Running title: Flavan-3-ol biosynthesis in spruce

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Flavan-3-ols in Norway spruce: Biosynthesis, accumulation and function in response to attack by the bark beetle-associated fungus Ceratocystis polonica

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Summary: Monomeric and polymeric flavan-3-ols are anti-fungal defense compounds in Norway spruce (Picea abies)

Keywords: proanthocyanidin, procyanidin, catechin, flavan-3-ol, leucoanthocyanidin reductase, Picea abies, Ceratocystis polonica
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SUMMARY

Proanthocyanidins (PAs) are common polyphenolic polymers of plants found in foliage, fruit, bark, roots, rhizomes and seed coats that consist 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, little is known about their biosynthesis and ecological roles in gymnosperms. In this study the genes encoding leucoanthocyanidin reductase (LAR), a branch-point enzyme involved in the biosynthesis of 2,3-trans-(+)-flavan-3-ols were identified and functionally characterized in Norway spruce (Picea abies), the most widespread and economically important conifer in Europe. In addition the accumulation of flavan-3-ols and PAs was investigated in spruce saplings after wounding or inoculation with the fungal pathogen Ceratocystis polonica, which is vectored by bark beetles and is usually present during fatal beetle attacks. Monomeric and dimeric flavan-3-ols were analyzed by reverse-phase high pressure liquid chromatography, while the size and subunit composition of larger PAs were characterized using a novel acid hydrolysis method and normal phase chromatography. Only flavan-3-ol monomers with 2,3-trans stereochemistry were detected in spruce bark; dimeric and larger PAs contained flavan-3-ols with both 2,3-trans and 2,3-cis stereochemistry. 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 the levels of 2,3-trans-(+)-catechin and PAs which are produced in the tree in response to fungal infection inhibit C. polonica growth and can therefore be considered chemical defense compounds.
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 consisting of a C_6-C_3-C_6 flavonoid skeleton. Flavan-3-ol units in PA chains differ structurally according to the stereochemistry of the asymmetric carbons on the C-ring (Fig. 1), with the two most common compounds being 2,3-trans-(+)-catechin or 2,3-cis-(-)-epicatechin. The structural diversity of these compounds is further enhanced by the substitution 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). For the formation of PAs, flavan-3-ol units are commonly linked by the C4 position of the extender units and the C8 position of the terminal unit (4 → 8) (Fig. 1), or less commonly by C4 and C6. The greatest source of structural variability in these compounds, however, is the length of the polymer (degree of polymerization), which varies from two to hundreds of monomer units per chain (for complete overview, see Ferreira and Slade, 2002).

Within angiosperms, monomeric flavan-3-ols and PAs appear to function in resistance against various biotic and abiotic stresses. Their role in providing protection against UV irradiation (Jaakola et al., 2004) and ozone (Karonen et al., 2006) by decreasing oxidative stress has been demonstrated in numerous plant species. These compounds are also well-studied as defenses against mammalian (Theodoridou et al., 2010) and insect herbivory (Feeny, 1970; Donaldson and Lindroth, 2004). Moreover, monomeric flavan-3-ols and PAs have been shown to negatively affect bacterial growth (Scalbert, 1991) as well as the 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 the biosynthesis of melanin (Chen et al., 2006), which is an important virulence factor in many plant pathogenic fungal species (Ebole, 2007). PAs 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 and other gymnosperms the functions of monomeric flavan-3-ols and PAs 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 are involved in the tree’s defensive response, given prior work in angiosperms.

The biosynthesis of flavan-3-ols and the accumulation of PAs have been studied in numerous economically important plant species (Bogs et al., 2005; Pfeiffer et al., 2006; Almeida et al., 2007; 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). Flavan-3-ols 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 leucoanthocyanidins (Fig. 1). For the biosynthesis of the 2,3-cis-(−) compounds (e.g. 2,3-cis-(−)-epicatechin), leucoanthocyanidins are converted to anthocyanidins by anthocyanidin synthase (ANS) and the products are then reduced by anthocyanidin reductase (ANR) (Xie et al., 2004). For the biosynthesis of 2,3-trans-type flavan-3-ols (e.g. 2,3-trans-(+)-catechin), leucoanthocyanidins are reduced directly to the corresponding flavan-3-ol (Tanner et al., 2003) by leucoanthocyanidin reductase (LAR). ANR and LAR are both NADPH/NADH-dependant isoflavone-like reductases belonging to the reductase-epimerase-dehydrogenase superfamily. Genes coding for these enzymes can occur as single genes or as gene families in different PA producing plant species (Paolocci et al., 2007; Bogs et al., 2005). However, not all plant species utilize both pathways for producing the monomeric units of their PAs (Xie et al., 2004).

Despite considerable efforts, the mechanism by which flavan-3-ol monomers are polymerized into PAs is not yet 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 oxidative coupling of flavan-3-ols or anthocyanidins has been proposed (Haslam, 1980). PA formation has been envisioned to occur by both enzyme-mediated and non-enzymatic mechanisms (Stafford, 1983).

Since conifers and angiosperms have diverged more than 300 million years ago, an understanding of the composition and biosynthesis of flavan-3-ols in conifers might lead to new insights into the formation as well as the biological function of these important compounds. In pursuit of this goal, we identified LAR genes involved in 2,3-trans-(+)-flavan-3-ol biosynthesis in the conifer species *Picea abies* (Norway spruce). The phylogenetic relationships of these sequences were investigated and they were functionally characterized *in vitro* by heterologous expression in a bacterial system and *in vivo* by overexpression in transgenic spruce. The most serious insect pest of Norway spruce is the spruce bark beetle *Ips typographus*, which co-invades host trees along with the fungal pathogen *Ceratocystis polonica*. We investigated the constitutive profiles of monomeric flavan-3-ols and PAs in Norway spruce bark and monitored their quantitative and qualitative changes after infection by *C. polonica* as well as changes in LAR transcripts. The effects of flavan-3-ols and PAs on *C. polonica* were also evaluated using *in vitro* assays with these compounds in concentrations similar to those present in spruce bark.

**RESULTS**

The flavan-3-ol content of Norway spruce (*P. abies*) bark increases after infection with *C. polonica*

In order to obtain an overview of flavan-3-ol composition in Norway spruce bark, we analyzed monomer, dimer and polymer concentrations in bark after infection with the bark beetle associated fungus *C. polonica*. Four-year-old spruce saplings were wounded and inoculated with two strains differing in virulence. Controls included unwounded saplings and those subjected to wounding without fungal inoculation. Bark tissue for analysis was harvested before treatment and 2, 7, 14 and 28 days after treatment.
The monomeric flavan-3-ols in Norway spruce bark consisted of mixtures of 2,3-
trans-(+)-catechin and 2,3-trans-(+)-gallocatechin with no detectable levels of 2,3-cis-(−)-
epicatechin or 2,3-cis-(−)-epigallocatechin (see Fig. 1 for structures). The levels of
catechin and gallocatechin (Fig. 2A) increased significantly over the experimental time-
course of 28 days in inoculated bark (p < 0.001). In controls that were wounded without
inoculation, the increase was much less. The levels of total monomers in wounded but
non-inoculated controls collected 2 days after treatment did not differ from those in
unwounded bark samples collected at the onset of the time-course (Supplemental figure
1). In fungal-inoculated samples, the ratio of catechin:gallocatechin changed from 11:1 at
2 days after infection to 20:1 at 28 days after infection (Fig. 2A, levels of individual
compounds are given in Supplemental figure S2). There were no differences in the
responses of spruce bark to infection by the avirulent (isolate 1) or virulent (isolate 2)
fungal strains.

The dimeric flavan-3-ols (=dimeric proanthocyanidins or dimeric PAs) that could
be detected were mainly two 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]. Dimeric prodelphinidins (trihydroxylated B-ring) with unknown
structures could also be detected, but were not included in this analysis. Concentrations of
the dimeric PAs, B1 and B3, increased significantly over the 28 day period (Fig. 2B,
levels of individual compounds are given in Supplemental figure S2) in inoculated and in
wounded control bark of spruce saplings (p < 0.001) compared to the non-wounded
control bark samples collected prior to treatment (Supplemental figure 1). The major
differences in dimer concentrations among the treatments were mainly explained by
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.

To measure the amount of polymeric flavan-3-ols (PAs), a method was developed
using reductive hydrolysis with trifluoroacetic acid and sodium cyanoborohydride to
yield monomeric units (Supplemental figure S3). The monomers detected in Norway
spruce bark tissue after hydrolysis were 2,3-trans-(+)-catechin and 2,3-trans-(+)-gallocatechin and high concentrations of 2,3-cis-(−)-epicatechin and 2,3-cis-(−)-epigallocatechin. Total monomers after hydrolysis increased significantly over the 28 day infection period in wounded control samples as well as in infected bark (p < 0.001) (Fig. 2C, levels of individual compounds are given in Supplemental figure S4) compared to the non-wounded control (Supplemental figure S1). However, hydrolysis yielded significantly more units with 2,3-trans stereochemistry in infected tissue than in wounded control bark (p < 0.001). No statistical difference in the 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 was 8:5:0.2:1 (epicatechin:catechin:gallocatechin:epigallocatechin) 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 flavan-3-ol polymerization in non-wounded, wounded and infected bark tissue, normal phase chromatography coupled to fluorescence detection (Kelm et al., 2006) was used (Supplemental figure S5). By employing this method, polymers up to 9-mers could be quantified (Fig. 2D). In all analyzed treatments, the abundance of polymers decreased with degree of polymerization (p < 0.0001). For example, there were significantly more trimers than tetramers based on the total number of monomer units allocated to each group. 28 days after infection, flavan-3-ol polymers were significantly more abundant in infected tissue than in the wounded control (p < 0.0001). There were no significant differences in the degree of polymerization between trees infected by the different fungal isolates.

A second experiment on two-year-old saplings was conducted to determine the effect of wounding on flavan-3-ol levels over a time course of 25 days (Supplemental figures S6 and S7). Comparison of wounded but not fungus-inoculated trees to non-wounded controls showed higher monomer, dimer and polymer levels in wounded vs. unwounded bark, but there were no statistically significant differences between the treatments. Wounded and non-wounded trees showed a similar increase (p < 0.001) in polymer levels over the 25 day time course, suggesting that polymers are formed from monomers continuously during this period (Supplemental figures S6 and S7).
Norway spruce has four leucoanthocyanidin reductase genes with distinct phylogenetic relationships

To identify leucoanthocyanidin reductase (LAR) genes that are involved in the synthesis of 2,3-trans-(+)-flavan-3-ols, BLAST sequence comparisons were carried out with previously reported LAR genes from angiosperms (Tanner et al., 2003; Pfeiffer et al., 2006) and Pinus taeda (Bogs et al., 2005). Searches of 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, four full-length LAR gene candidates could be amplified from P. abies cDNA by PCR.

Phylogenetic analysis (Supplemental data Alignment 1) revealed a clear evolutionary divergence between the translated LAR amino acid sequences and those of anthocyanidin reductase (ANR), which forms 2,3-cis-(−)-flavan-3-ols (about 10% sequence similarity) (Fig. 3). There was also a clear distinction between angiosperm and gymnosperm sequences for both enzyme classes. Deduced amino acid sequences for conifer LAR genes shared only 51-57% identity with LAR sequences from angiosperms, whereas sequences for P. abies LAR (PaLAR) were 79-87% identical and had 62-81% 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 63-65% similarity.

Two ANR-like orthologous sequences from P. abies (Treenomix database: Ralph et al., 2008) were 66% similar to each other and shared 62% and 70% sequence identity, respectively, with an uncharacterized ANR from Ginkgo biloba (Shen et al., 2006). ANR-like sequences from P. abies and selected angiosperm ANR had 50-57% sequence similarity.

Steady-state transcript levels were measured in wood, bark, stems, roots and needles of unwounded P. abies saplings for each of the four LAR candidate genes using quantitative real-time PCR (Fig. 3). LAR3 and LAR4 were highly expressed in all the tissues tested. LAR1 was highly expressed in roots and needles whereas LAR2 was not
expressed in great abundance in any of these tissues. LAR 3 and LAR 4 gene expression was lower in wood than in foliage, bark or roots (p < 0.001).

Norway spruce LAR proteins all catalyze leucoanthocyanidin reduction \textit{in vitro} and \textit{in vivo}

Functional characterization of the four putative LAR enzymes from \textit{P. abies in vitro} was accomplished via heterologous expression in a bacterial system. Catalytic activity of recombinant \textit{PaLAR} was determined \textit{in vitro} by incubating expressed enzymes with NADPH and the labile substrates leucocyanidin or leucodelphinidin. All \textit{PaLAR} preferentially accepted the substrate leucocyanidin and enzyme assays yielded 2,3-trans-\textit{(+)}-catechin for each of the four candidate \textit{PaLAR} enzymes (Fig. 4; Supplemental Fig. S8). The \textit{PaLAR} enzymes also accepted the trihydroxylated substrate leucodelphinidin, but only very low levels 2,3-trans-\textit{(+)}-gallocatechin were formed under the employed assay conditions. Optimal LAR activity with leucocyanidin was attained at 25°C at a pH of 7.5.

To compare \textit{PaLAR} activities with activities reported in the literature, \textit{MdLAR} from \textit{Malus domestica} (Pfeiffer et al., 2006) was cloned and expressed under the same conditions. \textit{PaLAR} 2, 3 and 4 had similar activities in \textit{in vitro} enzyme assays to \textit{MdLAR}, but the activity of \textit{PaLAR} 1 was much lower (p < 0.05) (Fig. 4; Supplemental Fig. S8).

An \textit{in vivo} LAR assay was also performed in which \textit{E. coli} was co-transformed with a LAR expression construct as well as a plasmid encoding a dihydroflavonol reductase enzyme (\textit{MdDFR}) from \textit{M. domestica} (Fischer et al., 2003). The assay was conducted by adding the dihydroflavonol substrate \textit{(+)}-taxifolin to the medium after induction of protein expression. Products were harvested from the medium and quantified after normalizing to the OD\textsubscript{600} of the bacterial culture. In this \textit{in vivo} assay bacteria with \textit{MdLAR} produced much higher levels of catechin from \textit{(+)}-taxifolin than the \textit{PaLAR} enzymes (Supplemental Fig. S8). Epimerase activity whereby \textit{PaLAR} produces both catechin and epicatechin (Pang et al., 2013) was not observed in this \textit{in vivo} assay or in the \textit{PaLAR} and \textit{MdLAR} \textit{in vitro} assays described above.
Norway spruce LAR 3 increases flavan-3-ol content after over-expression in transgenic spruce saplings

To confirm the in vitro activity of the P. abies LAR genes, embryonic Norway spruce tissue was transformed with PaLAR3 under the control of the inducible promoter *ubi1* using a disarmed *Agrobacterium tumefaciens* strain (Schmidt et al., 2010). PaLAR3 was selected for this experiment due to its high constitutive transcript accumulation (Fig. 3) and its high catalytic activity (Fig. 4). Three transgenic PaLAR3 seedling lines (line T1, line T2 and line T5) were selected. One-year-old transformed saplings grown in potting soil were used for transcript and metabolite analysis. LAR3 transcript levels were higher in the three transgenic lines than in the vector control line but this difference was only significant (p < 0.01) for lines T1 and T2 (Fig. 5A).

Total monomeric and polymeric flavan-3-ols were present in higher concentrations in the bark of transgenic LAR3 over-expressing lines than in the vector control line. The monomeric 2,3-trans-(+)-flavan-3-ols, catechin and gallocatechin (Fig. 5B), were more abundant in the three transgenic lines than in the vector control line (p < 0.001). Catechin levels and LAR3 transcript accumulation were positively correlated (Pearson’s r² = 0.32; p = 0.0053; Supplemental figure S9). Dimeric flavan-3-ols (Fig. 5C) were present at higher concentrations in line T2 and T5 (p < 0.001) than in line T1 and the vector control and ratios of PAB3 to PAB1 were similar in the vector control and the transgenic lines, with the catechin-catechin dimer PAB3 being more abundant in both types. However, dimers containing gallocatechin were more abundant in the transgenic lines than in the vector control (Supplemental figure S10). Polymeric flavan-3-ols were analyzed both as monomeric units following reductive acid-hydrolysis (Fig. 5D) and intact polymers using normal phase chromatography (Fig. 5E). Both methods showed that polymeric flavan-3-ols were significantly more abundant in lines T2 and T5 (p < 0.01) than in line T1 and the vector control. Moreover PAs from these lines contained very high concentrations of 2,3-cis-(−)-epicatechin (p < 0.001), but PAs from all transgenic lines did not contain significantly higher levels of flavan-3-ols with a tri-hydroxylated B-ring (gallocatechin and epigallocatechin) than the vector control line (p = 0.06).

Needles and roots of the LAR3 over-expressing T5 line and the vector control were also compared for LAR3 transcript abundance and flavan-3-ol content.
In accordance with the general pattern of LAR gene expression in different tissue types (Fig. 3), needles in both the T5 line as well as in the vector control had significantly higher \( \text{LAR}3 \) transcript abundance and flavan-3-ol content than samples from stems and root bark \((p < 0.05)\), while in roots and stems transcript and metabolite levels were similar. The T5 lines contained higher \( \text{LAR}3 \) transcript levels and flavan-3-ols in needles \((p < 0.05)\) than the vector control. In the roots, however, an opposing trend was observed where the vector control had higher levels of \( \text{LAR}3 \) transcripts \((p < 0.05)\) than in line T5, but similar levels of flavan-3-ols. The relative degree of polymerization of flavan-3-ols was similar in roots, bark and needles in both the vector control and the \( \text{LAR}3 \) over-expressing T5 line (Supplemental figure S11).

Inoculation with \( C. \text{ polonica} \) fungus increases the transcription of all four Norway spruce LAR genes

To determine if fungal inoculation activates LAR transcription in spruce, RNA from bark tissue of wounded and \( C. \text{ polonica} \)-inoculated spruce saplings was extracted and transcribed to cDNA. Transcript accumulation of \( \text{PaLAR} \), measured by quantitative real-time PCR, increased more in inoculated saplings than in wounded control saplings over a 28 day time course (Fig. 6). \( \text{PaLAR}1 \), \( \text{PaLAR}3 \) and \( \text{PaLAR}4 \) transcript levels increased significantly in inoculated saplings between 2 and 7 days post-inoculation \((p < 0.01)\) while transcript accumulation of \( \text{PaLAR}2 \) increased between 14 and 28 days after wounding or infection \((p = 0.04)\) compared to earlier time points.

Accumulation of \( \text{PaLAR}1 \), \( \text{PaLAR}2 \) and \( \text{PaLAR}4 \) transcripts in response to fungal inoculation were greater than in wounded saplings which were in turn greater than in unwounded controls. Levels of \( \text{PaLAR}1 \) 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 (assigned a relative transcript abundance of 1). Transcript accumulation of \( \text{PaLAR}2 \) was 2-fold higher in inoculated than in wounded saplings and between 180 to 200-fold higher than in bark of non-wounded saplings. \( \text{PaLAR}3 \), which had more than 20-fold higher steady-state transcript levels (Fig. 3) than \( \text{PaLAR}1 \), \( \text{PaLAR}2 \) and \( \text{PaLAR}4 \), did not increase in wounded vs. unwounded control saplings, but increased 3 to 4-fold in fungus-inoculated saplings. \( \text{PaLAR}4 \) increased between 10 and
30-fold compared to the wounded control and 20 to 60-fold compared to the non-
wounded controls.

To demonstrate general transcriptional activity of the entire flavan-3-ol
biosynthetic pathway, transcript accumulation of a subset of 7 chalcone synthase
(PaCHS) genes (Hammerbacher et al., 2011) was measured (Supplemental figure S12).
PaCHS gene expression closely followed the same patterns as PaLAR transcript
accumulation with an early and a late burst of transcription in fungus-infected bark,
which was lacking in the wounded controls.

**Norway spruce flavan-3-ols inhibit C. polonica growth on artificial medium**

In order to determine whether mixtures of Norway spruce monomeric flavan-3-ols
and PAs had activity against the fungus *C. polonica*, both fungal isolates used in this
study were grown on artificial nutrient medium amended with flavan-3-ol concentrations
similar to those observed in unwounded spruce bark or after 28 days of fungal infection.
Both isolates showed significantly lower growth rates on medium equivalent to 28 days
after *C. polonica* infection (p < 0.001) than medium equivalent to unwounded bark (Fig.
7). The growth rate of the virulent isolate (isolate 2; Hammerbacher et al., 2013) was
significantly higher than the growth of the avirulent isolate (isolate 1) on both the more
concentrated as well as the more dilute flavan-3-ol mixtures (p < 0.001). Curiously, on
medium without any flavan-3-ols the avirulent isolate grew significantly faster than the
virulent isolate (p < 0.001; data not shown).

**DISCUSSION**

Polymeric flavan-3-ols known widely as proanthocyanidins or condensed tannins,
are widely distributed in the plant kingdom, especially in woody species (Barbehenn and
Constabel, 2011), but there are still many open questions about their biosynthesis and
function, especially in conifers and other gymnosperms. Here we employed improved
analytical methods to characterize the monomeric and polymeric flavan-3-ol content of
bark from Norway spruce (*Picea abies*). In addition, investigation of the genes encoding
leucoanthocyanidin reductase (LAR) and corresponding proteins, branch point enzymes of flavan-3-ol biosynthesis, brought to light additional knowledge on the pathway regulation and evolution of monomeric and polymeric flavan-3-ol formation. The role of specific flavan-3-ols in defense against bark beetle associated Norway spruce pathogen was demonstrated by assays in artificial medium and the induction of LAR transcripts and accumulation of metabolites following fungal infection.

To analyze both monomeric flavan-3-ols and flavan-3-ol polymers, also known as proanthocyanidins (PAs), an integrative analytical approach was developed. While monomeric flavan-3-ols have been routinely analyzed in the past on a quantitative basis, previous analyses of PAs were largely based on colorimetric methods that are useful for broad comparison of structurally similar compounds but do not provide any information on PA structure, monomer composition or degree of polymerization (Porter et al., 1986; Mellway et al., 2009; de Pascual-Teresa et al., 2000; Almeida et al., 2007). More recent methods do not give strictly quantitative results due to incomplete hydrolysis of PA chains or the analysis of highly unstable molecules (Pang et al., 2007; Guyot et al., 1998).

In the present work a 3-step procedure was developed to more comprehensively analyze both monomeric and polymeric flavan-3-ols (PAs) in *P. abies* bark: (1) Monomeric and dimeric flavan-3-ols in plant extracts were quantified by reverse-phase HPLC. (2) PAs were completely hydrolyzed under acidic conditions in the presence of a strong reducing agent, thereby preventing oxidation and minimizing epimerization (Supplemental figure S3). Hydrolysis products were then separated by reverse-phase HPLC to yield quantitative and qualitative information on the composition of the individual monomeric units. (3) Intact PAs were separated by normal-phase chromatography to accurately determine their size distribution using a method developed by Kelm et al. (2006). Although specialized equipment was used to establish and verify the methodologies employed, such analyses could in principle be accomplished with just a single high pressure liquid chromatography (HPLC) instrument equipped with a fluorescence detector and 2 analytical grade columns. For the complete characterization of PAs, it is still necessary to deduce the sequence of monomeric units in each polymer and the types of linkages.
The main flavan-3-ol monomers in Norway spruce bark are 2,3-\textit{trans}-(+)-catechin (90-95\%) and 2,3-\textit{trans}-(+)-gallocatechin (5-10\%). These compounds are biosynthesized from leucoanthocyanidins by leucoanthocyanidin reductases (LAR). These enzymes are known to accept the substrates leucocyanidin or leucodelphinidin to form catechin or gallocatechin, respectively (Tanner et al., 2003). In sequenced white spruce and Sitka spruce transcriptomes (Ralph et al., 2008), four full-length \textit{LAR} mRNA sequences were identified by BLAST sequence comparisons and four orthologous \textit{LAR} transcript sequences were identified in Norway spruce. Angiosperms with smaller genomes than \textit{Picea} species such as apple and grape contain only two \textit{LAR} genes (Pfeiffer et al., 2006).

Phylogenetic analysis of \textit{LAR} enzymes from spruce revealed independent evolution of \textit{LAR} in angiosperms and gymnosperms. However, the active site regions (Alignment S2) of enzymes from both lineages were similar. Analysis of the crystal structure of \textit{V. vinifera} LAR by Mauge et al. (2010) revealed particular residues in the active site for NADPH binding and catalysis of leucoanthocyanidin reduction. All of the NADPH binding residues were similar in \textit{PaLAR} with the exception of residue 91 (\textit{V. vinifera} LAR numbering scheme) where in \textit{PaLAR2} and \textit{PaLAR3} a transition was observed from valine to methionine. In the \textit{V. vinifera} LAR active site Mauge et al. (2010) identified conserved histidine (position 123), tyrosine (position 137), serine (position 161) and isoleucine (position 171) residue which could be responsible for the enzyme’s catalytic activity. In the \textit{PaLAR} enzymes the histidine, serine and tyrosine residues were all conserved, but only \textit{PaLAR2} contained isoleucine in position 171. All other \textit{PaLAR} enzymes contained threonine at position 171. \textit{LAR} from \textit{D. uncinatum}, the first enzyme with \textit{LAR} activity discovered, had a cysteine residue at position 171. This might indicate that the amino acid at position 171 is not important for the reduction of leucoanthocyanidin.

Heterologously expressed \textit{PaLAR} enzymes accepted the substrates leucocyanidin and to a lesser extent leucodelphinidin, with a trihydroxylated B-ring, to produce 2,3-\textit{trans}-(+)-catechin or 2,3-\textit{trans}-(+)-gallocatechin, respectively. This activity was confirmed \textit{in vivo} by analysis of transgenic spruce lines over-expressing \textit{PaLAR3} which accumulated more catechin and epicatechin than the vector control.
While 3 – 4-fold higher flavan-3-ol concentrations were recorded in the transgenic LAR3 over-expressing lines compared to the vector controls, transcript levels of the LAR3 gene were only 1.7 - 2.9-fold higher than in the vector controls. However, considering that the LAR3 gene is highly expressed during steady-state conditions in spruce bark, a small change in gene expression could lead to significant fluctuations in flavan-3-ol concentrations. Another explanation for the lack of correlation between changes in gene expression and metabolite accumulation in transgenic seedlings might be the seasonal regulation of flavan-3-ol biosynthesis. In bilberry, for example, gene expression and flavonoid biosynthesis occurred predominantly early in the growth season after which the PA contents remained stable (Martz et al., 2010). Since in our study spruce saplings were harvested four months after their spring-flush, a similar seasonal rhythm could have led to differences in gene expression later in the season with larger differences in metabolite accumulation that reflect early season differences in biosynthetic rate. Furthermore, we were able to show a substantial increase of flavan-3-ol content in unwounded saplings over a time course of 25 days (Supplemental figures S6 and S7) substantiating the occurrence of seasonal shifts in flavan-3-ol biosynthesis in spruce. The overall complexity of this pathway may well be responsible for the incongruities between transcript levels of biosynthetic genes and the amounts of flavan-3-ol products.

Hydrolysis of spruce bark PAs of wild-type saplings treated with C. polonica as well as transgenic spruce seedlings yielded high levels of 2,3-cis-(−)-epicatechin and 2,3-cis-(−)-epigallocatechin that were significantly greater than their respective stereoisomers, 2,3-trans-(+)-catechin and 2,3-trans-(+)-gallocatechin. This pattern was consistent in fungal treated bark as well as untreated controls and in LAR over-expressing transgenics as well as in vector controls. Pang et al. (2013) reported epimerase activity for Camellia sinensis LAR which could potentially explain the correlation between elevated levels of 2,3-cis-(−)-isomers and LAR expression in Norway spruce. However, PaLAR enzymes were not observed to have epimerase activity in vitro. To further investigate a potential epimerase activity, PaLAR enzymes were expressed in E. coli in tandem with a dihydroflavonol reductase from M. domestica. The LAR activity was assayed in this system in vivo by adding the substrate (+)-taxifolin directly to the bacterial culture.
Taxifolin was transformed by the induced bacterial PaLAR and MdDFR expressing cultures to 2,3-trans-(+)-catechin without simultaneous production of the epimerized product, 2,3-cis-(-)-epicatechin.

Since free flavan-3-ol monomers in spruce have only the 2,3-trans-(+) stereochemistry, the 2,3-cis-(-)-isomers detected after hydrolysis must therefore be derived from extender units of the PA chains. PAs with 2,3-cis-(-)-epicatechin extender units have been reported in many plant species and are even more abundant than PAs with 2,3-trans-(+)-catechin extender units. However, it is still unclear how flavan-3-ol units of PAs with this configuration arise.

For plants producing 2,3-cis-(-)-epicatechin as a monomer, a specific pathway for the biosynthesis of the 2,3-cis-(-)-flavan-3-ol isomers has been described (Xie et al., 2004). Here a leucoanthocyanidin is converted by anthocyanidin synthase to the corresponding anthocyanidin which is further reduced by anthocyanidin reductase (ANR) to form the 2,3-cis-(-)-flavan-3-ol. We identified several ANR gene candidates in the spruce EST databases, but were not able to demonstrate any ANR activity upon heterologous expression of the encoded proteins in vitro using E. coli or S. cerevisiae. The absence of free monomeric epicatechin in extracts of Norway spruce also argues against the presence of 2,3-cis-(-)-flavan-3-ol-producing enzymes in this species. Therefore, the origin of epicatechin in spruce remains unknown. It could be formed during the polymerization itself.

Several reaction mechanisms have been proposed for the oxidative coupling of flavan-3-ol monomers during PA formation. The most widely accepted hypothesis is that 2,3-trans-(+)-leucoanthocyanidins are converted to carbocations via quinone methide intermediates, which then can be coupled to a terminal flavan-3-ol unit to form a growing PA chain (Creasy and Swain, 1965). However, this reaction mechanism does not account for the 2,3-cis stereochemistry of extender units of PAs in Norway 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 this unit in Norway spruce since the only free monomeric flavan-3-ols present in this species have a 2,3-trans stereochemistry. Reaction mechanisms where anthocyanidins, which can also occur in both stereoisomeric forms, are proposed as possible precursors for extender units.
in PA chains may offer a more probable model for PA biosynthesis in spruce. Haslam et al. (1980) suggested that anthocyanidins, present in acidic conditions as flavylium ions can be oxidized to quinone methides which can then 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 then be protonated to carbocations to form extender units of a PA chain (Haslam, 1977).

One of the main objectives of this study was to examine the changes in flavan-3-ol accumulation in Norway spruce during fungal infection. Earlier histological studies suggested that substances likely to be phenolics accumulated in phloem parenchyma cells in Norway spruce bark in response to infection by C. polonica (Nagy et al., 2004; Franceschi et al., 2005). More recently it was shown that these cells contain stilbenes and, after infection by C. polonica, also contain 2,3-trans-(+)-catechin (Li et al., 2012). By demonstrating that monomeric flavan-3-ols accumulate 4- to 6-fold in whole bark tissue following C. polonica infection, our current results are consistent with those from individual phloem parenchyma cells. The accumulation of flavan-3-ols also correlated well with increased transcript accumulation of the LAR genes involved in the formation of the flavan-3-ol monomer 2,3-trans-(+)-catechin, indicating a close relationship between LAR expression and flavan-3-ol biosynthesis. In addition, transcription of chalcone synthase (CHS) genes, which code for enzymes catalyzing an early step in flavan-3-ol biosynthesis, was up-regulated in treatments where flavan-3-ols were produced in high amounts (Supplemental Figure S12). This demonstrates that fungal infection increases the general transcriptional activity in the flavonoid pathway ultimately producing PAs.

Flavan-3-ols and PAs have been reported to function as anti-herbivore defense compounds on numerous occasions ever since the work of Feeny (1970). In our study, however, CHS and LAR gene expression and flavan-3-ol concentrations were much more elevated after infection by C. polonica than after simple wounding such as might be caused by herbivory. Furthermore, there were no statistically significant differences in concentrations of monomeric, dimeric or polymeric flavan-3-ols in wounded treatments when compared to unwounded controls over a time course of 25 days (Supplemental Figure S6). These data can be taken to suggest that flavan-3-ols are not responsive to
herbivory and so are not anti-herbivore defenses. However, mechanical wounding alone might not be a good mimic for insect or mammalian herbivory. In recent work on the phenylpropanoid-related genes of white spruce, Porth et al. (2011) showed that expression of CHS and LAR genes were also not correlated with resistance to herbivory, in this case by the pine weevil, *Pissodes strobi*.

Since fungal infection by the bark beetle-transmitted fungus *C. polonica* led to significantly higher LAR transcript accumulation and higher levels of monomeric and polymeric flavan-3-ols than wounding without infection, the biosynthesis of PAs likely functions as a defense against fungal infestation. In support of this hypothesis, growth of *C. polonica* on artificial medium was reduced when the fungus was cultured with mixtures of catechin and PAs equivalent to the levels found in induced sapling bark. Furthermore, Danielsson et al. (2011) showed a high positive correlation between LAR gene expression (LAR3 and LAR4 in this study) and spruce resistance to the root-rot fungus *Heterobasidion annosum sensu lato*.

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 also affect the growth of *C. polonica* by inhibiting melanin biosynthesis. *C. polonica* is not only frequently vectored by the spruce bark beetle, *I. typographus*, but fungal presence is highly correlated with successful invasion of the beetle and resulting tree death is essential for beetle reproduction (Franceschi et al., 2005). Thus by defending *P. abies* against *C. polonica* infection, flavan-3-ols would also help protect the tree against this serious insect pest as well. However, to define the role of flavan-3-ols as an anti-fungal defense more clearly, future studies should include growth assays of *C. polonica* on transgenic PA over-producing trees such as our LAR3 over-expressing lines, or on other transgenic trees where the pathway is silenced.

**Materials and Methods**
Identification of putative LAR genes from Picea EST collections

Protein LAR sequences from Malus domestica, Pyrus communis, Pinus taeda (Pfeiffer et al., 2006, Fischer et al., 2003) 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 Treenomix 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). In order to identify other possible LAR candidates not present in the Treenomix database the genomic sequences of Norway- and White spruce (Birol et al., 2013; Nysted et al., 2013) were screened using the four identified LAR sequences.

Cloning and sequencing PaLAR genes

RNA was purified from fresh bark tissue of four-year-old P. abies saplings 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, Carlsbad, CA, USA) and 50 pmol PolyT\(_{12-18}\) primer (Invitrogen). Primers were designed for candidate sequences by using the N-and C-terminal sequences of three putative LAR genes (Gateway\(^\text{TM}\) (Invitrogen) compatible) and two ANR genes from P. sitchensis and P. glauca as templates (primer sequences used are provided in Supplemental table S1). cDNA ends were also amplified independently using the rapid amplification of cDNA ends kit for LAR 1-3 (Clontech, Saint Germain en Laye, France). A fourth full length PaLAR gene candidate was identified in the Congenie database (www.congenie.org, Nysted et al., 2013) by blast searches. PaLAR cDNA were PCR amplified with primers (Supplemental table S1) using Platinum Taq\(^\text{TM}\) high fidelity DNA polymerase (Invitrogen) and purified with the QIAquick\(^\text{TM}\) PCR purification kit (Qiagen, Hilden, Germany). Gateway\(^\text{TM}\) entry clones were made by using BP clonase II and pDONR207 (Invitrogen) following the manufacturer’s protocol. 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.
Protein sequence analysis of PaLAR

LAR protein sequences from *P. abies* as well as protein sequences of LAR and ANR genes with confirmed enzyme function (Supplemental table S2) were aligned with the automatic alignment program MAFFT v 6 ([http://mafft.cbrc.jp/alignment/server/](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 (1992) matrix. The tree was searched with pair-wise elimination of alignment gaps. Statistical likelihood of tree branches was tested with 1000 bootstrap replicates.

Heterologous expression of PaLAR genes in *E. coli*

Four putative *PaLAR* pDONR207 constructs were cloned with LR clonase II (Invitrogen) according to the manufacturer’s instructions into the Gateway™ compatible expression vector pDest 16 (Invitrogen) which contains a GST tag 5’ of the N-terminus of the expressed protein. All constructs were verified by sequencing. BL21(DE3) chemically competent *E. coli* (Invitrogen) 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 ampicillin and grown for 12 hours at 30 °C. The 5 ml starter cultures were used to inoculate 100 ml LB medium supplemented with 1 µg ml⁻¹ kanamycin.

Bacterial cultures were grown for 12 hours at 18°C (220 rpm), induced with 0.5 mM IPTG and harvested 14 hours later by centrifugation. Bacteria were resuspended in 10 ml buffer containing 50 mM Bis-Tris (pH 7.5), 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT) and lysed by sonication for 4 minutes using 2 cycles at 65% power with a Bandelin Sonoplus HD 2070 sonifier (Bandelin Electronics, Berlin, Germany). Insoluble cell debris was removed from the lysate by centrifugation at 16000 g for 30 minutes at 4°C.
Expressed proteins were purified from the crude lysate by affinity chromatography with 1 ml Glutathione sepharose (GE Healthcare Life Sciences, Munich, Germany) according to the manufacturer’s instructions. 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).

**In vitro functional characterization of PaLAR expressed in E. coli**

The unlabelled substrates leucoanthocyanidin and leucodelphinidin were purchased from TransMit Flavonoid Forschung (Marburg, Germany). Recombinant LAR1, LAR2, LAR3 and LAR4 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 20 minutes at 25°C before the enzyme assay was stopped by addition of 1 volume of methanol and centrifugation. Negative control assays were initiated without substrate or with heat-denatured enzyme preparations. Low levels of catechin detected in control reactions were due to contamination of the substrate, which could easily be corrected by subtraction. The temperature optimum for LAR activity was determined at 25, 30 and 35°C. PH optima between pH 6 to pH 8.5 were determined by using 50 mM Bis-Tris. The already described LAR from *M. domestica* (Pfeiffer et al., 2006) was cloned and expressed using the above protocols and used as a positive control.

**In vivo functional characterization of PaLAR expressed in E. coli**

*In vivo* enzyme assays were initiated by co-transforming *E. coli* with LAR as well as *MdDFR* (dihydroflavonol reductase from *M. domestica*) which was cloned using the above protocols into the vector PH9 (Yu and Liu, 2006). The enzymes were expressed as above. Twelve h after inducing expression with IPTG, (+) taxifolin in DMSO was added to the culture medium to a final concentration of 100µg ml⁻¹. Cultures were harvested 3 h after addition of taxifolin. Bacteria were removed from the culture medium by
centrifugation. Ten ml medium was acidified with 100 µl of 0.1 N HCl and extracted with 3 volumes of ethyl acetate. The ethyl acetate extracts were evaporated under a stream of nitrogen gas and re-dissolved in 500 µl methanol for LC-ESI-MS/MS analysis.

**Genetic transformation of *P. abies* callus with *PaLAR3*, somatic embryogenesis and plant regeneration**

The *PaLAR3* pDONR207 was cloned with LR clonase II (Invitrogen) into the Gateway™ compatible binary vector pCAMGW (Schmidt et al., 2010). pCAMGW LAR3 or pCAMBIA 2301 (as a vector control) were transformed into *A. tumefaciens* strain C58/pMP90 (Schmidt et al., 2010) which were subsequently used to transform an embryonic *P. abies* cell culture (line 186/3c VIII ) as described by Schmidt et al. (2010). From the 6 transgenic lines obtained, line 1, line 2 and line 5 were selected for further experiments. Transgenic lines were maintained and regenerated into seedlings as described in Hammerbacher et al. (2011). Well developed plantlets were planted in soil substrate (3:1:1, fibric peat:vermiculite (2-3 mm grain size):perlite) in small plastic pots (4 x 4 cm).

**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% (wt/v) malt extract agar (MEA; Carl 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 replicate trees were used for each treatment per time point (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 -80°C.

**Quantitative real-time PCR**

Total RNA from inoculated treatment and wounded control bark as well as transgenic *PaLAR3* overexpressing 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) 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 *PaLAR1*, *PaLAR2*, *PaLAR3*, *PaLAR4* and *PaCHS* are given in supplemental table S3. PCR was performed using a Stratagene MX3000Pro 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 (Schmidt et al., 2010) gene (Primers in table S1) and was calculated from three technical replicates of five biological replicates. Relative transcript abundance was calibrated against the transcript abundance of five non-wounded control saplings.

**Extraction of phenolic compounds from spruce**

For extraction of phenolic compounds, Norway 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). After approximately 80 mg
dried tissue was extracted with 2 ml analytical grade methanol for 4 hours at 4°C, 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\(^{-1}\) chlorogenic acid (Sigma) as internal standard. For
LC-ESI-MS or hydrolysis of condensed tannins, samples were diluted five times (v/v)
with methanol. For LC-FLD samples were diluted 2 times (v/v) in acetonitrile.

**Liquid chromatography-fluorescence detection (LC-FLD)**

PAs 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. Compounds 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.

**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 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 (Bruker 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 $m/z$ between 50 and 1600 with an optimal target mass of 405 $m/z$. 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 was carried out between 200 and 2500 $m/z$ varying the optimal target mass according to the degree of polymerization.

Compounds were identified by mass spectrometry and by direct comparison with commercial standards, where available including 2,3-\textit{trans}-(+)-catechin, 2,3-\textit{trans}-(+)-gallocatechin, 2,3-\textit{cis}-(-)-epicatechin, 2,3-\textit{cis}(-)-epigallocatechin, PA B1 and PA B3. 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 were verified by analyzing serial dilutions of randomly selected samples. An external calibration curve created by linear regression was used for quantification of 2,3-\textit{trans}-(+)-catechin (Sigma) and 2,3-\textit{trans}-(+)-gallocatechin (Sigma) and PA B1 (Sigma). PA B3 was quantified relative PA B1. Process variability in different analyses was calculated relative to the internal standard.

\textbf{Reductive cleavage of proanthocyanidins}

PAs were cleaved in 800 µl reaction volumes containing 2.5% (v/v) trifluoroacetic acid (TFA) and 8% (v/v) 0.5 g ml$^{-1}$ sodium cyanoborohydrate in methanol. Reaction mixtures were heated to 65ºC for 15 minutes before adding additional 2.5% (v/v) TFA. Vials were sealed tightly and incubated at 65ºC overnight. Samples were dried under a
stream of nitrogen and re-dissolved in 800 µl methanol and analyzed using the Nucleodur Sphinx RP18ec column under the conditions already described, but detected with a UV DAD detector at 280 nm.

Validation of the hydrolysis method to quantify PAs was done by analyzing different amounts of partially purified grape proanthocyanidins (Laffort, Bordeaux, France; Supplemental figure S3). Although amounts of flavan-3-ol monomers detected after hydrolysis were slightly lower than expected, the precision error (based on degree of repeatability) was less than 10% and the error of accuracy due to incomplete hydrolysis or reduced recovery of hydrolyzed monomers was also less than 10% for all concentrations of PAs tested. The method also gave excellent linearity ($R^2 = 0.999$) between the amounts of PAs hydrolyzed and the amount of monomers detected within the range of 0.125 and 1 mg (Supplemental table S4).

**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 (Phenomenex, 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, gallocatechin and proanthocyanidin B1. For dimeric proanthocyanidins containing gallocatechin partially purified plant extracts were used for optimization. The ionspray 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 precursor ion → product ion: $m/z=299.9 \rightarrow 109.1$ (collision energy (CE )=34 V; declustering potential (DP) =-30 V) for catechin; $m/z=304.8 \rightarrow 179$ (CE =-28 V; DP =-390 V) for
gallocatechin; \( m/z \) 576.9 → 289.1 (CE -30 V; DP -50 V) for proanthocyanidin B1; \( m/z \) 609
592.9 → 125.1 (CE -52 V; DP -400 V) for the catechin:gallocatechin dimer; \( m/z \) 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.

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

The ratio of dry weight to fresh weight of spruce sapling bark was determined to
be 1.8 by weighing 6-year-old spruce bark prior and after oven-drying (n = 10). This was
used for calculations of metabolite concentrations in the artificial medium. For
determining fungal growth on catechin and polymeric PAs, mixtures containing
equivalent amounts of flavan-3-ols observed in spruce sapling bark before or after fungal
infection were assayed (induced levels: 6.7 mg ml\(^{-1}\) pure catechin standard and 4.5 mg
ml\(^{-1}\) pure grape PA standard; constitutive levels: 1.1 mg ml\(^{-1}\) catechin and 1.1 mg ml\(^{-1}\)
PAs). The growth medium was prepared by steam sterilizing water agar (2.5 % w vol\(^{-1}\))
amended with carrot juice (1.5 % w vol\(^{-1}\), Viva Vital, Maxhuette, Germany) and a
mixture of pure flavan-3-ols equivalent to constitutive or induced levels. Medium was
dispensed in Petri dishes (Ø=5.2 cm). After the medium solidified, an agar plug (Ø=4
mm) from a 14-day-old \( C. \) polonica stationary culture 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 until growth reached the margins of the Petri dish (n = 5).

Statistical analysis

Most experiments were analyzed with a multivariate repeated measures model
since this takes account of the correlation that exists between values of the same variable
measured at successive time points. The multivariate form was required because it
allows the examination of relationships among several dependent variables all measured
for the same tree (Sokal & Rohlf 1995), and allows the examination of relationships
between different variables.
Preliminary investigation showed that the data were not normal. Rank transformation was employed because it is particularly robust to non-normal errors, outliers, and several kinds of non-normal distributions (Iman, 1974). Because there was only one interaction in our model, and this was 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, flavan-3-ol metabolite occurrence and PA hydrolysis products were analyzed separately because direct conversion was not possible. Transgenic trees were analyzed using a one-way ANOVA on log transformed data. Fungal growth on Petri dishes was analyzed using linear regression. Differences in mean growth rate were calculated using a two-way ANOVA followed by Tukey’s post-hoc test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers KC589001 to KC589003.

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SUPPLEMENTAL DATA

Figure S1: Total flavan-3-ol monomers, dimers and polymers after hydrolysis of unwounded bark samples collected at the onset of the time-course (Figure 2) and of the wounded control samples collected 2 days post-wounding.

Figure S2: Relative concentrations of soluble monomeric and dimeric flavan-3-ols in spruce bark which was infected with C. polonica or wounded over a time course of 28 days. Catechin (A), galloallocatechin (B), proanthocyanidin B1 (m/z 577 (M-H)) (C), dimeric proanthocyanidin with m/z 593 (M-H) (D), dimeric proanthocyanidin with m/z...
577 (M-H) (E) and dimeric proanthocyanidin with m/z 609 (M-H) (F) were measured by LC-ESI-MS. Error bars represent standard errors.

**Figure S3**: Reductive hydrolysis of dimeric proanthocyanidin B1 yielded equal amounts of catechin (retention time: 10.9 min) and epicatechin (retention time: 11.2 min).

Hydrolysis of proanthocyanidin B2 yielded mostly epicatechin, but also small amounts of catechin.

**Figure S4**: Relative concentrations of hydrolysis products of proanthocyanidins formed in spruce bark during wounding and fungal infection over a time course of 28 days. Catechin (A), epicatechin (B), gallicatechin (C) and epigallicatechin (D) were measured with HPLC-UV. Error bars represent standard errors.

**Figure S5**: LC-FLD chromatogram of proanthocyanidin polymer distribution with mass spectra of each polymer detected.

**Figure S6**: Concentration in catechin equivalents of soluble monomeric, dimeric and polymeric flavan-3-ols in wounded and non-wounded control as well as wounded spruce bark over a time course of 25 days. No statistically significant differences were detected between wounded and control treatments which were measured prior to and after hydrolysis using LC-ESI-MS. Error bars represent standard errors.

**Figure S7**: Hydrolysis products of proanthocyanidins formed in spruce bark during wounding compared to non-wounded bark over a time course of 25 days. Catechin levels were slightly higher at 25 days than at 3 days (p = 0.004). No statistically significant differences were detected in epicatechin, gallicatechin and epigallicatechin levels between wounded and non-wounded control treatments. Error bars represent standard errors.

**Figure S8**: Characterization of LAR enzymes from *P. abies* using heterologous expression in *E. coli*. LAR from apple (*MdLAR*) was included as a positive control. Recombinant LAR enzymes accepted leucoanthocyanidin as substrate and formed catechin as products. (A) Extracted ion chromatograms for catechin after incubation of LAR enzyme (19 µg each) with leucoanthocyanidin for 20 minutes. A no substrate control with enzyme and a boiled-enzyme control with substrate are included. (B) *PaLAR* and *MdLAR* activity in *E. coli* which were co-transformed with *MdDFR*. The substrate (+)-taxifolin was added to the growth medium 12 hours after inducing protein expression with IPTG.
**Figure S9:** Correlation between catechin concentration and LAR3 transcript accumulation in LAR3 over-expressing lines and the vector control line ($R^2 = 0.32; p = 0.0058$).

**Figure S10:** Dimeric proanthocyanidins in the vector control and transgenic LAR3 overexpressor lines 1, 2 and 5.

**Figure S11:** *P. abies* line 5 over-expressing the LAR3 gene accumulates more flavan-3-ol metabolites in needles, stem and roots compared to the wild type. (A) PaLAR3 transcript accumulation and accumulation of (B) monomeric, (C) dimeric and (D and E) polymeric flavan-3-ols in the transgenic LAR3 over-expressing line and the vector control line. Transcript abundance (A) was measured by quantitative real-time PCR using CYBR Green for detection, normalized against PaUBI and calibrated against one vector control replicate. Monomeric (B) and dimeric (C) compounds were measured by LC-MS-MS. Polymers were measured after hydrolysis into catechin, gallocatechin, epicatechin and epigallocatechin and analyzed by LS-MS-MS (D). The degree of polymerization expressed as monomer equivalents was measured by LC-FLD (E). Error bars represent standard errors ($n = 5$).

**Figure S12:** Chalcone synthase (PaCHS) gene expression after wounding (control) or fungal inoculation during a time course of 28 days to illustrate general increases in the transcription in the flavonoid pathway in fungus-infected spruce bark ($p < 0.0005$) compared to the wounded control treatments. Relative transcript accumulation was measured with quantitative real-time PCR which was normalized against PaUBI (EF681766). Data was calibrated against the non-wounded control treatment. PCR primers were designed to amplify the whole CHS gene family from *P. abies* including CHS1 (JN400050), CHS2 (JN400051), CHS3 (JN400052), CHS4 (JN400053), CHS5 (JN400054), CHS6 (JN400055) and CHS8 (JN400057). The CHS7 gene was too dissimilar to include it in this analysis.

**Table S1:** Primers used for cloning of PaLAR genes

**Table S2:** Gene sequences used for construction of the phylogenetic tree.

**Table S3:** Primers used for quantitative real-time PCR.

**Table S4:** Validation of reductive hydrolysis of PAs using known standards from grape with equal catechin and epicatechin content. Hydrolysis was performed in triplicate. Recovery of catechin and epicatechin was quantified using HPLC-MS.
Alignment S1: CLUSTAL format of alignment used for construction of phylogenetic tree (Figure 3) for depicting the relationships of LAR and ANR amino acid sequences in gymnosperms and angiosperms. The alignment was created by the automated alignment software MAFFT (v7.045b).

Alignment S2: CLUSTAL format of alignment depicting the relationships of LAR amino acid sequences from *Picea abies*, *Picea glauca* and *Picea sitchensis* in relation to LAR from *Vitis vinifera* with known crystal structure and *Desmodium uncinatum* the first member of the protein family. Amino acids interacting with NADPH in the active site are highlighted in blue and amino acids interacting with leucoanthocyanidin are highlighted in pink. The numbering of active site amino acids follows the scheme for *V. vinifera* (Mauge et al., 2010). The alignment was created by the automated alignment software MAFFT (v7.045b).

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Figure legends

Figure 1: Biosynthesis of monomeric and polymeric flavan-3-ols via the flavonoid pathway. The mechanism by which monomeric flavan-3-ol units are incorporated as terminal and extender units of growing PA chains is not known. Abbreviations: CoA, coenzyme A; CHS, chalcone synthase; CHI, chalcone isomerase; FS, flavone synthase; F3H, flavanone-3-hydroxylase; F3’H, flavonol-3’-hydroxylase; F3’5’H, flavonol-3’5’-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin synthase; ANR, anthocyanidin reductase.

Figure 2: Content of monomeric and polymeric flavan-3-ols in bark of young Norway spruce saplings. Bark was infected with C. polonica or wounded without infection over a time course of 28 days. Presented are: (A) total monomers consisting of catechin and gallocatechin, (B) total dimers consisting of proanthocyanidin B1 and B3 and (C) total polymers after hydrolysis expressed as the total of monomeric units detected consisting of gallocatechin, catechin, epicatechin and epigallocatechin. The pie charts represent the ratio of the different compounds in each class as determined 2 and 28 days after inoculation of bark with isolate 2. For polymers the ratio of monomers detected after hydrolysis is depicted. (D) To compare the amounts of monomers, dimers and polymers together, the degree of polymerization of all flavan-3-ols at 28 days after the onset of infection is presented, with quantities expressed as monomer equivalents. The non-wounded control is based on day 2 measurements. Error bars represent standard errors (n = 5).
**Figure 3:** Phylogenetic relationships of *LAR* and *ANR* genes in gymnosperms and angiosperms and *LAR* expression in Norway spruce. Neighbor-joining tree of the deduced amino acid sequences of *P. abies* leucoanthocyanidin reductase (*PaLAR*) genes and sequences of characterized LAR enzymes from angiosperms were calculated using the JTT matrix. The tree was searched using the minimum evolution method with pair-wise elimination of alignment gaps. Gene expression in various tissue types without wounding or fungal inoculation was measured by quantitative real-time PCR using SYBR Green for detection and normalized against *PaUBI*. Error bars represent standard errors (n = 5).

**Figure 4:** Recombinant LAR enzymes accepted leucocyanidin as substrate and formed catechin as products. LAR from apple (*MdLAR*) was included as a positive control. Enzyme activity of *PaLAR* and *MdLAR* enzymes (19 µg each) is expressed in katal.

**Figure 5:** *P. abies* lines over-expressing the *LAR3* gene accumulate more flavan-3-ol metabolites. (A) *PaLAR3* transcript accumulation and accumulation of (B) monomeric, (C) dimeric and (D and E) polymeric flavan-3-ols in three transgenic LAR3 over-expressing lines and one vector control line. Transcript abundance (A) was measured by quantitative real-time PCR using CYBR Green for detection, normalized against *PaUBI* and calibrated against one vector control replicate. Monomeric (B) and dimeric (C) compounds were measured by LC-MS-MS. Polymers were measured after hydrolysis into catechin, gallo catechin, epicatechin and epigallocatechin and analyzed by LS-MS-MS (D). The degree of polymerization expressed as monomer equivalents was measured by LC-FLD (E). Error bars represent standard errors (n = 5).

**Figure 6:** *LAR* expression in Norway spruce bark is induced by *C. polonica* infection. Relative transcript accumulation of *PaLAR1* (A), *PaLAR2* (B), *PaLAR3* (C) and *PaLAR4* (D) in bark of 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. Error bars represent standard errors (n = 5).

**Figure 7:** Norway spruce flavan-3-ols inhibit *C. polonica* fungal growth at natural concentrations. Growth of *C. polonica* isolates on solid minimal medium containing mixtures of monomeric and polymeric flavan-3-ols similar to those in non-wounded spruce sapling bark (dilute flavan-3-ol mix containing 1.1 mg ml\(^{-1}\) catechin and 1.1 mg ml\(^{-1}\) PAs) or those in bark infected by *C. polonica* for 14 days (concentrated flavan-3-ol mix containing 6.7 mg ml\(^{-1}\) catechin and 4.5 mg ml\(^{-1}\) PAs). Error bars represent standard errors (n = 5).
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