocyanidins, the catalytic products of LAR, were also higher in the transgenic lines than in the vector control, hinting at a positive interaction between flavonoid and stilbene biosynthesis in spruce. However, more transgenic lines need to be evaluated to see if line 5 is representative of a general pleiotropic effect.

Genetic transformation, by altering transcript levels of biosynthetic genes, can also provide hints on how a metabolic pathway is regulated. In the bark of fungus-inoculated, wild-type *P. glauca* saplings, increases in *STS* transcript levels correlated well with the subsequent stilbene accumulation, suggesting regulation on the level of steady-state transcript. However, in the *PaSTS* overexpressing transgenic lines, *STS* transcripts were much higher in stems than in needles and higher in needles than in roots. These patterns did not correlate well with stilbene accumulation, which was highest in roots, intermediate in stems and lowest in needles. Such discrepancies may be due to differences in substrate availability or post-transcriptional regulation among organs, or to stilbene transport among organs. Further research is needed to understand what factors limit stilbene accumulation in both healthy and fungally-infected spruce. Stilbene formation may be adjusted to different levels

depending on the species of pathogen, degree of infection and the presence of other biotic and abiotic stresses.

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Identification of putative *STS* and *CHS* genes from spruce

Protein sequences from various gymnosperm *STS* (*Pinus strobus*: Raiber et al., 1995; *Pinus densiflora*: Kodan et al., 2001) and *CHS* (*Pinus sylvestris*: Fliegmann et al., 1991; *Ginkgo biloba*: Pang et al., 2004) were used to query *Picea sitchensis* and *Picea glauca* EST collections (Pavy et al., 2005; Ralph et al., 2008) by tBLASTn for candidate cDNA sequences. Open reading-frames from candidate sequences were detected using the software package DNA Star Version 8.02 (DNASTAR Inc., Madison, USA) and the absence of predicted signal peptides at the N-terminus was confirmed by SignalP software (http://www.cbs.dtu.dk/services).

2.5.2 Cloning and sequencing *PaSTS* and *PaCHS*

For RNA extraction, fresh bark tissue from four-year-old *Picea abies* saplings which were grown in an outdoor plot (clone 3369-Schongau, Samenklenge und Pflanzgarten Laufen, Germany) was ground to a fine powder. Total RNA was extracted using the method developed by Kolosova et al. (2004). One µg of total RNA was converted to cDNA in a 20 µl reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen, Darmstadt, Germany) and 50 pmol $PolyT_{(12\text{-}18)}$ primer (Invitrogen). Gateway™ compatible primers were designed for candidate sequences by using the N- and C-terminal sequences of putative *P. sitchensis* and *P. glauca STS* and *CHS* genes as templates (attB primer sequences are provided in table S2.1).

Pseudomature forms of *PaSTS* and *PaCHS* cDNA were PCR amplified with attB primers (Table S2.1) using Platinum Taq™ high fidelity DNA polymerase (Invitrogen) and purified with the QIAquick™ PCR purification kit (Qiagen, Hilden, Germany). Gateway™ entry clones were made by using BP clonase II and pDONR207 (Invitrogen) following the manufacturer's protocols.

pDONR207 constructs containing *PaSTS* and *PaCHS* genes were sequenced using 10 pmol of the vector specific primers pDONR F(5'-TCGCGTTAACGCTAGCATGGATCTC-3'), pDONR R (5'-GTAACATCAGAGATTTTGAGACAC-3') and the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism R 3100 sequencing system (Applied Biosystems, Darmstadt, Germany).

Sequences from each construct were assembled and translated into protein sequence using DNA Star software.

2.5.3 Protein sequence analysis of CHS and STS

STS and CHS protein sequences (Table S2.2) were aligned with the automatic alignment program MAFFT v 6 (mafft.cbrc.jp/alignment/server/) using the BLOSUM 62 scoring matrix with 1.53 gap opening penalty and an offset value of 1.

Phylogenetic analyses were conducted using MEGA v. 4 (Center for Evolutionary Medicine and Informatics, www.megasoftware.net) employing the neighbor-joining method. Evolutionary distances were calculated with the Poisson correction. The tree was searched using the close neighbor interchange algorithm with pair-wise elimination of alignment gaps. Statistical likelihood of tree branches was tested with 10,000 bootstrap replicates.

2.5.4 Heterologous expression of *PaSTS* genes in *E. coli*

Putative *PaSTS*1 and *PaSTS*2 pDONR207 constructs were cloned with LR clonase II (Invitrogen) according to the manufacturer's instructions into the Gateway® compatible expression vector pH9GW (Yu and Liu, 2006) coding for an N-terminal His-tag. Arctic Express ™ (DE3) chemically competent *E. coli* (Stratagene, La Jolla, CA, USA) were transformed with expression constructs. Single colonies were inoculated into 5 ml Luria-Bertani (LB) broth with 1 μg ml^{-1} kanamycin and grown for 16 hours at 24°C. The 5 ml starter cultures were used to inoculate 100 ml Overnight Express Instant TB Medium (Novagen, Madison, WI, USA) supplemented with 1% (v/v) glycerol and 1 μg ml^{-1} kanamycin.

Bacterial cultures were grown for 3 days at 12°C (220 rpm) and harvested by centrifugation. Bacteria were resuspended in 10 ml buffer containing 50 mM Bis-Tris (pH 7.2), 10% (v/v) glycerol, 0.5 mM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol (DTT) and disrupted by sonification (power 65%, 3 minutes, 2 cycles) using a Bandelin Sonoplus HD 2070 (Bandelin Electronics, Berlin, Germany). Insoluble cell debris was removed from the lysate by centrifugation at 4°C. Protein expression was confirmed by Western blot using an anti-histidine-horseradish peroxidase conjugate antibody (Novagen).

Expressed proteins were purified with a 1 ml His Trap ™ FF column (GE Healthcare, Munich, Germany) on an AEKTA 900 chromatography system (GE Healthcare). The column was washed with 50 mM Bis-Tris (pH 7.2) and 10% (v/v) glycerol and eluted with wash buffer amended with 220 mM imidazole. The eluted proteins were desalted into an assay buffer (50 mM Bis-Tris pH

7.2, 10% (v/v) glycerol, 1 mM DTT) on DG-10 desalting columns (Bio-Rad, Munich, Germany). The protein concentration was determined using the Bradford reagent (Bio-Rad).

2.5.5 Functional characterization of *Pa*STS

The aromatic coenzyme A esters cinnamoyl-CoA and *p*-coumaroyl-CoA were synthesized enzymatically using the methods described by Beuerle and Pichersky (2002). Caffeoyl-CoA and feruloyl-CoA were chemically synthesized following the methods from Brand et al. (2006). Identification of these compounds was confirmed by LC-ESI-MS.

Enzyme activities were assayed in 200 µl reaction volumes containing 150 µM malonyl-CoA (Sigma, Munich, Germany), 50 µM of individual aromatic coenzyme A esters and 26 µg purified enzyme. Reaction mixtures were incubated for 3 hours at 28°C and stopped by acidification with 50 µl of 0.1 N HCl and extracted with 3 volumes of ethyl acetate. The ethyl acetate extracts were evaporated under nitrogen gas flow and redissolved in 50 µl methanol for LC-ESI-MS analysis. Negative control assays were initiated with heat-denatured enzyme preparations.

Enzyme extracts from transgenic *P. abies* over-expressing *PaSTS*1 were prepared by extracting 100 mg bark tissue using the method of Martin et al. (2002). After pelleting plant debris, the extract was passed over a 1 ml reactive red agarose (Sigma) affinity chromatography column. The column was washed with 5 column volumes assay buffer and eluted with 2 column volumes assay buffer amended with 500 mM NaCl. STS activity was determined as above.

2.5.6 Functional characterization of *Pa*STS by co-expression with 4-coumaroyl-CoA ligase in *E. coli*

Arctic Express ™ (DE3) chemically competent *E. coli* (Stratagene) were co-transformed with either *PaSTS*1 or *PaSTS*2 expression constructs and pAC-4CL1 (Watts *et al*., 2006) coding for a 4-coumaroyl-CoA ligase from *Arachis hypogaea*. Overnight Express Instant TB Medium (Novagen) supplemented with 1% (v/v) glycerol, 1.5 µg ml^{-1} chloramphenicol and 1 µg ml^{-1} kanamycin (40 ml) was inoculated with transformed *E. coli* colonies.

Bacterial cultures were grown for 8 hours at 22°C followed by 4 days at 12°C before adding *p*-coumaric or caffeic acid in DMSO to the culture medium as substrates to a final concentration of 1 mM. Culture medium (1 ml) was harvested 24, 48 and 72 hours after addition of phenolic acids. After centrifuging the culture medium, the supernatant was acidified with 0.1 N HCl and extracted with one volume of ethyl acetate. After evaporation the extract was re-dissolved in 50 µl methanol for LC-ESI-MS analysis.

2.5.7 Genetic transformation of *P. abies* callus with *PaSTS*1

The *PaSTS*1 pDONR207 was cloned with LR clonase II (Invitrogen) into the Gateway™ compatible binary vector pCAMGW. pCAMGW *STS*1 or pCAMBIA 2301 (as vector control) were transformed into the chemically competent disarmed *A. tumefaciens* strain C58/pMP90 (Schmidt et al., 2010). An embryonic *P. abies* cell culture (line 186/3c VIII) was transformed as described by Schmidt et al. (2010). From the 6 kanamycin resistant transgenic lines obtained, line 5 and line 11 were selected for regeneration of seedlings and further experiments.

2.5.8 Somatic embryogenesis and plant regeneration

Transgenic embryonic tissue was maintained at 24°C in the dark and sub-cultured every 14 days. For plant regeneration, transgenic embryonic tissue was placed on semi-solid EMM1 medium (Walter et al., 1999) amended with 6 g l^{-1} gelrite (Duchefa, Amsterdam, Netherlands), 30 g l^{-1} sucrose and 15 mg l^{-1} abscisic acid for 2 weeks. The culture was then placed on EMM2 medium (4.5 g l^{-1} gelrite) (Walter et al., 1999) for 5 weeks. Embryos, 3-5 mm in size, were placed on a sterile nylon mesh in 3 wells of a six-well cell cluster. The other 3 wells were filled with sterile water to maintain humidity. Embryos were stored at 4°C for 7 days in the dark. For germination, developed embryos were transferred to MLV medium (Klimaszewska et al., 2005) with 40 g l^{-1} sucrose and 6 g l^{-1} gelrite (Duchefa). The cultures were placed for 6 weeks under low light conditions (5 µmol m^{-2} s^{-1}) for 16 h day^{-1} at 24°C. Somatic embryos were collected and placed horizontally on MLV medium with 6 g l^{-1} gelrite (Duchefa) under moderate light conditions (45 µmol m^{-2} s^{-1}). After three weeks plants were transferred to Magenta GA-7 (Sigma) plant culture boxes and placed under higher light conditions (100 µmol m^{-2} s^{-1}) to develop roots, epicotyls and needles. Well developed plantlets were planted in soil substrate (3:1:1, fibric peat:vermiculite (2-3 mm grain size):perlite) in small plastic containers (4 x 4 cm) (Figure 2.4).

Procedures from Klimaszewska *et al*. (2001) were used to generate the untransformed *P. glauca* line *Pg* 653.

2.5.9 Quantitative real-time PCR

Total RNA for all quantitative real-time PCR experiments was isolated with the Invitrap® Spin Plant RNA Mini Kit (Invitek, Berlin, Germany). RNA was quantified spectrophotometrically.

Reverse transcription of 1 µg RNA into cDNA was accomplished using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol $PolyT_{(12-18)}$ primer (Invitrogen). After cDNA was

diluted to 10% (v/v) with deionized water, 1 µl diluted cDNA was used as template for quantitative real-time PCR. PCR was performed with Brilliant SYBR Green QPCR Master Mix ™ (Stratagene) and 10 pmol of the forward primer 5'-GTGGCGAGCAGAACACAGACTTC-3' and 10 pmol of the reverse primer 5'-CAGCGATGGTACCTCCATGAACG-3', designed to amplify 140 base pairs of both *STS*1 and *STS*2 simultaneously. Primer sequences for *PaLAR*1, *PaLAR*2, *PaLAR*3 and *PaPAL* are given in table S2.3. PCR was performed using a Stratagene MX3000P thermocycler: 5 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by a melting curve analysis from 55°C to 95°C. Reaction controls included non-template controls and non-reverse transcribed RNA. Transcript abundance was normalized to the transcript abundance of the ubiquitin (Schmidt et al., 2010; GB:EF681766.1) gene (Table S2.3) and was calculated from three technical replicates each of at least four biological replicates. Relative transcript abundance for the transgenic *PaSTS*1 lines was calibrated against the transcript abundance of one biological replicate of the vector control. Relative transcript abundance of *PgSTS* in *P. glauca* was calibrated against a non-wounded control sample.

2.5.10 Extraction of phenolic compounds from spruce

Spruce tissue was ground to a fine powder in liquid nitrogen and lyophilized using a Alpha 1-4 LD plus freeze dryer (Martin Christ GmbH, Osterode, Germany). Approximately 5 mg dried tissue was extracted with 2 ml methanol for 4 hours at 4°C, then centrifuged at 2000 g and the supernatant recovered. Insoluble material was re-extracted with 1 ml methanol for 12 hours. The supernatants were combined and evaporated to dryness under a stream of nitrogen and re-dissolved in 1 ml methanol containing 50 µg/ml chlorogenic acid (Sigma) as internal standard. For LC-ESI-MS samples were diluted to 20% (v/v) with methanol.

2.5.11 Liquid chromatography-mass spectrometry with electrospray ionization (LC-ESI-MS)

Compounds were separated on a Nucleodur Sphinx RP18ec column (250 x 4.6 mm, particle size 5 µm) (Macherey-Nagel, Dueren, Germany) using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a flow rate of 1.0 ml min^{-1} at 25°C. Compound detection and quantification was accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Brucker Daltronics, Bremen, Germany) after diverting column flow-through in a ratio of 4:1. The mass spectrometer was operated as follows: skimmer voltage: 60 V; capillary voltage: 4200 V; nebulizer pressure: 35 psi; drying gas: 11 l min^{-1}; gas temperature: 330ºC.

Phenolic compounds from spruce and enzyme assay products were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0-1 min, 100% A; 1-15 min, 0-65% B; 15-18 min 100% B; and 18-22min 100% A. ESI-MS was operated in negative mode scanning $m\,z^{-1}$ between 50 and 1600 with an optimal target mass of 405 $m\,z^{-1}$. Capillary exit potential was kept at -121 V.

Compounds were identified by mass fragmentation spectra and by comparison to standards, where available. Brucker Daltronics Quant Analysis v.3.4 software was used for data processing and compound quantification using a standard smoothing width of 3 and Peak Detection Algorithm v. 2. Linearity in ionization efficiencies was verified by analyzing serial dilutions of randomly selected samples. External calibration curves for catechin (Sigma) and astringin were created by linear regression. Flavan-3-ol concentrations were determined relative to a catechin calibration curve and stilbene glycosides relative to astringin, which was extracted and purified from spruce bark following a previous protocol (Li et al., 2008). Quantification results were normalized relative to the internal standard.

Aromatic coenzyme A esters to be used as enzyme substrates were separated using 20 mM ammonium acetate (pH 4) and acetonitrile as mobile phases A and B respectively with the following elution profile: 0-20 min, 5-45% B; 20-21 min, 45-90% B; 21-23 min, 90% B; and 23-28 min, 5% B. ESI-MS was operated in alternating mode scanning between $m\,z^{-1}$ 50 and 1600 with an optimal target mass of 250 $m\,z^{-1}$. Capillary exit potential was kept at -109.8 V.

2.5.12 Treatment of *P. glauca* saplings with *C. polonica*

A *C. polonica* isolate (CMW 7749) provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (University of Pretoria, South Africa) was grown on 3% (w/v) malt extract agar (MEA; Carl Roth GmbH, Karlsruhe, Germany) for 12 days at 25ºC.

P. glauca saplings originating from embryonic tissue (*Pg* 653) were grown in a walk-in growth chamber for 2 years in 2 l pots under a light/temperature regime alternating between 4 months exposure to 16 hrs light at 25°C and 4 months exposure to 8 hrs light at 16°C. Inoculations with *C. polonica* were performed after saplings were acclimatized for 6 weeks to the higher light/temperature regime and had completed their 'spring' flush.

A bark plug, 4 mm in diameter, was removed from the lower part of the sapling between the second and third branch-whorl with a cork borer. A 4 mm plug from the *C. polonica* culture was placed into the wound with the mycelium oriented toward the wood surface and sealed with parafilm. For the wounded control treatment, plugs of sterile MEA were inserted.

Bark tissue from inoculated and wounded saplings was harvested 5 days, 15 days and 25 days after the onset of the experiment. Four saplings were sacrificed per time point for each treatment. Bark material was flash-frozen in liquid nitrogen and stored at -80ºC.

2.5.13 Statistical analysis

Graphical representations of results are presented as mean ± standard error.

Statistical significance of differences in *STS* transformed lines was determined using a one-way analysis of variance (ANOVA) on log-transformed data. Significance of differences in the *C. polonica* inoculation trial was determined using a two-way ANOVA. Differences in means were calculated using Tukey's post-hoc pair-wise comparisons test at a 95% confidence level. Analyses were conducted using the open source software R v. 2.81 (www.r-project.org) and the LAERCIO package for pair-wise comparisons.

3. Research Chapter II

Bark beetle vectored fungus detoxifies polyphenolic defenses of its conifer host

3.1 ABSTRACT

Norway spruce (*Picea abies*) is frequently subject to severe attacks by the scolytid bark beetle *Ips typographus* and its microbial associate, the blue stain fungus, *Ceratocystis polonica*. Spruce trees have been shown to protect themselves against bark beetle attack by production of terpenoid resins, but it is less clear how they defend themselves against the fungal associate. Spruce bark possesses polyphenolic compounds called stilbenes with known antifungal properties, and stilbene biosynthesis was found to be up-regulated during *C. polonica* attack on spruce based on elevated transcript levels of stilbene synthase genes. However, stilbene accumulation itself has been shown to decline during attack due to conversion of these phenolics to deglycosylated, oxidized and dimeric products by the fungus. Chromatographic separation of a *C. polonica* protein extract confirmed that these metabolites arose from specific fungal enzyme activities. Comparison of two *C. polonica* strains that differ in their major pathways of stilbene breakdown showed that more rapid metabolism of stilbenes is associated with increased virulence in the tree.

3.2 INTRODUCTION

Norway spruce (*Picea abies*), a dominant tree species in European boreal, montane and sub-alpine ecosystems, is frequently subject to severe attacks by the scolytid bark beetle *Ips typographus*. During attacks, these gregarious beetles often introduce fungal pathogens into their hosts. One of the most virulent spruce pathogens associated with *I. typographus* is the blue staining fungus, *Ceratocystis polonica*. This beetle-fungus complex is thought to act synergistically (Franceschi et al., 2005) to kill trees, where the beetles damage the cambium by feeding and the fungus interrupts water flow in the vascular bundles. However, it is still unclear how bark beetles benefit from association with this virulent, necrotrophic fungus (Paine et al., 1997) and whether trees possess any defenses against the fungus.

Norway spruce trees have effective structural and chemical defense strategies against low density bark beetle attacks. The best known example of chemical defense in *P. abies* is oleoresin (Martin et al., 2002). This viscous mixture of terpenoids stored in specialized ducts has been shown to increase in quantity and change in composition during defense responses. A less well-studied defense mechanism in spruce is the production and storage of phenolic compounds in specialized cells in the phloem parenchyma (PP-cells). During a defense response, elicited by wounding, methyl jasmonate or *C. polonica* infection, PP-cells expand and the inclusion bodies develop higher autofluorescence thought to indicate the presence of phenolic compounds (Franceschi et al., 2000). It is, therefore, likely that PP-cells form a protective barrier against pest species invading the tree's cambial zone.

Stilbenes are important components of the extensive array of phenolic compounds synthesized by tree species from the family Pinaceae (Underwood and Pierce, 1992). These compounds have been reported to have anti-fungal properties and been shown to significantly contribute to plant disease resistance (Chong et al., 2009). In the genus *Picea*, the stilbene glucosides astringin and isorhapontin (Figure 3.1a) occur in high concentrations in bark, roots and foliage. Two stilbene synthase (STS) enzymes have been recently described in Norway spruce, which contribute to the formation of these compounds (Figure 3.1a).These enzymes play a role in tree defense, since it has been shown that fungal elicitation can induce *STS* transcription and enzyme accumulation (Brignolas et al., 1995). However, contradictory reports on stilbene glucoside accumulation in spruce bark during fungal infection have been published. On the one hand, significant increases in astringin were observed after elicitation of *Picea glauca* saplings with an avirulent *C. polonica* isolate. On the other hand, stilbene concentrations in mature Norway spruce accessions were stable or decreased during the course of *C. polonica* infections (Brignolas et al., 1995; Virii et al., 2001).

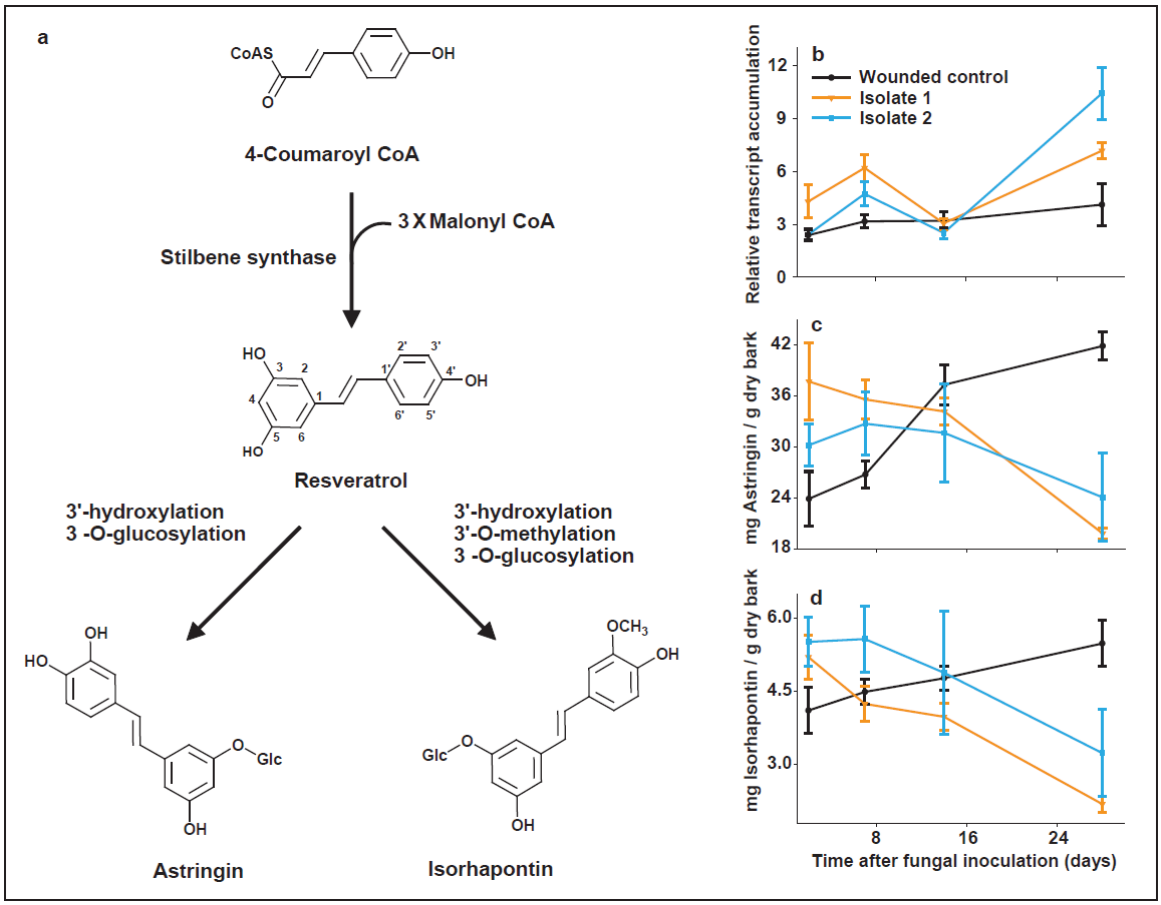


Figure 3.1: Stilbene biosynthesis and accumulation in response to fungal infection. (a) Stilbene biosynthetic pathway in *P. abies*. **(b)** Relative transcript accumulation of *P. abies* stilbene synthase (*PaSTS*) genes in response to infection by two *C. polonica* isolates or to inoculation with sterile agar (control). Concentrations of the stilbene glucosides **(c)** astringin and **(d)** isorhapontin after wounding or infection by *C. polonica*. Transcript accumulation of *PaSTS* was measured by quantitative real-time PCR using an ubiquitin gene for normalization and non-wounded saplings for calibration. Astringin and isorhapontin concentrations were measured by LC-ESI-MS. Error bars represent standard errors (n = 5 replicates per treatment per time point).

After host infection, fungi follow different strategies to gain access to host nutrients. Biotrophic fungi, which acquire nutrients from living cells, penetrate the host causing minimal damage to infected cells (Voegele and Mendgen, 2003). On the other hand, necrotrophs such as *C. polonica* lyse cells in order to release nutrients which are then assimilated by the fungi (Oliver and Solomon, 2010). However, during lysis of plant cells vaculolar contents, rich in potentially anti-fungal plant defense compounds, come into direct contact with fungal hyphal tips. To the best of our knowledge, it is not known what defense compounds Norway spruce deploys against *C. polonica* and

whether or not this specialized, bark beetle vectored, necrotrophic pathogen of *P. abies* is at least partially resistant to them.

In this work, we demonstrate that *C. polonica* can circumvent the anti-fungal activity of stored and *de novo* synthesized stilbenes in spruce during the infection process. Although high levels of *STS* transcripts accumulate in fungal-infected Norway spruce bark, a net loss of stilbene glucosides was detected at the site of infection. This stilbene deficit was explained by fungal biotransfomation of host compounds. These enzyme-driven biotransformations include the formation of stilbene dimers, aglycones and ring-opened lactone oxidation products. We could also show that different *C. polonica* isolates followe different stilbene biotransformation strategies with rapid metabolism being associated with increased fungal virulence.

3.3 RESULTS

3.3.1 Effect of fungal inoculation on *STS* transcription and stilbene accumulation in Norway spruce phloem

Norway spruce saplings were wounded and inoculated with two different *C. polonica* isolates or with sterile agar (control treatment) and bark tissue was harvested for analysis 2, 7, 14 or 28 days afterwards. Transcript accumulation of *PaSTS*, measured by quantitative real-time PCR, increased significantly in fungal-inoculated saplings ($p < 0.0005$) compared to the sterile-agar inoculated control saplings at 7 days and again at 28 days post-inoculation (Figure 3.1b). In control saplings the increase in *PaSTS* transcript accumulation over the time course of the experiment was non-significant. Transcript abundances in saplings infected by *C. polonica* isolate 1 did not differ from transcript abundances in saplings infected by isolate 2.

Levels of the stilbene glucosides, astringin (Figure 3.1c) and isorhapontin (Figure 3.1d), increased in the bark of sterile agar-inoculated control saplings over the time-course of the experiments, while in fungal-inoculated saplings a decrease in stilbenes could be observed. Significant differences in total stilbenes during the time course could be detected between 14 and 28 days post-inoculation ($p = 0.042$) in both the inoculated treatments and the control.

In order to determine if *C. polonica* was the agent responsible for reduced stilbene levels in infected bark, isolates 1 and 2 were grown in nutrient broth amended with 2 mg/ml astringin. A significant decrease in astringin was observed in the fungal culture medium compared to the sterile astringin amended control medium ($p < 0.0001$) over a time course of 72 hours (Figure 3.2). The rate

of astringin decrease in the medium was significantly higher for isolate 2 compared to isolate 1 ($p < 0.0001$).

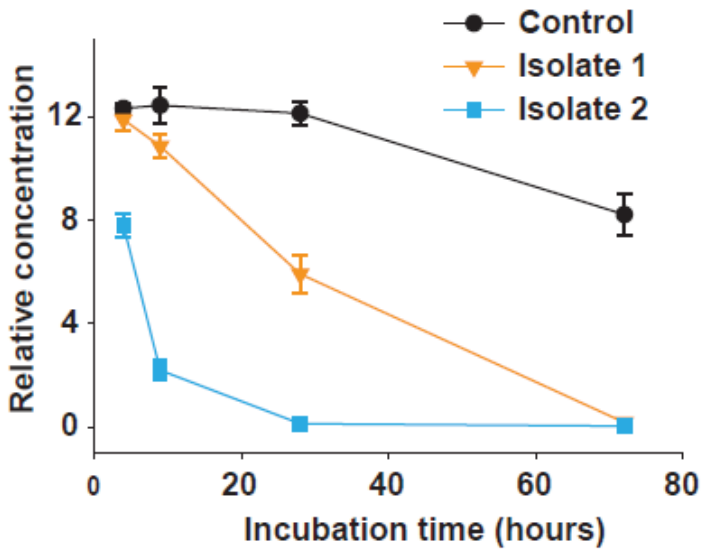


Figure 3.2: *In* vitro biotransformation of astringin by the fungus *C. polonica*. Depletion of astringin added to artificial medium by two isolates of *C. polonica* over a period of 72 hours compared to changes in astringin concentration in sterile medium (n = 4 replicates per isolate per time point).

3.3.2 Structural elucidation of fungal biotransformation products

Several products of fungal astringin biotransformation were detected in fungal culture filtrates (Figure 3.3) including ring-opened lactones **(1** and **4)**, the deglucosylated piceatannol **(2)** as well as dimeric stilbenes **(3**, **5** and **6)**.

Monomeric derivatives. Separation of the major biotransformation metabolites was achieved by solid phase extraction on RP-18 material followed by HPLC-SPE chromatography and resulted in the isolation of compounds **(1)** to **(6)** (Figure 3.3).

The molecular formula of compound **(4)** was determined to be $C_{14}H_{12}O_6$ on the basis of its positive-mode ESI-HRMS (*m* z^{-1} 277.0709 $[M+H]^+$, calcd. for $C_{14}H_{13}O_6$ 277.0707). ^{1}H-NMR showed a typical *trans*-double bond pattern (both J_{H-H} 16.5 Hz) characteristic for a stilbene-related structure at 6.99 and 7.05 ppm, respectively. However, both signals were shifted to lower field compared to the parent structure of astringin, indicating a major change in the substitution of the aromatic rings. A pattern of a pseudo-triplet at 6.28 ppm (1H equivalent) and a broad singlet at 6.53 ppm (2H equivalents) revealed the loss of the β-*D*-glucose moiety, resulting in a symmetrical 3,5-dihydroxy substituted phenyl ring. HMBC correlation spectroscopy showed the connection to the trans-double

bond system at a carbon shift of 140.99 ppm (corresponding to a 7.04 ppm proton resonance, see supplementary table S3.1). HMBC correlations from the second trans-double bond proton at 6.99 ppm to an unsaturated methine at 115.47 ppm (proton resonance at 6.12 ppm) and a saturated methine at 81.57 ppm (corresponding to a proton at 5.733 ppm), as well as to a quarternary carbon at 168.47 ppm indicated structural changes of the former aromatic ring system. H-H-COSY correlations from the saturated methine proton at 5.74 ppm (*d*, J_{H-H} 8.6 Hz) to a methylene group at 2.44 ppm (*dd*, J_{H-H} 9/16 Hz) and 2.99 ppm (*dd*, J_{H-H} 3/16 Hz), respectively, together with HMBC correlations at 175.48 and 174.86 ppm led to the conclusion that the structure of the former aromatic ring system contained a but-2-en-4-olide, an α,β-unsaturated-γ-lactone with a CH_2COOH substituent (Supplementary Figure S3.1). Similar structures were described as veratryl alcohol degradation products derived from a reaction with lignin peroxidase (Schmidt et al., 1989; Shimada et al., 1987; Leisola et al., 1985), as synthetic products from stilbene oxidation (Beziudenhout et al., 1990), and as metabolites isolated from peanut kernels (Sobolev et al., 2006). All other spectral data were consistent with this structure.
Compound **(1)** has a molecular composition of $C_{20}H_{22}O_{11}$ based on negative mode ESI-HRMS (m/z 437.1094 $[M-H]^-$, calcd. for $C_{20}H_{21}O_{11}$ 437.1084). Comparison of the proton NMR data with reference data for astringin showed an intact 3,5-disubstituted phenyl ring with the glucose substituent, but the absence of aromatic signals for the 3',4'-dihydroxy substituted phenyl ring. The signal pattern closely resembled that of compound **(4)**. Thus, the structure of **(1)** was determined to be a β-*D*-glucosylated derivative of **(4)**. The structure of **(2)** was revealed to be that of the de-glucosylated derivative of astringin, called *E*- and *Z*-piceatannol based on comparison of its MS spectra and proton NMR data to those in the literature (Li et al., 2007).

Dimeric derivatives. The molecular composition of the isolated dimeric metabolite **(6)** was $C_{40}H_{42}O_{18}$, based on negative mode ESI-HRMS ($m\ z^{-1}$ 809.2297 $[M-H]^-$, calcd. for $C_{40}H_{41}O_{18}$ 809.2293). NMR spectroscopy revealed them to be identical with the formerly isolated piceasides A, B, G and H (Li et al., 2008) (supplementary Figure S3.2 and S3.3). Dimeric derivatives with molecular mass $m\ z^{-1}$ 648.61 and 486.47 were revealed to be piceasides A, B, G or H lacking one or both glucose moieties respectively based on ^{1}H-NMR spectra as well as low resolution MS.

3.3.3 Biotransformation of astringin by *C. polonica* in culture

Although all 6 biotransformation products could be detected in the cultures of both fungal isolates, the major pathways of stilbene breakdown differed between the two isolates. The lactone **(1)** (Figure 3.3, Table 3.1) was produced at significantly higher levels by isolate 2 than isolate 1 ($p < 0.0001$). Lactone **(4)**, was formed at lower concentrations but was also detected at higher levels in isolate 2 cultures than in cultures from isolate 1 ($p < 0.0001$).

E- and *Z*-piceatannol **(2)** were produced at higher levels by isolate 1 than by isolate 2 ($p < 0.0001$). A linear increase in piceatannol concentration could be observed between 4 and 28 hours incubation time in cultures colonized by isolate 1, whereas a decrease in piceatannol content was noted in cultures colonized by isolate 2. Changes in piceatannol content in culture media of both fungi were statistically significant ($p < 0.0001$).

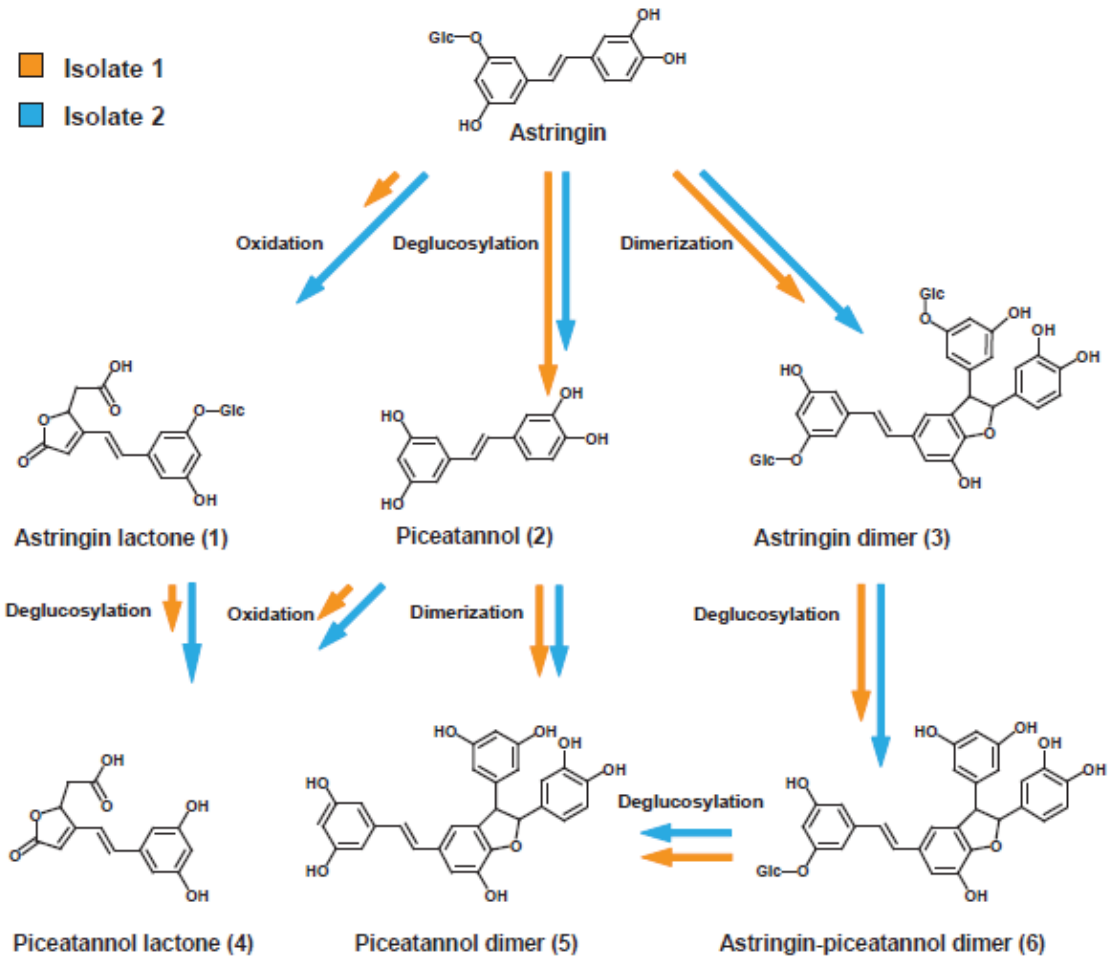


Figure 3.3: Stilbene biotransformation pathways of *C. polonica* isolate 1 and isolate 2. The length of the arrows denote the relative velocity of each reaction in the first 4 hours after astringin was added to the growth media of fungal isolates.

Dimeric astringin **(3)** was observed in culture medium with fungus as well as in non-inoculated control medium. In sterile control cultures as well as in cultures containing isolate 1, a gradual increase in astringin dimer concentrations was observed. Levels of dimeric astringin were significantly higher in the fungal cultures than in the control ($p = 0.0006$). In cultures containing isolate 2 the highest concentration of astringin dimer was observed 4 hours after the onset of the

experiment. At later time points of the experiment a gradual decrease in astringin dimers could be detected in the isolate 2 culture medium. Biotransformation of astringin to piceatannol dimers **(5)** and astringin-piceatannol dimers **(6)** followed the same kinetics observed for dimeric astringin with statistically significant differences between fungal isolates ($p < 0.0001$).

Table 3.1: Astringin biotransformation of *C. polonica*. The percentage (± SD) of substrate recovered in individual biotransformation products 4 and 28 hours after adding astringin to *C. polonica* isolate 1 and isolate 2 (n = 4 replicates per isolate per time point).

	Isolate 1		Isolate 2	
	4 Hours	**28 Hours**	**4 Hours**	**28 Hours**
Astringin lactone (1)	0.05 ± 0.02	0.13 ± 0.04	6.54 ± 1.47	9.01 ± 1.28
Piceatannol (2)	10.3 ± 1.14	20.7 ± 4.94	3.93 ± 0.74	0.32 ± 0.27
Astringin dimer (3)	3.92 ± 0.50	13.9 ± 2.92	22.3 ± 3.21	10.1 ± 4.36
Piceatannol lactone (4)	0.05 ± 0.01	0.08 ± 0.01	0.15 ± 0.04	0.54 ± 0.20
Piceatannol dimer (5)	0.14 ± 0.02	1.12 ± 0.31	0.16 ± 0.01	0.15 ± 0.43
Astringin-piceatannol dimer (6)	1.34 ± 0.87	15.0 ± 3.95	4.56 ± 0.87	3.83 ± 3.77
Total identified stilbene metabolites	15.8 ± 2.53	50.9 ± 12.2	37.6 ± 6.63	24.0 ± 10.3
Remaining astringin	77.5 ± 4.46	46.6 ± 11.6	53.7 ± 8.67	0.94 ± 0.27

In isolate 1 cultures, 15 % of the initial astringin substrate was converted to biotransfomation products within the first 4 hours, whereas 37% of the astringin was transformed by isolate 2. The amount of recovered biotransformation products increased over the next 24 hours to 50% in cultures of isolate 1, but decreased to 24% in cultures of isolate 2. After 28 hours of incubation, almost all of the substrate could be recovered in the form of astringin or identified biotransformation products in the isolate 1 culture medium. However, in isolate 2 cultures only 25% of the astringin added to the culture medium initially was recovered, mainly as biotransformation products.

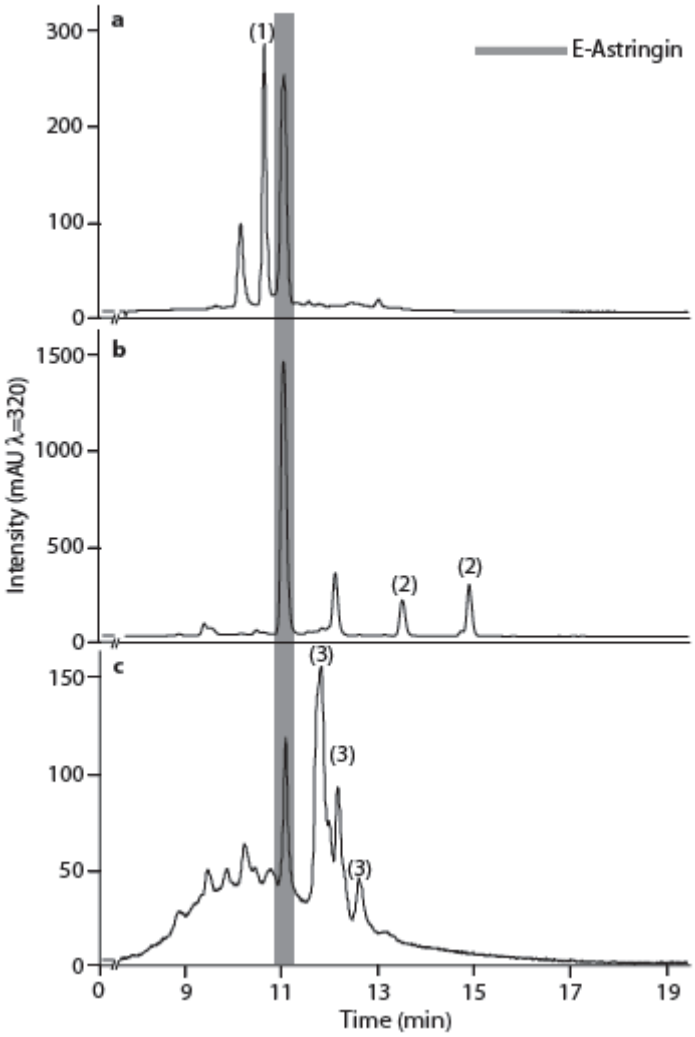


Figure 3.4: Stilbene biotransformation activity in partially purified protein fractions from *C. polonica*. Enzyme activities observed in three separate protein fractions after chromatographic separation with affinity chromatography and anion exchange. **(a)** Fraction that oxidized astringin to astringin lactone (**1**) was eluted by low salt concentration from the anion exchange column. **(b)** Fraction that deglycosylated astringin to form E- and Z- piceatannol (**2**) eluted in flow-through fraction from affinity chromatography on concanavalin A. **(c)** Fraction that eluted from the anion exchange column with high salt formed astringin dimers (**3**).

3.3.4 Demonstration of *C. polonica* enzyme activities, involved in stilbene biotransformation

To verify that the metabolism of stilbenes was carried out by *C. polonica* the relevant enzyme activities were sought in fungal extracts. By partial 2-step purification of soluble protein extract from *C. polonica*, separate enzyme activities could be recovered for the three different types of

biotransformation reactions observed in the *in vivo* study. In the first purification step enzymes were purified by affinity chromatography with concanavalin A sepharose, a matrix to which glycoproteins selectively bind. Catalytic activities for both stilbene dimerization and formation of ring-opened stilbene lactones were recovered from enzymes bound to this matrix and eluted with α-D-methylglucopyranoside. The protein fraction which did not bind to the lectin showed β-glucosidase activity (Figure 3.4b) forming *E*- and *Z*-piceatannol **(2)** from astringin.

Proteins recovered from affinity chromatography were further purified by anion exchange chromatography. Protein fractions eluted from the anion exchanger by 200mM NaCl showed catechol dioxygenase activity (Figure 3.4a) forming the ring-opened lactone **(1)** from astringin. A fraction eluted from the anion exchanger by higher salt concentration exhibited laccase activity (Figure 3.4c) forming astringin dimers **(3)**.

3.3.5 *In vitro* stilbene biotransformation activity of induced and un-induced *C. polonica*

Ceratocystis polonica isolates 1 and 2 were grown for 4 days in medium containing crude, polyphenol rich extract from spruce to induce stilbene biotransformation pathways. Protein extracts from induced and non-induced mycelium were then used in enzyme assays for stilbene biotransformation activity in a time course over a period of 8 hours. Enzymes from isolate 2 transformed astringin more efficiently than isolate 1 in the induced and un-induced state (Table 3.2). Conversion to the lactone **(1)** ($p = 0.001$), aglycone piceatannol **(2)** ($p < 0.0001$), as well as stilbene dimers **(3)** ($p = 0.09$) was higher in enzyme assays from isolate 2 than from isolate 1.

Table 3.2: Enzymatic stilbene biotransformation. Enzyme reaction rates (μg product g fresh mycelium^{-1} hour^{-1}) ± SD of *C. polonica* isolate 1 and isolate 2 for various metabolic reactions of astringin. Isolates were induced by incubation with 40 μg spruce extract per ml culture (n = 4 replicates per isolate per time point).

	Isolate 1		Isolate 2	
	Control	**Induced**	**Control**	**Induced**
Oxidation (1)	0.021 ± 0.003	0.025 ± 0.004	0.043 ± 0.022	0.187 ± 0.043
Deglycosylation (2)	0.112 ± 0.024	0.266 ± 0.027	0.411 ± 0.068	0.688 ± 0.035
Dimerization (3)	0.112 ± 0.093	0.263 ± 0.014	0.223 ± 0.056	0.275 ± 0.033

Induction of fungal mycelium with spruce extract lead to a 4-fold increase in the rate of lactone formation by the protein extract from isolate 2 ($p = 0.003$). No change in the rate of lactone

formation could be observed for enzymes from induced isolate 1. A 2-fold increase in piceatannol-forming β-glucosidase activity was noted for the enzyme fraction from isolate 1 ($p < 0.0001$) after induction. β-Glucosidase activity for isolate 2 was 3-fold higher in the un-induced state than for isolate 1, but showed only a 1.5-fold increase after induction. Enzymes from induced isolate 1 revealed a 2-fold increase in dimer formation compared to the uninduced state ($p = 0.02$). No changes in dimer formation could be observed for isolate 2, but activity was similar to that of induced isolate 1.

3.3.6 Fungal growth in spruce saplings and on artificial medium containing astringin

To determine if stilbene transformation was associated with fungal virulence in spruce, the growth rate of the two isolates on artificial medium and in spruce bark was compared. On solid minimal medium the *in vitro* growth rate of *C. polonica* isolate 2 was higher than the growth rate of isolate 1 ($p < 0.0001$). Growth rates of both isolates were lower in medium amended with 100 µg astringin per ml medium ($p < 0.0001$) than in non-amended control medium. The relative growth rate on astringin-amended medium was lower for isolate 1 than for isolate 2 (Figure 3.5).

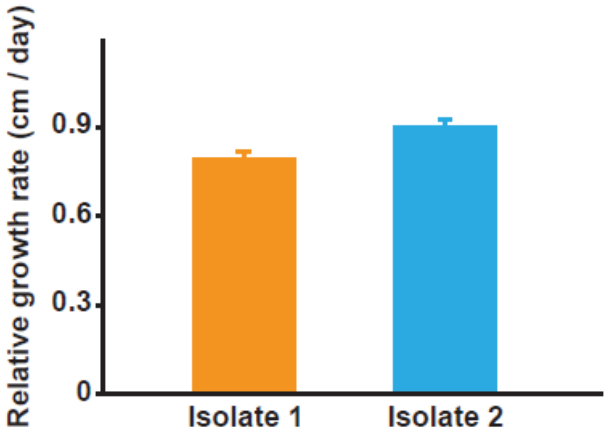


Figure 3.5: Growth rate of *C. polonica* in the presence of stilbenes. *In vitro* growth rate of two *C. polonica* isolates on solid minimal medium in the presence of astringin is presented relative to growth without astringin. Error bars represent standard errors (n = 10 replicates per isolate per treatment).

Growth of *C. polonica* isolate 1 and isolate 2 in the cambial zone of spruce saplings was measured 28 days after inoculation to estimate lesion length. There were highly significant differences between lesion lengths created by isolate 1 and isolate 2 ($p < 0.0001$) (Figure 3.6). Lesion lengths created by isolate 2 were more than twice as long as those created by isolate 1 indicating the greater virulence of this strain in spruce bark.

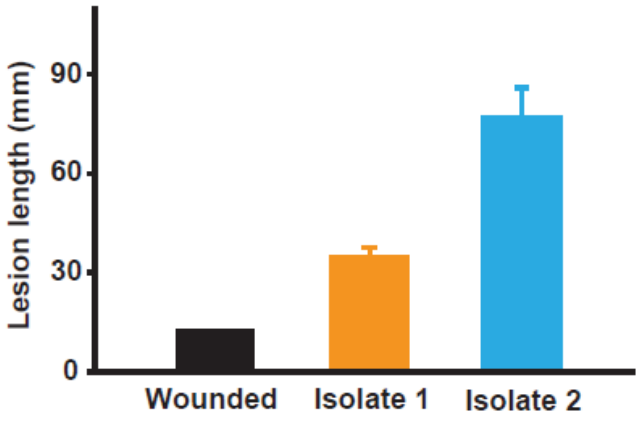


Figure 3.6: Measurement of fungal virulence. Lesion lengths of two *C. polonica* isolates were measured in the cambium 28 days after inoculation on eight year old spruce saplings (n = 5 replicates per isolate).

3.4 DISCUSSION

During *C. polonica* infection of spruce saplings the *STS* transcript levels showed a two-phase induction pattern, similar to *STS* induction patterns described for numerous other species (Wiese et al., 1994; Preisig-Mueller et al., 1999). However, levels of the stilbene glucosides astringin and isorhapontin gradually decreased in bark over the infection period. Similar observations have also been made by Brignolas et al. (1995), who showed that stilbene levels in spruce decreased during *C. polonica* infection in spite of increased STS enzyme activity in infected tissues. These findings suggest that *C. polonica* alters the stilbene content of host tissue during infection and colonization.

Stilbenes are considered anti-fungal compounds because they act as inhibitors of fungal growth by interfering with microtubule assembly (Woods et al., 1995), disrupting plasma membranes and uncoupling electron transport in fungal spores and germ tubes (Pont and Petzet, 1990). It is therefore to be expected that *C. polonica* developed mechanisms to circumvent the deleterious effects of these anti-fungal compounds produced in a host with whom the fungus has co-evolved over millions of years. We could confirm this by showing that *C. polonica* could deplete stilbenes by various biotransformation processes, which explains the decrease in stilbene levels in spruce bark after infection, even though their biosynthesis was up-regulated.

Partially purified *C. polonica* enzyme extracts showed that the stilbene biotransformation processes were catalyzed by a number of fungal enzymes. Furthermore it was shown that these enzymes were induced by a crude methanol extract prepared from *P. abies*. The induction of

detoxification pathways has also been reported in other phytopathogenic fungi, most notably laccases responsible for stilbene dimerization in *B. cinerea* after induction by phenolic compounds and pectin (Gigi et al., 1980). Similarly, transcription of genes originating from a cluster involved in the β-ketoadipate pathway were highly expressed in *Cochliobolus heterostrophus*, the causal agent of Southern corn leaf blight, in response to caffeic acid (Shanmugam et al., 2010). However, the induction of detoxification pathways seems to be species-specific. For example, *Grosmannia clavigera*, a bark-beetle vectored pine pathogen which is phylogenetically closely related to *C. polonica*, revealed no significant induction of genes putatively involved in polyphenol detoxification (Hesse-Ocre et al., 2010).

The tetrahydroxystilbene glucoside, astringin, which is produced in high amounts by *P. abies*, was transformed by *C. polonica* in culture. The major biotransformation products were identified as astringin-lactones, piceatannol and dimeric stilbenes using NMR spectroscopy and LC-MS. Similar reactions of stilbenes have been reported previously. Lactones, for example, can be formed by oxidative ring cleavage by dioxygenase enzymes (Harwood and Parales, 1996) and aglycones are formed by β-glucosidases that typically cleave β1→4 glucoside bonds (Morant et al., 2008). Furthermore, dimerization of stilbenes has been demonstrated in cell-free systems with oxidative enzymes, such as peroxidases and laccases (Breuil et al., 1998).

The structural similarities between the astringin-lactone formed upon fungal biotransformation and the lactone formed as an intermediary step in the β-ketoadipate pathway hint at the possibility of further catabolic degradation of astringin by *C. polonica*. The β-ketoadipate pathway plays a central role in the catabolic degradation of aromatic compounds, derived from lignin, as an energy source used by soil bacteria and fungi (Harwood and Parales, 1996). Spruce tissue is a poor substrate for fungal nutrition, containing low levels of protein and high levels of hard-to-digest polymers such as cellulose and lignin. Utilizing astringin, which is produced by spruce for anti-fungal defense, as an energy source might increase the fitness of *C. polonica*. Proliferation of fungal isolates with more rapid pathways for catabolic degradation of soluble phenolic compounds may, therefore, be greater than for isolates with low catabolic rates. In addition, the metabolic cost of detoxification of anti-fungal stilbenes might be outweighed if such a process ultimately creates energy for growth and reproduction.

Ring opening and lactonization is a logical early step of stilbene catabolism in *C. polonica*, since it appears to represent a detoxification process. Stilbenes exert their toxicity on fungi by diffusing through membranes into vegetative and reproductive structures (Adrian et al., 1998). Ring-opening and lactonization increase the polarity of astringin, which could lead to reduced diffusion into fungal cells and therefore reduced toxicity. Increasing the polarity of plant defense compounds

has already been shown to be a successful detoxification mechanism in other plant pathogens (Esaki et al., 1998; Weltring and Barz, 1980).

The deglycosylation of astringin to form aglucones may be catalyzed by β-glucosidase enzymes. This process has been previously reported in *C. polonica* infected bark (Virii et al., 2001). Deglucosylation is a seemingly disadvantageous phytoalexin biotransformation strategy as this process releases a less polar molecule with a higher potential biological activity. In fact, one of the best studied plant defense strategies is the activation of glucosylated phytoanticipins by β-glucosidases when cell compartments are disrupted by herbivore or pathogen attack (Morant et al., 2008). Furthermore, glucosylation of plant defense compounds is a known detoxifying mechanism of certain plant pathogens (Pedras et al., 2004). However, in a nutrient-poor environment it might be advantageous for fungal pathogens with high tolerance to stilbenes to carry out deglucosylation reactions in order to utilize the glucose moieties of astringin as an energy source.

Ceratocystis polonica also transforms astringin and its aglucone into dehydrodimers. Similar stilbene biotransformations mediated by laccase or peroxidase enzymes have been reported from the grape pathogen *Botritis cinerea* (Breuil et al., 1998; Rodrigues-Bonilla et al., 2011). Dimerization of resveratrol and pterostilbenes has been shown to be an oxidative process involving the 4'hydroxyl-group of the stilbene skeleton. It has been suggested that dehydrodimers are less toxic than monomeric stilbenes due to a reduction in solubility of the dimeric compounds, allowing the fungus to escape from contact with these phytoalexins (Breuil et al., 1999). Deglucosylation of dimeric compounds could lead to a further reduction in solubility. This supports the argument that, contrary to expectation, deglucosylation of stilbenes might be beneficial for *C. polonica*.

Phytoalexin biotransformation has been shown to be an important virulence factor for other plant pathogens. For example, the virulence of *B. cinerea* isolates could be correlated directly with their ability to dimerize stilbenes (Sbaghi et al., 1996). Similarly, the virulence of pathogens on *Pisum sativum* depends on fungal ability to detoxify the pea phytoalexin, pisatin (Vanetten et al., 2001). In this study we could show that *C. polonica* isolates used more than one pathway to detoxify host defense compounds and that virulence correlated well with the rate of stilbene biotransformation. This study also revealed that certain combinations of constitutive and inducible stilbene biotransformation strategies appear to be more favorable than others.

The avirulent isolate showed high β-glucosidase and laccase activity in culture, forming mainly piceatannol and dimeric stilbenes as astringin-derived metabolites. These pathways were also induced in response to metabolites extracted from spruce. The virulent isolate, on the other hand, produced higher amounts of piceatannol and stilbene dimers, as well as stilbene lactones. However, induction by application of spruce extract revealed only an increase in stilbene lactone formation.

Constitutive levels of enzymes for deglycosylation and dimerization were in any case higher in the virulent isolate than in the avirulent isolate. Virulence in *C. polonica* is therefore associated with a high initial activity for stilbene deglucosylation and dimerization (which decreases solubility and thus toxicity) followed by lactonization, which may be an early step in the β-ketoadipate pathway for degradation of the stilbene core structure for substrates for growth and respiration.

Furthermore, stilbene biotransformation by *C. polonica* may also be beneficial to associated bark beetle larvae. It has been shown that in the semi-alkaline conditions of insect guts (Balogun, 1969), catechol groups such as those present in the astringin core structure are spontaneously or enzymatically rearranged to form reactive quinones (Lin et al., 2010; Haruta et al., 2001). Amino acids that contain nucleophilic centers such as sulfhydryl and amino groups are susceptible to alkylation by these quinones (Son et al., 2010; Felton et al., 1992). Protein alkylation may reduce the quality of ingested food or even directly destabilize the peritrophic membrane (Barbehenn et al., 2008) in the bark beetle gut. Lactonization of catechol groups by *C. polonica* should prohibit quinone formation and thus protect bark beetles from the harmful effects of these stilbenoid defense compounds.

Although *C. polonica* and the bark beetle *I. typographus* co-occur, the basis for this mutualistic behavior is still largely unknown. It has been proposed that the fungus benefits by being dispersed to new hosts and gaining entry into the tree. The beetle on the other hand, is thought to benefit if the fungus helps weaken or kill the tree. The findings of this study provide indications that the beetle-fungus mutualism may also be based on the fungal metabolism of host defenses. Breakdown of stilbenes to less toxic products may increase the growth of bark beetle larvae.

3.5 MATERIALS AND METHODS

3.5.1 Biotransformation of astringin by *C. polonica*

Two *C. polonica* isolates (CMW 7749 = isolate 1 and CMW 7135 = isolate 2) provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (University of Pretoria, South Africa) were grown on 2% (w/v) malt extract agar (MEA) for 14 days at 25°C. Agar plugs (Ø=4 mm) from stationary cultures were placed in 15 ml test tubes for biotransformation assays. Then 2 ml sterile 2% (v/v) malt extract amended with astringin to a final concentration of 2 mg ml^{-1} was added to the test tubes and incubated at 28°C with shaking at 220 rpm. Negative control treatments contained astringin-amended medium without fungus and fungus grown in medium without astringin. Cultures were harvested 4 hours, 8 hours, 24 hours and 72 hours after the onset of the experiment.

Biotransformation processes were stopped with 50 µl HCl (2 N). The internal standard apigenin-3-O-glucoside was added to a final concentration of 0.1 mg/ml prior to analysis by LC-ESI-MS. The concentrations of biotransformation products were calculated relative to the internal standard.

3.5.2 Partial purification of fungal proteins responsible for stilbene biotransformation

Isolates 1 and 2 were cultured in 10% (v/v) carrot juice liquid culture for 6 days, harvested by centrifugation and lyophilized. Dried material was finely ground using a vibrating ball mill. Powdered mycelium (0.5 g) was extracted with 10 ml extraction buffer (50 mM Tris, pH 7.5, 5 mM ascorbic acid, 5 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MnCl_2$, 0.5 M NaCl, 10% glycerol, 1% polyvinylpyrrolidone (MW 360,000), 4% polyvinylpolypyrrolidone and 0.1% Tween 20) at 4 °C for 30 minutes shaking. Crude fungal protein extract in extraction buffer was loaded onto an open column of concanavalin A sepharose (GE Healthcare, Munich, Germany) with a bed volume of 5 ml. The protein extract was incubated with the concanavalin A matrix for 30 minutes at 4 °C before washing un-bound proteins off with 5 bed volumes of washing buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 10% glycerol). Elution of bound proteins was achieved using 2 column volumes of washing buffer amended with 500 mM α-D-methylglucopyranoside. Proteins from the column eluate were desalted at 4°C into 50 mM MOPSO (pH 6.8) containing 10% glycerol using a HiPrepTM 26/10 (GE Healthcare) desalting column on an AEKTA 900 chromatography system (GE Healthcare). The desalted protein fraction was loaded onto a 5 ml DEAE sepharose column and washed with 2 column volumes DEAE washing buffer (50 mM MOPSO, pH 6.8, 10% glycerol) at a flow rate of 5 ml/min. Proteins were eluted from the column with DEAE wash buffer adjusted with NaCl using a step-wise gradient (100 mM NaCl, 200 mM NaCl, 300 mM NaCl, 500 mM NaCl and 1 M NaCl). Elution steps and fraction volumes were 10 ml. Stilbene biotransformation activity was determined for crude extracts as well as for proteins that were eluted from the concanavalin A and DEAE sepharose columns. Samples from the flow-through of both columns were also tested for astringin biotransformation activity. Enzyme activities were assayed in 300 µl reaction volumes containing 200 µl enzyme from the purification steps described above and 100 µg astringin in DEAE washing buffer. Reaction mixtures were incubated at 30°C for 4 hours before stopping the reaction with 10 µl 0.1 M HCL. After removing the protein by centrifugation, 20 µl reaction mixture was analyzed by LC-ESI-MS.

3.5.3 *In vitro* astringin biotransformation assay

Mycelium was cultured as above for 3 days. Each culture (10 ml) was sub-cultured in 50 ml 10% (v/v) carrot medium. To induce biosynthesis of enzymes for stilbene biotransformation, the medium of 4 biological replicates was amended with 2 mg crude spruce extract. As controls, 4 other replicates were sub-cultured without adding spruce extract. Sub-cultured mycelium was harvested after 4 days by centrifugation and ground to a fine powder using a mortar and pestle. Then 100 mg of ground mycelium was extracted with 5 ml extraction buffer as above. Insoluble material was removed from extracts by centrifugation, and the resulting supernatant filtered with a syringe filter with exclusion size of 0.2 µm.

Astringin biotransformation activity was assayed at 30ºC in 2 ml reaction volumes containing fungal protein in extraction buffer and 100 µg ml^{-1} astringin. A 200 µl sub-sample was taken from each assay reaction 45 minutes, 2 hours, 4 hours and 8 hours after the assays were initiated. The biotransformation reaction was stopped and analyzed as above.

3.5.4 Inoculation of *P. abies* saplings with *C. polonica*

Two *C. polonica* isolates were grown on 2% (w/v) malt extract agar (MEA) for 12 days at 25°C. Eight-year-old *P. abies* saplings originating from the 3369-Schongau clone (Samenklenge und Pflanzengarten Laufen, Germany) were grown in an outdoor plot for four years prior to the experiment. Inoculations of saplings with *C. polonica* were performed three weeks after their 'spring'-flush (10 June 2008). A bark plug, 8 mm in diameter, was removed between the second and third branch-whorl from the upper part of the sapling with a cork borer. An 8 mm plug from one of the two *C. polonica* cultures was placed into the wound with the mycelium oriented toward the wood surface and sealed with parafilm. For the control treatment, plugs of sterile MEA were inserted into the wound.

Bark tissue samples from inoculated and wounded saplings were harvested 2, 7, 14 and 28 days after the onset of the experiment. Fungal lesions were measured with a caliper. Five saplings were sacrificed per time point for each treatment. Bark material was flash-frozen immediately after harvest in liquid nitrogen and stored at -80ºC.

3.5.5 Quantitative real-time PCR of *STS* from *P. abies*

Total RNA from inoculated treatment and wounded control bark was isolated with the Invitrap ® Spin Plant RNA Mini Kit (Invitek, Berlin, Germany) following the protocols of the manufacturer except that an additional DNA digestion step was included (RNase Free DNase set,

Qiagen). RNA was quantified by spectrophotometry. Reverse transcription of 1 µg RNA into cDNA was achieved by using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol $PolyT_{(12-18)}$ primer (Invitrogen) in a reaction volume of 20 µl. cDNA was diluted to 10% (v/v) with deionized water. One µl diluted cDNA was used as template for quantitative real-time PCR in a reaction mixture containing Brilliant SYBR Green QPCR Master Mix ® (Stratagene), 10 pmol forward and 10 pmol reverse primer. *PaSTS* transcripts were amplified using the forward primer 5'-GTGGCGAGCAGAACACAGACTTC-3' and the reverse primer 5'-CAGCGATGGTACCTCCATGAACG-3'. This primer pair was designed to amplify 140 base pairs of both *STS*1 and *STS*2 simultaneously. PCR was performed using a Stratagene MX3000P thermocycler using the following cycling parameters: 5 min at 95°C followed 40 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, and a melting curve analysis from 55°C to 95°C. Reaction controls included non-template controls as well as non-reverse transcribed RNA. *STS* gene abundance was normalized to the abundance of the ubiquitin gene (Schmidt et al., 2010) (GenBank accession number EF681766) amplified with the forward primer 5'-GTTGATTTTTGCTGGCAAGC-3' and the reverse primer 5'-CACCTCTCAGACGAAGTAC-3'. Relative transcript abundance was calculated from three technical replicates of five biological replicates and calibrated against the transcript abundance of five non-wounded control saplings.

3.5.6 Metabolite extraction from spruce

For extraction of phenolic compounds, spruce tissue was ground to a fine powder under liquid nitrogen and lyophilized. Approximately 40 mg dried tissue was extracted with 2 ml analytical grade methanol for 4 hours at 4°C. Insoluble material was pelleted by centrifugation and the supernatant was recovered. Insoluble material was re-extracted with 1.5 ml methanol for 16 hours. Extracts were combined and evaporated to dryness under a stream of nitrogen. Dried samples were re-dissolved in 1 ml methanol containing 100 µg/ml of the internal standard apigenin glucoside. For LC-ESI-MS samples were diluted to 20% (v/v) with methanol.

3.5.7 LC-ESI-MS

Compounds from analytical samples and enzyme assays were separated on a Nucleodur Sphinx RP18ec column with dimensions of 250 X 4.6 mm and a particle size of 5 µm (Macherey Nagel, Dueren, Germany) using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA). The total mobile phase flow rate for chromatographic separation was 1.0 ml min^{-1}. The column temperature was maintained at 25°C. Phenolic compounds from spruce and fungal

biotransformations were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B respectively with the following elution profile: 0-1 min, 100% A; 1-25 min, 0-65% B in A; 25-28 min 100% B; and 28.1-32 min 100% A. Products from enzyme assays were separated with the elution profile: 0-1 min, 100% A; 1-18 min, 0-100% B in A; 18-19 min 100% B; and 19.1-22 min 100% A.

Compound detection and quantification was accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Brucker Daltronics, Bremen, Germany). Flow coming from the column was diverted in a ratio of 4:1 before entering the mass spectrometer electrospray chamber. ESI-MS was operated in negative mode scanning $m\,z^{-1}$ between 50 and 1600 with an optimal target mass of 405 $m\,z^{-1}$. The mass spectrometer was operated using the following specifications: skimmer voltage: 60 V; capillary voltage: 4200 V; nebulizer pressure: 35 psi; drying gas: 11 l min^{-1}; gas temperature: 330 ºC. Capillary exit potential was kept at -121 V.

Brucker Daltronics Quant Analysis v.3.4 software was used for data processing and compound quantification using a standard smoothing width of 3 and Peak Detection Algorithm v. 2. Linearity in ionization efficiencies was verified by analyzing serial dilutions of randomly selected samples. Ion suppression was controlled for by calculating analyte:internal standard ratios in serial diluted samples in which the internal standard was maintained at the same concentration. An external calibration curve for astringin was created by linear regression. Process variability in different analyses was calculated relative to the internal standard.

3.5.8 Sample preparation for NMR

Culture medium samples from biotransformation experiments were subjected to solid phase extraction on RP-18 material. After loading, the columns were washed with water, dried with nitrogen gas and finally eluted with methanol. The methanol extracts were separated by means of HPLC-SPE chromatography and peaks of interest were collected by on-line trapping. The HPLC-SPE system consisted of an Agilent 1100 chromatography system and a J&M photodiode array detector (DAD, detection 200-650 nm) connected to a Spark Prospekt 2 solid-phase extraction (SPE) device (Spark Holland, Emmen, The Netherlands), equipped with HySphere resin GP cartridges (10 × 2 mm, 10 μm). Separations were performed with linear gradient elution (water (A) and methanol (B), both solvents containing 0.1% formic acid, starting with 100% A at 0 min, kept isocratic for 1 min, within 20 min to 100% B, kept isocratic for 5 min, back to 100% A in two min and finally 7 min at 100% A for re-equilibration) on A Macherey-Nagel Isis RP-18e column (MN Germany, 250x4 mm). The make-up flow for SPE trapping was set to 2.5 ml min^{-1} using Millipore-grade water. The SPE cartridges were subsequently dried using pressurized nitrogen. Gradient grade MeCN was used to

extract the trapped analytes from the GP cartridges into HPLC glass vials. After evaporation to dryness using nitrogen gas, the samples were reconstituted with 80 µl deuterated methanol and transferred into 2 mm i.d. capillary NMR tubes and subjected to NMR measurements. Hystar 3.2 software was used to coordinate the LC-SPE experiments and Topspin™ 2.1 software was used to control the NMR spectrometer and to perform data processing.

3.5.9 NMR and HRESIMS analysis

^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, TOCSY, HMBC, and HSQC spectra were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany), operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C. A TCI cryoprobe (5 mm) was used to measure spectra at a probe temperature of 300 K. Spectra are referenced to the residual signal of MeOD-d_4 at δ 3.31. Capillary tubes (2 mm i.d.) were used for all NMR measurements.

HRESIMS was recorded on a UPLC–MS/MS system consisting of an Ultimate 3000 series RSLC (Dionex, Idstein, Germany) system, and an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UPLC was performed using a Dionex Acclaim C18 Column (150 × 2.1 mm, 2.2 µm) at a constant flow rate of 300 µl min^{-1}. A binary solvent system of H_2O (solvent A) and MeCN (solvent B), both containing 0.1% formic acid, was used as follows. 0 min: 20% B, 6 min: 95% B, 10 min: 95% B, then brought back to the initial conditions and held for 4 more min in order to re-equilibrate the column for the next injection. Full-scan mass spectra were generated using 30,000 resolving power, the mass accuracy was better than 3 ppm.

3.5.10 Fungal growth in the presence of astringin

Petri-dishes with synthetic nutrient agar (Nirenberg and O'Donnell, 1998) amended with astringin were prepared by cooling the autoclaved medium to 55°C and adding astringin in ethanol to a final concentration of 100 µg ml^{-1} medium. Medium for negative control treatments was amended with ethanol only. Medium (25 ml) was dispensed in Petri-dishes (Ø=10 cm). After the medium set, agar plugs (Ø=4 mm) from 14-day-old *C. polonica* stationary cultures (CMW 7749 or CMW 7135) were placed in the middle of each Petri-dish. Cultures were sealed with Parafilm and incubated at 26°C. Diameters of the expanding fungal cultures were measured every 24 hours for 5 days. Growth rates were calculated using the slope of linear growth curves and relative growth on astringin was calibrated against the mean growth rate of each isolate on control medium.

3.5.11 Statistical analysis

Graphical representations of results are presented as mean ± standard error. Tabulated results are presented as mean ± standard deviation. Normality of data was tested for with the Shapiro-Wilk test.

Statistical significance of differences in *PaSTS* transcript accumulation and stilbene concentrations in living trees was determined using a two-way analysis of variance (ANOVA) on log-transformed data. Differences between fungal biotransformations of astringin as well as differences of fungal growth on astringin amended medium were analyzed with the non-parametric Kruskal-Wallis test, as data could not be normalized.

Differences in fungal growth in spruce were analyzed using a one-way ANOVA on square root-transformed data. Differences in reaction rates of fungal biotransformation of astringin on enzyme level were analyzed using two-way ANOVAs of untransformed or reciprocally transformed data. Following ANOVA, differences in means were calculated using Tukey's post-hoc pair-wise comparisons test at a 95% confidence level. Analyses were conducted using the open source software R (v. 2.81) and the LAERCIO package for Tukey's pair-wise comparisons.

4. Research Chapter III

Flavan-3-ol biosynthesis in spruce in response to attack by the bark beetle associated fungus *Ceratocystis polonica*

4.1 ABSTRACT

Proanthocyanidins (PAs) which are produced by plants in foliage, fruit, bark and seed coats are ubiquitous polymeric compounds consisting of flavan-3-ol units, such as 2,3-*trans*-(+)-catechin and 2,3-*cis*-(-)-epicatechin. Although the biosynthesis of flavan-3-ols has been studied in angiosperms such as grapevine and alfalfa, very little is known about their ecological roles and biosynthesis in conifer species. To investigate the biosynthesis of these compounds in the economically and environmentally important conifer species, *Picea abies* (Norway spruce), genes encoding enzymes called leucoanthocyanidin reductases (LAR), which are responsible for the biosynthesis of 2,3-*trans*-(+)-flavan-3-ols, were identified and functionally characterized both *in vitro* and *in vivo* by overexpression in transgenic spruce. This was accompanied by analysis of the flavan-3-ol and PA content of spruce saplings which were wounded or artificially inoculated with the bark beetle associated fungus *Ceratocystis polonica*, the causal agent of an economically important forestry disease in Europe. Reverse-phase high pressure liquid chromatography (HPLC) was employed for the analysis of monomeric and dimeric flavan-3-ols in combination with a novel acid hydrolysis method and normal phase size exclusion chromatography for the analysis of polymers. Whereas spruce bark was found to contain flavan-3-ol units with only 2,3-*trans*-stereospecificity, dimeric and polymeric PAs contained flavan-3-ols with both 2,3-*trans*-(+)- and 2,3-*cis*-(-)-stereospecificity. Levels of monomers as well as PAs with a higher degree of polymerization increased dramatically in spruce bark after infection by *C. polonica*. In accordance with their role in the biosynthesis of 2,3-*trans*-(+)-flavan-3-ols, transcript abundance of *PaLAR* genes also increased significantly during fungal infection. Bioassays with *C. polonica* revealed that 2,3-*trans*-(+)-catechin and PAs act as agents inhibiting fungal growth and thus can be considered chemical defense compounds in *P. abies*.

4.2 INTRODUCTION

Proanthocyanidins (PAs), also known as condensed tannins, are oligomeric or polymeric natural products produced in the vegetative structures of most woody plant species as well as in the seed coats of many herbaceous plants (Dixon et al., 2005). The basic structural units of these compounds are flavan-3-ols, based on a C_6-C_3-C_6 flavonoid skeleton. Flavan-3-ol units in PA chains can differ structurally according to the stereochemistry of the asymmetric carbons on the C-ring, which are commonly either 2,3-*trans* giving (+)-catechin or 2,3-*cis* giving (-)-epicatechin (Figure 4.1). The structural diversity of PAs is further enhanced by changes in the structure of the B-ring, which can be monohydroxylated (2,3-*cis*-(-)-epiafzelechin or 2,3-*trans*-(+)-afzelechin), dihydroxylated (2,3-*cis*-(-)-epicatechin or 2,3-*trans*-(+)-catechin) or trihydroxylated (2,3-*cis*-(-)-epigallocatechin or 2,3-*trans*-(+)-gallocatechin). In the formation of PAs, flavan-3-ol units are commonly linked from the C4 position of the extender units to the C8 position of the terminal unit (referred to as a 4 → 8 linkage) (Figure 4.1), or less common is a C4 → C6 linkage. The greatest source of structural variability in these compounds, however, is the length of the polymer (degree of polymerization), as PAs can vary in length from dimers to hundreds of monomer units per chain (for complete overview, see Ferreira and Slade, 2002).

Within angiosperms, flavan-3-ols and PAs appear to function as protectants against various biotic and abiotic stresses. Their role in providing tolerance to UV irradiation (Jaakola et al., 2004) and ozone fumigation (Karonen et al., 2006) by decreasing oxidative stress has been demonstrated in numerous plant species. These compounds are also reported to be constitutive defenses against mammalian (Theodoridou et al., 2010) and insect herbivory (Feeny, 1970; Donaldson and Lindroth, 2004). Moreover, PAs and flavan-3-ols have been shown to negatively affect bacterial growth (Scalbert, 1991) as well as transcription of quorum-sensing-regulated genes which are necessary for bacterial biofilm formation (Vandeputte et al., 2010). These compounds can also inhibit fungal spore germination (Andebrhan et al., 1995) and suppress melanin biosynthesis (Chen et al., 2006) which is an important virulence factor in many plant pathogenic fungal species (Ebole, 2007). PAs and flavan-3-ols have also been studied for many years for their biomedical applications in the prevention of oxidative stress (Nichols and Katiyar, 2010) and inhibition of cholesterol accumulation (Blade et al., 2010) in mammals. However, in conifers the functions of flavan-3-ols and PAs during insect or pathogen attack are poorly studied, despite their abundance in economically important and widespread genera such as spruce and pine. In spruce, it has been suggested that phenolic compounds may play a pivotal role in defense against herbivores and pathogens due to the appearance of fluorescent inclusion bodies in the phloem parenchyma cells of pathogen challenged bark (Franceschi

et al., 2005). Since PAs are abundant constituents of spruce bark, it is likely that these compounds might be involved in this apparent defensive response.

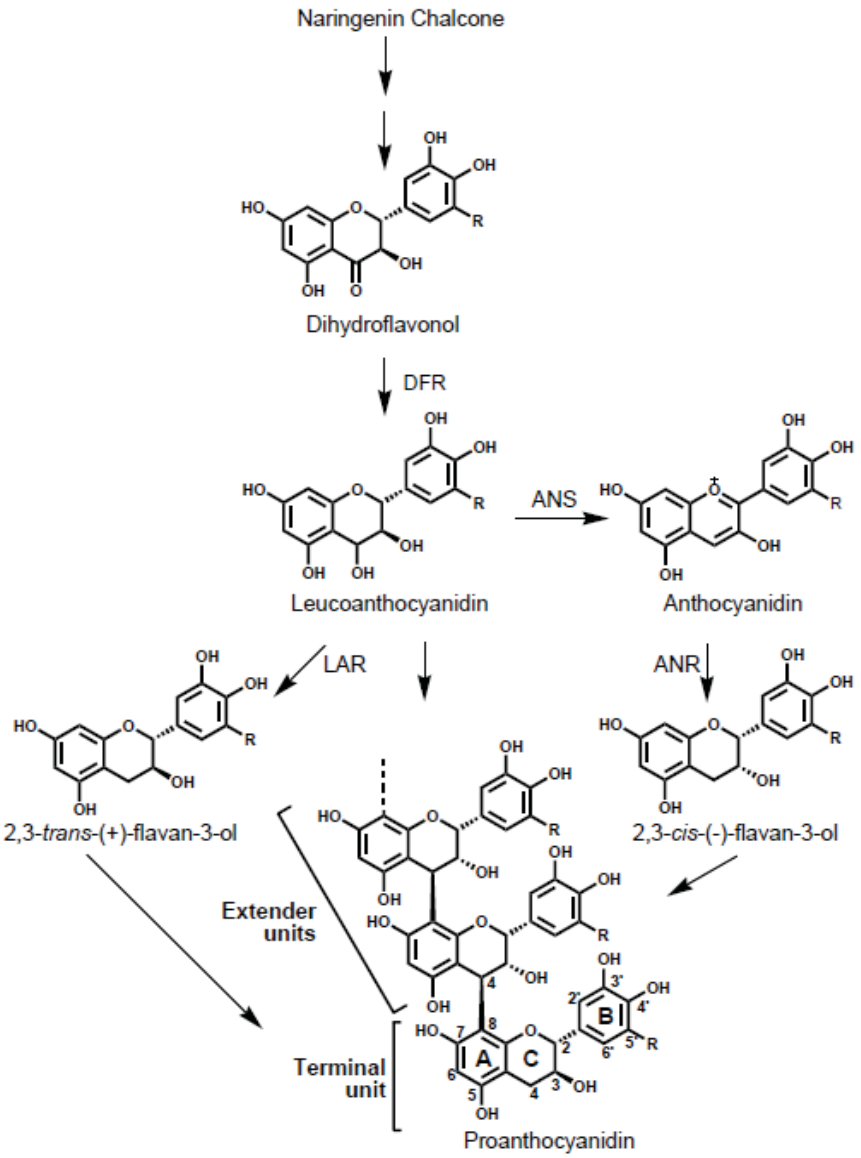


Figure 4.1: Biosynthesis of proanthocyanidins, 2,3-*trans*-(+)- and 2,3-*cis*-(-)-flavan-3-ols. Dihydroflavonol reductase (DFR) reduces dihydroflavonol to leucoanthocyanidin which serves as substrate for leucoanthocyanidin reductase (LAR) to form 2,3-*trans*-(+)-flavan-3-ols or for anthocyanidin synthase (ANS) to form anthocyanidin. Anthocyanidin is reduced by anthocyanidin reductase (ANR) to form 2,3-*cis*-(-)-flavan-3-ols. Flavan-3-ols appear to form terminal as well as extender units of proanthocyanidins and leucoanthocyanidins have been proposed to form extender units of growing proanthocyanidin chains.

The biosynthesis of flavan-3-ols and the accumulation of PAs have been studied in angiosperm species of the Vitaceae (Bogs et al., 2005), Rosaceae (Pfeiffer et al., 2006; Almeida et al., 2007) and Fabaceae (Xie et al., 2004; Pang et al., 2009) as well as in the model plant *Arabidopsis thaliana* (Xie et al., 2004; Kitamura et al., 2010). Monomers of PAs are produced in the last steps of the flavonoid pathway. Depending on the stereochemistry of the asymmetric carbons on the C-ring, there are two biosynthetic routes for the formation of flavan-3-ols from the common substrate, leucoanthocyanidin (Figure 4.1). For the biosynthesis of the 2,3-*cis*-(-)-flavan-3-ol (e. g. 2,3-*cis*-(-)-epicatechin), leucoanthocyanidin is converted to anthocyanidin by anthocyanidin synthase (ANS). In the second phase of this 2-step process, anthocyanidin is reduced by anthocyanidin reductase (ANR) to form the flavan-3-ol (Xie et al., 2004). For the biosynthesis of 2,3-*trans*-(+)-flavan-3-ol (e. g. 2,3-*trans*-(+)-catechin), leucoanthocyanidin is reduced to the corresponding flavan-3-ol (Tanner et al., 2003) by leucoanthocyanidin reductase (LAR) (Figure 4.1). ANR and LAR are both NADPH/NADH-dependent isoflavone-like reductases belonging to the reductase-epimerase-dehydrogenase superfamily. Genes coding for these enzymes can occur as single copies or as gene families in different PA producing plant species (Bogs et al., 2005; Paolocci et al., 2007). Although the biosynthesis of flavan-3-ols has only been documented for a few plant species, it has become clear that not all plant species utilize both pathways for the production of PA units (Xie et al., 2004).

Despite considerable efforts, the mechanism by which PA polymers are formed is presently not well understood. The most commonly accepted hypothesis is that leucoanthocyanidins form extender units, which are oxidatively coupled, via quinone methides, to a flavan-3-ol terminal unit (Creasy and Swain, 1965). Alternatively, formation of PAs by non-enzymatic oxidative coupling of flavan-3-ols or anthocyanidins has been proposed (Haslam, 1980). However, because PA composition is very specific for individual plant species, an enzyme-mediated mechanism for PA polymerization has also been envisioned (Stafford, 1983).

To date very little is known about the biosynthesis and structure of monomeric and polymeric flavan-3-ols in conifers. Studying the biosynthesis and accumulation of these compounds in conifer tree species, whose biosynthetic routes could be ancestral to angiosperm pathways, might lead to greater understanding and knowledge in this field, which is of interest to both plant biochemistry and biomedical research. In consequence we identified *LAR* genes involved in 2,3-*trans*-(+)-flavan-3-ol biosynthesis in the conifer species *Picea abies* (Norway spruce) and studied their function, both *in vitro* and *in vivo* by overexpression in transgenic spruce. To gain insights into the biological relevance of flavan-3-ols and PAs in *P. abies*, we determined the constitutive flavan-3-ol and PA profiles in spruce bark using novel analytical approaches and monitored the quantitative and qualitative changes

in these compounds after infection by the bark beetle-associated blue stain fungus (*Ceratocystis polonica*).

4.3 RESULTS

4.31 Flavan-3-ol content in Norway spruce (*P. abies*) bark infected with *C. polonica*

In order to determine if *P. abies* modulates its monomeric, dimeric and polymeric flavan-3-ol content in response to infection by the bark beetle associated fungus *Ceratocystis polonica*, four-year old spruce saplings were wounded and inoculated with two strains of this fungus (isolate 1 and isolate 2) differing in virulence. Controls included unwounded saplings and those subjected to wounding without fungal inoculation. Bark tissue was harvested for analysis 2, 7, 14 and 28 days after treatment.

Monomeric flavan-3-ols in spruce bark consisted of mixtures of 2,3-*trans*-(+)-catechin and 2,3-*trans*-(+)-gallocatechin with no detectable levels of 2,3-*cis*-(-)-epicatechin. Levels of catechin and gallocatechin (Figure 4.2A) increased significantly over the experimental time-course of 28 days in inoculated as well as in wounded control bark ($p < 0.001$), but higher levels of both flavan-3-ols were observed in inoculated bark when compared to wounded control bark ($p < 0.001$). There were no differences in the responses of spruce bark to infection by the virulent and avirulent fungal isolates. The ratio of catechin:gallocatechin changed from 11:1 at 2 days after infection to 20:1 at 28 days after infection (Figure 4.2A).

Dimeric PAs that could be detected in spruce bark were mainly the procyanidins (dihydroxylated B-ring) PA B1 (2,3-*cis*-(-)-epicatechin-(4β→8)- 2,3-*trans*-(+)-catechin) and PA B3 (2,3-*trans*-(+)-catechin-(4β→8)- 2,3-*trans*-(+)-catechin). Low levels of dimeric prodelphinidins (trihydroxylated B-ring) could also be detected, but were not quantified. Concentrations of the dimeric PAs, B1 and B3 increased significantly over the 28 day period (Figure 4.2B) in inoculated and in wounded control bark of spruce saplings ($p < 0.001$). Major changes in dimer concentrations in treatments were due mainly to significantly higher PA B1 content in infected bark compared to concentrations in wounded controls ($p < 0.001$). Increases in PA B3 were similar in inoculated and wounded bark over the time course of the experiment ($p = 0.055$). Between 2 days and 28 days post-inoculation the ratio of PA B1:PA B3 changed in inoculated bark more than 10-fold from 0.11:1 to 1.28:1.

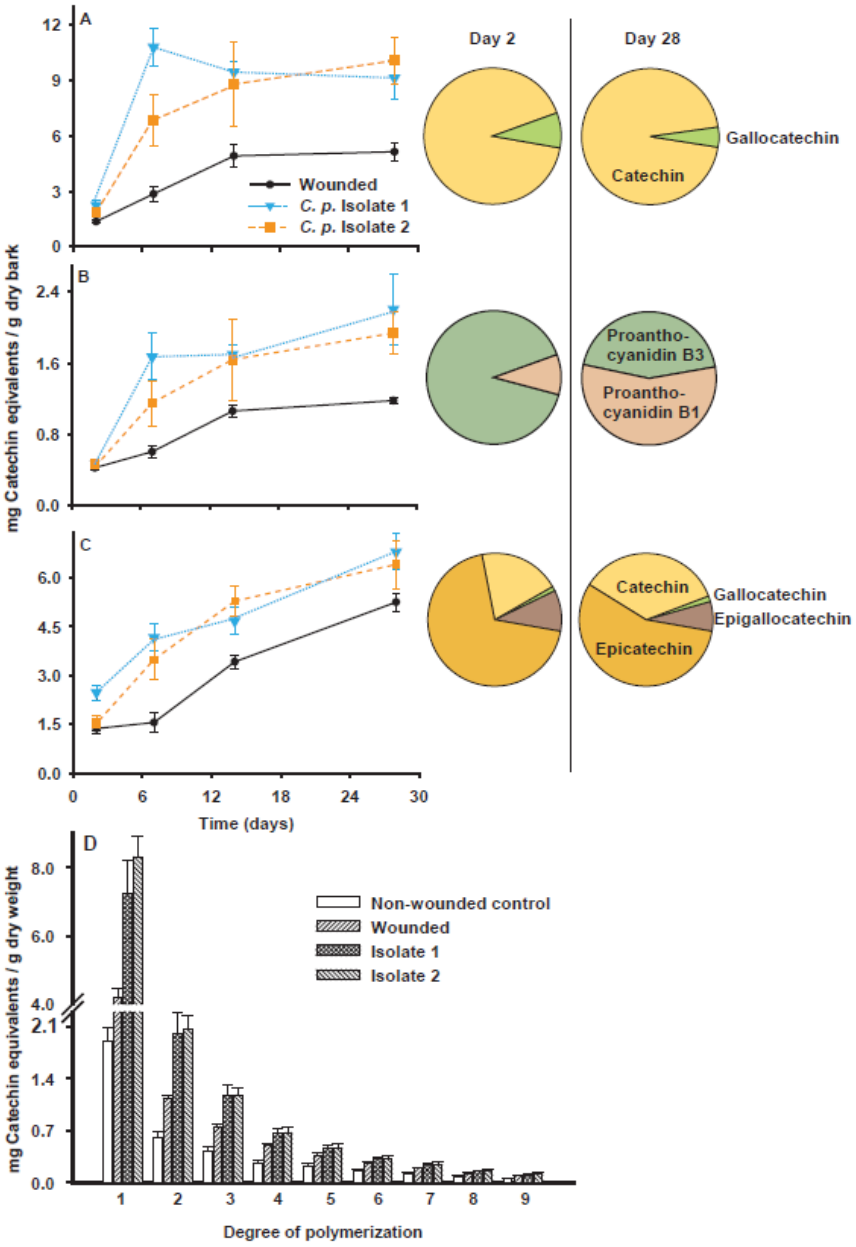


Figure 4.2: Concentration of soluble monomeric and polymeric flavan-3-ols in spruce bark which was infected with *C. polonica* or wounded over a time course of 28 days. Total monomers consisting of catechin and gallocatechin **(A)** and total dimers consisting of proanthocyanidin B1 and B3 **(B)** are presented. Total polymers are expressed as the total of monomeric units detected after hydrolysis; monomers detected were gallocatechin, catechin, epigallocatechin and epicatechin **(C)**. The pie charts represent the ratio of the different soluble monomeric flavan-3-ols **(A)**, dimeric proathocyanidins **(B)** and the monomeric flavan-3-ols detected after hydrolysis **(C)** as determined 28 days after inoculation for isolate 2 infected spruce bark. The degree of polymerization **(D)** of flavan-3-ols at 28 days after the onset of infection is expressed as monomer-equivalents. Error bars represent standard errors.

A method was developed to measure the amount of polymeric PAs as total monomeric units after complete hydrolysis. Monomers detected in spruce bark tissue after hydrolysis were 2,3-*trans*-(+)-catechin and 2,3-*trans*-(+)-gallocatechin and high concentrations 2,3-*cis*-(-)-epicatechin and 2,3-*cis*-(-)-epigallocatechin. Total monomers after hydrolysis (Figure 4.2C) increased significantly over the 28 day infection period in wounded control as well as in infected bark ($p < 0.001$). However, hydrolysis yielded significantly more units with 2,3-*trans* stereochemistry in infected tissue than in wounded control bark ($p < 0.001$). No statistically different levels of the 2,3-*cis* stereoisomers (epicatechin and epigallocatechin) could be observed between infected and wounded control bark. At 28 days after infection the ratio of hydrolyzed monomers epicatechin:catechin:gallocatechin: epigallocatechin was 8:5:0.2:1 in infected spruce bark, which differs significantly from the 7:2:0.1:1 ratio observed 2 days post-inoculation.

In order to determine the degree of polymerization in non-wounded, wounded and infected bark tissue, size exclusion chromatography coupled to fluorescence detection (FLD) (Kelm et al., 2006) was conducted (Figure 4.3).

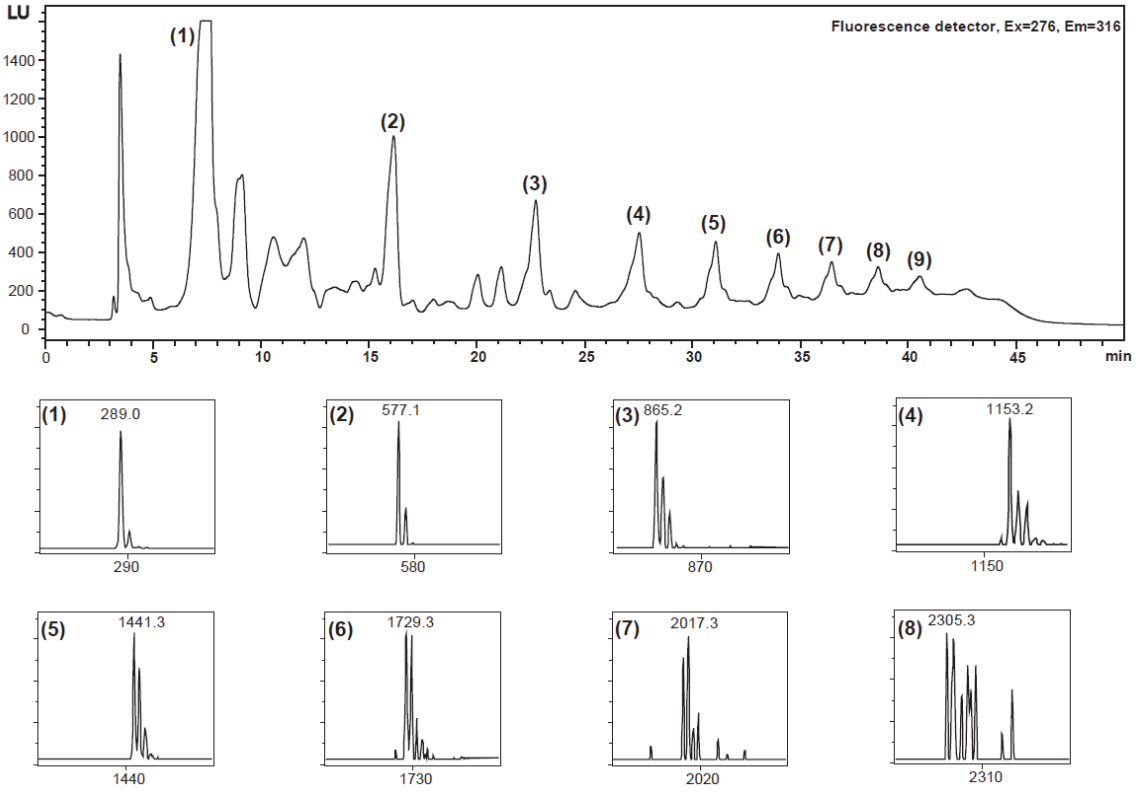


Figure 4.3: LC-FLD chromatogram of proanthocyanidin polymers with mass spectra of each polymeric compound detected.

By using this method polymers up to 9-mers could be quantified (Figure 4.2D). The abundance of polymers in all analyzed treatments decreased with degree of polymerization ($p<0.0001$). There were for example significantly more monomer units allocated to trimers than to tetramers. At 28 days after infection flavan-3-ols with degree of polymerization up to a 9-mer were significantly higher in infected tissue than in the wounded control ($p < 0.0001$), whereas the wounded control treatment contained significantly higher levels than the non-wounded controls ($p < 0.0001$). There were, however, no significant differences in the degree of polymerization between trees infected by the different fungal isolates.

4.3.2 Identification of spruce leucoanthocyanidin reductase genes and their phylogenetic relationships

In order to study the biosynthesis of flavan-3-ols in Norway spruce, *LAR* gene candidates were identified by BLAST sequence comparisons with known genes from angiosperms (Tanner et al., 2003; Pfeiffer et al., 2006). Searching more than 180,000 expressed sequence tags from *Picea sitchensis* and 250,000 from *Picea glauca* in the Treenomix database (Ralph *et al*., 2008) revealed numerous distinct contigs from both spruce species with similarity to *LAR* coding regions from angiosperm species. By using sequences from *P. glauca* and *P. sitchensis* as templates for primer design, three full-length *LAR* candidates could be amplified from *P. abies* cDNA by PCR. Predictive algorithms suggested that N-terminal signal sequences were absent in candidate *LAR* sequences.

Phylogenetic analysis revealed a clear evolutionary divergence between ANR and LAR amino acid sequences (which shared about 10% sequence similarity) (Figure 4.4). There was also a clear distinction between angiosperm and gymnosperm sequences for both enzyme classes. Conifer LAR protein sequences shared only between 51% and 57% sequence identity with LAR sequences from angiosperms whereas deduced amino acid sequences for *Pa*LAR were between 79% and 87% identical and had 62% to 64% similarity to an uncharacterized LAR sequence from *Pinus taeda* (Bogs et al., 2005). Phylogenetic analysis also revealed separate sub-clades for LAR protein sequences from the Rosaceae (Pfeiffer et al., 2006; Almeida et al., 2007) and the Fabaceae (Tanner et al., 2003), which showed between 63% and 65% similarity.

Two *Pa*ANR-like candidate sequences were 66% similar and shared 62% and 70% sequence identity with an uncharacterized ANR from *Ginkgo biloba* (Shen et al., 2006). ANR-like protein sequences from *P. abies* and selected angiosperm ANR had 50% to 57% sequence similarity.

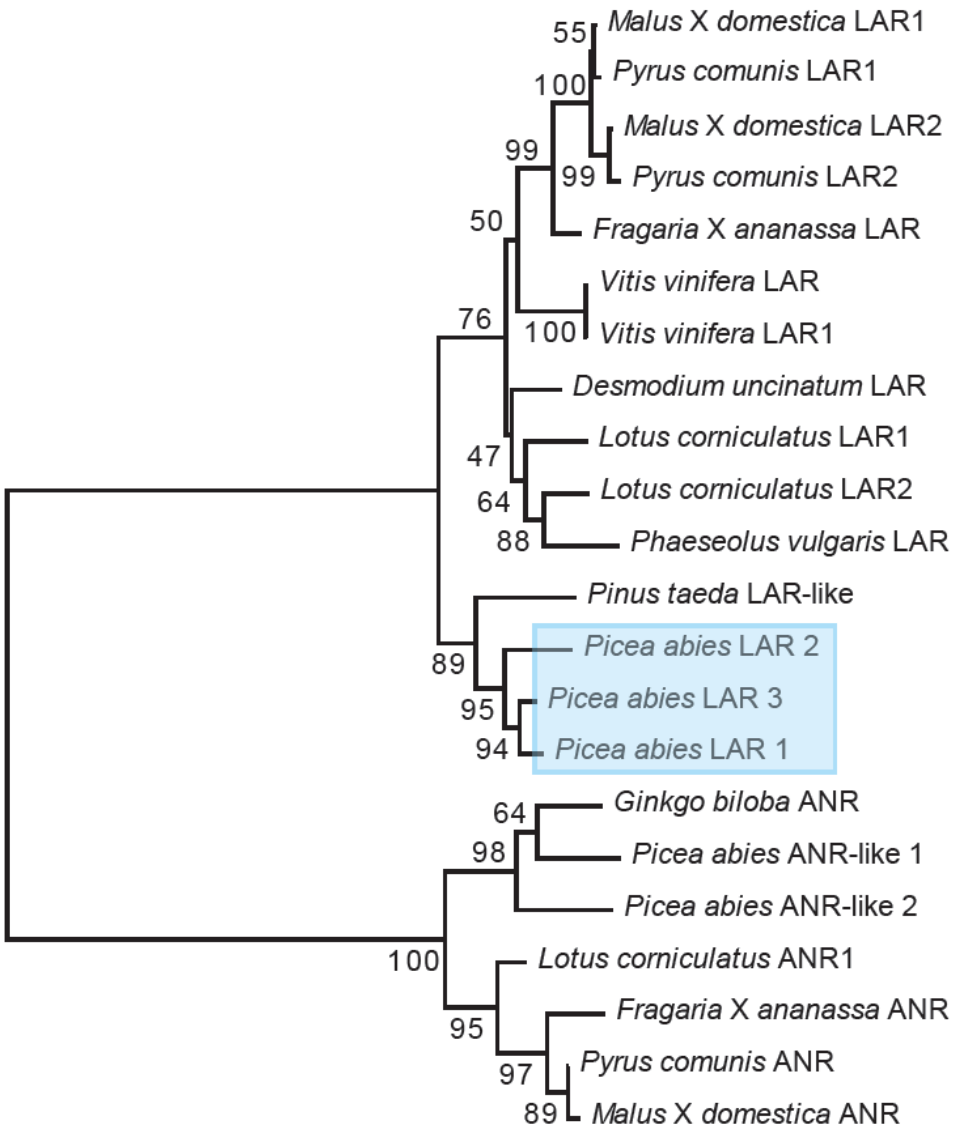


Figure 4.4: Neighbor-joining tree of the deduced amino acid sequences of *PaLAR* and sequences of characterized LAR enzymes from angiosperms. Evolutionary distances were calculated using the JTT matrix. The tree was constructed using the minimum evolution method with pair-wise elimination of alignment gaps. Bootstrap values are based on 10,000 replicate trees.

4.3.3 *In vitro* functional characterization of recombinant *Pa*LAR

Functional characterization of the three putative LAR enzymes from *P. abies* was accomplished via heterologous expression in a bacterial system. SDS-PAGE as well as Western blotting revealed that the 41 kDa subunit of these homodimeric enzymes was present in low concentrations. Catalytic activity of recombinant *Pa*LAR was determined by incubating expressed enzymes with NADPH and leucocyanidin or leucodelphinidin. All three *Pa*LAR accepted the

substrate leucocyanidin and yielded 2,3-*trans*-(+)-catechin (Figure S4.1). None of the *Pa*LAR enzymes accepted the trihydroxylated substrate leucodelphinidin under the assay conditions employed, which could form 2,3-*trans*-(+)-gallocatechin. Optimal LAR activity with leucocyanidin was attained at 25°C at a pH of 7.5 (Figure S4.1). Low levels of catechin detected in control reactions were due to contamination in the substrate, which could easily be corrected for by simple subtraction.

4.3.4 *In vivo* characterization of *Pa*LAR enzyme function by over-expression of *PaLAR*3 in transgenic *P. abies*

To confirm the *in vivo* activity of our LAR genes, embryonic spruce tissue (line 186/3c VIII) was transformed with *PaLAR*3 under the control of the inducible promoter *ubi*1 using a disarmed *Agrobacterium tumefaciens* strain (Schmidt et al., 2010). Two transgenic *PaLAR*3 seeding lines (line 1 and line 2) were obtained. One-year-old transformed saplings grown in potting soil were used for transcript and metabolite analysis.

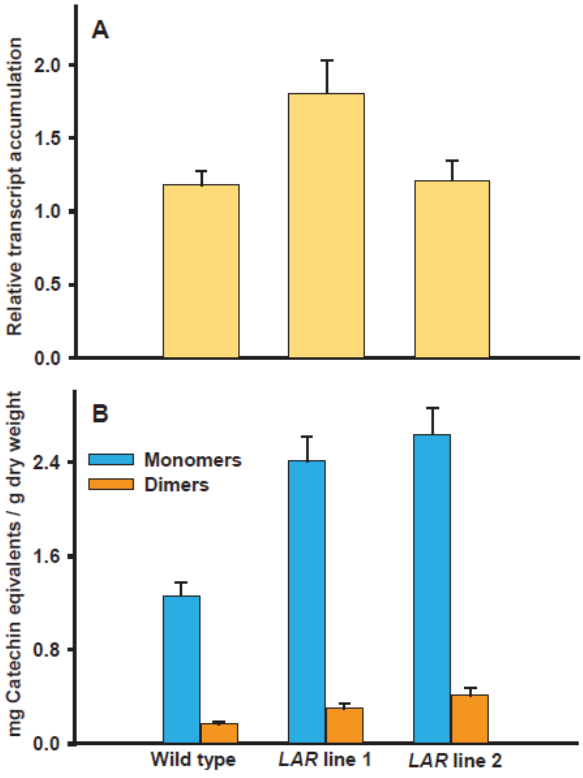


Figure 4.5: *PaLAR*3 transcript accumulation (A) and metabolite accumulation (B) in wild type and two transgenic *LAR*3 over-expressing lines. Transcript abundance was measured by quantitative real-time PCR using CYBR green for detection, normalized against *PaUBI* and calibrated against one wild-type replicate. Metabolites were measured by LC-FLD. Error bars represent standard errors.

Relative transcript accumulation of *PaLAR*3 in transgenic seedling stems was determined by quantitative real-time PCR. *PaLAR*3 transcript abundances were approximately 1.56-fold higher in *PaLAR*3 line1 than in *PaLAR*3 line 2 and wild-type seedlings ($p = 0.03$) (Figure 4.5 A). Monomeric flavan-3-ols as well as PA concentrations (polymers not shown) in both *PaLAR*3 lines were significantly higher than in the wild-type ($p < 0.009$), providing evidence that the *PaLAR*3 gene encodes a functional LAR enzyme (Figure 4.5 B).

4.3.5 Effect of fungal inoculation on *LAR* transcription

To determine if fungal inoculation leads to the activation of *LAR* transcription in spruce, RNA from bark tissue of wounded and inoculated spruce saplings was extracted and transcribed to cDNA. Transcript accumulation of *PaLAR*, measured by quantitative real-time PCR, increased in inoculated saplings compared to the wounded control saplings (Figure 4.6). *PaLAR*1 and *PaLAR*3 transcript levels increased significantly in inoculated saplings between 2 and 7 days post-inoculation ($p < 0.01$) while transcript accumulation of *PaLAR*2 increased at 28 days after wounding or infection ($p = 0.04$).

Increases in transcript accumulation of *PaLAR*1 and *PaLAR*2 in response to wounding were amplified by fungal inoculation. Levels of *PaLAR*1 mRNA in inoculated saplings were 8 to 12 fold higher than in the wounded control and between 2000 and 6000-fold higher than in non-wounded saplings. Transcript accumulation of *PaLAR*2 was 2-fold higher in inoculated saplings than in the wounded saplings and between 180 to 200-fold higher than in bark of non-wounded saplings. There were no differences in *PaLAR*3 transcript accumulation in the bark of wounded control and non-wounded saplings, but there was a 3 to 4-fold increase in transcript accumulation in the bark of inoculated saplings.

4.3.6 Growth of *C. polonica* in the presence of catechin and proanthocyanidins

Growth of *C. polonica* isolate 2 (Figure 4.7) plated on medium containing 100 µg 2,3-*trans*-(+)-catechin or PAs was significantly lower than on medium without flavan-3-ols ($p < 0.001$). Fungal growth on medium containing catechin was also significantly lower than growth on medium containing PAs ($p < 0.001$). There were no significant differences in relative growth between isolates 1 and 2 on control, catechin or PA medium.

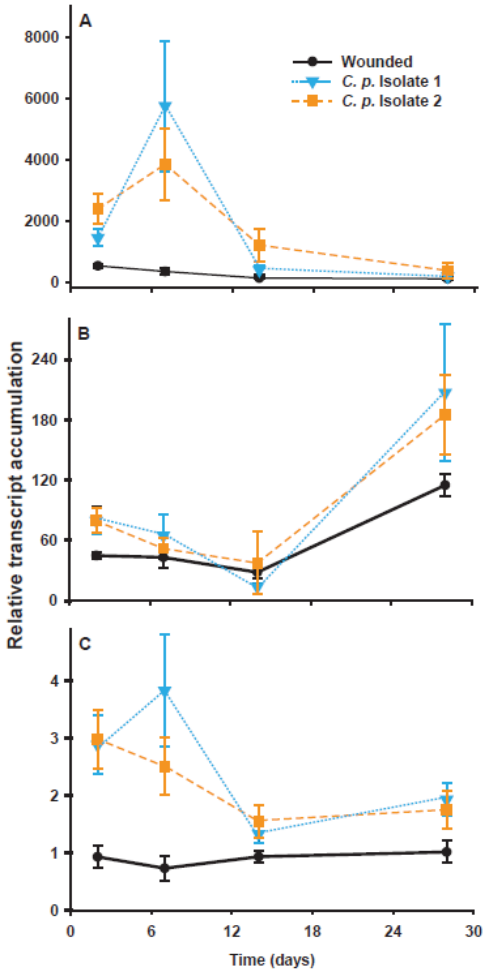


Figure 4.6: Relative transcript accumulation of *PaLAR*1 (**A**), *PaLAR*2 (**B**) and *PaLAR*3 (**C**) in bark from clonal spruce saplings over a time course of 28 days after wounding or inoculation with two *C. polonica* isolates. Transcript abundance was measured by quantitative real-time PCR using CYBR green for detection, normalized against *PaUBI* and calibrated against a non-wounded bark sample from the same clone.

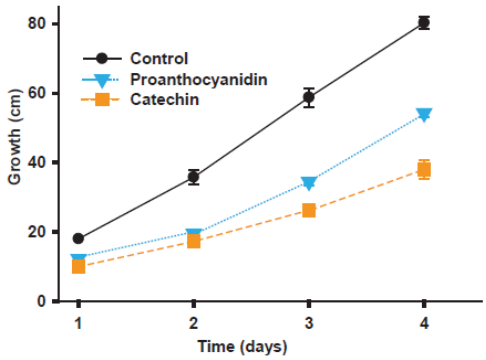


Figure 4.7: Growth of *C. polonica* isolate 2 on solid medium containing100 µg/ml of a complex mixture of grape proanthocyanidins or 100 µg/ml catechin. Error bars represent standard errors.

4.4 DISCUSSION

4.4.1 Analysis of proanthocyanidins (PA) and flavan-3-ols

To learn more about the polyphenolic composition of *Picea abies* and the regulation of phenolic biosynthesis during infection by the bark beetle associated fungus *Ceratocystis polonica*, we sought to analyze constitutive and fungus-induced profiles of flavan-3-ols and PAs in treated trees at various time points after infection. Methods were established for elucidating the chemical structure and abundance of monomeric and dimeric flavan-3-ols, while for the polymeric PAs the monomeric composition and degree of polymerization was determined (up to 9-mers). The current work used a 3-step procedure analysis of PAs in *P. abies*: (1) Monomeric and dimeric flavan-3-ols in plant extracts were analyzed and quantified by reverse-phase HPLC. (2) Polymeric flavan-3-ols were completely hydrolyzed under acidic conditions in the presence of a strong reducing agent, thereby preventing oxidation and minimizing epimerization. Hydrolysis products were then analyzed by reverse-phase HPLC to yield quantitative and qualitative information on the composition of the individual flavan-3-ol units in PAs. (3) PAs were separated by normal-phase chromatography to accurately determine the size distribution of oligomers using a method developed by Kelm et al. (2006). Although high resolution mass spectrometry was employed to establish and verify analytical methodologies, the routine analysis of PAs can be accomplished using these methods with only a single high pressure liquid chromatography (HPLC) instrument equipped with a fluorescence detector and 2 different analytical grade HPLC columns.

Previous studies had quantified PAs by making use of colorimetric methods, such as the acid-butanol test (Porter et al., 1986; Mellway et al., 2009) and the dimethylaminocinnamaldehyde test (de Pascual-Teresa et al., 2000; Almeida et al., 2007). Although these methods can deliver accurate quantitative measurements when the correct controls and standards are included in the analysis, they do not yield information on PA structure, composition and degree of polymerization. Methods have also been developed to combine the quantitative colorimetric assays with HPLC-UV to determine PA composition (Pang et al., 2008). However, these methods as well as the commonly used thiolysis method (Guyot et al., 1998), only yield indirect, non-quantitative information on the degree of polymerization of PAs due to incomplete hydrolysis or quantification of highly unstable molecules.

4.4.2 Biosynthesis of flavan-3-ols in spruce

The flavan-3-ols, 2,3-*trans*-(+)-catechin (90-95%) and 2,3-*trans*-(+)-gallocatechin (5-10%), are the predominant monomers extracted from spruce bark. The enzymes responsible for the synthesis

of these compounds are leucoanthocyanidin reductases (LAR), which accept the substrates leucocyanidin or leucodelphinidin to form catechin or gallocatechin, respectively (Tanner et al., 2003). Three *LAR* transcript sequences were identified in Norway spruce. *In vitro*, LAR enzymes accepted the substrate leucocyanidin to produce 2,3-*trans*-(+)-catechin. To confirm the *in vitro* characterization, two lines of transgenic spruce overexpressing *PaLAR*3 were developed. Transgenic lines synthesized significantly more gallocatechin, catechin and PAs than the wild type, providing further evidence that the candidate genes from spruce encode active LAR enzymes.

Although significantly higher flavan-3-ol concentrations were recorded in both transgenic LAR3 overexpressing lines than in the wild type, transcript accumulation of the *LAR*3 gene in line 2 was similar to that of the wild type. A possible explanation for this discrepancy is that sampling did non coincide with the major period of biosynthesis. In bilberry, for example, gene expression in the flavonoid pathway was significantly higher in spring and early summer than at later time points, although flavan-3-ol and PA accumulation remained high during the later part of the season (Martz et al., 2010). In this study seedlings were harvested for analysis four months after their spring growth flush. This may have been too late to detect major differences in gene expression of flavan-3-ol biosynthetic enzymes, although differences in accumulation were still evident.

After hydrolysis of spruce bark PAs, high levels of 2,3-*cis*-(-)-epicatechin and 2,3-*cis*-(-)-epigallocatechin were released. However, the free flavan-3-ol monomers from spruce are 2,3-*trans*-(+)-isomers. The 2,3-*cis*-(-)-isomers detected after hydrolysis of PAs may therefore represent extender units of PA chains. Although PAs with 2,3-*cis*-(-)-epicatechin are significantly more abundant in nature than PAs with 2,3-*trans*-(+)-catechin extender units, it is not known how the epicatechin extender units in the PA polymers arise.

Plants producing 2,3-*cis*-(-)-epicatechin as monomers, dimers and polymers, contain a pathway for the biosynthesis of the 2,3-*cis*-(-)-flavan-3-ol isomers separate from the 2,3-*trans*-flavan-3-ols. In this pathway leucoanthocyanidin is first converted to anthocyanidin by anthocyanidin synthase, and the anthocyanidin is reduced by anthocyanidin reductase (ANR) to form the 2,3-*cis*-(-)-flavan-3-ol. The absence of free monomeric epicatechin in spruce extracts, however, argues against the presence of epicatechin producing enzymes in this tree species. The origin of epicatechin in the PA polymers in spruce is therefore still an open question.

Several reaction mechanisms have been proposed for the oxidative coupling of PAs. The most widely accepted hypothesis is that 2,3-*trans*-(+)-leucoanthocyanidins are converted to carbocations via quinone methide intermediates which can be coupled to a terminal flavan-3-ol unit to form a growing PA chain (Creasy and Swain, 1965). However, this reaction mechanism can not account for the 2,3-*cis* stereochemistry of extender units of PAs in spruce. Similarly, a reaction

mechanism employing monomeric flavan-3-ols as extender units as proposed by Oszmianski and Lee (1990) would not explain the altered stereochemistry of the 2,3-*cis*-(-)-epicatechin. Reaction mechanisms where anthocyanidins, which can occur in both stereoisomeric forms, are proposed as possible precursors for extender units in PA chains offer a more probable model for PA biosynthesis in spruce. Haslam et al. (1980) suggested that anthocyanidins, that are present in acidic conditions as flavylium ions can be oxidized to quinone methides, which can form carbocations for PA chain elongation. An enzyme-mediated alternative to this hypothesis is that anthocyanidins are reduced to flav-3-en-3-ols by ANR (Xie et al., 2004; Dixon et al., 2005), which can be oxidized to carbocations to form extender units of a PA chain (Haslam, 1977).

4.4.3 Regulation of PA biosynthesis in spruce in response to fungal infection

One of the main objectives of this study was to examine the regulation of flavan-3-ol biosynthesis in spruce during biotic stress such as wounding or fungal infection. Earlier histological studies revealed that polyphenolic-like substances accumulated in phloem parenchyma cells in defense-induced spruce bark (Nagy et al., 2004; Franceschi et al., 2005). Since flavan-3-ol and PA accumulation were never directly measured in these phloem parenchyma cells, it was still an open question whether these compounds were involved in the defense response. This study, however, clearly demonstrated that PAs and flavan-3-ols are inducible defense compounds in spruce which accumulate more than 4-fold in bark tissue over time and may be responsible for the anatomical changes previously observed in the phloem parenchyma during wounding or fungal infection. It could also be shown that increased accumulation of these metabolites correlated with increased transcript levels of genes involved in the biosynthesis of 2,3-*trans*-(+)-catechin.

Simple wounding induced significantly lower LAR transcript accumulation as well as lower levels of monomeric and polymeric flavan-3-ols than infection by the bark beetle transmitted fungus, *C. polonica*. This suggests that biosynthesis of PAs may have evolved in spruce as a defense mechanism against fungal infection. In support of this hypothesis, growth of *C. polonica* was reduced when the fungus was cultured on medium containing catechin or PAs. Flavan-3-ols have been reported to inhibit melanin (black pigmentation) biosynthesis (Yamakoshi et al., 2003; Chen et al., 2006), which is an important virulence factor for many pathogenic fungi (Liu and Nizet, 2009). Since *C. polonica* is a black melanin containing fungus (Wang et al., 2010) flavan-3-ols could be highly effective defense compounds inhibiting an essential pathway in this economically important tree pathogen.

4.5 EXPERIMENTAL PROCEDURES

4.5.1 Identification of putative *LAR* genes from *Picea*

Protein LAR sequences from *Malus domestica*, *Pyrus comunis* (Pfeiffer et al., 2006) as well as an anthocyanidin reductase sequence from *Ginkgo biloba* (Shen et al., 2006) were used to screen *P. sitchensis* and *P. glauca* EST collections in the TREENOMICS database (Ralph et al., 2008) for candidate cDNA sequences using tBLASTn. Open reading-frames from candidate sequences were detected manually using the software package DNA Star Version 8.02 (DNASTAR Inc., Madison, USA) and the absence of predicted signal peptides at the N-terminus was confirmed by SignalP software (http://www.cbs.dtu.dk/services).

4.5.2 Cloning and sequencing *PaLAR*

RNA was extracted from fresh bark tissue from four-year-old *P. abies* saplings according to the method developed by Kolosova et al. (2004). One µg of total RNA was converted to cDNA in a 20 µl reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 50 pmol $PolyT_{(12-18)}$ primer (Invitrogen).

Primers were designed for *P. abies* candidate sequences by using the N-and C-terminal sequences of three putative *LAR* genes (Gateway™ compatible) and two *ANR* genes (pYES™ vector compatible) from *P. sitchensis* and *P. glauca* as templates (primer sequences are provided in supplementary table S4.1). Pseudomature forms of *PaLAR* cDNA were PCR amplified with primers (Table S4.1) using Platinum Taq™ high fidelity DNA polymerase (Invitrogen) and purified with the QIAquick™ PCR purification kit (Qiagen, Hilden, Germany). Gateway™ entry clones were made by using BP clonase II and pDONR207 (Invitrogen) following the manufacturer's protocols.

pDONR207 constructs containing *PaLAR* genes were sequenced using 10 pmol of gene specific primers and the BigDye Terminator v 3.1 Cycle Sequencing Kit on an ABI Prism R 3100 sequencing system (Applied Biosystems, Darmstadt, Germany). Sequences from each construct were assembled and translated into protein sequence using DNA Star software.

4.5.3 Protein sequence analysis of *Pa*LAR

LAR protein sequences from *P.abies* as well as protein sequences of LAR and ANR genes with confirmed enzyme function (Table S4.2) were aligned with the automatic alignment program MAFFT v 6 (http://mafft.cbrc.jp/alignment/server/) using the BLOSUM 62 scoring matrix with 1.53 gap opening penalty and an offset value of 1.

Phylogenetic analyses were conducted using MEGA v. 4 (Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA) employing the Minimum Evolution method. Evolutionary distances were calculated with the Jones, Taylor and Thornton matrix. The tree was searched with pair-wise elimination of alignment gaps. Statistical likelihood of tree branches was tested with 10,000 bootstrap replicates.

4.5.4 Heterologous expression of *PaLAR* genes in *E. coli*

Three putative *PaLAR* pDONR207 constructs were cloned with LR clonase II (Invitrogen) according to the manufacturer's instructions into the Gateway™ compatible expression vector pH9GW (Yu and Liu, 2006) which contains 9 histidine residues 5' of the N-terminus of the expressed protein. All constructs were verified by sequencing. Arctic Express™ (DE3) chemically competent *E. coli* cells (Stratagene, La Jolla, CA, USA), which co-express cold adapted chaperonins to overcome protein misfolding and insolubility, were transformed with the expression constructs. For protein expression, single colonies were inoculated into 5 ml Luria-Bertani (LB) broth with 1 μg ml^{-1} kanamycin and grown for 16 hours at 24 °C. The 5 ml starter cultures were used to inoculate 100 ml Overnight Express Instant TB Medium (Novagen, Madison, WI, USA) supplemented with 1% (v/v) glycerol and 1 μg ml^{-1} kanamycin.

Bacterial cultures were grown for 3 days at 12°C (220 rpm) and harvested by centrifugation. Bacteria were resuspended in 10 ml buffer containing 50 mM Bis-Tris (pH 7.5), 10% (v/v) glycerol, 0.5 mM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol (DTT) and disrupted by sonification for 3 minutes using 2 cyles at 65% power using a Bandelin Sonoplus HD 2070 sonifier (Bandelin Electronics, Berlin, Germany). Insoluble cell debris was removed from the lysate by centrifugation at 16,000 rpm for 30 minutes at 4°C. The presence of the expressed protein in the *E. coli* lysate was confirmed by Western blot using an anti-histidine-horseradish peroxidase conjugated antibody (Novagen, Nottingham, UK) and chemo-illuminescence for visualization.

Expressed proteins were purified from the crude lysate by affinity chromatography with a 1 ml His Trap ™ FF column (GE Healthcare Life Sciences, Munich, Germany) on an AEKTA 900 chromatography system (GE Healthcare) using a wash buffer containing 50 mM Bis-Tris (pH 7.5) and 10% (v/v) glycerol. Histidine-tagged proteins were eluted from the column with wash buffer amended with 250 mM imidazole. Fractions containing the expressed proteins were desalted into an assay buffer (50 mM Bis-Tris pH 7.5, 10% (v/v) glycerol, 1 mM DTT) on DG-10 desalting columns (Bio-Rad, Munich, Germany) and stored at -20°C. The protein concentration was determined using the Bradford reagent (Bio-Rad).

4.5.5 *In vitro* functional characterization of *Pa*LAR expressed in *E. coli*

The unlabelled substrates leucoanthocyanidin and leucodelphinidin were purchased from TransMit Flavonoid Forschung (Marburg, Germany). Recombinant LAR1, LAR2 and LAR3 enzyme activities were assayed in 200 µl reaction volumes containing 19 µg purified enzyme, 500 nmol NADPH (Carl Roth GmbH, Karlsruhe, Germany) and 200 nmol leucoanthocyanidin or leucodelphinidin in assay buffer. Reaction mixtures were incubated for 3 hours at 28°C before the enzyme assay was stopped by acidification with 10 µl of 0.1 N HCl and extracted with 3 volumes of ethyl acetate after addition of 10 µg ml^{-1} apigenin-7-O-glucoside (Carl Roth) as an internal standard. The ethyl acetate extracts were evaporated under a stream of nitrogen gas and re-dissolved in 50 µl methanol for LC-ESI-MS analysis. Negative control assays were initiated without substrate or with heat-denatured enzyme preparations. The temperature optimum for LAR activity was determined at 25, 30 and 35°C. pH optima were determined by using 50 mM Bis-Tris at pH 6 to pH 8.5.

4.5.6 Genetic transformation of *P. abies* callus with *PaLAR*3

The *PaLAR*3 pDONR207 was cloned with LR clonase II (Invitrogen) into the Gateway™ compatible binary vector pCAMGW (Schmidt *et al*., 2010). pCAMGW *LAR*3 or pCAMBIA 2301 (as a vector control) were transformed into the chemically competent disarmed *Agrobacterium tumefaciens* strain C58/pMP90 (Schmidt et al., 2010) and then used to transform an embryonic *P. abies* cell culture (line 186/3c VIII) as described by Schmidt et al. (2010). From the 6 kanamycin resistant transgenic lines obtained, line 1 and line 2 were selected for further experiments.

4.5.7 Somatic embryogenesis of *P. abies* and plant regeneration

Transgenic embryonic tissue was maintained at 24°C in the dark and sub-cultured every 14 days. For plant regeneration, transgenic embryonic tissue was placed on semi-solid EMM1 medium (Walter et al., 1999) amended with 6 g L^{-1} gelrite (Duchefa, www.duchefa.com), 30 g L^{-1} sucrose and 15 mg L^{-1} abscisic acid for 2 weeks. The culture was then placed on EMM2 medium (4.5 g L^{-1} gelrite) (Walter et al., 1999) for 5 weeks. Embryos, 3-5 mm in size, were placed on a sterile nylon mesh in 3 wells of a six-well cell cluster. The other 3 wells were filled with sterile water to maintain humidity. Embryos were stored at 4°C for 7 days in the dark. For germination, developed embryos were transferred to MLV medium (Klimaszewska et al., 2005) with 40 g L^{-1} sucrose and 6 g L^{-1} gelrite (Duchefa). The cultures were placed for 6 weeks under low light conditions (5 µmol m^{-2} s^{-1}) for 16 h day^{-1} at 24°C. Somatic embryos were collected and placed horizontally on MLV medium with 6 g L^{-1} gelrite (Duchefa) under moderate light conditions (45 µmol m^{-2} s^{-1}). After three weeks, plants were

transferred to Magenta GA-7 (Sigma) plant culture boxes and placed under higher light conditions (100 µmol m^{-2} s^{-1}) to develop roots, epicotyls and needles. Well developed plantlets were planted in soil substrate (3:1:1, fibric peat:vermiculite- 2-3 mm grain size:perlite) in small plastic containers (4 x 4 cm).

4.5.8 Inoculation of *P. abies* saplings with *C. polonica*

Two *C. polonica* isolates (CMW 7749 = isolate 1 and CMW 7135 = isolate 2) provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (University of Pretoria, South Africa) were grown on 2% (w/v) malt extract agar (MEA; Karl Roth GmbH) for 12 days at 25°C in the dark.

Eight-year-old *P. abies* saplings originating from the 3369-Schongau clone (Samenklenge und Pflanzengarten Laufen, Germany) were grown in an outdoor plot for four years prior to the experiment. Inoculations of saplings with *C. polonica* were performed three weeks after their 'spring'-flush (10 June 2008). A bark plug, 8 mm in diameter, was removed between the second and third branch-whorl from the upper part of the sapling with a cork borer. An 8 mm plug from one of the two *C. polonica* cultures was placed into the wound with the mycelium oriented toward the wood surface and sealed with parafilm. For the wounded control treatment, plugs of sterile MEA were inserted into the wound.

Bark tissue samples from inoculated and wounded saplings were harvested 2 days, 7 days, 14 days and 28 days after the onset of the experiment. Five saplings were sacrificed per time point for each treatment (Control, CMW 7749 and CMW 7135) to follow a multivariate repeated measures model experimental design. Bark material was flash-frozen immediately after harvest in liquid nitrogen and stored at -80ºC. Five replicate trees were used for each treatment per time point.

4.5.9 Quantitative real-time PCR

Total RNA from inoculated treatment and wounded control bark as well as transgenic *PaLAR*3 over-expresser lines and wild-type controls was isolated with the Invitrap™ Spin Plant RNA Mini Kit (Invitek, Berlin, Germany) following the protocols of the manufacturer except that an additional DNA digestion step was included (RNase Free DNase set, Qiagen). RNA was quantified by spectrophotometry. Reverse transcription of 1 µg RNA into cDNA was achieved by using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol $PolyT_{(12-18)}$ primer (Invitrogen) in a reaction volume of 20 µl. After cDNA was diluted to 10% (v/v) with deionized water, 1 µl diluted cDNA was used as template for quantitative real-time PCR in a reaction mixture containing Brilliant

SYBR Green QPCR Master Mix™ (Stratagene), 10 pmol forward and 10 pmol reverse primer. Primer sequences for *PaLAR*1, *PaLAR*2 and *PaLAR*3 are given in supplementary table S4.3. PCR was performed using a Stratagene MX3000P thermocycler using the following cycling parameters: 5 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by a melting curve analysis from 55°C to 95°C. Reaction controls included non-template controls as well as non-reverse transcribed RNA. Transcript abundance was normalized to the transcript abundance of the ubiquitin gene (Schmidt et al., 2010) (Table S4.3) and was calculated from three technical replicates each of five biological replicates. Relative transcript abundance was calibrated against the transcript abundance of five non-wounded control saplings.

4.5.10 Extraction of phenolic compounds from spruce

For extraction of phenolic compounds, spruce tissue was ground to a fine powder in liquid nitrogen and lyophilized at 0.34 mbar pressure using an Alpha 1-4 LD plus freeze dryer (Martin Christ Gmbh, Osterode, Germany). Approximately 80 mg dried tissue was extracted with 2 ml analytical grade methanol for 4 hours at 4°C, then the extract was centrifuged at 3200 g and the supernatant was recovered. Insoluble material was re-extracted with 1.5 ml methanol for 16 hours. Supernatants were combined and evaporated to dryness under a stream of nitrogen. Dried samples were re-dissolved in 1 ml methanol containing 100 µg ml^{-1} apigenin glucoside (Sigma) as internal standard. For LC-ESI-MS or hydrolysis of condensed tannins, samples were diluted five-fold (v/v) with methanol. For LC-FLD samples were diluted 20-fold (v/v) in acetonitrile.

4.5.11 Liquid chromatography-fluorescence detection (LC-FLD)

Condensed tannins were separated on a LiChrosphere diol column with dimensions of 250 X 4 mm and a particle size of 5 µm (Merck, Darmstadt, Germany) using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) employing a modified method previously described by Kelm et al. (2006). Briefly, the total mobile phase flow rate for chromatographic separation was 1.2 ml min^{-1}. The column temperature was maintained at 30°C. Tannins were separated using acetonitrile:acetic acid (98:2) and methanol:water:acetic acid (95:3:2) as mobile phases A and B, respectively, with the following elution profile: 0-35 min, 0-40% B in A; 35-40 min 40% B, 40-45 min 40-0% B and 45.1-50 min 0% B. Eluent was monitored by fluorescence detection with excitation at 276 nm and emission at 316 nm.

4.5.12 Liquid chromatography-mass spectrometry with electrospray ionization (LC-ESI-MS)

Compounds to be analyzed were separated on a Nucleodur Sphinx RP18ec column with dimensions of 250 X 4.6 mm and a particle size of 5 µm (Macherey Nagel, Dueren, Germany) using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a flow rate of 1.0 ml min^{-1}. The column temperature was maintained at 25°C. Phenolic compounds were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0-1 min, 100% A; 1-25 min, 0-65% B in A; 25-28 min 100% B; and 28-32 min 100% A.

Compound detection and quantification was accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Brucker Daltronics, Bremen, Germany). Flow coming from the column was diverted in a ratio of 4:1 before entering the mass spectrometer electrospray chamber. ESI-MS was operated in negative mode scanning $m\,z^{-1}$ between 50 and 1600 with an optimal target mass of 405 $m\,z^{-1}$. The mass spectrometer was operated using the following specifications: skimmer voltage, 60 V; capillary voltage, 4200 V; nebulizer pressure, 35 psi; drying gas, 11 l min^{-1}; gas temperature, 330ºC. Capillary exit potential was kept at -121 V.

For mass determination of PAs, the same chromatographic separation method was used as for LC-FLD, using a flow rate of 1ml min^{-1}. To enhance ionization, 10 mmol l^{-1} ammonium acetate in methanol was added to the column eluent at a flow rate of 0.1 ml min^{-1} using an infusion pump. The mass spectrometer was operated using the same specifications as for the analysis of small molecules, except scanning between 200 and 2500 $m\,z^{-1}$ varying the optimal target mass according to the degree of polymerization of the PA that needed to be detected.

Compounds were identified by mass spectrometry and by comparison with commercial standards, where available. Brucker Daltronics Quant Analysis v.3.4 software was used for data processing and compound quantification using a standard smoothing width of 3 and Peak Detection Algorithm v. 2. Linearity in ionization efficiencies was verified by analyzing serial dilutions of randomly selected samples. An external calibration curve created by linear regression was used for quantification of PA B1, catechin and gallocatechin (Sigma). Process variability in different analyses was calculated relative to the internal standard.

4.5.13 Hydrolysis of condensed tannins

Condensed tannins in bark extracts and enzyme assays were hydrolyzed in 800 µl reaction volumes containing 2.5% (v/v) trifluroacetic acid (TFA) and 8% (v/v) 0.5 g ml^{-1} sodium cyanoborohydrate in methanol. Reaction mixtures were heated to 65°C for 15 minutes before adding another 2.5% (v/v) TFA. Vials were sealed tightly and incubated at 65°C overnight. Samples were dried under a stream

of nitrogen and re-dissolved in 800 µl methanol and analyzed using the chromatographic separation method described for LC-ESI-MS and detected at 280 nm using a UV-DAD detector.

Verification of the hydrolysis method for quantification of condensed tannins was achieved using dilutions from 1 mg ml^{-1} to 0.0125 mg ml^{-1} of pure proanthocyanidin B1 (Sigma) and proanthocyanidin B2 (Sigma) (Figure S4.2 and Figure S4.3) as well as partially purified grape proanthocyanidins (Laffort, Bordeaux, France). Recovery studies were conducted by adding pure catechin to hydrolysis mixtures. Hydrolysis method development was performed in 200 µl reaction volumes as described above and measured by LC-ESI-MS-MS for more accurate quantification.

4.5.14 Liquid chromatography-tandem mass spectrometry (LC-ESI-MS-MS)

Chromatography was performed on an Agilent 1200 HPLC system (Agilent). Separation was achieved on a 100 X 4.6 mm Kinetex C18 column with particle size of 2.6 µm (Penomenex, Aschaffenburg, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-1 min, 100% A; 1-7 min, 0-65% B in A; 7-8 min 65-100% B in A; 8-9 min 100% B and 9-10 min 100% A. The total mobile phase flow rate was 1.5 ml min^{-1}. The column temperature was maintained at 25°C.

An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards of catechin and proanthocyanidin B1. For dimeric proanthocyanidins containing gallocatechin, partially purified plant extracts were used for optimization. The ion spray voltage was maintained at -4500 V. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 10 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion formation as follows: $m\,z^{-1}$ 299.9 →109.1 (collision energy, CE -34 V; declustering potential, DP -30 V) for catechin; $m\,z^{-1}$ 304.8 →179 (CE -28 V; DP -390 V) for gallocatechin; $m\,z^{-1}$ 576.9 →289.1 (CE -30 V; DP -50 V) for proanthocyanidin B1; $m\,z^{-1}$ 592.9 →125.1 (CE -52 V; DP -400 V) for the catechin:gallocatechin dimer; $m\,z^{-1}$ 609 →125.1 (CE -50 V; DP -45 V) for the gallocatechin dimer. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity of compound detection for quantification was verified by external calibration curves for catechin and proanthocyanidin B1. Flavan-3-ol concentrations were determined relative to the catechin calibration curve (Figure S4.3).

4.5.15 Fungal growth in the presence of flavan-3-ol derivatives

Petri-dishes with carrot agar amended with flavan-3-ol derivatives were prepared by autoclaving 10% carrot juice (v/v) and 3% (wt/v) agar per liter of water. After cooling the medium to 55°C, catechin or grape proanthocyanidin in ethanol (10mg ml^{-1}) was added to a final concentration of 100 µg ml^{-1}. Medium for negative control treatments was amended with ethanol only. Each Petri dish (Ø=10 cm) contained 25 ml medium. After the medium solidified, an agar plug (Ø=4 mm) from 14-day-old *C. polonica* stationary cultures (CMW 7749 or CMW 7135) was placed in the middle of each Petri dish, sealed with Parafilm and incubated at 26°C in the dark. Fungal growth was measured every 24 hours for 4 days.

4.5.16 Statistical analysis

The multivariate repeated measures model experimental design allows for the correlation between values of the same variable measured at successive time points. The multivariate form is required when several dependent variables are measured for the same entity, *i.e.* clonal trees (Sokal & Rohlf 1995), and allows the examination of relationships between different variables.

Preliminary investigation showed that the data was not normal and contained numerous outliers. Such data cannot be analyzed appropriately without transformation and so rank transformation was carried out (Iman, 1974), which is particularly robust to non-normal errors, outliers, and several kinds of non-normal distributions. Because there is only one interaction in our model, and this is rarely significant, we were not troubled by the increased Type 1 error caused by applying rank transformation to models with many interactions (Sawilowsky, 1990). The data for gene expression, small molecules and hydrolysis products were analyzed separately because there is no direct conversion possible among them.

Transcript and metabolite accumulation in transgenic trees were analyzed using a one-way ANOVA on log transformed data.

5. CONCLUDING DISCUSSION

Norway spruce (*Picea abies*), an economically and environmentally important tree species in Europe, is often attacked by the bark beetle species *Ips typographus* and the beetle-vectored blue stain fungus *Ceratocystis polonica*. Apart from terpenoid resin-based defense strategies, Norway spruce contains autofluorescent rings of cells in the phloem parenchyma. These cells have been identified as important components of systemic acquired resistance against fungal infection (Franceschi et al., 2005). Due to the autofluorescent nature of the inclusion bodies of these phloem parenchyma cells, it has been suggested that their contents could be aromatic polyphenolic compounds such as stilbene glucosides and proanthocyanidins, which have been described in spruce bark in earlier studies. However, very little is known about the biosynthesis, the structural diversity and biological roles of polyphenolic compounds in Norway spruce. In this thesis the biosynthetic pathway for the formation of tetrahydroxystilbene glucosides as well as the last steps of the proanthocyanidin pathway in Norway spruce were elucidated. Furthermore, this research showed that these compounds are involved in anti-fungal defense against the bark beetle vectored blue stain fungus *C. polonica*. It was also shown that *C. polonica* detoxifies defense-related host stilbenes using biotransformation stategies which were predetermined by the fungus' virulence during host infection.

5.1 *Picea abies* stilbene synthases form a trihydroxylated product, resveratrol, which is an intermediate in the biosynthesis of the tetrahydroxystilbene glucosides, astringin and isorhapontin

Tetrahydroxystilbene glucosides are abundant polyphenols in Norway spruce bark. In order to learn more about their involvement in the spruce defense response against fungal infection, the pathway leading up to their biosynthesis was elucidated. Stilbenes are synthesized via condensation of three acetate units from malonyl-coenzyme A (CoA) to a CoA-activated phenolic acid by stilbene synthase (STS), which results in formation of a linear tetraketide. Ring closure, to form the stilbene skeleton is achieved via an intramolecular aldol condensation coupled to the loss of CO_2 (Austin et al., 2004). For the formation of dihydroxylated or trihydroxylated stilbenes the STS enzymes from pine or grape accept the non-hydroxylated substrate cinnamoyl CoA (Fliegmann et al., 1992) or the monohydroxylated substrate *p*-coumaroyl CoA (Lanz et al., 1991), respectively. It has been suggested that for the biosynthesis of the tetrahydroxystilbenes which are predominantly present in Norway spruce, dihydroxylated substrates should be necessary (Chong et al., 2009; Rolfs and Kindl, 1984).

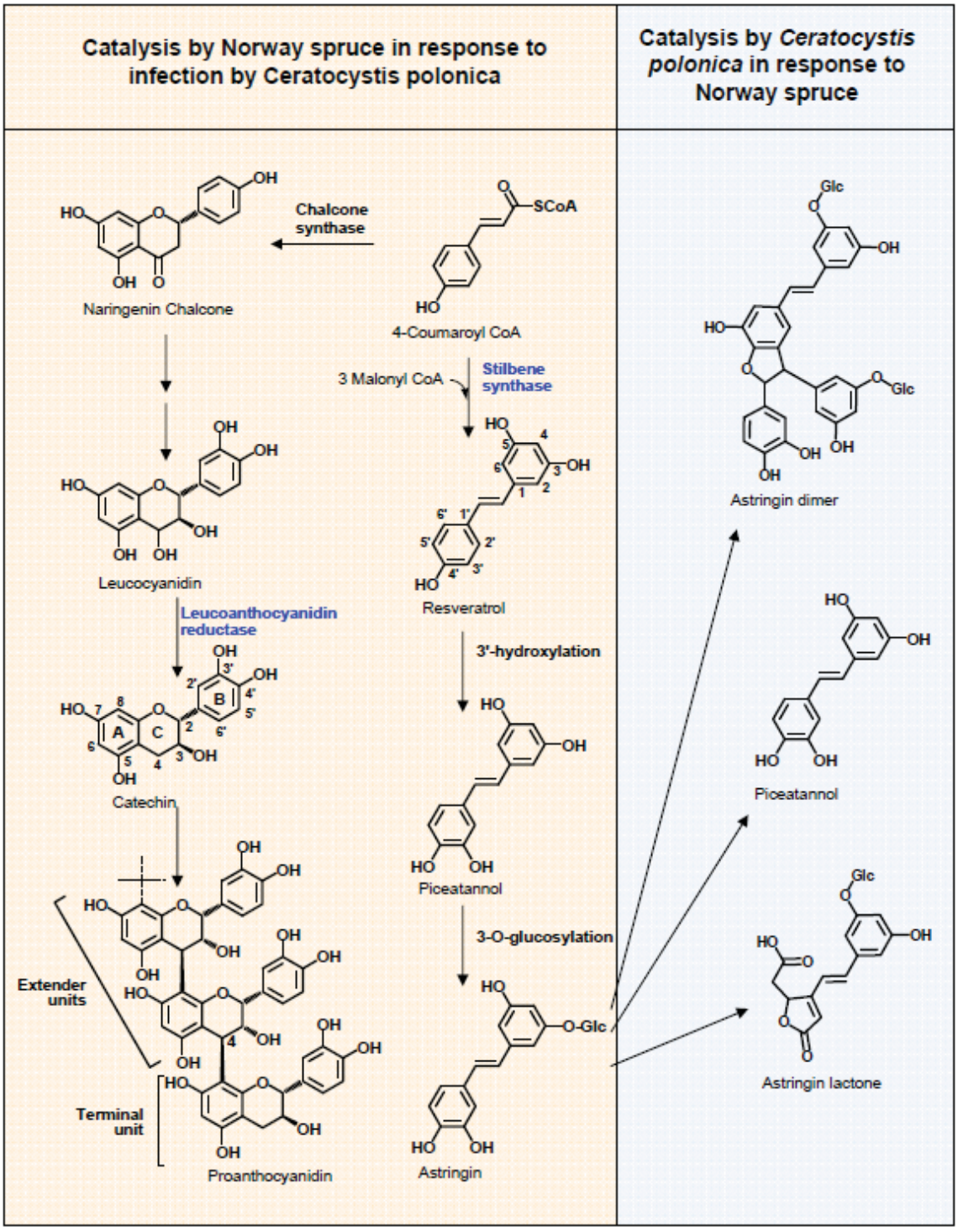


Figure 5.1: Graphical abstract of all the pathways elucidated in this thesis. Enzymes highlighted in blue have been characterized *in vivo*.

Spruce STS enzyme function was studied by using numerous approaches such as *in vitro* characterization of heterologously and natively expressed STS, *in vivo* characterization of stilbenes

produced by *Escherichia coli* which were genetically engineered to produce spruce-specific stilbenes and by using transgenic spruce overexpressing native STS enzymes. The different methodologies led to accumulation of evidence that 3,5,3',4'-tetrahydroxystilbenes are only produced after the STS-mediated formation of a 3,5,4'-trihydroxystilbene, resveratrol, from the substrate *p*-coumaroyl-CoA. The STS product, resveratol which is present only in very low quantities in spruce tissue, must be further modified by 3'-hydroxylation and 3-O-glucosylation to yield astringin, the major stilbene produced in spruce. Our proposed pathway for tetrahydroxystilbene formation could in future studies be further validated by the identification and characterization of spruce stilbene 3' hydroxylases, an as yet undescribed group of P450 monooxygenase enzymes.

5.2 Although proanthocyanidin polymers in spruce contain high levels of flavan-3-ols with 2,3-*cis*-(-)-stereospecificity, monomers have exclusively 2,3-*trans*-(+)-stereospecificity

Even though polymeric flavan-3-ols known as proanthocyanidins, are of great ecological importance in Norway spruce, their biosynthesis and metabolite profiles in this tree species were not well studied. In order to learn more about the involvement of these compounds in the spruce defense response against fungal infection their biosynthesis was studied and metabolite profiles of monomeric as well as polymeric flavan-3-ols were analyzed in spruce bark. As traditional methodology for proanthocyanidin analysis yields inadequate information on metabolite quantity, structure and degree of polymerization, a suite of tools were developed for the analysis and quantification of these compounds. A new and integrative approach was chosen, combining reverse-phase high pressure liquid chromatography (HPLC) for the analysis of monomeric and dimeric flavan-3-ols with a novel reductive acid hydrolysis method as well as size exclusion chromatography for the analysis of polymers.

Analysis of monomeric flavan-3-ols revealed that Norway spruce exclusively produces monomers with 2,3-*trans*-stereospecificity, such as 2,3-*trans*-catechin. Consequently, leucoanthocyanidin reductase (LAR) enzymes from spruce, which catalyze the last step in the pathway synthesizing 2,3-*trans*-(+)-catechin were characterized in this study. This was done *in vitro* using heterologously expressed enzymes as well as *in vivo* by analysis of flavan-3-ol profiles in transgenic spruce overexpressing a native LAR gene. Further proof of the function of *Pa*LAR lies in the correlation between *PaLAR* gene expression and proanthocyanidin accumulation in spruce bark.

Unexpectedly, the analysis of spruce proanthocyanidins revealed that they contain flavan-3-ols with both 2,3-*trans*- as well as 2,3-*cis*-stereospecificity. To synthesize flavan-3-ols with 2,3-*cis*-stereospecificity, such as 2,3-*cis*-epicatechin, enzymes known as anthocyanidin reductases (ANR) are necessary (Xie et al., 2004). Angiosperms studied thus far contain either only 2,3-*cis*-epicatechin and

produce 2,3-*cis*-epicatechin polymers, (Xie et al., 2004; Pang et al., 2007), or contain both 2,3-*cis*-epicatechin and 2,3-*trans*-catechin and produce polymers consisting of mixtures of 2,3-*trans*-catechin and 2,3-*cis*-epicatechin (Bogs et al., 2005; Paolocci et al., 2007). Consequently, angiosperms synthesizing 2,3-*cis*-epicatechin contain only ANR enzymes, whereas angiosperms producing both 2,3-*trans*-catechin and 2,3-*cis*-epicatechin contain both ANR and LAR enzymes. Due to the fact, that we did not characterize any ANR enzymes in this study, it is unclear, if flavan-3-ols with 2,3-*cis*-stereospecificity are made in Norway spruce and channeled directly to proanthocyanidin biosynthesis, or if there is another mechanism by which units with 2,3-*cis*-stereospecificity arise in proanthocyanidin chains. Although much progress was made in the analysis of proanthocyanidins and the elucidation of biosynthetic routes leading to the formation of monomeric flavan-3-ols in this work, future studies are needed to determine how the 2,3-*cis*-(-)-units are formed in proanthocyanidin chains in spruce. None the less, Norway spruce is the first plant described with only 2,3-*trans*-stereospecific monomers but both 2,3-*trans*- and 2,3-*cis*-stereospecific flavan-3-ols in the polymers.

5.3 Genetic transformation of spruce is a useful tool for the characterization of conifer enzymes involved in the biosynthesis of polyphenolic compounds

In this study the heterologous expression of Norway spruce enzymes involved in polyphenol biosynthesis and their *in vitro* characterization was not always easy to accomplish. Active enzymes could only be obtained from heterologous systems when expressed at very low temperatures by specialized *E. coli* strains co-expressing cold resistant chaperonins to aid in folding, and even there the activity was usually low. On the other hand, transformed spruce overexpressing biosynthetic genes readily synthesized the expected metabolites at higher levels than the wild type or vector control lines with no detectable pleiotropic effects. An added advantage of using transgenic spruce overexpressing native genes for enzyme characterization is that the contribution of the gene to the tree's phenotype can be evaluated. For example, the use of stably transformed spruce in the functional characterization of stilbene synthases was critical to demonstrate that the STS enzymes, although only making resveratrol as an intermediate product, still contribute to the formation of the major stilbenes in the plant.

Overexpressing native genes in spruce requires regulation of gene expression by a weak promoter, as transformations with genes involved in natural product biosynthesis using the strong 35S promoter lead to non-viable explants (unpublished data-Trevor Fenning). However, the use of a weak promoter leads to secondary control of transcript levels of the introduced gene or of the encoded protein which is often unpredictable (unpublished data-Axel Schmidt). For example, this phenomenon could be observed in the analysis of *PaLAR*3 overexpressing spruce lines used for the *in vivo*

characterization of this gene. While significantly higher levels of flavan-3-ols and proanthocyanidins were detected in *PaLAR*3 overexpressing lines than in the wild type, transcript abundance of *PaLAR*3 in the transgenic seedlings was similar to the wild type. This discrepancy between gene expression and metabolite accumulation is an indication that the regulation of the expression of the introduced gene is superseded by the tree's own regulatory mechanisms for flavan-3-ol biosynthesis. Higher levels of flavan-3-ols in the *LAR*3 overexpressing spruce seedling lines, probably originated due to expression of the introduced *LAR*3 gene and might have accumulated at a developmental stage prior to tissue analysis.

5.4 Stilbene glucosides and flavan-3-ols are produced by spruce as chemical defense compounds against infection by the bark-beetle-associated blue stain fungus *Ceratocystis polonica*

One of the main objectives of this research was to elucidate if polyphenols are inducible chemical defense compounds in Norway spruce. Earlier histological studies revealed that substances with polyphenol-like fluorescence accumulated in phloem parenchyma cells in defense-induced spruce bark (Nagy et al., 2004; Franceschi et al., 2005). Since stilbene glucoside, flavan-3-ol and proanthocyanidin accumulation was never directly measured in these anatomical studies, it was still an open question whether these compounds were involved in these histological changes. Other studies in which spruce phenolic compounds were quantified after trees were challenged by wounding or fungal inoculation, often rendered unclear or contradictory data on the involvement of these compounds in the tree's defense response (Brignolas et al., 1995; Virii et al., 2001). Research conducted in this thesis, however, convincingly shows that Norway spruce, in addition to the broad array of resin-based chemical defenses, also produces polyphenolic compounds as a defense strategy against infection by the fungal bark beetle associate, *C. polonica*.

The biosynthesis of stilbene glucosides as well as the biosynthesis of flavan-3-ols leading to proanthocyanidin formation was studied at the transcriptional level in spruce bark at different time points after *C. polonica* infection. Stilbene synthase (*STS*) and leucoanthocyanidin reductase (*LAR*) transcript abundance significantly increased after fungal bark infection compared to transcript abundance in wounded bark. Interestingly, both *STS* and *LAR* genes were induced both early in the infection process (2-7 days post-inoculation) and at a late phase at 28 days post-inoculation. These data, as well as the fact that levels of flavan-3-ols were higher in transgenic spruce over-expressing *STS*, provides circumstantial evidence that stilbene and flavan-3-ol biosynthesis might be co-regulated in Norway spruce. Similar transcriptional co-regulation of genes involved in flavonoid and stilbene biosynthesis was reported by Ralph et al. (2006) in Sitka spruce challenged by spruce budworm. In order to fully elucidate the regulation of genes of defense-compounds in spruce, studies

on regulatory elements encoded by genomic DNA as well as characterization of relevant transcription factors have to be conducted.

In accordance with increased transcript accumulation of genes encoding enzymes responsible for stilbene glucoside, flavan-3-ol and proanthocyanidin biosynthesis, these metabolites increased initially in *C. polonica* infected spruce bark. However, while flavan-3-ol and proanthocyanidin levels increased steadily over 28 days post-inoculation, levels of stilbene glucosides decreased during the later stages of *C. polonica* infection. Similar observations have also been made by Brignolas et al. (1995), who showed that stilbene levels in spruce decreased during *C. polonica* infection in spite of increased STS enzyme activity in infected tissues, while levels of flavan-3-ols increased. Accordingly, possible tree-defense-response-related mechanisms by which levels of soluble stilbenes could decrease in bark tissue were investigated. However, no evidence was found that oxidative polymerization of stilbenes by plant-derived enzymes (Li et al., 2007) or oxidative coupling of these compounds to cell wall polymers (Lange et al., 1994) occurred in Norway spruce during fungal infection (data not shown).

Another possible process resulting in reduced stilbene levels in infected spruce bark is the fungal biotransformation of these compounds. Specialized strains of fungi and bacteria have been shown to detoxify a wide array of aromatic xenobiotcis. It has also been shown that specialist pathogenic fungi evolved strategies to detoxify phytoalexins produced by their hosts (Vanetten et al., 1989). It is therefore conceivable that *C. polonica*, a specialist fungal phytopathogen, might have evolved strategies to circumvent the toxic effects of these phytoalexins produced by its host tree.

5.5 Host derived stilbene biotransformation by *C. polonica* may be an important fungal virulence factor

The tetrahydroxystilbene glucoside, astringin, which is the major stilbene produced by *P. abies*, was transformed by *C. polonica* in culture to astringin-lactones, piceatannol and dimeric stilbenes. This provides an explanation for the reduced levels of astringin in infected host tissue. Furthermore, virulence of *C. polonica* towards spruce correlated with the rate of astringin biotransformation. By comparing biotransformation strategies of a virulent and an avirulent strain, this study also revealed that certain combinations of constitutive and inducible stilbene biotransformation strategies appear to be more favorable for the fungus than others. For example, the virulent fungal isolate showed high enzyme activity for stilbene dimerization in culture. Upon exposure to host phenolic compounds, however, this fungal isolate also showed significant increases in stilbene lactonization activity.

5. Concluding Discussion

The structural similarities between the astringin-lactone formed upon fungal biotransformation by the virulent *C. polonica* isolate and the lactone formed as an intermediary step in the β-ketoadipate pathway (Harwood and Parales, 1996) hint at the possibility of further catabolic degradation of astringin by this fungus. The β-ketoadipate pathway, which plays a central role in the catabolic degradation of aromatic compounds derived from lignin, might be used by *C. polonica* as an energy source during its saprophytic growth stages in dead wood, which is poor in nutrients. Stilbenes are anti-fungal compounds with multiple modes of action, such as interference with microtubule assembly (Woods et al., 1995), disruption of plasma membranes and uncoupling of electron transport in fungal spores and germ tubes (Pont and Pezet, 1990). For this reason, fungi may not be able to acquire resistance to these compounds by single mutations, as has been shown for many other anti-fungal compounds (Hollomon and Brent, 2009). Using biotransformation strategies to lower the concentration of stilbenes during infection and colonization might, therefore, be a more effective form of resistance.

The avirulent *C. polonica* isolate formed extremely low concentrations of the astringin lactone. This result indicates that the β-ketoadipate pathway could be involved in catabolic processes for energy production to a lesser degree in the avirulent isolate than in the virulent fungal isolate. The avirulent isolate would, therefore, need to exploit a different nutrient source. In consequence, we could show that the highest stilbene biotransformation activity detected in this isolate was the β-deglucosylation of astringin to form piceatannol and glucose which can be employed for the production of energy for growth and reproduction. Deglucosylation of astringin to form piceatannol may, however, be a disadvantageous biotransformation strategy as this process releases a less polar molecule that may have higher biological activity. Possession of such a suspect biotransformation strategy might form part of the explanation why the avirulent isolate grows less than the virulent isolate within living spruce bark. However, to compensate for higher production of piceatannol, the avirulent isolate revealed elevated stilbene dimerization activity. It has been suggested that dimers are less toxic than monomeric stilbenes due to a reduction in solubility of the dimeric compounds (Breuil et al., 1999), allowing the fungus to escape from contact with these phytoalexins. Deglucosylated dimeric compounds are even less soluble and low solubility could partially compensate for increased reactivity.

Although much has been learned about spruce stilbene biotransformation by *C. polonica* and its correlation with fungal virulence, certain important questions still need to be addressed, for example: (I) Although circumstantial evidence hint at the involvement of the β-ketoadipate pathway in stilbene detoxification, this has not been shown by direct experimentation. To show the involvement of this pathway in stilbene detoxification ^{13}C-labled stilbenes should be offered to fungi

as a sole nutrient source, followed by quantification of fungal respiration by measuring $^{13}CO_2$ evolution. (II) It is possible that spruce stilbenes are only pro-toxins, which are activated by fungal biotransformation to become more toxic to *C. polonica*. Bioassays are therefore necessary to determine how the different biotransformation products identified in this study affect *C. polonica* growth. (III) Evidence has accumulated in this study that stilbene biotransformation is an important virulence factor of *C. polonica* during host infection and colonization. However, in order to attain greater understanding of the mechanisms by which stilbene biotransformation contributes to pathogenicity, molecular tools have to be developed to identify and characterize fungal genes involved in stilbene biotransformation and to silence or overexpress these genes in *C. polonica* to determine their *in vivo* role during spruce infection and colonization. (IV) Spruce also produces other phenolic compounds, such as isorhapontin, catechin and taxifolin, with structural similarities to astringin. It would be of interest to determine if these compounds are also transformed by *C. polonica* using similar reaction mechanisms.

5.6 Stilbene biotransformation could be beneficial to *C. polonica* as well as to its bark beetle associate, *Ips typographus*

Although stilbenes are studied for their health-promoting properties in mammalian systems, they may have negative effects on bark beetles and their fungal associates throughout all developmental stages. The anti-proliferative, pro-apoptotic (Torres et al., 2003; Athar et al., 2009) and hypolipidemic (Paceasciak et al., 1995) functions of stilbenes in mammalian systems could contribute to retarded development of the larval stages of *I. typographus* as well as the vegetative and reproductive stages of *C. polonica*. Furthermore, potential stilbene-driven inhibition of melanogenesis (Gillbro and Olsson, 2010) could affect bark beetle and fungal survival since melanin in the insect exoskeleton or in the cell walls of *C. polonica* is necessary for protection against UV radiation and desiccation. Stilbenes may also affect the nutritional quality of food ingested by bark beetles and fungi due to protein alkylation by reactive quinones which can originate from catechol groups such as those present in the astringin core structure (Barbehenn et al., 2008). Reactive quinones originating from astringin may even directly destabilize the peritrophic membrane in the bark beetle gut or fungal cell membranes.

Although *C. polonica* and the bark beetle *I. typographus* co-occur, the basis for this mutualistic behavior is still largely unknown. It has been suggested that the fungus depletes the trees defensive capacities during an attack to allow the next generation of bark beetles to develop unimpeded by induced tree defenses (Paine et al., 1997). However, the findings in this thesis, with reference to the potential detrimental effects of stilbenes on both bark beetles and *C. polonica*,

provide indications that the beetle-fungus mutualism may be partially based on the stilbene detoxifying abilities of the fungus.

5.7 Polyphenols in the context of the whole array of spruce defense compounds

Conifers of the family Pinaceae produce a great number of natural products that have been shown to be involved in tree defense against biotic stress. Positive correlations could be drawn between concentrations of diverse natural compounds in conifers and tree resistance to insects and fungi in numerous studies on phenolic and terpenoid based defense mechanisms (Franceschi et al., 2002; Byun-McKay et al., 2006). The most well studied group of compounds involved in conifer defense against insect herbivory and fungal attack are the mono-, sesqui- and diterpenoids, which together form oleoresin (reviewed by Zulak and Bohlmann, 2010). However, there are also many studies showing that single natural products had no effect on herbivores or pathogens attacking conifers (reviewed by Paine et al., 1997) or even showed negative correlations between tree resistance and compound concentration (Clark et al., 2010). It is therefore difficult to draw conclusions on the importance of individual natural products in conifer defense, since the efficacy of a compound depends on numerous aspects, including the genotype and physiological state of the tree, the attacking organism and its population dynamics, and the abiotic conditions prevailing during an attack. However, in evolutionary terms a high diversity of defense compounds allows greater plasticity in defense responses and can confer horizontal resistance or tolerance to attack by a great number of herbivores and pathogens (Raffa and Berryman, 1987).

In this thesis it was shown that the less well studied but highly abundant flavan-3-ols, proanthocyanidins and stilbenes also play an important role in the conifer defense response to wounding and fungal infection. However, besides the compounds studied in this thesis, spruce produces many more polyphenolic compounds such as flavonols, dihydroflavonols, phenolic acids, lignans and neolignans, the ecological functions of which have not yet been studied during biotic stress.

6. GENERAL SUMMARY

The family Pinaceae (order Coniferales) includes many environmentally and economically important softwood tree genera. The most important Pinaceae species endemic to Europe and Northern Asia is the Norway spruce tree (*Picea abies*). This tree species is frequently subject to severe attacks by the scolytid bark beetle *Ips typographus* and its microbial associate, the blue stain fungus, *Ceratocystis polonica*. Spruce trees appear to have structural and chemical defense strategies against invasion by the beetle-fungus complex, including cells with autofluorescent inclusion bodies arranged in rings within the phloem parenchyma, that may form a chemical barrier against invasion by *I. typographus* and *C. polonica*. It has been suggested that these cells could contain polyphenolic compounds, such as stilbene glucosides and proanthocyanidins (polymeric flavan-3-ols), which have been isolated from spruce bark in earlier studies. However, the biosynthesis of these compounds and the regulation of their formation during invasion by bark beetles and their fungal associates is largely unknown. The objective of this research, therefore, was the elucidation of the pathways leading to the formation of stilbene glucosides, flavan-3-ols and proanthocyanidins in spruce and their role in the defense of this tree against infection by the bark beetle associate *C. polonica*.

To investigate the biosynthesis of stilbene glucosides in Norway spruce, two highly similar stilbene synthase (*STS*) genes, *PaSTS*1 and *PaSTS*2, were identified, which differed from angiosperm STSs. Several types of i*n vitro* and *in vivo* assays revealed that *P. abies* STSs catalyze the condensation of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to yield the trihydroxystilbene resveratrol, but do not directly form the dominant spruce stilbenes, which are tetrahydoxylated. However, in transgenic Norway spruce over-expressing *PaSTS*1, significantly higher amounts of the tetrahydroxystilbene glycosides, astringin and isorhapontin, were produced. This result suggests that the first step of stilbene biosynthesis in spruce is the formation of resveratrol which is further modified by hydroxylation, O-methylation and O-glucosylation.

To investigate the biosynthesis of proanthocyanidins in Norway spruce, three genes encoding enzymes responsible for the biosynthesis of flavan-3-ols were identified and functionally characterized as 2,3-*trans*-catechin producing leucoanthocyanidin reductase enzymes (*LAR*) both by *in vitro* enzyme characterization as well as by overexpression in transgenic spruce. Analysis of chemical profiles of monomeric, dimeric as well as polymeric proanthocyanidins in spruce bark by using novel methodologies revealed that proanthocyanidin monomers in spruce have exclusively 2,3-*trans*-(+)-stereospecificity, while polymers contain high levels of flavan-3-ols with 2,3-*cis*-(-)-stereospecificity.

6. Summary

The regulation of stilbene glucoside and proanthocyanidin biosynthesis during fungal attack was investigated by inoculating spruce saplings with *C. polonica* and analyzing bark with different degrees of infection. Transcript abundance of *PaSTS* as well as *PaLAR* genes increased significantly during fungal infection. Levels of monomeric as well as polymeric proanthocyanidins also increased dramatically during the infection process. However, levels of stilbene glucosides increased only transiently during the first few days of infection and then decreased in infected spruce bark tissue. This stilbene deficit was explained by fungal biotransformation of these compounds.

Transformation of host stilbenes by *C. polonica* lead to the formation of stilbene dimers, stilbene-lactones and piceatannol. Upon exposure to host phenylpropanoids enzyme activities for stilbene biotransformation was up-regulated in *C. polonica*. When two strains of *C. polonica* were tested, the rate of stilbene biotransformation was significantly higher in the more virulent fungal stain. In addition, the two strains of *C. polonica* showed different biotransformation strategies, producing different stilbene biotransformation product profiles at different rates. *In vitro* studies also showed a decrease of fungal growth in the presence of stilbenes and proanthocyanidins. While the growth-inhibition of both virulent and avirulent *C. polonica* strains was similar in the presence of proanthocyanidins, the relative growth of the avirulent strain decreased significantly in the presence of stilbenes. This demonstrates that biotransformation of host-derived defense compounds has a significant effect on fungal pathogenicity during host infection and colonization, and should be regarded as a form of host compound detoxification.

7. Zusammenfassung

Zur Familie der Pinaceae (Ordnung Coniferales) gehören mehrere ökologisch als auch ökonomisch bedeutsame Baumarten. Die am weitersten verbreitete Art der Pinaceae ist die Gemeine Fichte (*Picea abies*). Dieser Baum ist sehr häufig vom Borkenkäfer (*Ips typographus*) und einem mit ihm assoziierten Pilz, dem Blaufäulepilz (*Ceratocystis polonica*), befallen. Die Kolonisation eines Baumes hat in der Regel dessen Absterben zur Folge. Um sich vor der Bedrohung zu schützen, haben Fichten chemische Abwehrmechanismen entwickelt. Einer dieser Mechanismen ist die Bildung spezieller autofluoreszenter Zellen im phloematischen Parenchym. Diese Zellen enthalten phenolische Verbindungen wie Stilbenglucoside oder Proanthocyanidine die ein Vordringen des Borkenkäfers bzw. des Pilzes erschweren. Die Biosynthese dieser Verbindungen sowie die Regulation der Biosynthese während eines *I. typographus/C. polonica*-Befalls ist bisher noch weitgehend unerforscht. Die vorliegende Dissertation beinhaltet die Aufklärung der Biosynthese der Stilbenglucoside, Flavan-3-ole und Proanthocyanidine in der Gemeinen Fichte und beschreibt die Funktion der Verbindungen bei der chemischen Verteidigung

Um die Biosynthese der Stilbenglucoside näher zu untersuchen, wurden zwei sehr ähnliche STS-Gene, *PaSTS*1 und *PaSTS*2, identifiziert, die äußerst unterschiedlich gegenüber den STS aus Angiospermen sind. Eine Anzahl von *in vitro* und *in vivo* Enzymtests ergab, dass diese STS nur die Kondensationreaktion von *p*-Cumaryl-CoA und drei Molekülen Malonyl-CoA katalysieren, was zur Bildung des Tetrahydroxystilbens Resveratrol führt. Demgegenüber wurden aber in *PaSTS*1-überexprimierender transgener Fichte signifikant höhere Mengen an den Tetrahydroxystilbenglykosiden, Astringin und Isorhapontin, gefunden. Dies lässt den Schluss zu, dass der erste Schritt der Stilbenbiosynthese in Fichte, die Bildung von Resveratrol ist, welches später dann durch Hydroxylierung, O-Methylierung oder O-Glucosilierung zu den in der Fichtenborke am meisten vorkommenden Stilbeneglucosiden Astringin und Isorhapontin umgewandelt wird.

Die Biosynthese der Proanthocyanidine (Flavan-3-ole) in der Fichte wurde näher charakterisiert, indem 3 Leucoanthozyanidin Reduktasen (*LAR*) isoliert wurden, die für die Biosynthese der Proanthocyanidin-Monomere verantwortlich waren. Die Funktionalität konnte dabei durch *in vitro* Charakterisierung als auch durch Überexpression in transgener Fichte gezeigt werden. Neu etablierte chemische Analysen zur Stereochemie der monomeren, dimeren und als auch polymeren Proanthocyanidine zeigten dabei, dass Monomere aus der Fichtenborke ausschließlich in der 2,3-*trans*-(+) Form, Polymere aber dagegen hauptsächlich in der 2,3-*cis*-(-) Form vorkommen.

Die Regulation der Stilbenglucoside und Proanthocyanidine (Flavan-3-ole) während des Pilzbefalles wurde mittels Pilzinokulation von *C. polonica* in die Fichtenborke und anschließender

Analyse der Borke in den unterschiedlichen Befallsgraden näher untersucht. Dabei wurde festgestellt, dass die Transkriptanzahl der *PaSTS* als auch die der *PaLAR*, ebenso wie der Gehalt an mono- und polymeren Proanthocyanidin während der Pilzinfektion signifikant erhöht war. Allerdings erhöhten sich die Mengen der Stilbenglucoside im infizierten Borkengewebe nur während der ersten Infektionstage und nahmen danach wieder ab. Dieser Abfall konnte weiterführend durch Versuche erklärt werden, die zeigten, dass *C. polonica* in der Lage ist, die abgelagerten als auch *de novo* gebildeten Stilbenglucoside im Phloemparenchym enzymatisch umzuwandeln. Stilbenglucoside der Fichte können durch *C. polonica* in Stilben-Dimere, Stilben-Lactone oder Piceatannol enzymatisch konvertiert werden, was sich auch in einer höheren Aktivität der jeweils daran beteiligten Enzyme äußerte. Als zwei *C. polonica* Stämme getestet wurden, zeigte der virulente Stamm eine höhere Aktivität ebenso wie qualitative und quantitative Unterschiede in den Abbauprodukten gegenüber dem nicht-virulenten Stamm. Weitere i*n vitro* Studien belegten ein geringeres Wachstums von *C. polonica* in Gegenwart von Stilbenenglucosiden und Proanthocyanidinen (Flavan-3-olen). Während die Wachstums-Inhibierung von virulentem und avirulentem Stamm in Gegenwart von Proanthocyanidin vergleichbar war, verringerte sich das Wachstum des avirulenten Stammes in Gegenwart von Stilbenen signifikant. Die in der vorliegenden Arbeit vorgestellten Ergebnisse zeigen, dass der Abbau bzw. die Umwandlung von pflanzlichen Abwehrsubstanzen durch den Pilz einen entscheidenden Einfluss auf den Ablauf einer Infektion bzw. einer weiteren Besiedelung haben kann, und demzufolge wahrscheinlich als eine Form der Entgiftung von pflanzlichen Sekundärmetaboliten einzuschätzen ist.

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9.1 Supplementary materials: Research Chapter 1

Supplementary methods: Quantitative genomic PCR

Needle tissue of *PaSTS*1 Line 5, *PaSTS*1 Line 11 and the vector control was ground to a fine powder under liquid nitrogen. Genomic DNA was isolated from the tissue powder using the method developed by Wright *et al*. (2010) and diluted to a concentration of 100 ng μl^{-1}. PCR was performed with Brilliant SYBR Green QPCR Master Mix ® (Stratagene), 100 ng genomic DNA and 10 pmol forward and 10 pmol reverse primer. *PaSTS* was amplified using the forward primer 5'-CTCGAGATGTCTTCCTCATCTCGTCC-3' and the reverse primer 5'-CCTTCCGTCAGTTCAAAATCTCCGAC -3', designed to amplify 190 base pairs from both *STS*1 and *STS*2 simultaneously. PCR thermocycles were run as follows: 15 min at 95°C followed by 40 cycles of 45 s at 95°C, 30 s at 60°C and 30 s at 72°C using a Stratagene MX3000P thermocycler. *STS* gene abundance was normalized to the abundance of the ubiquitin gene (Schmidt et al., 2010) (GenBank accession number EF681766) amplified with the forward primer 5'-CCCTCGAGGTAGAGTCATCG-3' and the reverse primer 5'-CCAGAGTTCTCCCTTTACTTG-3'. Primer efficiencies were calculated according to Pfaffl (2001). Primer specificity was confirmed by melting curve analysis from 55°C to 95°C and by cloning and sequencing 20 amplicons for each primer pair. Relative *STS* gene copy number in transgenic *PaSTS*1 over-expressing lines was calibrated against the vector control. Each gene abundance represents the rounded average of four biological replicates, each of which is represented by three technical replicates.

9. Supplementary Material

Table S2.1. Forward and reverse attB primers for amplifying and cloning *PaSTS* and *PaCHS* into pDONR207. attB forward and reverse sequences are attached to the 5'end of the sequence specific primer regions

	Forward Primer	**Reverse primer**
attB sequence	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-
STS 1	attBF-CAATGTCTGCAGGAATGACTGTTG-3'	attBR-ATTATGGAAGGAGAACGCTCTTAAGAAC-3'
STS 2	attBF-GAATGACTGTTGATTTGGAAAC-3'	attBR-ATTATGGAAGGAGAACGCTCTTAAGAAC-3'
CHS 1	attBF-GCATGTCTCAGAATTTGGGTTTG-3'	attBR-ATCACTGCAGGGGAACGCTCTTGAG-3'
CHS 2	attBF-GCATGTCTCAGAATTTGGGTTTG-3'	attBR-TTCAATCAGTGCAGGGAACGCTCCTG-3'
CHS 3	attBF-TTATGGCTGGAGGACTGATGGCG-3'	attBR-ATCACTGCAGGGGAACACTCTTCAG-3'
CHS 4	attBF-TTATGGCTGGAGGACTGATGGCG-3'	attBR-ATCACTGCATGGGAACGCTTTTCAG-3'
CHS 5	attBF-TTATGGCTGGAGGACTGATGGCG-3'	attBR-ATTATTGCAGGGGAACGCTCTTGAG-3'
CHS 6	attBF-TTATGGCTGGAGGAATCATGGAG-3'	attBR-ATTATTGCAGGGGAACGCTCTTGAG-3'
CHS 7	attBF-ATATGCCTGCTGGAATGAAGGTGG-3'	attBR-CCTATTGCAGAGGGACGCTCTTGAGAAC-3'
CHS 8	attBF-GCATGCCTGGGACTTTGGGTTTGG-3'	attBR-GTTATTGCGGGCAGGGGACGCTCTTG-3'
CHS 9	attBF-GAATGATGAAGGATCTGGAGG-3'	attBR-ATCACTGCAGGGGAACACTCTTC-3'

Table S2.2. NCBI accession numbers of CHS and STS sequences used for phylogenetic analysis.

Organism	*Gene*	NCBI accession number	Reference
Vitis vinifera	*CHS*	CAA53583.1	Sparvoli et al., 1994
Vitis vinifera	*RVS 1*	P28343.2	Melchior and Kindl, 1990
Vitis vinifera	*RVS 2*	P51071.1	Melchior and Kindl, 1991
Arachis hypogaea	*CHS*	AAU43217.1	Condori et al., 2009
Arachis hypogaea	*RVS 1*	1Z1EA	Shomura et al., 2005
Arachis hypogaea	*RVS 2*	1Z1FA	Shomura et al., 2005
Arachis hypogaea	*RVS 3*	P51069.1	Lanz et al., 1991
Medicago sativa	*CHS*	AAA02824.1	Junghans et al., 1993
Pisum sativum	*CHS*	CAA44935.1	Ichinose et al., 1992
Trifolium subterraneum	*CHS*	AAA1876.1	Arioli et al., 1994
Glycine max	*CHS*	AAB01004.1	Akada and Dube, 1995
Pyrus communis	*CHS*	AAX16494.1	Fischer et al., 2007
Malus X *domestica*	*CHS*	AAX16492.1	Fischer et al., 2007
Ginkgo biloba	*CHS*	AAS21057	Pang et al., 2004
Pinus densiflora	*STS*	BAA94593.1	Kodan et al., 2002
Pinus densiflora	*STS*	BAA89667.1	Kodan et al., 2002
Pinus strobus	*STS*	CAA87013.1	Raiber et al., 1995
Pinus strobus	*STS*	CAA87012.1	Raiber et al., 1995
Picea sitchensis	*STS 1*	ABR17893.1	
Picea sitchensis	*STS 2*	XXXXXXXX	
Picea glauca	*STS 1*	XXXXXXXX	
Picea glauca	*STS 2*	XXXXXXXX	
Picea abies	*STS 1*	XXXXXXXX	
Picea abies	*STS 2*	XXXXXXXX	
Picea sitchensis	*CHS 1*	ABK23800.1	
Picea sitchensis	*CHS 2*	ABK24675.1	
Picea sitchensis	*CHS 3*	ABK25228.1	
Picea sitchensis	*CHS 4*	ABK25266.1	
Picea sitchensis	*CHS 5*	ABK23777.1	
Picea sitchensis	*CHS 6*	ABK25340.1	
Picea sitchensis	*CHS 7*	ABK24577.1	
Picea sitchensis	*CHS 8*	ABK25158.1	
Pinus sylvestris	*CHS*	CAA43166.1	Fliegmann et al., 1992
Picea glauca	*CHS 1*	XXXXXXXX	

Picea glauca	*CHS 2*	XXXXXXXX
Picea glauca	*CHS 3*	XXXXXXXX
Picea glauca	*CHS 4*	XXXXXXXX
Picea glauca	*CHS 5*	XXXXXXXX
Picea glauca	*CHS 6*	XXXXXXXX
Picea glauca	*CHS 7*	XXXXXXXX
Picea glauca	*CHS 8*	XXXXXXXX
Picea abies	*CHS 1*	XXXXXXXX
Picea abies	*CHS 2*	XXXXXXXX
Picea abies	*CHS 3*	XXXXXXXX
Picea abies	*CHS 4*	XXXXXXXX
Picea abies	*CHS 5*	XXXXXXXX
Picea abies	*CHS 6*	XXXXXXXX
Picea abies	*CHS 7*	XXXXXXXX
Picea abies	*CHS 8*	XXXXXXXX

Table S2.3: Forward and reverse primers for quantitative real-time PCR

	Forward Primer	**Reverse primer**
PaUBI	5'-GTTGATTTTTGCTGGCAAGC-3'	5'-CACCTCTCAGACGAAGTAC-3'
***PaLAR*1**	5'-GAACTGGCAGCCATATGGGAGACC-3'	5'-CTGTAATAAAGTTCAGAGGCCTCG-3'
***PaLAR*2**	5'-ACAAGAACTTTTGCATTTAGCCG-3'	5'-GAAATCTCTGGATATAGTTGTGAC-3'
***PaLAR*3**	5'-GGGCATCACGATCTAGAGGTCTG-3'	5'-GGATGGTAAATAGAGGAAGACGAGTC-3'
PaPAL	5'-GTACTTCAGTAGGAGCAGCACTGG-3'	5'-GACATACTCCATGATCGCTGCGG-3'

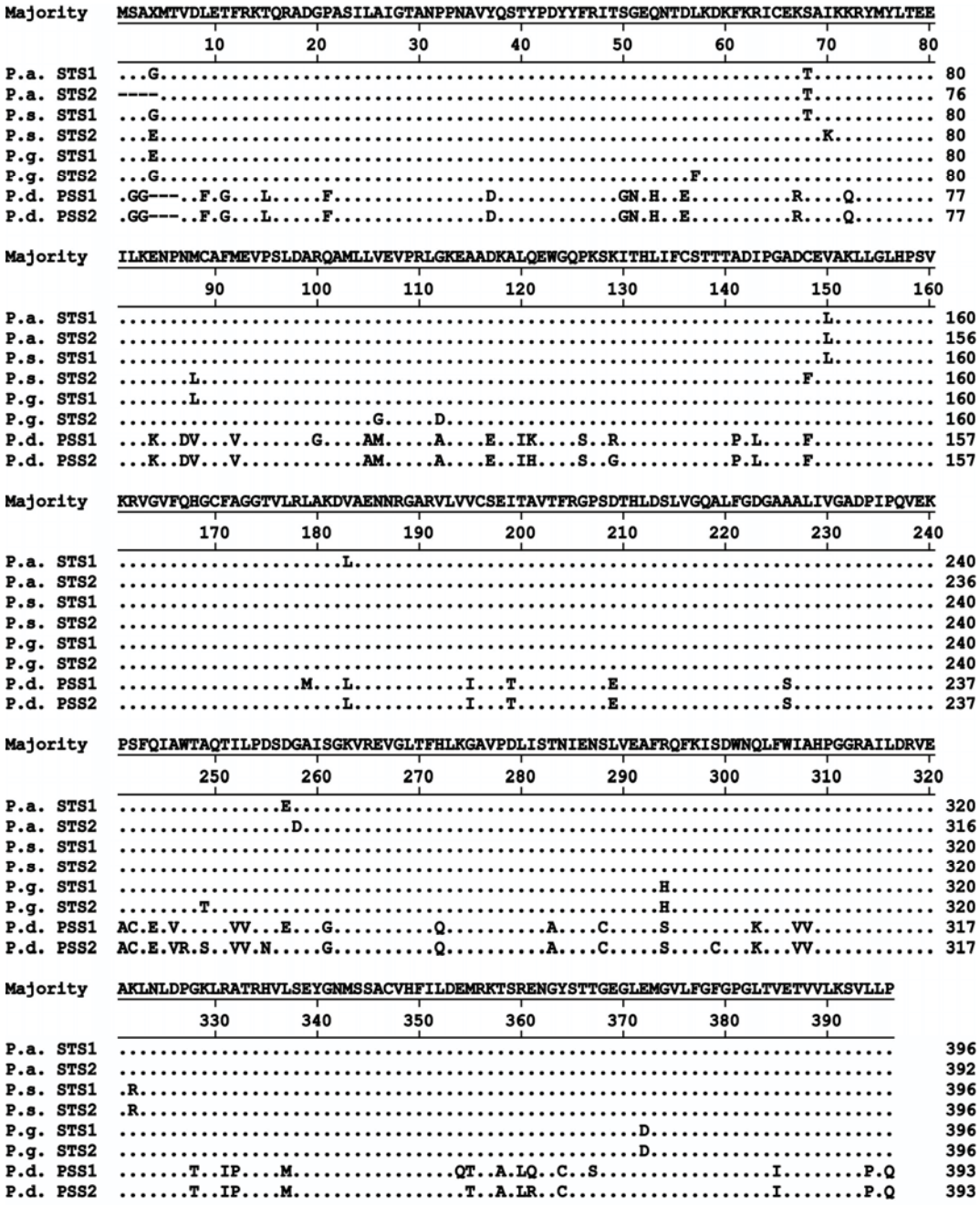

Figure S2.1. Amino acid sequence alignment of STS from *P. abies* (P.a.), *P. sitchensis* (P.s.), *P. glauca* (P.g.) and pinosylvin synthase (PSS) enzymes from *P. densiflora* (P.d.). Dots represent amino acids which are conserved in all presented sequences.

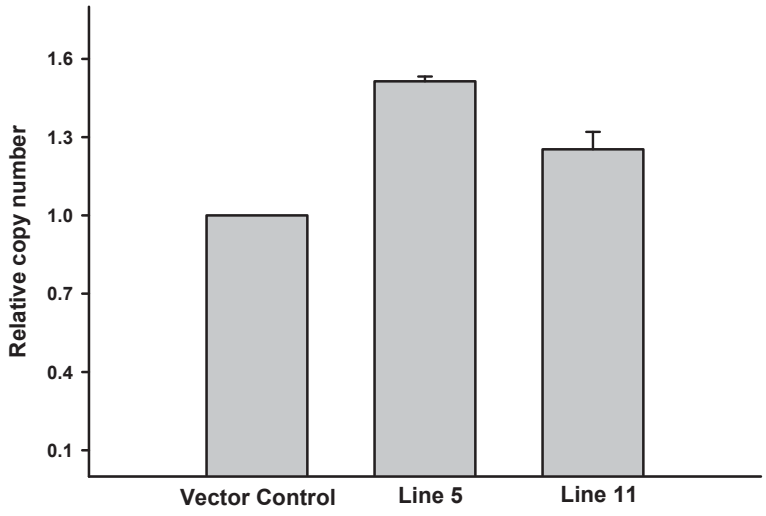

Figure S2.2. Relative *PaSTS* gene copy number in transgenic *PaSTS*1 lines and vector control.

9.2 Supplementary materials: Research Chapter 2

Table S3.1. Chemical shifts of the new compounds (**1**) and (**4**)

Position	δ_H (**1**)[a, c]	δ_c (**1**)[b, c]	δ_H (**4**)[a, d]	δ_c (**4**)[b, d]
1		172.48		175.48
2	6.271 (*s*)	114.61	6.122 (*s*)	115.47
3		165.29		168.07
4	5.584 (*d*, 8.9)	78.26	5.733 (*d*, 8.6)	81.57
5	7.037 (*d*, 16.0)	118.23	6.986 (*d*, 16.5)	118.82
6	7.128 (*d*, 16.0)	138.51	7.049 (*d*, 16.5)	140.99
7		137.96		138.70
8	6.830 (*d*, 2.0)	105.74	6.530 (*d*, 2.1)	107.15
9		159.00		160.12
10	6.458 (*t*, 2.0, 2.0)	105.42	6.283 (*t*, 2.1, 2.1)	105.45
11		158.60		160.12
12	6.646 (*s*)	108.92	6.530 (*d*, 2.1)	107.15
13a	2.545 (*ddd*, 16.6, 8.9, 2.6)	38.06	2.992 (*dd*, 16.2, 2.9)	41.87
13b	3.10 (*m*)	38.06	2.442 (*dd*, 16.1, 9.0)	41.87
14		170.90		174.86
1'	4.805 (*dd*, 7.5, 3.1)	100.41		
2'	3.20 (*overlap*)	73.06		
3'	3.24 (*overlap*)	76.50		
4'	3.14 (*overlap*)	69.53		
5'	3.30 (*overlap*)	76.99		
6'a	3.45 (*m*)	60.42		
6'b	3.70 (*m*)	60.42		

[a)] proton resonances: shift value [ppm] (*multiplicity*, coupling constants [Hz])

[b)] carbon resonances: shift value [ppm]

[c)] in CD_3OD

[d)] in d_6-DMSO

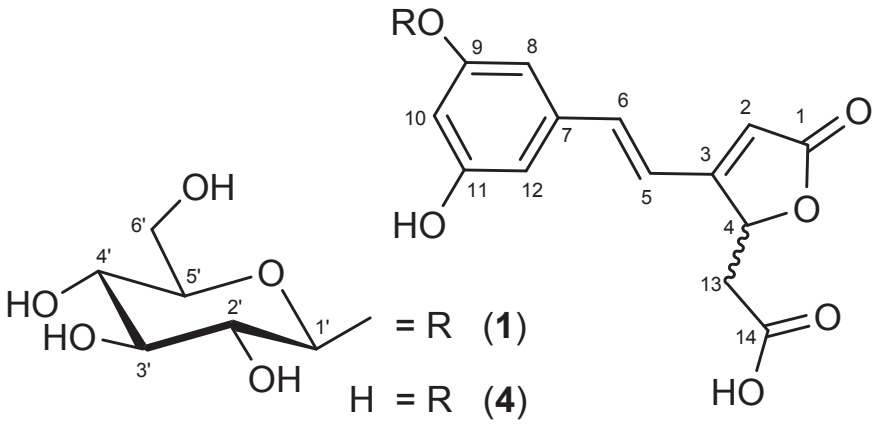

Figure S3.1. Monomeric astringin transformation products isolated from *in-vitro*-biotransformation experiments

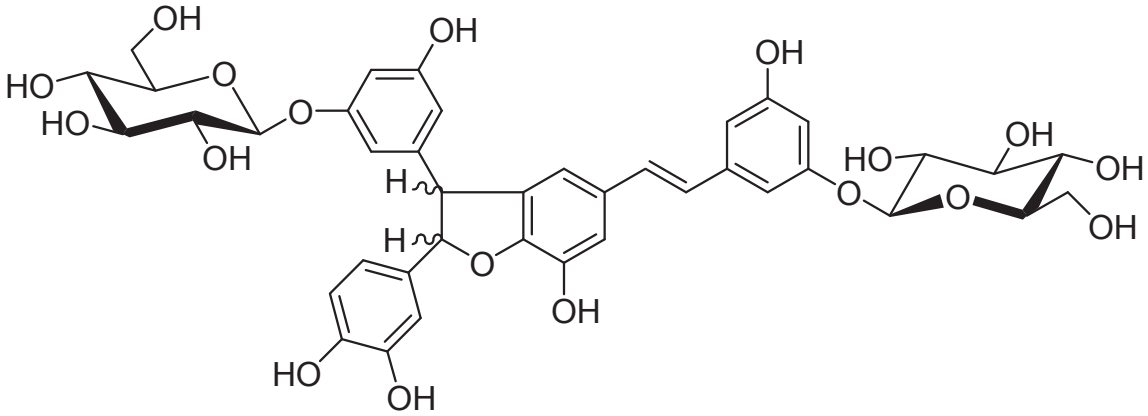

Figure S3.2. Dimeric astringin transformation products isolated from *in-vitro*-biotransformation experiments (Piceasides A and B)

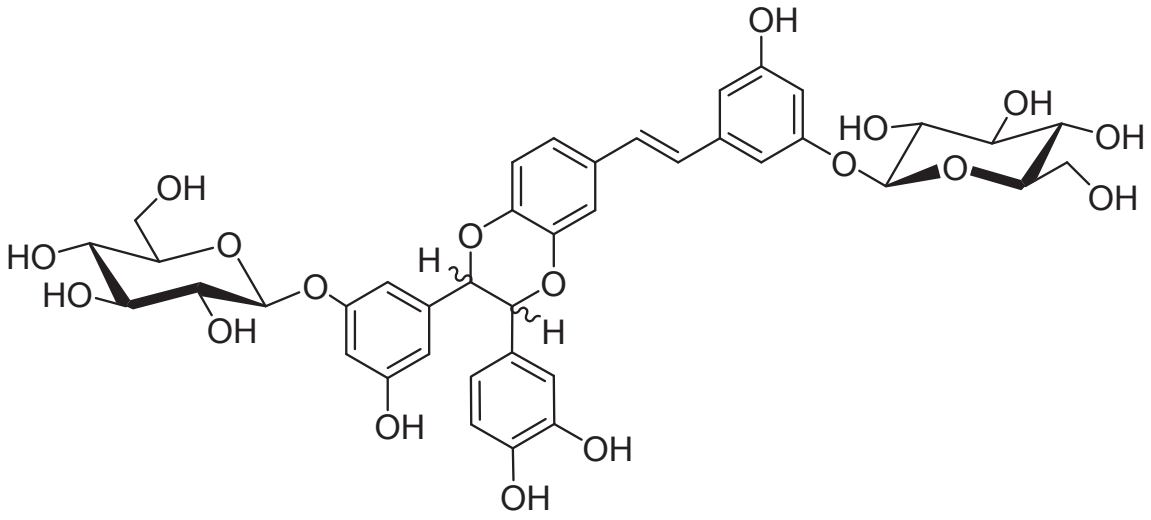

Figure S3.3. Dimeric astringin transformation products isolated from *in-vitro*-biotransformation experiments (Piceasides G and H)

9.3 Supplementary materials: Research Chapter 3

Table S4.1. Forward and reverse primers for amplifying and cloning *LAR* and *ANR* into pDONR207 or pYES. attB forward and reverse sequences are attached to the 5'end of the sequence specific primer regions

	Forward Primer	Reverse primer
attB	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-
LAR 1	attBF-CCATGGCGGCCTGTGGAACTG-3'	attBR-GGTGGGAAATGCCAACGTTTAGTG-3'
LAR 2	attBF-CCATGGCCAGCAGTAGAATACTAG-3'	attBR-GGAAATTCGAAGATGTACAAGCAAC-3'
LAR 3	attBF-CAATGGCGGTCTGTGGAACTGAAG-3'	attBR-GCAGATATTCGTCGAAAAATTCATC-3'
ANR 1	5'-GAAAAATGGCAGCAACGATCAGCAATGGC-3'	5'-GTAAGCAAGCAAGCCCTTGGCCTTGG-3'
ANR 2	5'-GAACCATGGCGGTCTGTGGAAC-3'	5'-CACAGATATTCGTCGAAAAATTCATCTAC-3'

Table S4.2. NCBI accession numbers of LAR and ANR sequences used for phylogenetic analysis.

Organism	Gene	NCBI accession number	Reference
Malus domestica	*LAR* 1	DQ139836	Pfeiffer et al., 2006
Malus domestica	*LAR* 2	DQ139837	Pfeiffer et al., 2006
Malus domestica	*ANR*	AAZ79363	Pfeiffer et al., 2006
Pyrus comunis	*LAR* 1	DQ251190	Fischer et al., 2007
Pyrus comunis	*LAR* 2	DQ251191	Fischer et al., 2007
Pyrus comunis	*ANR*	ABB77695	Pfeiffer et al., 2006
Fragaria X ananassa	*LAR* 1	DQ834906	Almeida et al., 2007
Fragaria X ananassa	*ANR*	ABG76842	Almeida et al., 2007
Vitis vinifera	*LAR* 1	BN000698	Bogs et al., 2005
Vitis vinifera	*LAR* 2	3I6IA	Mauge et al., 2010
Desmodium unicatum	*LAR* 1	Q84V83	Tanner et al., 2003
Lotus corniculatus	*LAR* 1	DQ49103	Paolocci et al., 2007
Lotus corniculatus	*LAR* 2	DQ49105	Paolocci et al., 2007
Lotus corniculatus	*ANR*	ABC71337	Paolocci et al., 2007
Phaeseolus vulgaris	*LAR* 1	BN000698	Bogs et al., 2005
Pinus taeda	*LAR*-like	BN000697	Bogs et al., 2005
Ginkgo biloba	*ANR*	AAU95082	Shen et al., 2006
Picea abies	*LAR* 1		
Picea abies	*LAR* 2		
Picea abies	*LAR* 3		
Picea abies	*ANR*-like		
Picea abies	*ANR*-like		

Table S4.3: Forward and reverse primers for quantitative real-time PCR

	Forward Primer	Reverse primer
PaUBI	5'-GTTGATTTTTGCTGGCAAGC-3'	5'-CACCTCTCAGACGAAGTAC-3'
PaLAR1	5'-GAACTGGCAGCCATATGGGAGACC-3'	5'-CTGTAATAAAGTTCAGAGGCCTCG-3'
PaLAR2	5'-ACAAGAACTTTTGCATTTAGCCG-3'	5'-GAAATCTCTGGATATAGTTGTGAC-3'
PaLAR3	5'-GGGCATCACGATCTAGAGGTCTG-3'	5'-GGATGGTAAATAGAGGAAGACGAGTC-3'

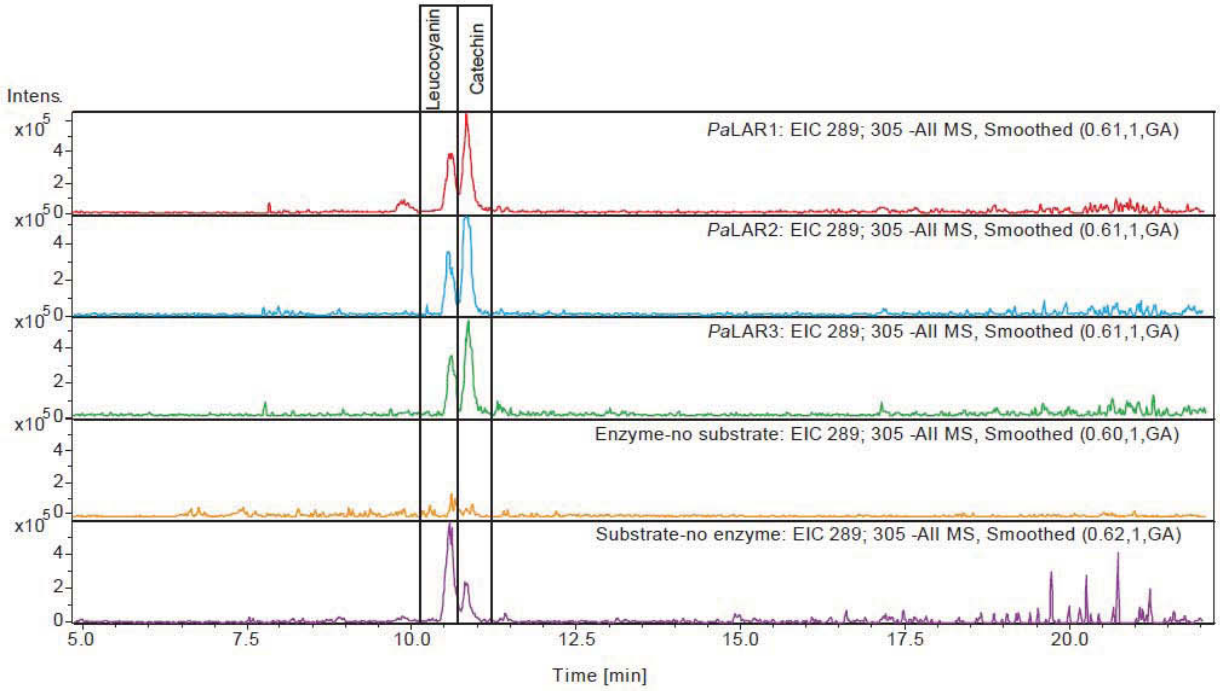

Figure S4.1: Characterization of LAR enzymes from *P. abies* expressed in *E. coli*. Recombinant LAR enzymes accepted leucoanthocyanidins as substrates and released catechin as products. Extracted ion chromatograms (EIC) from enzyme-no substrate controls as well as from substrate-no enzyme controls are included.

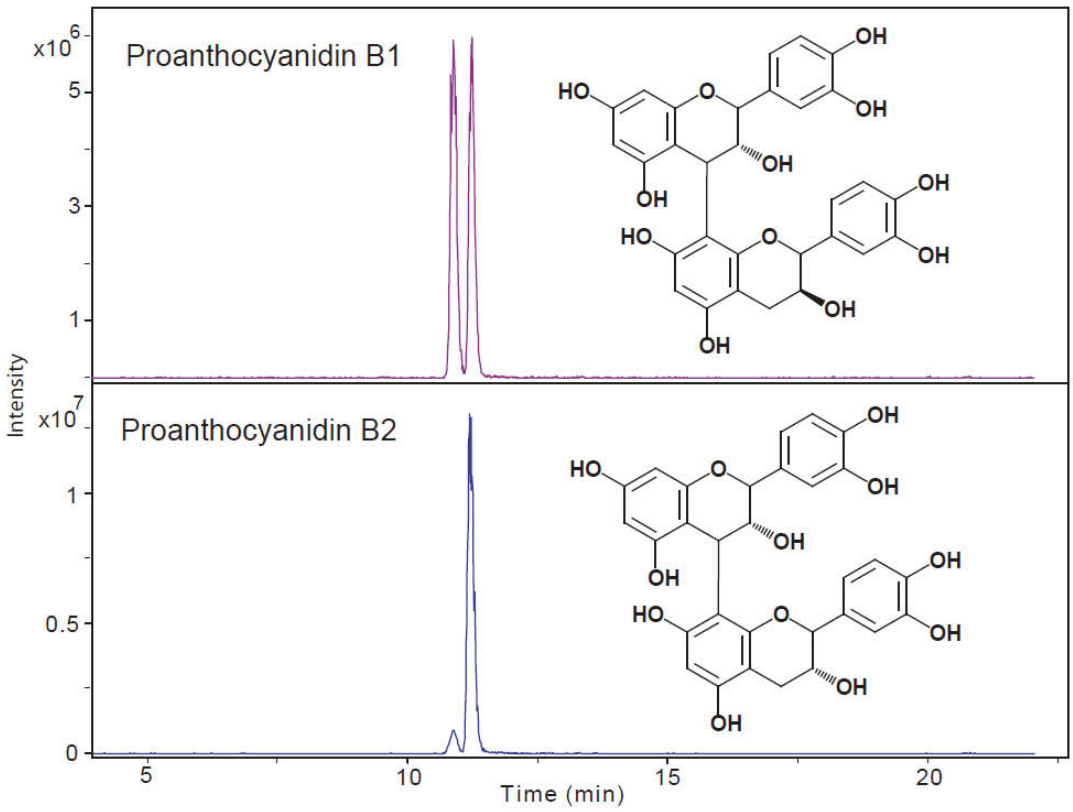

Figure S4.2: Reductive hydrolysis of dimeric proanthocyanidin B1 yielded equal amounts of catechin (retention time: 10.9) and epicatechin (retention time: 11.2). Hydrolysis of proanthocyanidin B2 yielded mostly epicatechin, but also small amounts of catechin.

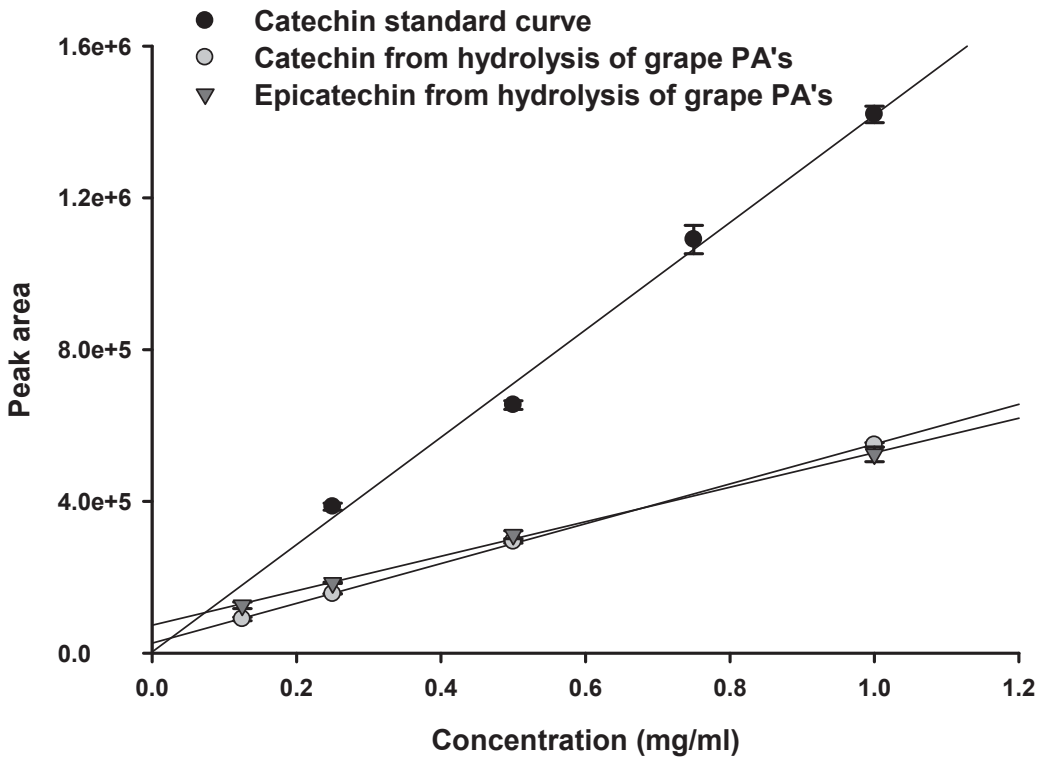

Figure S4.3: Linear response curve after reductive hydrolysis of increasing concentrations of partially purified proanthocyanidins from grape skins. Quantification of hydrolysis products was accomplished by LC-ESI-MS-MS.

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Abridged Curriculum Vitae

Personal information

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Nationality	Germany / South Africa
Date of birth	08. March 1976
Gender	Female
Marital status	Married

Education and training

Dates	2006-Current
Title of qualification awarded	PhD Studentship
Thesis title	Biosynthesis of polyphenols in Norway spruce as a defense strategy against attack by fungus *Ceratocystis polonica*
Name and type of organisation providing education and training	Max Planck Institute for Chemical Ecology, Department of Biochemistry, Hans Knoell Strasse 8, 07745 Jena Germany
Dates	2003-2005
Title of qualification awarded	**Magister Scientiae Agriculturae**
Thesis Title	The ecology and epidemiology of *Fusarium circinatum*, the causal agent of pitch canker in pine
Name and type of organisation providing education and training	Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa
Dates	1998-2002
Title of qualification awarded	**Baccalaureus Scientiae Agriculturae**
Principal subjects covered	Microbiology, Genetics, Biochemistry, Botany, Molecular biology, Soil science, Agronomy-Horticulture, Statistics
Name and type of organisation providing education and training	University of Pretoria, South Africa

11. Curriculum Vitae

Personal skills and competences

Mother tongue: German

Other language(s): English (writing, reading, verbal communication excellent)
Afrikaans (writing, reading, verbal communication excellent)
Dutch (reading and rudimentary verbal communication)
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Publications

Beyaert, I., Köpke, D., Stiller, J., Hammerbacher, A., Yoneya, K., Schmidt, A., Gershenzon, J., Hilker, M. (in press). Can insect egg deposition 'warn' a plant of future feeding damage by herbivorous larvae? PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON SERIES B-BIOLOGICAL SCIENCES

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Oral presentations

Hammerbacher A., Paetz C., Fenning T., Bohlmann J., Gershenzon J., Schmidt A. The role of stilbene glycosides in *Picea abies* defense against the bark beetle associated fungus *Ceratocystis polonica*. 2009 IUFRO Tree Biotechnology Conference, Whistler, CA, Jul 2009

Poster presentations

Hammerbacher A., Paetz C., Gershenzon J., Schmidt A. Stereospecific proanthocyanidin coupling by fungal oxidoreductases in *Picea abies* during *Atta*ck by the bark beetle associated fungus *Ceratocystis polonica*. 9th International Symposium on Peroxidases, OxiZymes, Leipzig, DE, Jun 2010

Hammerbacher A., Paetz C., Fenning T., Bohlmann J., Gershenzon J., Schmidt A. Virulence of the bark beetle associated fungus, *Ceratocystis polonica*, in Norway spruce (*Picea abies*) is associated with metabolism of host defenses. Gordon Research Conference - Plant-Herbivore Interaction, Gordon Research Conferences, Galveston, TX, US, Feb 2010

Hammerbacher A., Paetz C., Fischer T., Fenning T., Bohlmann J., Gershenzon J., Schmidt A. Proanthocyanidin production in *Picea abies* during infection by the bark beetle-associated fungus *Ceratocystis polonica*. 2009 IUFRO Tree Biotechnology Conference, Whistler, CA, Jul 2009

12. Selbständigkeitserklärung

Die zurzeit gültige Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst und nur unter Verwendung der angegebenen Hilfsmittel erstellt. Alle verwendeten Quellen wurden angegeben. Alle Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind benannt.

Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen.

Die vorliegende Arbeit wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch bei einer anderen Hochschule als Dissertation eingereicht.

Jena, den

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Almuth Hammerbacher