

Flavan-3-ols in Norway Spruce: Biosynthesis, Accumulation, and Function in Response to Attack by the Bark Beetle-Associated Fungus *Ceratocystis polonica*¹[C][W][OPEN]

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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, 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 Norway spruce saplings after wounding or inoculation with the fungal pathogen *Ceratocystis polonica*, which is vectored by bark beetles (*Ips typographus*) 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 Norway spruce *LEUCOANTHOCYANIDIN REDUCTASE* genes also increased significantly during fungal infection. Bioassays with *C. polonica* revealed that the levels of 2,3-trans-(+)-catechin and PAs that are produced in the tree in response to fungal infection inhibit *C. polonica* growth and can therefore be considered chemical defense compounds.

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 C₆-C₃-C₆ 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 2 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)

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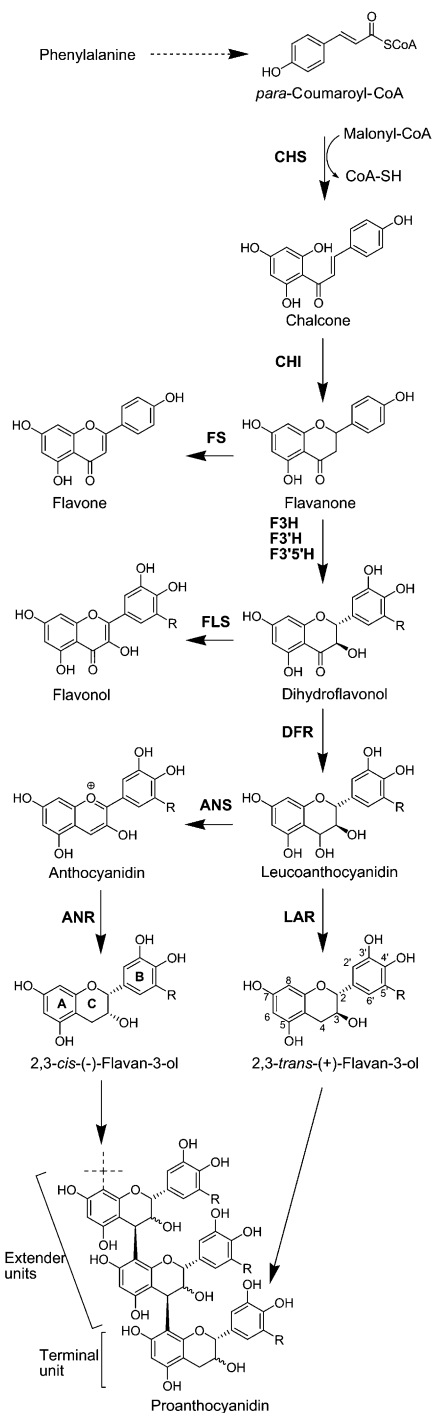
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	R = H	R = OH
Flavonol	Quercetin	Myricetin
Dihydroflavonol	Dihydroquercetin	Dihydromyricetin
Anthocyanidin	Cyanidin	Delphinidin
Leucoanthocyanidin	Leucocyanidin	Leucodelphinidin
2,3-cis-(-)-flavan-3-ol	Epicatechin	Epigallocatechin
2,3-trans-(+)-flavan-3-ol	Catechin	Gallocatechin

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

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 that 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 (Ebbolle, 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 (Bladé 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 *Picea* and *Pinus*. In spruce (*Picea* spp.), 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). Because 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 (Xie et al., 2004; Bogs et al., 2005; Pfeiffer et al., 2006; Almeida et al., 2007; Pang et al., 2009) as well as in the model plant *Arabidopsis* (*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, 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-dependent isoflavone-like reductases

chains is not known. CHI, Chalcone isomerase; FS, flavone synthase; F3H, flavanone-3-hydroxylase; F3'H, flavanone-3'-hydroxylase; F3'5'H, flavanone-3',5'-hydroxylase; FLS, flavonol synthase; ANS, anthocyanidin synthase.

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 (Bogs et al., 2005; Paolucci et al., 2007). 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 nonenzymatic mechanisms (Stafford, 1983).

Because 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 a conifer species, Norway spruce (*Picea abies*). 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-infects 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 Bark Increases after Infection with *C. polonica*

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 d after treatment.

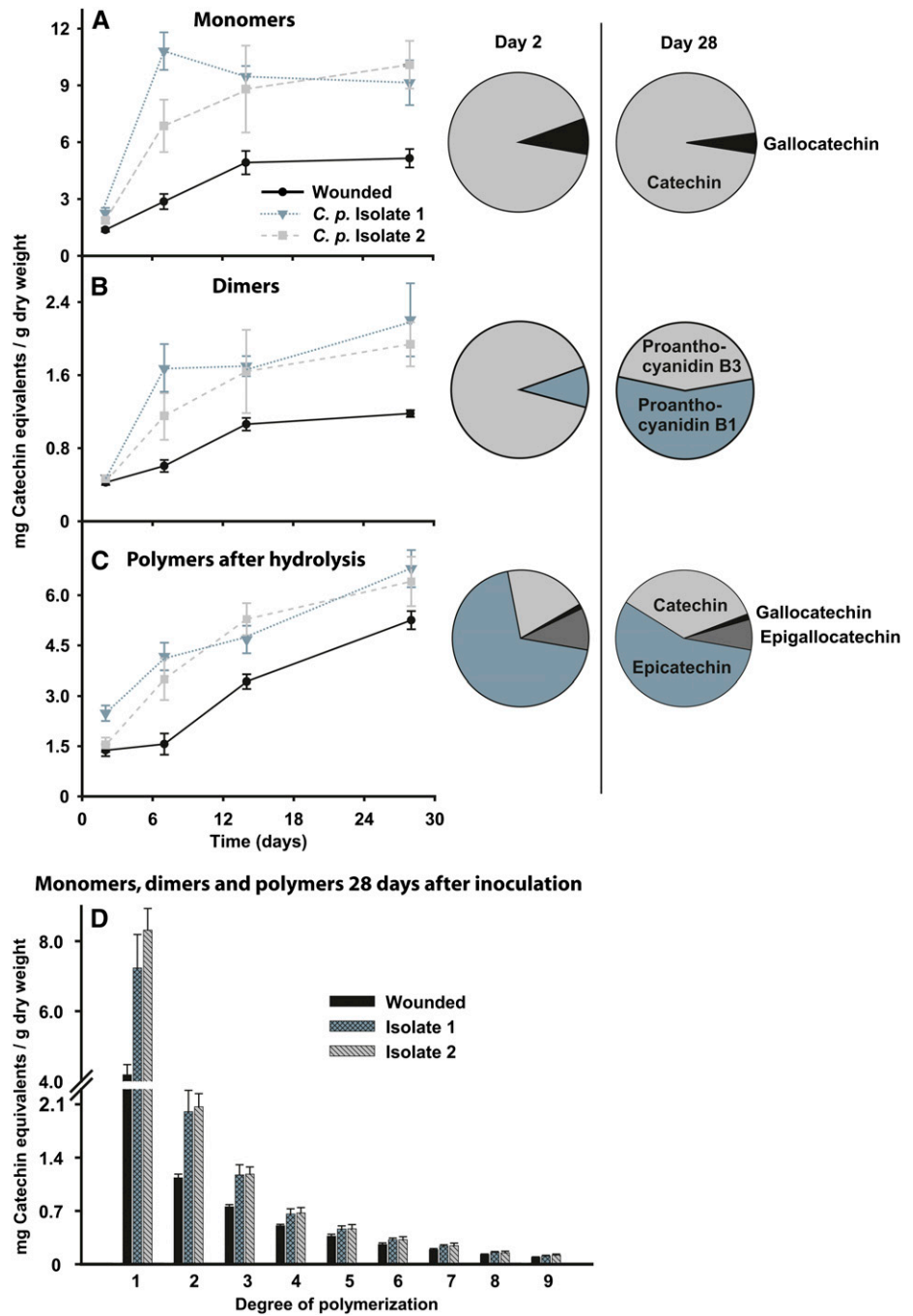
The monomeric flavan-3-ols in Norway spruce bark consisted of mixtures of 2,3-trans-(+)-catechin and

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 d 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 noninoculated controls collected 2 d after treatment did not differ from those in unwounded bark samples collected at the onset of the time course (Supplemental Fig. S1). In fungal-inoculated samples, the ratio of catechin:gallocatechin changed from 11:1 at 2 d after infection to 20:1 at 28 d after infection (Fig. 2A; levels of individual compounds are given in Supplemental Fig. 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 PAs) that could be detected were mainly two procyanidins (dihydroxylated B-ring), PA B1 [2,3-cis-(–)-epicatechin-(4 β →8)- 2,3-trans-(+)-catechin] and PA B3 [2,3-trans-(+)-catechin-(4 β →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-d period (Fig. 2B; levels of individual compounds are given in Supplemental Fig. S2) in inoculated and in wounded control bark of spruce saplings ($P < 0.001$) compared with the nonwounded control bark samples collected prior to treatment (Supplemental Fig. S1). The major differences in dimer concentrations among the treatments were mainly explained by significantly higher PA B1 content in infected bark compared with 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 and 28 d postinoculation, the ratio of PA B1 to 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 cyanoborohydrate to yield monomeric units (Supplemental Fig. 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-d 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 Fig. S4) compared with the nonwounded control (Supplemental Fig. 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 d

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 d. Present are total monomers consisting of catechin and gallocatechin (A), total dimers consisting of PA B1 and B3 (B), and total polymers after hydrolysis expressed as the total of monomeric units detected consisting of gallocatechin, catechin, epicatechin, and epigallocatechin (C). The pie charts represent the ratio of the different compounds in each class as determined 2 and 28 d 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 d after the onset of infection is presented, with quantities expressed as monomer equivalents. The nonwounded control is based on day 2 measurements. Error bars represent *ses* (*n* = 5). [See online article for color version of this figure.]



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 d postinoculation.

To determine the degree of flavan-3-ol polymerization in nonwounded, wounded, and infected bark tissue, normal phase chromatography coupled to fluorescence detection (FLD; Kelm et al., 2006) was used (Supplemental Fig. 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. Twenty-eight 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 2-year-old saplings was conducted to determine the effect of wounding on flavan-3-ol levels over a time course of 25 d (Supplemental Figs. S6

and S7). Comparison of wounded but not fungus-inoculated trees to nonwounded controls showed higher monomer, dimer, and polymer levels in wounded versus unwounded bark, but there were no statistically significant differences between the treatments. Wounded and nonwounded trees showed a similar increase ($P < 0.001$) in polymer levels over the 25-d time course, suggesting that polymers are formed from monomers continuously during this period (Supplemental Figs. S6 and S7).

Norway Spruce Has Four LAR Genes with Distinct Phylogenetic Relationships

To identify 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 ESTs from Sitka spruce (*Picea sitchensis*) and 250,000 from white spruce (*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 white spruce and Sitka spruce as templates for primer design, four full-length LAR gene candidates could be amplified from Norway spruce complementary DNA (cDNA) by PCR.

Phylogenetic analysis (Supplemental Alignment S1) revealed a clear evolutionary divergence between the translated LAR amino acid sequences and those of 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% to 57% identity with LAR sequences from angiosperms, whereas sequences for Norway spruce LAR (PaLAR) were 79% to 87% identical and had 62% to 81% similarity to an uncharacterized LAR sequence from *P. taeda* (Bogs et al., 2005). Phylogenetic analysis also revealed separate subclades 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% to 65% similarity.

Two ANR-like orthologous sequences from Norway spruce (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 Norway spruce and selected angiosperm ANR had 50% to 57% sequence similarity.

Steady-state transcript levels were measured in wood, bark, stems, roots, and needles of unwounded Norway spruce 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. *LAR3* and *LAR4* gene expression was lower in wood than in foliage, bark, or roots ($P < 0.001$).

Norway Spruce LAR Proteins All Catalyze Leucoanthocyanidin Reduction in Vitro and in Vivo

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

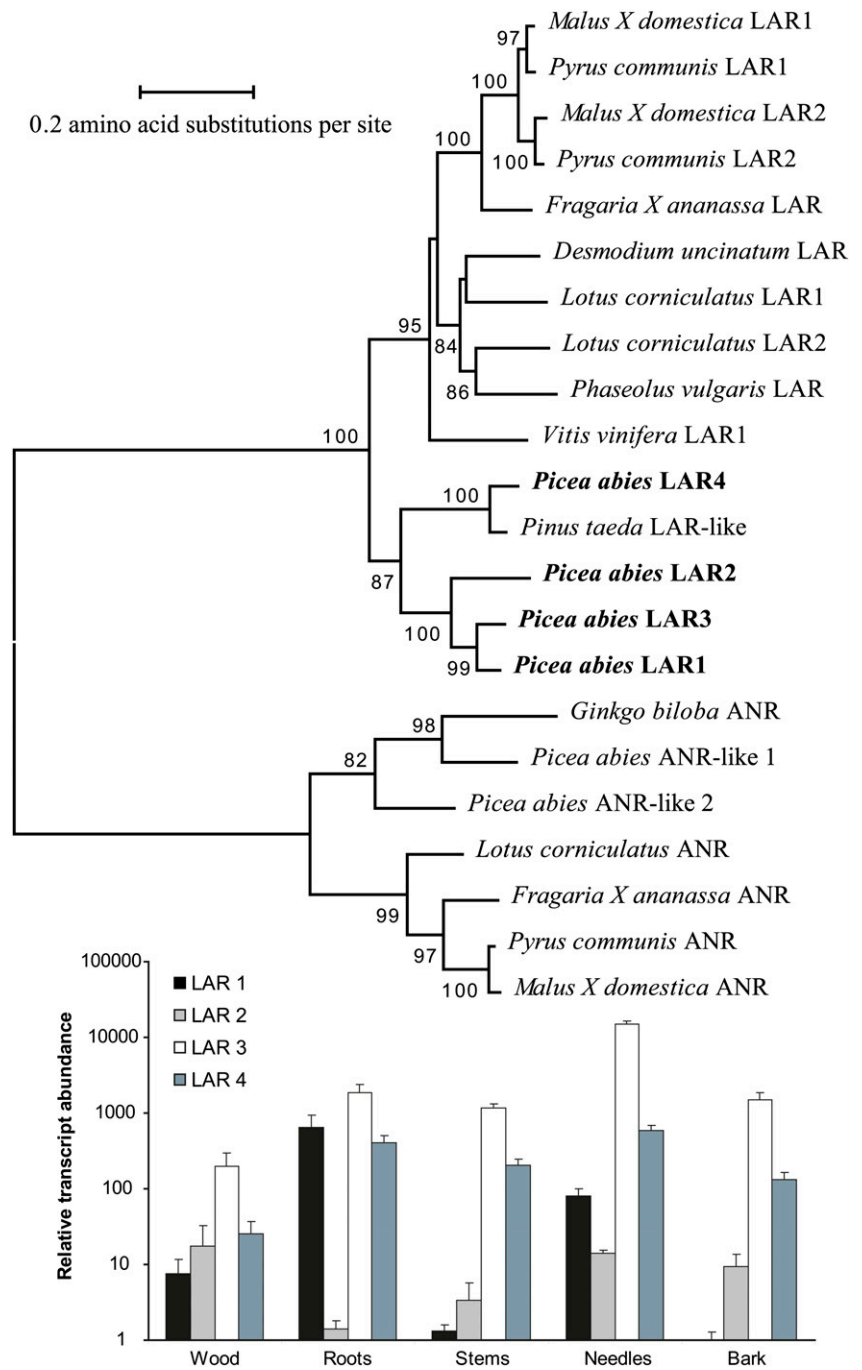
To compare PaLAR activities with activities reported in the literature, MdLAR from apple (*Malus domestica*; Pfeiffer et al., 2006) was cloned and expressed under the same conditions. PaLAR2, PaLAR3, and PaLAR4 had similar activities in in vitro enzyme assays to MdLAR, but the activity of PaLAR1 was much lower ($P < 0.05$; Fig. 4; Supplemental Fig. S8).

An in vivo LAR assay was also performed in which *Escherichia coli* was cotransformed with a LAR expression construct as well as a plasmid encoding a dihydroflavonol reductase enzyme (MdDFR) from apple (Fischer et al., 2003). The assay was conducted by adding the dihydroflavonol substrate (+)-taxifolin to the medium after induction of protein expression. Products were harvested from the medium and quantified after normalizing to the optical density at 600 nm of the bacterial culture. In this in vivo assay, bacteria with MdLAR produced much higher levels of catechin from (+)-taxifolin than the PaLAR enzymes (Supplemental Fig. S8). Epimerase activity whereby PaLAR produces both catechin and epicatechin (Pang et al., 2013) was not observed in this in vivo assay or in the PaLAR and MdLAR in vitro assays described above.

Norway Spruce LAR3 Increases Flavan-3-ol Content after Overexpression in Transgenic Spruce Saplings

To confirm the in vitro activity of the Norway spruce LAR genes, embryonic Norway spruce tissue was transformed with PaLAR3 under the control of the inducible promoter *ubiquitin1* (*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

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 *PaLAR* genes and sequences of characterized *LAR* enzymes from angiosperms were calculated using the Jones, Taylor, Thornton matrix. The tree was searched using the minimum evolution method with pairwise 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 *ses* ($n = 5$). [See online article for color version of this figure.]



its high catalytic activity (Fig. 4). Three transgenic *PaLAR3* seedling lines (lines T1, T2, and 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* overexpressing 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 correlation coefficient $R^2 = 0.32$; $P = 0.0053$; Supplemental Fig. S9). Dimeric flavan-3-ols (Fig. 5C) were present at higher concentrations in lines T2 and T5 ($P < 0.001$) than in line T1, and the vector control and ratios of PA B3 to PA B1 were similar in the vector control and the transgenic lines, with the

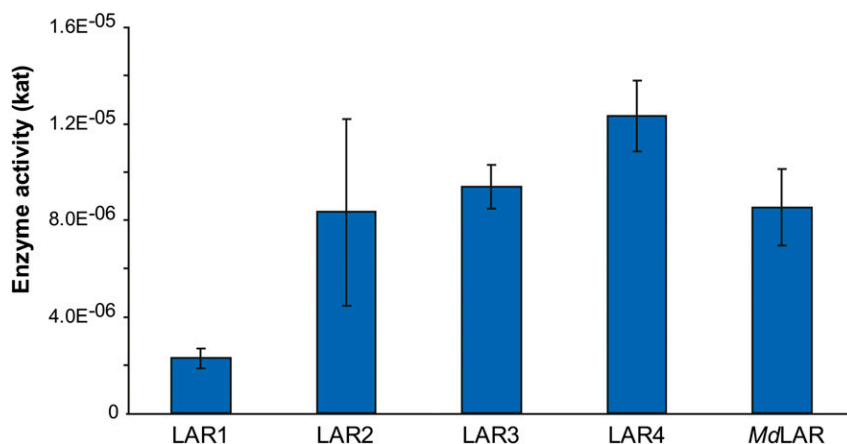


Figure 4. Recombinant LAR enzymes accepted leucocyanidin as substrate and formed catechin as products. MdLAR was included as a positive control. Enzyme activity of PaLAR and MdLAR enzymes (19 μ g each) is expressed in katal. [See online article for color version of this figure.]

catechin-catechin dimer PA B3 being more abundant in both types. However, dimers containing gallo catechin were more abundant in the transgenic lines than in the vector control (Supplemental Fig. 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 trihydroxylated B-ring (gallocatechin and epigallocatechin) than the vector control line ($P = 0.06$).

Needles and roots of the *LAR3* overexpressing T5 line and the vector control were also compared for *LAR3* transcript abundance and flavan-3-ol content (Supplemental Fig. S11). 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 *LAR3* 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 *LAR3* 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 *LAR3* 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 *LAR3* overexpressing T5 line (Supplemental Fig. S11).

Inoculation with *C. 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. polonica*-inoculated spruce saplings was extracted and transcribed to cDNA. Transcript

accumulation of *PaLAR*, measured by quantitative real-time PCR, increased more in inoculated saplings than in wounded control saplings over a 28-d time course (Fig. 6). *PaLAR1*, *PaLAR3*, and *PaLAR4* transcript levels increased significantly in inoculated saplings between 2 and 7 d postinoculation ($P < 0.01$), while transcript accumulation of *PaLAR2* increased between 14 and 28 d after wounding or infection ($P = 0.04$) compared with earlier time points.

Accumulation of *PaLAR1*, *PaLAR2*, and *PaLAR4* transcripts in response to fungal inoculation were greater than in wounded saplings, which were in turn greater than in unwounded controls. Levels of *PaLAR1* mRNA in inoculated saplings were 8- to 12-fold higher than in the wounded control and between 2,000- and 6,000-fold higher than in nonwounded saplings (assigned a relative transcript abundance of 1). Transcript accumulation of *PaLAR2* was 2-fold higher in inoculated than in wounded saplings and between 180- and 200-fold higher than in bark of nonwounded saplings. *PaLAR3*, which had more than 20-fold higher steady-state transcript levels (Fig. 3) than *PaLAR1*, *PaLAR2*, and *PaLAR4*, did not increase in wounded versus unwounded control saplings but increased 3- to 4-fold in fungus-inoculated saplings. *PaLAR4* increased between 10- and 30-fold compared with the wounded control and 20- to 60-fold compared with the nonwounded controls.

To demonstrate general transcriptional activity of the entire flavan-3-ol biosynthetic pathway, transcript accumulation of a subset of seven chalcone synthase (*PaCHS*) genes (Hammerbacher et al., 2011) was measured (Supplemental Fig. 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

To determine whether mixtures of Norway spruce monomeric flavan-3-ols and PAs had activity against

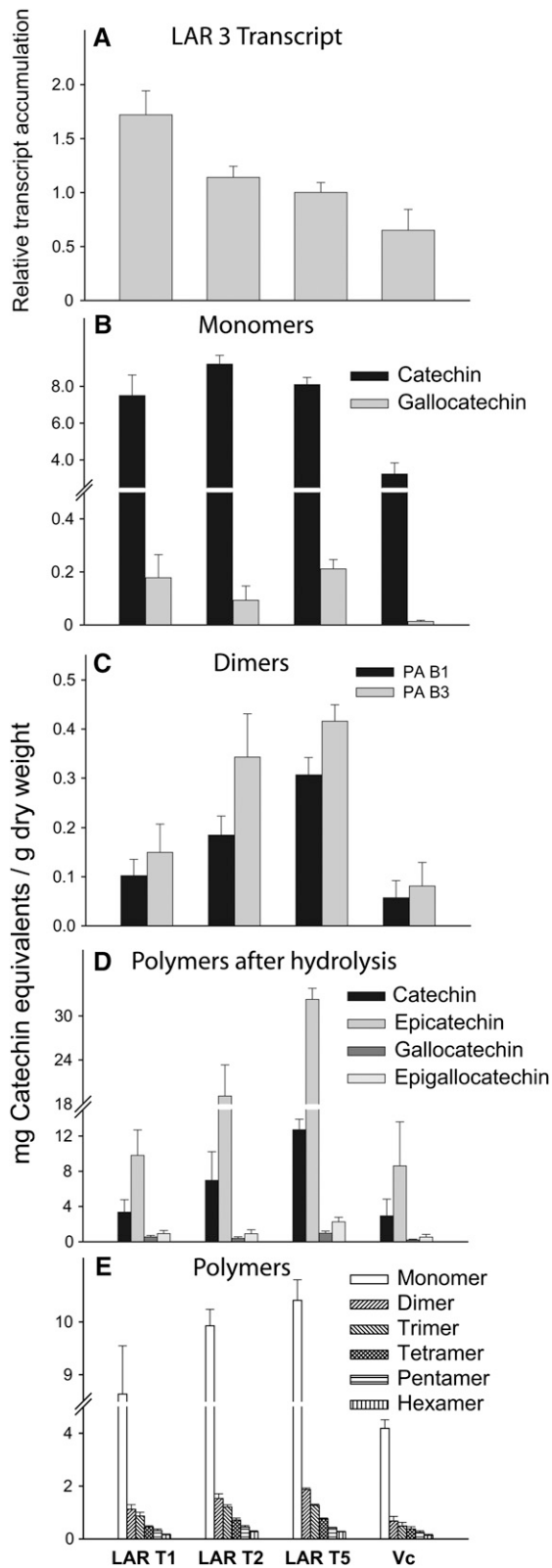


Figure 5. Norway spruce lines overexpressing the *LAR3* gene accumulate more flavan-3-ol metabolites. *PaLAR3* transcript accumulation (A) and accumulation of monomeric (B), dimeric (C), and polymeric flavan-3-ols (D and E) in three transgenic *LAR3*-overexpressing lines

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 d of fungal infection. Both isolates showed significantly lower growth rates on medium equivalent to 28 d 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 PAs or condensed tannins are widely distributed in the plant kingdom, especially in woody species (Barbehenn and Peter 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. In addition, investigation of the genes encoding *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 a 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 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; de Pascual-Teresa et al., 2000; Almeida et al., 2007; Mellway et al.,

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. D, Polymers were measured after hydrolysis into catechin, gallocatechin, epicatechin, and epigallocatechin and analyzed by LC-MS/MS. E, The degree of polymerization expressed as monomer equivalents was measured by LC-FLD. Error bars represent ses ($n = 5$).

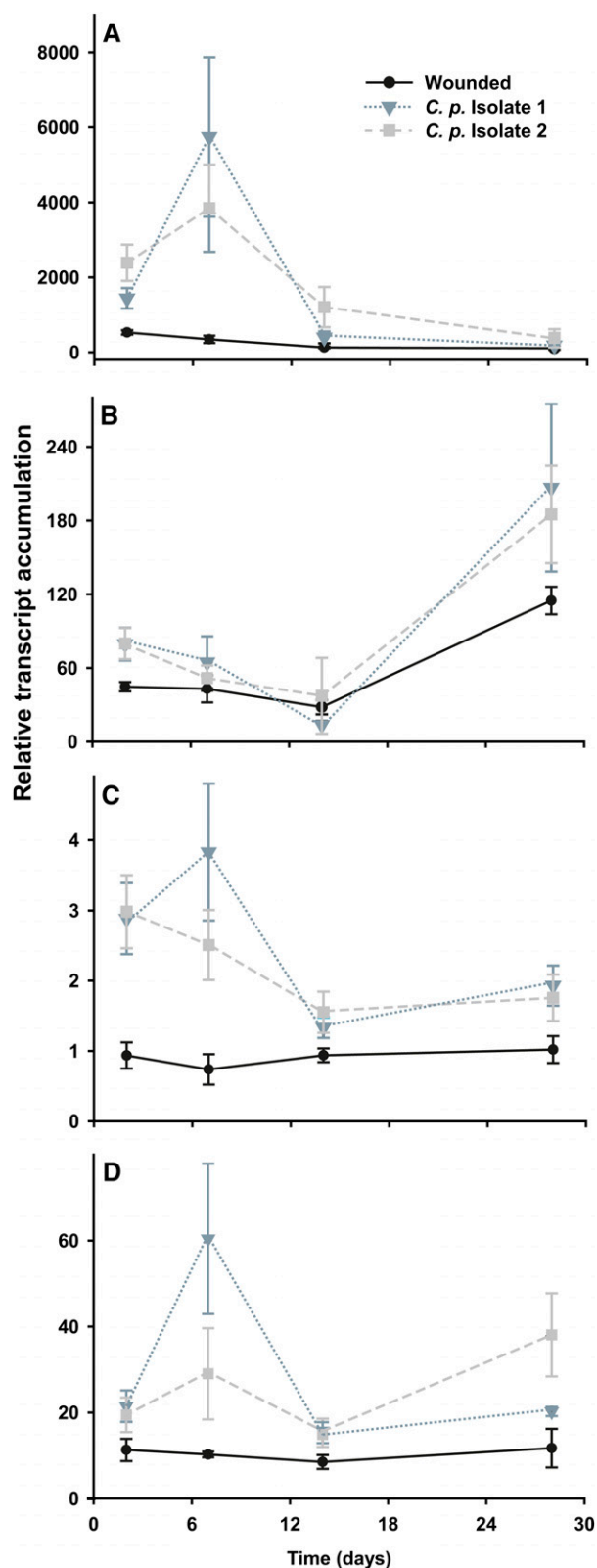


Figure 6. *LAR* expression in Norway spruce bark is induced by *C. polonica* infection. Relative transcript accumulation of *PaLAR1* (A), *PaLAR2* (B), *PaLAR3* (C), and *PaLAR4* (D) in bark of spruce saplings over a time course of 28 d after wounding or inoculation with two

2009). More recent methods do not give strictly quantitative results due to incomplete hydrolysis of PA chains or the analysis of highly unstable molecules (Guyot et al., 1998; Pang et al., 2007). In this work, a three-step procedure was developed to more comprehensively analyze both monomeric and polymeric flavan-3-ols (PAs) in Norway spruce bark. First, monomeric and dimeric flavan-3-ols in plant extracts were quantified by reverse-phase HPLC. Second, PAs were completely hydrolyzed under acidic conditions in the presence of a strong reducing agent, thereby preventing oxidation and minimizing epimerization (Supplemental Fig. S3). Hydrolysis products were then separated by reverse-phase HPLC to yield quantitative and qualitative information on the composition of the individual monomeric units. Third, 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 HPLC instrument equipped with a fluorescence detector and two 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-trans-(+)-catechin (90%–95%) and 2,3-trans-(+)-gallocatechin (5%–10%). These compounds are biosynthesized from leucoanthocyanidins by 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 *LAR* mRNA sequences were identified by BLAST sequence comparisons and four orthologous *LAR* transcript sequences were identified in Norway spruce. Angiosperms with smaller genomes than *Picea* species such as apple and grape (*Vitis vinifera*) contain only two *LAR* genes (Pfeiffer et al., 2006).

Phylogenetic analysis of *LAR* enzymes from spruce revealed independent evolution of *LAR* in angiosperms and gymnosperms. However, the active site regions (Supplemental Alignment S2) of enzymes from both lineages were similar. Analysis of the crystal structure of grape *LAR* by Maugé 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 *PaLAR* with the exception of residue 91 (grape *LAR*

C. polonica isolates. Transcript abundance was measured by quantitative real-time PCR using CYBR Green for detection, normalized against *PaUBI*, and calibrated against a nonwounded bark sample from the same clone. Error bars represent *ses* ($n = 5$). [See online article for color version of this figure.]

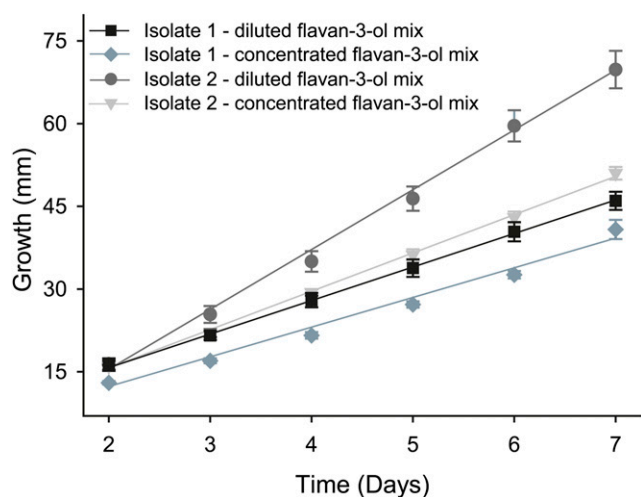


Figure 7. Norway spruce flavan-3-ols inhibit *C. polonica* fungal growth at natural concentrations. Growth of *C. polonica* isolates on solid minimal medium containing mixtures of monomeric and polymeric flavan-3-ols similar to those in nonwounded spruce sapling bark (dilute flavan-3-ol mix containing 1.1 mg mL^{-1} catechin and 1.1 mg mL^{-1} PAs) or those in bark infected by *C. polonica* for 14 d (concentrated flavan-3-ol mix containing 6.7 mg mL^{-1} catechin and 4.5 mg mL^{-1} PAs). Error bars represent *ses* ($n = 5$). [See online article for color version of this figure.]

numbering scheme), where in PaLAR2 and PaLAR3 a transition was observed from Val to Met. In the grape LAR active site, Maugé et al. (2010) identified conserved His (position 123), Tyr (position 137), Ser (position 161), and Ile (position 171) residues, which could be responsible for the enzyme's catalytic activity. In the PaLAR enzymes, the His, Ser, and Tyr residues were all conserved, but only PaLAR2 contained Ile in position 171. All other PaLAR enzymes contained Thr at position 171. LAR from *Desmodium uncinatum*, the first enzyme with LAR activity discovered, had a Cys residue at position 171. This might indicate that the amino acid at position 171 is not important for the reduction of leucoanthocyanidin.

Heterologously expressed PaLAR enzymes accepted the substrates leucoanthocyanidin and to a lesser extent leucodelphinidin, with a trihydroxylated B-ring, to produce 2,3-trans-(+)-catechin or 2,3-trans-(+)-gallocatechin, respectively. This activity was confirmed in vivo by analysis of transgenic spruce lines overexpressing PaLAR3, which accumulated more catechin and epicatechin than the vector control.

While 3- to 4-fold higher flavan-3-ol concentrations were recorded in the transgenic LAR3-overexpressing lines compared with the vector controls, transcript levels of the LAR3 gene were only 1.7- to 2.9-fold higher than in the vector controls. 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 (*Vaccinium myrtillus*), 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). Because, in our study, spruce saplings were harvested 4 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 d (Supplemental Figs. 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-overexpressing transgenics as well as in vector controls. Pang et al. (2013) reported epimerase activity for *Camellia sinensis* LAR that 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 apple. 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.

Because 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 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 *Saccharomyces 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 because 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 (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 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 *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 Fig. 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 antiherbivore 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 with unwounded controls over a time course of 25 d (Supplemental Fig. S6). These data can be taken to suggest that flavan-3-ols are not responsive to herbivory and so are not antiherbivore 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*.

Because 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). Because *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, 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 Norway spruce 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 antifungal defense more clearly, future studies should include growth assays of *C. polonica* on transgenic PA-overproducing trees such as our *LAR3*-overexpressing lines or on other transgenic trees where the pathway is silenced.

MATERIALS AND METHODS

Identification of Putative LAR Genes from *Picea* spp. EST Collections

Protein LAR sequences from apple (*Malus domestica*), *Pyrus communis*, and *Pinus taeda* (Fischer et al., 2003; Pfeiffer et al., 2006), as well as an ANR sequence from *Ginkgo biloba* (Shen et al., 2006) were used to screen Sitka spruce (*Picea sitchensis*) and white spruce (*Picea 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). To identify other possible LAR candidates not present in the Treenomix database, the genomic sequences of Norway spruce (*Picea abies*) and white spruce (Biol et al., 2013; Nystedt et al., 2013) were screened using the four identified LAR sequences.

Cloning and Sequencing PaLAR Genes

RNA was purified from fresh bark tissue of 4-year-old Norway spruce saplings using the method developed by Kolosova et al. (2004). One microgram of total RNA was converted to cDNA in a 20- μ L reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen) 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 [Invitrogen] compatible) and two ANR genes from Sitka spruce and white spruce as templates (primer sequences used are provided in Supplemental Table S1). cDNA ends were also amplified independently using the RACE kit for LAR1, LAR2, and LAR3 (Clontech). A fourth full-length PaLAR gene candidate was identified in the Congenie database (<http://www.congenie.org>; Nystedt et al., 2013) by blast searches. PaLAR cDNA were PCR amplified with primers (Supplemental Table S1) using Platinum Taq high-fidelity DNA polymerase (Invitrogen) and purified with the QIAquick PCR purification kit (Qiagen). Gateway 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). Sequences from each construct were assembled and translated into protein sequence using DNA Star software.

Protein Sequence Analysis of PaLAR

LAR protein sequences from Norway spruce 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 version 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 version 4 (Center for Evolutionary Medicine and Informatics) employing the Minimum Evolution method. Evolutionary distances were calculated with the Jones et al. (1992) matrix. The tree was searched with pairwise elimination of alignment gaps. Statistical likelihood of tree branches was tested with 1,000 bootstrap replicates.

Heterologous Expression of PaLAR Genes in *Escherichia 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 glutathione S-transferase 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 broth with 1 μ g mL⁻¹ ampicillin and grown for 12 h at 30°C. The 5-mL starter cultures were used to inoculate 100 mL Luria-Bertani medium supplemented with 1 μ g mL⁻¹ kanamycin.

Bacterial cultures were grown for 12 h at 18°C (220 rpm), induced with 0.5 mM isopropylthio- β -galactoside, and harvested 14 h 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 and lysed by sonication for 4 min using two cycles at 65% power with a Bandelin Sonoplus HD 2070 sonifier (Bandelin Electronics). Insoluble cell debris was removed from the lysate by centrifugation at 16,000g for 30 min at 4°C.

Expressed proteins were purified from the crude lysate by affinity chromatography with 1 mL Glutathione Sepharose (GE Healthcare Life Sciences) 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 dithiothreitol) on DG-10 desalting columns (Bio-Rad) 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 unlabeled substrates leucoanthocyanidin and leucodelphinidin were purchased from TransMit Flavonoid Forschung. Recombinant LAR1, LAR2, LAR3, and LAR4 enzyme activities were assayed in 200- μ L reaction volumes containing 19 μ g purified enzyme, 500 nmol NADPH (Carl Roth GmbH), and 200 nmol leucoanthocyanidin or leucodelphinidin in assay buffer. Reaction mixtures were incubated for 20 min at 25°C before the enzyme assay was stopped by addition of one 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°C, 30°C, and 35°C. pH optima between pH 6 and pH 8.5 were determined by using 50 mM Bis-Tris. The already described LAR from apple (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 cotransforming *E. coli* with LAR as well as *MdDFR* (dihydroflavonol reductase from apple), which was cloned using the above protocols into the vector PH9 (Yu and Liu, 2006). The enzymes were expressed as above. Twelve hours after inducing expression with isopropylthio- β -galactoside, (+) taxifolin in dimethyl sulfoxide was added to the culture medium to a final concentration of 100 μ g mL⁻¹. Cultures were harvested 3 h after addition of taxifolin. Bacteria were removed from the culture medium by centrifugation. Ten milliliters medium was acidified with 100 μ L of 0.1 N HCl and extracted with three volumes of ethyl acetate. The ethyl acetate extracts were evaporated under a stream of nitrogen gas and redissolved in 500 μ L methanol for liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) analysis.

Genetic Transformation of Norway Spruce 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) was transformed into *Agrobacterium tumefaciens* strain C58/pMP90 (Schmidt et al., 2010), which was subsequently used to transform an embryonic Norway spruce cell culture (line 186/3c VIII) as described by Schmidt et al. (2010). From the six transgenic lines obtained, lines 1, 2, and 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 × 4 cm).

Inoculation of Norway Spruce Saplings with *Ceratocystis 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 (Carl Roth GmbH) for 12 d at 25°C in the dark.

Eight-year-old Norway spruce saplings originating from the 3369-Schongau clone (Samenklänge und Pflanzengarten Laufen) were grown in an outdoor plot for 4 years prior to the experiment. Inoculations of saplings with *C. polonica* were performed 3 weeks after their spring flush (June 10, 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 malt extract agar were inserted into the wound.

Bark tissue samples from inoculated and wounded saplings were harvested 2, 7, 14, and 28 d 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 (Invitex) 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 PolyT₁₂ 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) and 10 pmol forward and 10 pmol reverse primer. Primer sequences for *PaLAR1*, *PaLAR2*, *PaLAR3*, *PaLAR4*, and *PaCH5* 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 nontemplate controls as well as nonreverse-transcribed RNA. Transcript abundance was normalized to the transcript abundance of the ubiquitin (Schmidt et al., 2010) gene (primers in Supplemental Table S1) and was calculated from three technical replicates of five biological replicates. Relative transcript abundance was calibrated against the transcript abundance of five nonwounded 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 millibar pressure using an Alpha 1-4 LD Plus freeze dryer (Martin Christ GmbH). After approximately 80 mg dried tissue was extracted with 2 mL analytical grade methanol for 4 h at 4°C , the extract was centrifuged at 3,200g, and the supernatant was recovered. Insoluble material was reextracted with 1.5 mL methanol for 16 h. Supernatants were combined and evaporated to dryness under a stream of nitrogen. Dried samples were redissolved in 1 mL methanol containing 100 μg mL⁻¹ chlorogenic acid (Sigma) as internal standard. For LC-ESI-mass spectrometry (MS) or hydrolysis of condensed tannins, samples were diluted five times (v/v) with methanol. For LC-FLD, samples were diluted two times (v/v) in acetonitrile.

LC-FLD

PAs were separated on a LiChrosphere diol column with dimensions of 250×4 mm and a particle size of 5 μm (Merck) using an Agilent 1100 series HPLC 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 to 35 min, 0% to 40% B in A; 35 to 40 min, 40% B; 40 to 45 min, 40% to 0% B; and 45.1 to 50 min, 0% B. Eluent was monitored by FLD with excitation at 276 nm and emission at 316 nm.

LC-ESI-MS

Compounds to be analyzed were separated on a Nucleodur Sphinx RP18ec column with dimensions of 250×4.6 mm and a particle size of 5 μm

(Macherey Nagel) 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 to 1 min, 100% A; 1 to 25 min, 0% to 65% B in A; 25 to 28 min, 100% B; and 28 to 32 min, 100% A.

Compound detection and quantification was accomplished with an Esquire 6000 ESI ion trap mass spectrometer (Bruker Daltonics). 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 a mass-to-charge ratio (m/z) between 50 and 1,600 with an optimal target mass of 405 m/z . The mass spectrometer was operated using the following specifications: skimmer voltage, 60 V; capillary voltage, 4,200 V; nebulizer pressure, 35 pounds per square inch (psi); drying gas, 11 L min⁻¹; and gas temperature, 330°C . 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 1 mL 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 2,500 m/z , varying the optimal target mass according to the degree of polymerization.

Compounds were identified by MS and by direct comparison with commercial standards, where available, including 2,3-trans-(+)-catechin, 2,3-trans-(+)-gallocatechin, 2,3-cis-(–)-epicatechin, 2,3-cis-(–)-epigallocatechin, PA B1, and PA B3. Bruker Daltonics Quant Analysis version 3.4 software was used for data processing and compound quantification using a standard smoothing width of 3 and Peak Detection Algorithm version 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 2,3-trans-(+)-catechin (Sigma), 2,3-trans-(+)-gallocatechin (Sigma), and PA B1 (Sigma). PA B3 was quantified relative to PA B1. Process variability in different analyses was calculated relative to the internal standard.

Reductive Cleavage of PAs

PAs were cleaved in 800 μL reaction volumes containing 2.5% (v/v) trifluoroacetic acid and 8% (v/v) 0.5 g mL⁻¹ sodium cyanoborohydrate in methanol. Reaction mixtures were heated to 65°C for 15 min before adding additional 2.5% (v/v) trifluoroacetic acid. Vials were sealed tightly and incubated at 65°C overnight. Samples were dried under a stream of nitrogen and redissolved in 800 μL methanol and analyzed using the Nucleodur Sphinx RP18ec column under the conditions already described but detected with a UV diode array detector at 280 nm.

Validation of the hydrolysis method to quantify PAs was done by analyzing different amounts of partially purified grape (*Vitis vinifera*) PAs (Laffort; Supplemental Fig. 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).

LC-ESI-MS/MS

Chromatography was performed on an Agilent 1200 HPLC system. Separation was achieved on a 100- \times 4.6-mm Kinetex C18 column with particle size of 2.6 μm (Phenomenex). Formic acid (0.05% [v/v]) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was as follows: 0 to 1 min, 100% A; 1 to 7 min, 0% to 65% B in A; 7 to 8 min, 65% to 100% B in A; 8 to 9 min, 100% B; and 9 to 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 PA B1. For dimeric PAs containing gallocatechin, partially purified plant extracts were used for optimization. The

ion spray voltage was maintained at $-4,500$ 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 was used to monitor analyte precursor ion \rightarrow 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 \rightarrow 289.1 (CE, -30 V; DP, -50 V) for PA B1; m/z 592.9 \rightarrow 125.1 (CE, -52 V; DP, -400 V) for the catechin:gallocatechin dimer; and m/z 609 \rightarrow 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 PA 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/v]) amended with carrot (*Daucus carota*) juice (1.5% [v/v]; Viva Vital) and a mixture of pure flavan-3-ols equivalent to constitutive or induced levels. Medium was dispensed in petri dishes (diameter = 5.2 cm). After the medium solidified, an agar plug (diameter = 4 mm) from a 14-d-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 h until growth reached the margins of the petri dish ($n = 5$).

Statistical Analysis

Most experiments were analyzed with a multivariate repeated measures model because 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 and 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 nonnormal errors, outliers, and several kinds of nonnormal 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 posthoc test.

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

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Total flavan-3-ol monomers, dimers, and polymers after hydrolysis of unwounded bark samples collected at the onset of the time course (Fig. 2) and of the wounded control samples collected 2 d postwounding.

Supplemental Figure S2. Relative concentrations of soluble monomeric and dimeric flavan-3-ols in spruce bark that was infected with *C. polonica* or wounded over a time course of 28 d.

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

Supplemental Figure S4. Relative concentrations of hydrolysis products of PAs formed in spruce bark during wounding and fungal infection over a time course of 28 d.

Supplemental Figure S5. LC-FLD chromatogram of PA polymer distribution with mass spectra of each polymer detected.

Supplemental Figure S6. Concentration in catechin equivalents of soluble monomeric, dimeric, and polymeric flavan-3-ols in wounded and nonwounded control as well as wounded spruce bark over a time course of 25 d.

Supplemental Figure S7. Hydrolysis products of PAs formed in spruce bark during wounding compared with nonwounded bark over a time course of 25 d.

Supplemental Figure S8. Characterization of LAR enzymes from Norway spruce using heterologous expression in *E. coli*.

Supplemental Figure S9. Correlation between catechin concentration and *LAR3* transcript accumulation in *LAR3*-overexpressing lines and the vector control line ($R^2 = 0.32$; $P = 0.0058$).

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

Supplemental Figure S11. Norway spruce line 5 overexpressing the *LAR3* gene accumulates more flavan-3-ol metabolites in needles, stem, and roots compared with the wild type.

Supplemental Figure S12. *PaCHS* gene expression after wounding (control) or fungal inoculation during a time course of 28 d to illustrate general increases in the transcription in the flavonoid pathway in fungus-infected spruce bark ($P < 0.0005$) compared with the wounded control treatments.

Supplemental Table S1. Primers used for cloning of *PaLAR* genes.

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

Supplemental Table S3. Primers used for quantitative real-time PCR.

Supplemental Table S4. Validation of reductive hydrolysis of PAs using known standards from grape with equal catechin and epicatechin content.

Supplemental Alignment S1. CLUSTAL format of alignment used for construction of phylogenetic tree (Fig. 3) for depicting the relationships of LAR and ANR amino acid sequences in gymnosperms and angiosperms.

Supplemental Alignment S2. CLUSTAL format of alignment depicting the relationships of LAR amino acid sequences from Norway spruce, white spruce, and Sitka spruce in relation to LAR from grape with known crystal structure and *D. uncinatum*, the first member of the protein family.

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