# Biosynthesis of the Major Tetrahydroxystilbenes in Spruce, Astringin and Isorhapontin, Proceeds via Resveratrol and Is Enhanced by Fungal Infection[1][W][OA]

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Stilbenes are dibenzyl polyphenolic compounds produced in several unrelated plant families that appear to protect against various biotic and abiotic stresses. Stilbene biosynthesis has been well described in economically important plants, such as grape (*Vitis vinifera*), peanut (*Arachis hypogaea*), and pine (*Pinus* species). However, very little is known about the biosynthesis and ecological role of stilbenes in spruce (*Picea*), an important gymnosperm tree genus in temperate and boreal forests. To investigate the biosynthesis of stilbenes in spruce, we identified two similar stilbene synthase (*STS*) genes in Norway spruce (*Picea abies*), *PaSTS1* and *PaSTS2*, which had orthologs with high sequence identity in sitka (*Picea sitchensis*) and white (*Picea glauca*) spruce. Despite the conservation of STS sequences in these three spruce species, they differed substantially from angiosperm STSs. Several types of in vitro and in vivo assays revealed that the *P. abies* STSs catalyze the condensation of *p*-coumaroyl-coenzyme A and three molecules of malonyl-coenzyme A to yield the trihydroxystilbene resveratrol but do not directly form the dominant spruce stilbenes, which are tetrahydroxylated. However, in transgenic Norway spruce overexpressing *PaSTS1*, significantly higher amounts of the tetrahydroxystilbene glycosides, astringin and isorhapontin, were produced. This result suggests that the first step of stilbene biosynthesis in spruce is the formation of resveratrol, which is further modified by hydroxylation, *O*-methylation, and *O*-glucosylation to yield astringin and isorhapontin. Inoculating spruce with fungal mycelium increased STS transcript abundance and tetrahydroxystilbene glycoside production. Extracts from STS-overexpressing lines significantly inhibited fungal growth in vitro compared with extracts from control lines, suggesting that spruce stilbenes have a role in antifungal defense.

Stilbenes are plant secondary metabolites consisting of two phenol moieties linked by a $C_2$ bridge (Fig. 1). These compounds are produced by species from a number of unrelated gymnosperm and angiosperm plant families, including the Pinaceae, Cyperaceae, Vitaceae, Polygonaceae, Betulaceae, Fabaceae, and Poaceae (Chong et al., 2009). Although the basic stilbene structure is widespread in plants, diverse species-specific substitution patterns exist. For example, pine (*Pinus*) species produce 3,5-dihydroxystilbene [pinosylvin; **(5)** in Fig. 1] as a constituent of heartwood and roots (Chiron et al., 2000). Species from the Vitaceae, Fabaceae, and Poaceae form mainly 3,5,4′-trihydroxystilbene [resveratrol **(6)**] (Sparvoli et al., 1994; Shomura et al., 2005; Sobolev et al., 2007). Spruce (*Picea*) species synthesize the more complex 3,5,3′,4′-tetrahydroxystilbene 3-glucopyranoside [astringin **(10)**] and 3,5,4′-trihydroxy-3′-methoxystilbene 3-glucopyranoside [isorhapontin **(11)**] (Toscano-Underwood and Pearce, 1991; Lieutier et al., 2003).

Although much has been learned about stilbene biosynthesis in recent years (Chong et al., 2009; Jeandet et al., 2010), the formation of tetrahydroxy-substituted stilbenes [such as **(7)**, **(8)**, **(10)**, and **(11)** in Fig. 1] is still unresolved. The stilbene skeleton is synthesized via condensation of three acetate units from malonyl-CoA to a CoA-activated phenolic acid by an enzyme known as stilbene synthase (STS), which results in the formation of a linear tetraketide intermediate. Ring closure to form the stilbene product is achieved via an intramolecular aldol condensation coupled to the loss of $CO_2$ (Austin et al., 2004). STSs belong to the large polyketide synthase (PKS) enzyme family, whose best known representative in plants is chalcone synthase, catalyst of the first step in flavonoid biosynthesis. For the

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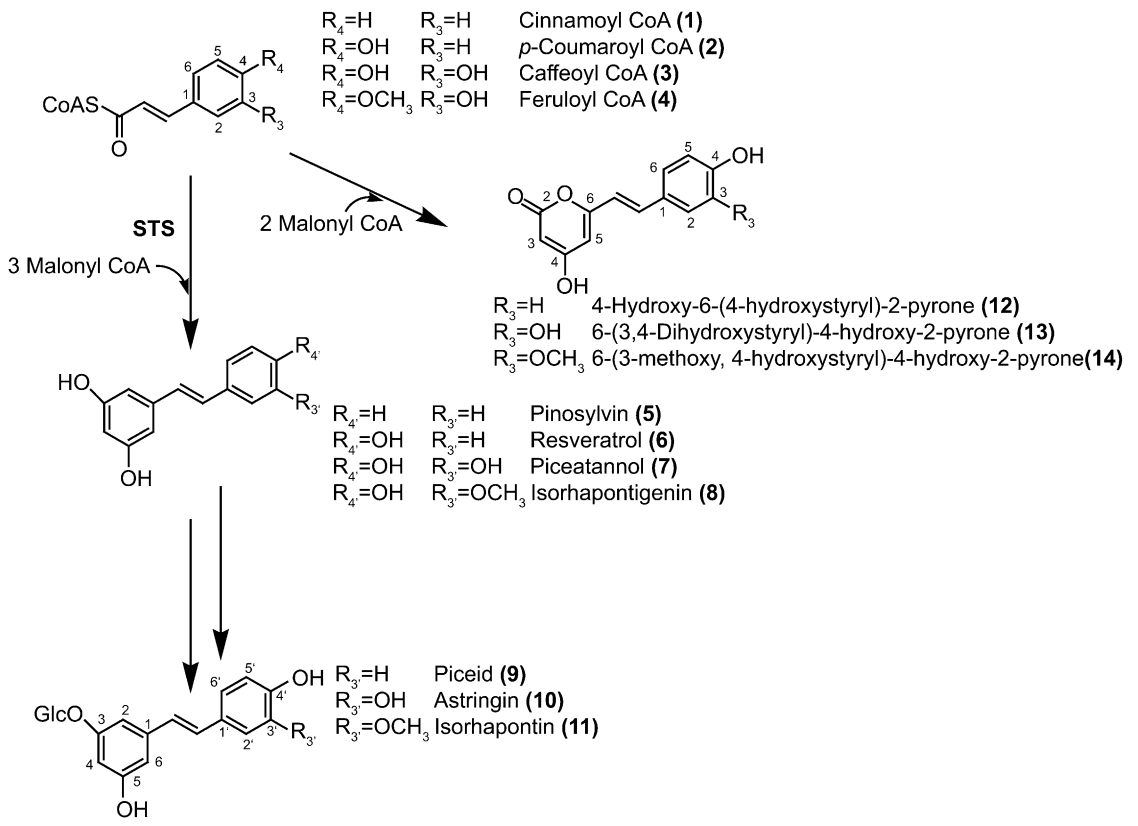


**Figure 1.** Potential intermediates, products, and by-products of stilbene biosynthesis in spruce. Compounds **(12)** to **(14)** are derailment products of STS catalysis.

synthesis of simple dihydroxystilbenes **(5)** and trihydroxystilbenes **(6)**, the CoA-activated phenolic acid that is incorporated into the tetraketide determines the substitution pattern of the stilbene formed (Austin and Noel, 2003). However, it is not known if complex tetrahydroxystilbenes, such as those produced by spruce, originate from the incorporation of more highly substituted CoA-activated phenolic acids into the initial tetraketide or from initial formation of the basic stilbene ring system and subsequent oxidation.

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

The objective of this study was to learn more about the pathway of tetrahydroxystilbene formation in spruce as well as their role in tree defense. We isolated and sequenced two genes encoding STS enzymes (STS1 and STS2) from *Picea abies*, *Picea glauca*, and *Picea sitchensis*. In vitro enzyme assays revealed that these STSs synthesize the trihydroxystilbene resveratrol **(6)**, which is not commonly observed in high concentrations in spruce tissue. However, by overexpressing *PaSTS1* in *P. abies*, we could demonstrate that resveratrol is an intermediate in tetrahydroxystilbene glycoside biosynthesis in spruce. By measuring transcript accumulation and stilbene content after

fungal inoculation of bark tissue, as well as testing fungal growth in vitro on stilbene-containing medium, we could also show that STS enzymes in spruce are involved in antifungal defense responses.

## RESULTS

### Identification of Spruce STS Genes and Their Phylogenetic Relationships

In order to study the stilbene biosynthetic pathway in spruce, *STS* gene candidates plus putative sequences for the very similar chalcone synthase (*CHS*) genes were identified using BLAST searches of spruce transcriptome resources with known sequences of the *PKS* family. Search of more than 180,000 ESTs from *P. sitchensis* and 250,000 from *P. glauca* in the Treenomix database (Ralph et al., 2008) revealed two distinct contigs from each spruce species with high similarity to *STS* from *Pinus densiflora* (Kodan et al., 2002; Supplemental Fig. S1). Additionally, more than eight distinct contigs were discovered for each spruce species that had high similarity to *CHS* from *Pinus sylvestris* (Fliegmann et al., 1992).

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

Comparisons of these STS and CHS sequences among spruce species demonstrated high conservation within the genus. Deduced amino acid sequences for STS1 and STS2 were 99% identical within species and 98% identical between species (Supplemental Fig. S1). Outside the genus, spruce STSs showed between 81% and 84% sequence identity to previously characterized pine STSs encoding pinosylvin synthases (Raiber et al., 1995; Kodan et al., 2002).

Amino acid sequences for CHS were 96% to 99% identical within and 95% to 100% identical between spruce species, with the exception of CHS7, which showed only 81% to 84% sequence identity to other CHSs within the genus. Outside the genus, spruce CHSs showed 95% to 97% sequence identity to biochemically characterized CHSs from *P. sylvestris* (Fliegmann et al., 1992).

Phylogenetic analysis revealed a clear evolutionary divergence between PKS from angiosperms and the Pinaceae (Fig. 2). In addition, the application of neighbor-joining algorithms resulted in separate clusters for CHS and STS in the Pinaceae ($P = 0.02$). Within the Pinaceae STS cluster, enzymes from spruce grouped with high bootstrap support into a separate subcluster from *Pinus* STS enzymes ($P < 0.001$) but gave no resolution of orthologs. Angiosperm PKS separated into clusters according to the taxonomic affinities of their species of origin.

### In Vitro Characterization of Recombinant Spruce STSs Expressed in Bacteria

Functional characterization of the two putative STSs from *P. abies* (PaSTS1 and PaSTS2) was accomplished via heterologous expression in a bacterial system. SDS-PAGE as well as western blotting revealed low-level expression of these 45-kD proteins, which form homodimeric enzymes (data not shown). Catalytic activity was determined by incubation with malonyl-CoA and a phenylpropanoid-CoA ester (Fig. 3). Reaction of the two PaSTSs with *p*-coumaroyl-CoA **(2)** yielded the stilbenes (*E*)- and (*Z*)-resveratrol **(6)**, but neither enzyme converted cinnamoyl-CoA **(1)**, caffeoyl-CoA **(3)**, and feruloyl-CoA **(4)** to stilbene products (Table I).

Instead, caffeoyl-CoA and feruloyl-CoA afforded (*E*)- and (*Z*)-6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone **(13)** and 6-(3 methoxy,4-hydroxystyryl)-4-hydroxy-2-pyrone **(14)**, respectively (Table I). Incubation with *p*-coumaroyl-CoA yielded the corresponding (*E*)- and (*Z*)-4-hydroxy-6-(4-hydroxystyryl)-2-pyrone **(12)**. These "derailment products" arise from two consecutive condensation reactions with malonyl-CoA and spontaneous cyclization after release from the active site (Austin and Noel, 2003). To form stilbenes, STS enzymes normally catalyze three consecutive condensations of the phenylpropanoid-CoA ester with malonyl-CoA followed by ring closure and $CO_2$ loss. The formation of derailment products in variable amounts prevented the measurement of relevant kinetic parameters.

### In Vivo Characterization of Spruce STSs by Coexpression with 4-Coumaroyl-CoA Ligase in *Escherichia coli*

Enzyme activities of PaSTS1 and PaSTS2 were assayed in vivo in *E. coli* engineered to produce potential substrates. This was achieved by the addition of phenolic acid starter units to the medium of *E. coli* coexpressing *PaSTS* along with 4-coumaroyl-CoA ligase from peanut (Watts et al., 2006), which produced CoA esters in the bacterium. Addition of *p*-coumaric acid to the growth medium of such cultures resulted in the production of resveratrol **(6)**, which was released back into the medium without the formation of detectable derailment products. However, addition of caffeic acid yielded the derailment products (*E*)- and (*Z*)-6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone **(13)** but no stilbenes (Table I). Ferulic acid could not be tested as a substrate in this system due to the substrate specificity of the 4-coumaroyl-CoA ligase.

### In Vivo Characterization of Spruce STS Function by Overexpression of *PaSTS1* in *P. abies*

To determine the enzymatic activity of spruce STSs in planta, embryogenic tissue from *P. abies* was transformed with *PaSTS1* under the control of the

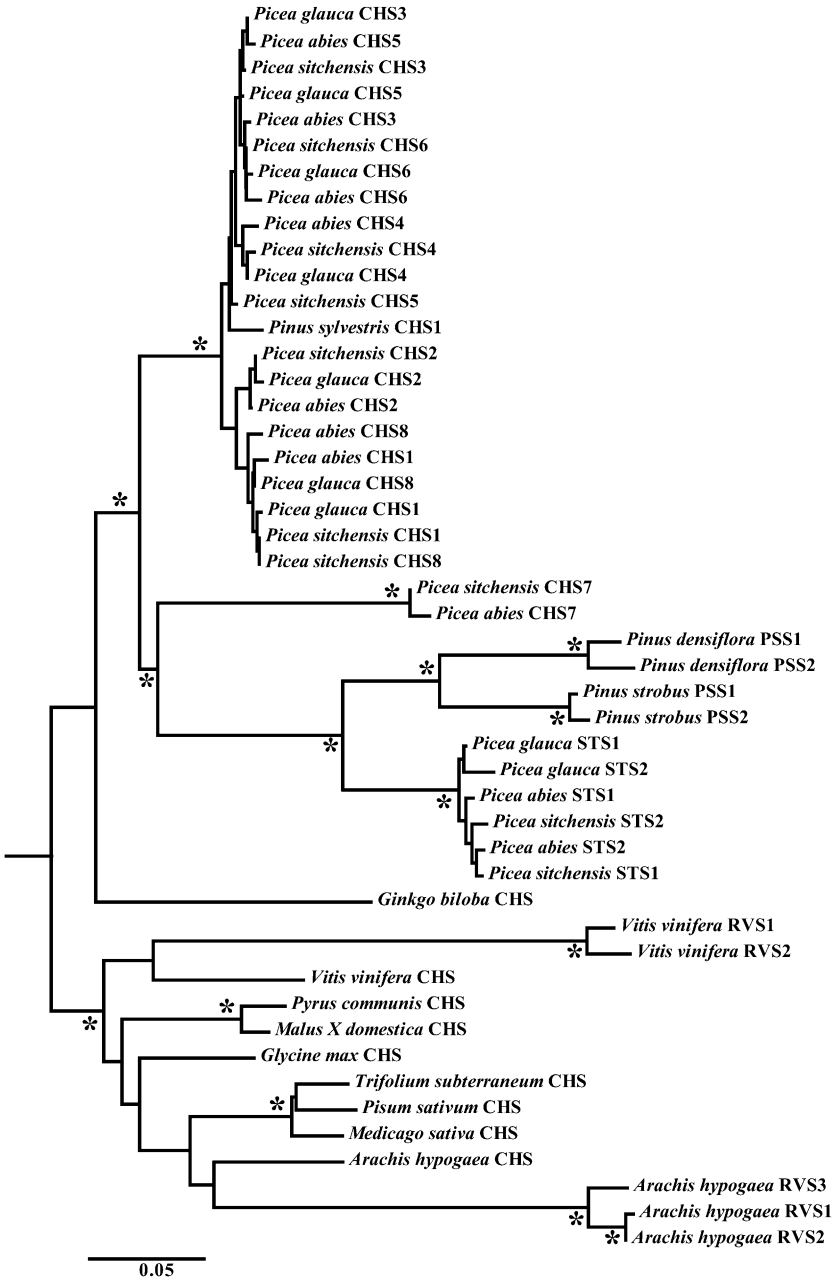


**Figure 2.** Phylogenetic relationships of STS amino acid sequences. A neighbor-joining tree of the PaSTS and representative STS and CHS sequences from angiosperm and gymnosperm species is shown. Branches supported with more than 95% confidence are indicated by asterisks. Some STSs whose products have been previously characterized are resveratrol synthase (RVS) and pinosylvin synthase (PSS). The use of a similar numerical nomenclature for the three different species does not imply that these genes are orthologs, as patterns of orthology could not be resolved in the absence of a genome-wide analysis.

inducible promoter *ubi1* using a disarmed *Agrobacterium tumefaciens* strain. Two transgenic *PaSTS1* callus lines (lines 5 and 11) and one empty vector control line were obtained, from which somatic embryos were produced by abscisic acid application under low-light conditions. Shoots and roots were regenerated under moderate light conditions in the absence of plant growth regulators. One-year-old transformed seedlings grown in potting soil were used for transcript and metabolite analysis.

Relative transcript accumulation of *PaSTS* (*PaSTS1* and *PaSTS2* were measured together by quantitative real-time PCR) was significantly higher in both *STS1* overexpression lines than in the vector control line ($P < 0.001$). *PaSTS* transcript abundance in bark tissue was on average 30-fold higher in line 5 and 6-fold higher in line 11 than in the vector control line (Fig. 4A). Relative *PaSTS* transcript abundance was also measured in needles, stems, and roots of line 5 and the vector control line. *PaSTS* transcript abundance was significantly higher in stems ($P < 0.01$) than in roots in both lines. Needles contained transcript levels intermediate between stems and roots (Fig. 5A).

Stilbene glycoside concentrations in both *PaSTS1* lines were significantly higher than in the vector control line ($P < 0.01$), providing further evidence that the *PaSTS1* gene encodes a functional STS (Fig. 4B). Concentrations of astringin **(10)** [3,5,3′,4′-tetrahy-

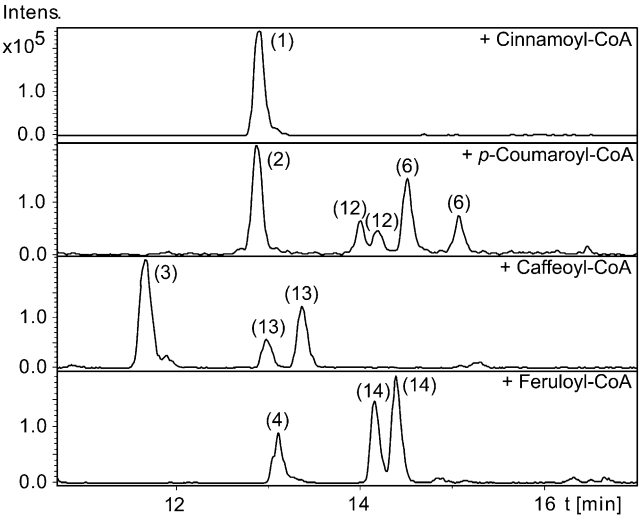


**Figure 3.** In vitro assay of *E. coli*-expressed *P. abies* STS. Representative LC-ESI-MS traces (extracted ion chromatograms) are given for reactions of PaSTS supplied with malonyl-CoA and a phenylpropanoid-CoA as substrates. With cinnamoyl-CoA **(1)**, no products were detected (top panel). The substrate *p*-coumaroyl-CoA **(2)** yielded the derailment products (*E*)- and (*Z*)-6-(4-hydroxystyryl)-4-hydroxy-2-pyrone **(12)** as well as (*E*)- and (*Z*)-resveratrol **(6)** (second panel). Caffeoyl-CoA **(3)** yielded (*E*)- and (*Z*)-6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone **(13)** (third panel). Feruloyl-CoA **(4)** yielded (*E*)- and (*Z*)-6-(3 methoxy,4-hydroxystyryl)-4-hydroxy-2-pyrone **(14)** (bottom panel). Compounds were identified by comparisons of mass spectra with those of standards.

droxystilbene 3′-glucopyranoside] and isorhapontin **(11)** [3,5,4′-trihydroxy-3′-methoxystilbene 3-glucopyranoside] in bark tissue were more than 9-fold higher in line 5 and more than 5-fold higher in line 11 than in the vector control. Stem and root tissue from *PaSTS1* line 5 and the vector control contained significantly higher concentrations of stilbene glycosides (Fig. 5B) than the needle tissue ($P < 0.0001$). Other stilbenes, including resveratrol **(6)**, piceid [resveratrol 3-glucopyranoside **(9)**], as well as the aglycones of astringin and isorhapontin [piceatannol **(7)** and isorhapontigenin **(8)**], could be detected in bark and root tissue of the *PaSTS1* lines and the vector control line using liquid chromatography-mass spectrometry (LC-MS) but could not be accurately quantified due to their low concentrations (Table I).

To detect STS activities in transgenic spruce bark, crude extracts were enriched through reactive-red affinity chromatography. As in the previously described in vitro assays with *E. coli* expressing PaSTS1 and PaSTS2, the partially purified STS fraction from *P. abies* overexpressing *PaSTS1* produced resveratrol **(6)** with the substrate *p*-coumaroyl-CoA **(2)** but only derailment products with the substrates caffeoyl-CoA **(3)** and feruloyl-CoA **(4)**.

### Effect of Transformation with *PaSTS1* on Other Pathways of Phenolic Metabolism in Spruce

To investigate whether *PaSTS1* overexpression influenced the biosynthesis of other *P. abies* phenolics, we measured the accumulation of flavan-3-ols, the other major group of phenolics in this species besides stilbenes, and quantified gene transcripts encoding early and late biosynthetic enzymes in flavan-3-ol formation. We measured the transcript abundance of a *P. abies* gene encoding phenylalanine ammonia lyase (PAL), the first step in the phenylpropanoid pathway (Noel et al., 2005), but transcript levels did not differ between *PaSTS1*-overexpressing lines and the vector control line (Fig. 6A). However, for leucoanthocyanidin reductase (LAR), which catalyzes the last step in flavan-3-ol biosynthesis (Tanner et al., 2003), relative transcript abundance of one of three putative *PaLAR* genes was significantly higher in the vector control line and in line 5 ($P < 0.0001$) than in line 11. No significant differences were observed for transcript levels of the other two *PaLAR* genes between the two transgenic *PaSTS1*-overexpressing lines and the vector control. Quantification of the products of LAR enzymes, catechin and proanthocyanidin dimers, revealed that concentrations of these metabolites were significantly higher in *PaSTS1* line 5 ($P < 0.001$) than in line 11 and the vector control (Fig. 6B).

### Effect of Fungal Inoculation on STS Transcription and Stilbene Glycoside Biosynthesis in *P. glauca* Saplings

To determine if fungal inoculation leads to the activation of stilbene biosynthesis in spruce, 2-year-old *P. glauca* (white spruce) saplings were wounded and inoculated with an avirulent strain of the bark beetle (*Ips typographus*)-associated fungus *Ceratocystis polonica*. Controls included unwounded saplings and those subjected to wounding without fungal inoculation. Given the ability of *C. polonica* to metabolize

**Table I.** *Products formed by PaSTS1 and PaSTS2 from various substrates under in vitro and in vivo conditions*

Structures of numbered compounds are given in Figure 1. Products considered to be artifacts are listed in italics. 4CL, 4-Coumaroyl-CoA ligase.

| Substrate | Products | | |
|---|---|---|---|
| | In Vitro Assay with Phenylpropanoid-CoA Esters (Protein Expressed in *E. coli*) | In Vivo Formation from Phenylpropanoid Acids (Protein Expressed in *E. coli* with 4CL) | In Vitro Assay with Phenylpropanoid-CoA Esters (Protein Expressed in *P. abies*) |
| 1 | None | None | None |
| 2 | 6, *12* | 6 | 6, *12* |
| 3 | *13* | *13* | *13* |
| 4 | *14* | Not tested | *14* |

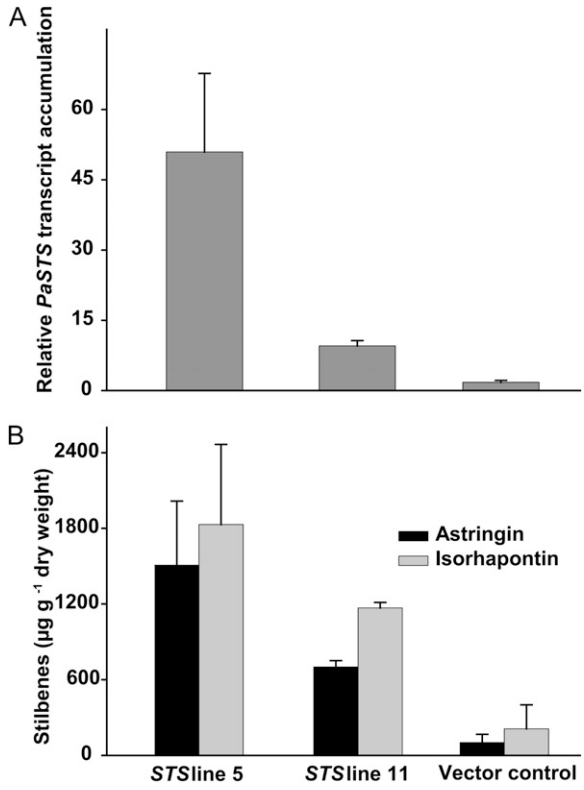


**Figure 4.** Transformation with STS increased stilbene biosynthesis. Relative transcript accumulation of *PaSTS1* and *PaSTS2* (A) and mean accumulation of the two major stilbenes, astringin and isorhapontin (B), in bark tissue of two lines of *P. abies* transformed with *PaSTS1* and a vector control line are shown. Depicted are means ± SE of at least four replicates.

the stilbenes of its host, *P. abies* (A. Hammerbacher, A. Schmidt, B. Schneider, J. Bohlmann, W.A. Brand, J. Gershenzon, and C. Paetz, unpublished data), the nonhost *P. glauca* was used in this experiment to test if fungal inoculation leads to elevated stilbene levels. *P. glauca* is not a natural host of *C. polonica* due to nonoverlapping geographic distribution.

Bark tissue was harvested for analysis 5, 15, or 25 d after treatment. While no lesions or typical "reaction zones" (Krokene et al., 2001) could be observed in the inner bark of the inoculated saplings 25 d after the onset of the experiment, the level of *STS* transcript in inoculated *P. glauca* bark increased significantly between days 5 and 15 ($P < 0.001$). Compared with the nonwounded control, transcript levels in inoculated saplings were on average 3-fold higher 5 d post inoculation and 8-fold higher 15 d post inoculation. At 25 d post inoculation, transcript levels were only 1.5-fold higher than in the nonwounded control. Saplings that were wounded without inoculation exhibited similar, although less pronounced, changes in STS transcript accumulation. Compared with the nonwounded control, relative transcript levels only reached a maximum 3-fold increase 15 d post wounding (Fig. 7B).

Fungal inoculation resulted in a significant increase in stilbene concentration in bark ($P < 0.001$) 15 to 25 d post inoculation. Compared with nonwounded controls, total stilbene levels were 3-fold higher in inoculated bark (Fig. 7A). In wounded but not fungus-inoculated saplings, only a small, statistically insignificant increase in total stilbene content could be detected. The increase in stilbenes was principally due to increases in the glycoside astringin, not in isorhapontin, which remained nearly constant over the time course of the experiment (data not shown).

### Effect of Stilbene-Containing Spruce Extracts and Pure Tetrahydroxystilbenes on Fungal Growth

In order to determine whether spruce stilbenes had biological activity against the fungus *C. polonica*, artificial medium was prepared containing extract obtained from either the STS-overexpressing line 5 or the empty vector control line. The fungus was plated on the respective medium and radial growth was measured. The growth rate (Fig. 8A) of the *C. polonica* isolate was significantly higher on the medium with extract from the vector control seedlings than on the medium containing extract from the transgenic STS line ($P < 0.001$).

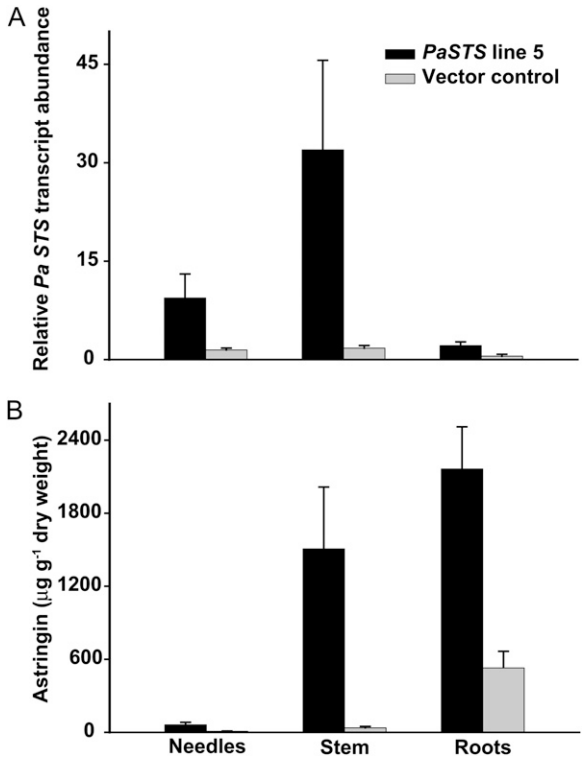


**Figure 5.** Organ-specific effects of STS transformation. Relative transcript accumulation of *PaSTS1* and *PaSTS2* (A) and accumulation of the stilbene astringin (B) in various organs of *PaSTS* transgenic line 5 relative to the vector control line are shown. Depicted are means ± SE of at least four replicates.

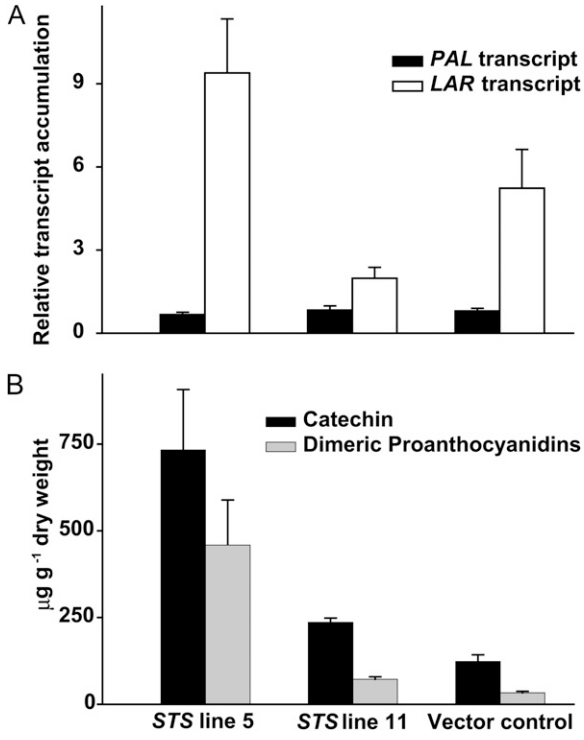


**Figure 6.** Effect of STS transformation on flavan-3-ol metabolism. Relative transcript accumulation of *P. abies* genes encoding PAL and LAR genes (A) and mean catechin and proanthocyanidin dimer accumulation (B) in a vector control line and two lines of *P. abies* transformed with *PaSTS1* are shown. Depicted are means ± SE of at least four replicates.

To determine if the growth effects of the extracts were actually due to differences in their stilbene content, an additional experiment was carried out where *C. polonica* was grown on artificial nutrient medium amended with stilbene concentrations similar to those found in the two spruce lines. The growth rate (Fig. 8B) of the fungus was significantly higher on the medium with 0.1 mg $mL^{-1}$ astringin (equivalent to the total stilbene content per gram fresh weight in the bark of the empty vector control line) than on the medium containing 1 mg $mL^{-1}$ astringin (equivalent to the concentration per gram fresh weight in bark of the transgenic STS line 5; $P < 0.001$).

## DISCUSSION

### Coding Regions of STSs in the Genus *Picea* Are Highly Conserved

To learn more about the biosynthesis of stilbenes in the genus *Picea*, we investigated the genes encoding STS, which form the basic stilbene skeleton by condensing three molecules of malonyl-CoA with one phenylpropanoid-CoA molecule. Two genes were identified, *STS1* and *STS2*, that had high sequence similarity within the three species studied, *P. abies*, *P. glauca*, and *P. sitchensis*.

Fossil records and phylogenetic analysis reveal that the genus *Picea* originated in western North America in the Paleocene era, about 62 million years ago (LePage, 2001), with *P. sitchensis* as the ancestral species (Ran et al., 2006). Spruce appears to have radiated from North America westward to Asia and Europe, with *P. abies* originating from a recent speciation event in the Pliocene era, about 5 million years ago (LePage, 2001; Ran et al., 2006). At the amino acid level, STS sequences from *P. glauca*, *P. sitchensis*, and *P. abies* were highly conserved, with 99% similarity within taxa and 98% sequence similarity between taxa (Supplemental Fig. S1; Supplemental Materials and Methods S1). The high similarity of STSs from the ancestral *P. sitchensis* to that of the more recently evolved *P. abies* and *P. glauca* indicates that genes for stilbene biosynthesis originated prior to the diversification of *Picea* and that these genes most likely fulfill the same function in all three spruce species studied.

The genes most closely related to *STS* in plants are the *CHS* genes and members of the *PKS* gene family. CHS enzymes employ the same substrates as STS but produce the $C_{15}$ flavonoid skeleton instead of the $C_{14}$ stilbene skeleton. Phylogenetic analysis of the known conifer STSs (from the genera *Picea* and *Pinus*) together

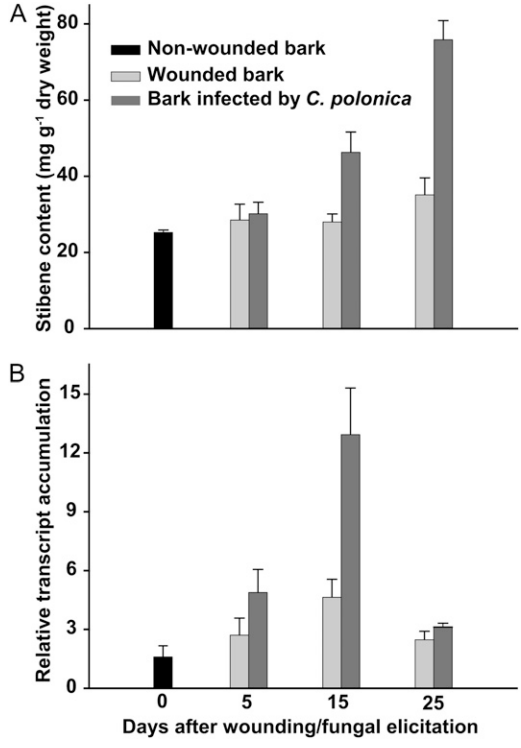


**Figure 7.** Fungal induction of stilbene biosynthesis. Total stilbene accumulation (A) and *PgSTS1* and *PgSTS2* transcript accumulation (B) in nonwounded, wounded, and *C. polonica*-inoculated bark tissue of *P. glauca* saplings are shown. Depicted are means ± SE of at least four replicates.

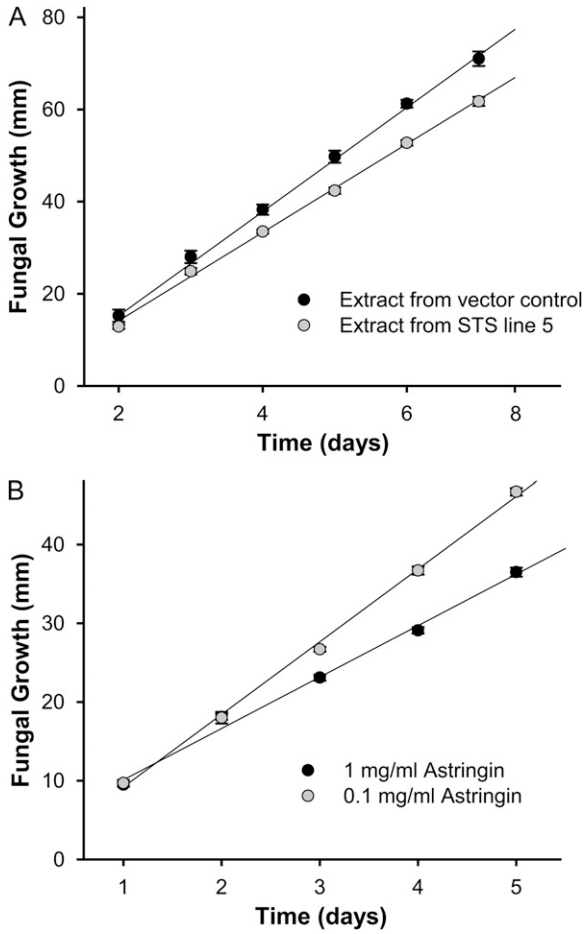


**Figure 8.** Growth of *C. polonica* on water agar amended with extract from *PaSTS*-overexpressing line 5 or the empty vector control line (A) and with 1 or 0.1 mg of astringin per mL of minimal medium (B). Depicted are means ± SE of at least four replicates.

with CHS revealed that enzymes from the Pinaceae form separate clusters from angiosperm enzymes with the same function. This separation implies independent origins of STS after divergence of the angiosperm and gymnosperm lineages. In accordance with the phylogeny of the plant PKS family as reconstructed by Tropf et al. (1994), our analysis shows that PKSs in general separate into clusters that are based less on catalytic function and more on the taxonomic affinities of the species from which the genes were isolated. Our results provide further evidence that STS likely evolved from CHS on multiple occasions (Tropf et al., 1995) within stilbene-producing lineages rather than originating from a single common ancestor.

The presence of multiple STS genes in individual species of the pine and spruce genera suggests several levels of control over stilbene formation. For example, in *P. sylvestris*, four *STS* genes that are almost identical have been described that are under the control of distinct promoters that activate transcription in response to different environmental cues and at different time intervals after a single stimulus (Preisig-Müller et al., 1999). This level of complexity is further enhanced by the existence of multiple copies of the same *STS* gene in the genome with multiple 3′ untranslated regions. For example, *P. densiflora* contains three conserved *STS* genes with 12 different 3′ untranslated regions (Kodan et al., 2002). Attempts to determine gene copy numbers in *P. abies* using quantitative real-time PCR suggested the presence of numerous copies of both *STS1* and *STS2* genes in the spruce genome (Supplemental Fig. S2). It is conceivable, therefore, that, similar to pine, spruce may also possess multiple *STS* genes that modulate stilbene biosynthesis differently in response to a range of internal and external factors.

### Stilbene Biosynthesis in Spruce Is a Defense Mechanism against Fungal Pathogens

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

It has been shown that the introduction of STSs into non-stilbene-producing species such as poplar (Seppänen et al., 2004), wheat (Serazetdinova et al., 2005), and alfalfa (Hipskind and Paiva, 2000) led to increased fungal resistance. However, it was unclear whether increased stilbene levels in spruce, which already produces a basal level of these compounds, would also lead to increased resistance against the bark beetle-associated fungus *C. polonica*. Since *C. polonica* rarely produces spores (Harrington and Wingfield, 1998), the common procedure for testing resistance to such a fungus would be direct inoculation of a wounded spruce stem with a mycelial plug (Christiansen and Solheim, 1990). However, the transgenic spruce seedlings used in this study were too young to create a sufficiently large wound on the stem for fungal inoculation. Therefore, the biological activity of stilbenes against *C. polonica* was studied on artificial medium.

Medium amended with extracts from a transgenic STS-overexpressing line inhibited fungal growth when

compared with medium amended with extracts from empty vector control seedlings. That this result was due to differences in stilbene content was confirmed by comparing medium amended with stilbene concentrations similar to those observed in bark of the STS-overexpressing line with medium amended with stilbene concentrations similar to those in the bark of the empty vector control seedlings. Thus, the tetrahydroxystilbenes of spruce are not only induced on fungal infection, but higher concentrations can help defend against fungal pathogens, just as they have been previously shown to defend various angiosperms and pine against pathogenic fungi (Jeandet et al., 2010).

### *P. abies* STSs Form a Trihydroxylated Product, Resveratrol, Which Is an Intermediate in the Biosynthesis of the Tetrahydroxy Stilbenes Astringin and Isorhapontin

The most abundant stilbenes in spruce are the 3,5,3′,4′-tetrahydroxystilbene glycosides astringin **(10)** and isorhapontin **(11)**. STSs condense phenylpropanoid-CoA esters with three molecules of malonyl-CoA to form the stilbene ring system (Austin and Noel, 2003). Several possible biosynthetic pathways can be envisioned that differ according to the substrate specificity of the STS reaction and the timing of oxidation (Fig. 9). The first possibility (I) involves STS-catalyzed formation of a 3,5-dihydroxystilbene, pinosylvin, from cinnamoyl-CoA. This 3,5-dihydroxystilbene product could then be further modified by 3′- and 4′-hydroxylation and glucosylation to give astringin **(10)**, while additional 3′-*O*-methylation is required to yield isorhapontion **(11)**. Another possibility (II) involves STS formation of a 3,5,4′-trihydroxystilbene, resveratrol **(6)**, from *p*-coumaroyl-CoA, which could be further modified by 3′-hydroxylation and glucosylation (and 3′-*O*-methylation) to give astringin (and isorhapontin). A third possibility (III) is STS formation of a 3,5,3′,4′-tetrahydroxystilbene, piceatannol **(7)**, from caffeoyl-CoA (Chong et al., 2009), which could be further transformed by glucosylation (and 3′-*O*-methylation). Feruloyl-CoA could also serve as a STS substrate to give a product that does not require 3′-*O*-methylation.

Of the phenylpropanoid-CoA substrates offered, the recombinant spruce STSs, PaSTS1 and PaSTS2, accepted only *p*-coumaroyl-CoA, producing the 3,5,4′-trihydroxy product resveratrol. Cinnamoyl-CoA, caffeoyl-CoA, and feruloyl-CoA were not converted to stilbene products. However, both caffeoyl-CoA and feruloyl-CoA, but not cinnamoyl-CoA, were converted to styrylpyrones. These compounds are considered to arise from the condensation of the phenylpropanoid-CoA ester with two malonyl-CoA units, followed by premature release from the active site and cyclization before the third condensation can occur. The formation of such derailment products indicates that the STS cannot convert the respective phenylpropanoid-CoA substrate to a stilbene, probably due to limitations in the size of the active site cavity (Jez et al., 2002). Styrylpyrones can also arise from STSs due to suboptimal in vitro conditions that lead to distortions in the active site cavity (Jez et al., 2002). In our work, styrylpyrone products were also formed in assays with *p*-coumaroyl-CoA along with stilbenes. However, the substrates caffeoyl-CoA and feruloyl-CoA gave only styrylpyrone products and no stilbenes; thus, they are unlikely to be native substrates of spruce STSs. Styrylpyrones have never been observed in planta. Moreover, the PaSTS enzymes in genetically engineered *E. coli* expressing a 4-coumaroyl-CoA ligase assayed in vivo and the PaSTS enzymes in the bark of *P. abies* overexpressing PaSTS1 assayed in vitro both converted *p*-coumaroyl-CoA only to the stilbene resveratrol without any styrylpyrone formation, while caffeoyl-CoA and feruloyl-CoA were only converted to styrylpyrones.

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

Although *P. abies* STS1 and STS2 produced the trihydroxystilbene aglycone resveratrol in vitro and in vivo, the major stilbenes accumulated in most spruce accessions are the tetrahydroxystilbene glycosides astringin and isorhapontin (Toscano-Underwood and Pearce, 1991; Lieutier et al., 2003). To determine if resveratrol is the precursor of the major stilbenes, *P. abies* was genetically engineered to overexpress PaSTS1. Overexpression lines had higher *PaSTS* transcript levels and produced significantly more astringin and isorhapontin than a control line transformed with an empty vector. Resveratrol levels, on the other hand, remained consistently low in both overexpression and vector control lines. Thus, PaSTS1 and PaSTS2 are involved in the biosynthesis of the major *P. abies* stilbenes, astringin and isorhapontin. Our in vitro characterization of PaSTS indicates that the first step of the pathway is the formation of resveratrol. This intermediate is then modified by 3′-hydroxylation, 3′-*O*-methylation, and 3-*O*-glycosylation to yield the major stilbene glycosides accumulated in spruce.

In rhubarb (*Rheum rhaponticum*), a similar pathway for the production of the major stilbene, 3,5,3′-trihydroxy-3′-methoxystilbene-3-*O*-glucoside, from resveratrol was proposed after in vivo substrate-feeding experiments (Rupprich et al., 1980). However, in this species, resveratrol and another likely intermediate, resveratrol-3-*O*-glucoside, appear in high concentrations in the rhizome (Püssa et al., 2009). In contrast, in both wild-type *P. abies* as well as transgenic lines overexpressing the PaSTS1 resveratrol synthase, only minute amounts of resveratrol and its 3-*O*-glucoside were detected. This could indicate that metabolite channeling (Winkel-Shirley, 1999) of resveratrol to the next pathway enzyme, a stilbene 3′-hydroxylase, occurs in *P. abies*, as has been shown for flavonoid biosynthesis in Arabidopsis (*Arabidopsis thaliana*; Burbulis and Winkel-Shirley, 1999).

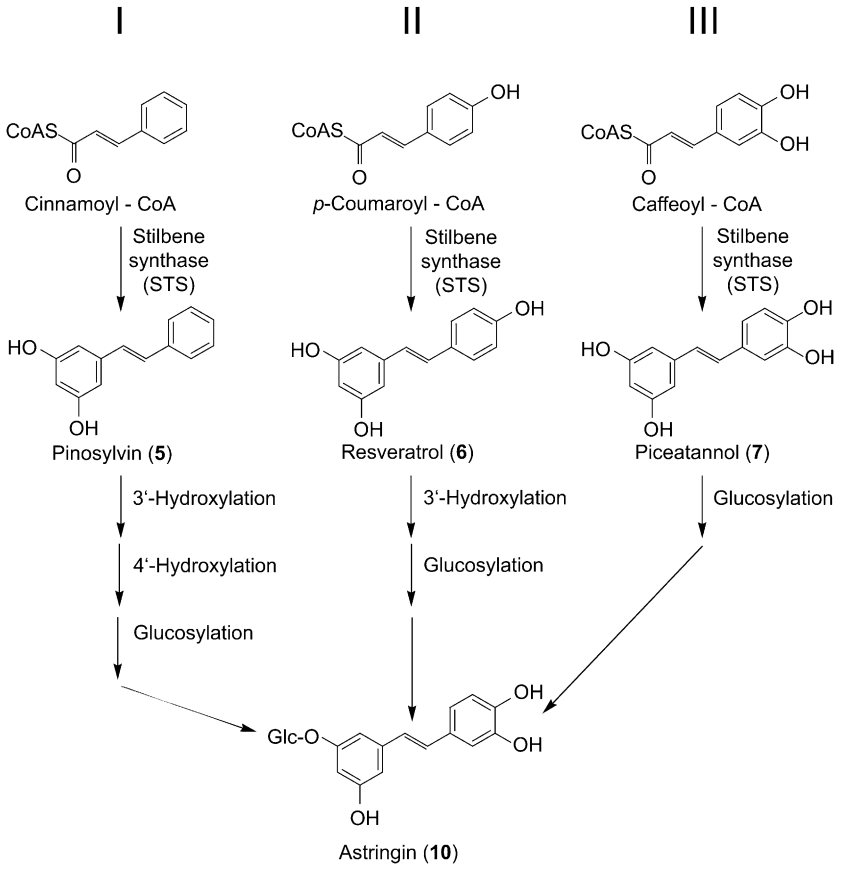


**Figure 9.** Biosynthetic pathway for astringin formation in *P. abies* depends on the substrate specificity of STS. Both STS1 and STS2 were found to utilize only *p*-coumaroyl-CoA as substrate, indicating that the most likely pathway is II.

STS enzymes evolved from CHS by gene duplication and neofunctionalization. Therefore, it is not surprising that both enzymes share the same substrate, *p*-coumaroyl-CoA. Thus, in both stilbene and flavonoid biosynthesis, further oxidation, *O*-methylation, or other modifications at positions on the aromatic ring derived from the phenylpropanoid-CoA substrate must occur in later steps of the pathway.

### Genetic Transformation of Spruce with PaSTS Provided New Insights into Phenylpropanoid Biosynthesis in the Pinaceae

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

Overexpression of STS also provided hints on how the metabolic pathway is regulated. In the bark of fungus-inoculated wild-type *P. glauca* saplings, increases in *STS* transcript levels correlated well with the subsequent stilbene accumulation, suggesting regulation by the level of steady-state transcript. However, in the *PaSTS*-overexpressing transgenic lines, *STS* transcripts were much higher in stems than in needles and higher in needles than in roots. These patterns did not correlate well with stilbene accumulation, which was highest in roots, intermediate in stems, and lowest in needles. Such discrepancies may be due to differences in substrate availability or post-transcriptional regulation among organs or to stilbene transport among organs.

## CONCLUSION

This study demonstrated that spruce STS enzymes, although only making resveratrol as their intermediate

product, still contribute to the formation of the major tetrahydroxylated stilbenes in the tree. It could also be shown that fungal infection induced the tree to produce higher levels of *STS* transcript and tetrahydroxylated stilbene glycosides and that these compounds have antifungal activity. However, additional research is needed to understand what factors limit stilbene accumulation in both healthy and fungally infected spruce, as stilbene formation may be adjusted to different levels depending on the species of pathogen, the degree of infection, and the presence of other biotic and abiotic stresses. In addition, further investigation is required to determine whether any of the other roles proposed for stilbenes in plants (e.g. antiherbivore protection, allelopathy, and resistance to oxidative stress) can be supported. The ability to manipulate stilbene concentrations independently of other factors, as in the transgenic spruce lines described here, will be valuable in pursuing such work.

## MATERIALS AND METHODS

### Identification of Putative *STS* and *CHS* Genes from Spruce

Protein sequences from various Pinaceae *STSs* (*Pinus strobus* [Raiber et al., 1995] and *Pinus densiflora* [Kodan et al., 2001]) and *CHS* (*Pinus sylvestris* [Fliegmann et al., 1992] and *Ginkgo biloba* [Pang et al., 2004]) were used to query *Picea sitchensis* and *Picea glauca* EST collections (Pavy et al., 2005; Ralph et al., 2008) by tBLASTn for candidate cDNA sequences. Open reading frames from candidate sequences were detected using the software package DNA Star version 8.02 (DNASTAR), and the absence of predicted signal peptides at the N terminus was confirmed by SignalP software (http://www.cbs.dtu.dk/services).

### Cloning and Sequencing *PaSTS* and *PaCHS*

For RNA extraction, fresh bark tissue from 4-year-old *Picea abies* saplings that were grown in an outdoor plot (clone 3369-Schongau; Samenklenge und Pflanzgarten, Laufen, Germany) was ground to a fine powder. Total RNA was extracted using the method developed by Kolosova et al. (2004). One microgram of total RNA was converted to cDNA in a 20-$\mu$L reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol of poly(T)$_{12-18}$ primer (Invitrogen). Gateway-compatible primers were designed for candidate sequences by using the N- and C-terminal sequences of putative *P. sitchensis* and *P. glauca STS* and *CHS* genes as templates (attB primer sequences are provided in Supplemental Table S1).

Pseudomature forms of *PaSTS* and *PaCHS* cDNA were PCR amplified with attB 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 protocols.

pDONR207 constructs containing *PaSTS* and *PaCHS* genes were sequenced using 10 pmol of the vector-specific primers pDONR F (5′-TCGCGTTAACGCTAGCATGGATCTC-3′) and pDONR R (5′-GTAACATCAGAGATTTTGAGACAC-3′) and the BigDye Terminator version 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 CHS and STS

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

Phylogenetic analyses were conducted using MEGA version 4 (Center for Evolutionary Medicine and Informatics; Tamura et al., 2007) employing the neighbor-joining method. Evolutionary distances were calculated with the Poisson correction. The tree was searched using the close-neighbor interchange algorithm with pairwise elimination of alignment gaps. The statistical likelihood of tree branches was tested with 10,000 bootstrap replicates.

### Heterologous Expression of *PaSTS* Genes in *Escherichia coli*

Putative *PaSTS1* and *PaSTS2* pDONR207 constructs were cloned with LR Clonase II (Invitrogen) according to the manufacturer's instructions into the Gateway-compatible expression vector pH9GW (Yu and Liu, 2006), coding for an N-terminal His tag. Arctic Express (DE3) chemically competent *E. coli* (Stratagene) was transformed with expression constructs. Single colonies were inoculated into 5 mL of Luria-Bertani broth with 1 $\mu$g mL$^{-1}$ kanamycin and grown for 16 h at 24°C. The 5-mL starter cultures were used to inoculate 100 mL of Overnight Express Instant TB Medium (Novagen) supplemented with 1% (v/v) glycerol and 1 $\mu$g mL$^{-1}$ kanamycin.

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

Expressed proteins were purified with a 1-mL His Trap FF column (GE Healthcare) on an AEKTA 900 chromatography system (GE Healthcare). The column was washed with 50 mM Bis-Tris (pH 7.2) and 10% (v/v) glycerol and eluted with wash buffer amended with 220 mM imidazole. The eluted proteins were desalted into an assay buffer (50 mM Bis-Tris, pH 7.2, 10% [v/v] glycerol, and 1 mM dithiothreitol) on DG-10 desalting columns (Bio-Rad). The protein concentration was determined using the Bradford reagent (Bio-Rad).

### Functional Characterization of PaSTS

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

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

Enzyme extracts from transgenic *P. abies*-overexpressing *PaSTS1* were prepared by extracting 100 mg of bark tissue using the method of Martin et al. (2002). After pelleting plant debris, the extract was passed over a 1-mL reactive red agarose (Sigma) affinity chromatography column. The column was washed with 5 column volumes of assay buffer and eluted with 2 column volumes of assay buffer amended with 500 mM NaCl. STS activity was determined as above.

### Functional Characterization of PaSTS by Coexpression with 4-Coumaroyl-CoA Ligase in *E. coli*

Arctic Express (DE3) chemically competent *E. coli* (Stratagene) was cotransformed with either *PaSTS1* or *PaSTS2* expression constructs and pAC-4CL1 (Watts et al., 2006), coding for a 4-coumaroyl-CoA ligase from peanut (*Arachis hypogaea*). Overnight Express Instant TB Medium (Novagen) supplemented with 1% (v/v) glycerol, 1.5 $\mu$g mL$^{-1}$ chloramphenicol, and 1 $\mu$g mL$^{-1}$ kanamycin (40 mL) was inoculated with transformed *E. coli* colonies.

Bacterial cultures were grown for 8 h at 22°C followed by 4 d at 12°C before adding *p*-coumaric or caffeic acid in dimethyl sulfoxide to the culture medium as substrate to a final concentration of 1 mM. Culture medium (1 mL) was harvested 24, 48, and 72 h after the addition of phenolic acids. After centrifuging the culture medium, the supernatant was acidified with 0.1 N HCl and extracted with 1 volume of ethyl acetate. After evaporation, the extract was redissolved in 50 $\mu$L of methanol for LC-ESI-MS analysis.

## Genetic Transformation of *P. abies* Callus with *PaSTS1*

The *PaSTS1* pDONR207 was cloned with LR Clonase II (Invitrogen) into the Gateway-compatible binary vector pCAMGW. pCAMGW *STS1* or pCAMBIA 2301 (as vector control) was transformed into the chemically competent disarmed *Agrobacterium tumefaciens* strain C58/pMP90 (Schmidt et al., 2010). An embryonic *P. abies* cell culture (line 186/3c VIII) was transformed as described by Schmidt et al. (2010). From the six kanamycin-resistant transgenic lines obtained, line 5 and line 11 were selected for regeneration of seedlings and further experiments.

## Somatic Embryogenesis and Plant Regeneration

Transgenic embryonic tissue was maintained at 24°C in the dark and subcultured every 14 d. For plant regeneration, transgenic embryonic tissue was placed on semisolid EMM1 medium (Walter et al., 1999) amended with 6 g $L^{-1}$ Gelrite (Duchefa), 30 g $L^{-1}$ Suc, and 15 mg $L^{-1}$ abscisic acid for 2 weeks. The culture was then placed on EMM2 medium (4.5 g $L^{-1}$ Gelrite; Walter et al., 1999) for 5 weeks. Embryos (3–5 mm in size) were placed on a sterile nylon mesh in three wells of a six-well cell cluster. The other three wells were filled with sterile water to maintain humidity. Embryos were stored at 4°C for 7 d in the dark. For germination, developed embryos were transferred to modified Litvay medium (Klimaszewska et al., 2005) with 40 g $L^{-1}$ Suc and 6 g $L^{-1}$ Gelrite. The cultures were placed for 6 weeks under low-light conditions (5 $\mu$mol $m^{-2}$ $s^{-1}$) for 16 h per day at 24°C. Somatic embryos were collected and placed horizontally on modified Litvay medium with 6 g $L^{-1}$ Gelrite under moderate-light conditions (45 $\mu$mol $m^{-2}$ $s^{-1}$). After 3 weeks, plants were transferred to Magenta GA-7 (Sigma) plant culture boxes and placed under higher light conditions (100 $\mu$mol $m^{-2}$ $s^{-1}$) to develop roots, epicotyls, and needles. Well-developed plantlets were planted in soil substrate (3:1:1 fibric peat:vermiculite [2–3 mm grain size]:perlite) in small plastic containers (4 × 4 cm).

Procedures from Klimaszewska et al. (2001) were used to generate the untransformed *P. glauca* line Pg653.

## Quantitative Real-Time PCR

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

Reverse transcription of 1 $\mu$g of RNA into cDNA was accomplished using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol of poly$(T)_{12-18}$ primer (Invitrogen). After cDNA was diluted to 10% (v/v) with deionized water, 1 $\mu$L of diluted cDNA was used as template for quantitative real-time PCR. PCR was performed with Brilliant SYBR Green QPCR Master Mix (Stratagene) with 10 pmol of the forward primer 5′-GTGGCGAGCAGAACACAGACTTC-3′ and 10 pmol of the reverse primer 5′-CAGCGATGGTACCTCCATGAACG-3′, designed to amplify 140 bp of both *STS1* and *STS2* simultaneously. Primer sequences for *PaLAR1*, *PaLAR2*, *PaLAR3*, and *PaPAL* are given in Supplemental Table S3. PCR was performed using a Stratagene MX3000P thermocycler: 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by a melting curve analysis from 55°C to 95°C. Reaction controls included nontemplate controls and non-reverse-transcribed RNA. Transcript abundance was normalized to the transcript abundance of the ubiquitin (Schmidt et al., 2010; GB:EF681766.1) gene (Supplemental Table S3) and was calculated from three technical replicates each of at least four biological replicates. Relative transcript abundance for the transgenic *PaSTS1* lines was calibrated against the transcript abundance of one biological replicate of the vector control. Relative transcript abundance of *PgSTS* in *P. glauca* was calibrated against a nonwounded control sample.

## Extraction of Phenolic Compounds from Spruce

Spruce tissue was ground to a fine powder in liquid nitrogen and lyophilized using an Alpha 1-4 LDplus freeze dryer (Martin Christ). Approximately 5 mg of dried tissue was extracted with 2 mL of methanol for 4 h at 4°C and then centrifuged at 2,000*g*, and the supernatant was recovered. Insoluble material was reextracted with 1 mL of methanol for 12 h. The supernatants were combined and evaporated to dryness under a stream of nitrogen and redissolved in 1 mL of methanol containing 50 $\mu$g $mL^{-1}$ chlorogenic acid (Sigma) as an internal standard. For LC-ESI-MS, samples were diluted to 20% (v/v) with methanol.

## LC-ESI-MS

Compounds were separated on a Nucleodur Sphinx RP18ec column (250 × 4.6 mm; particle size of 5 $\mu$m; Macherey-Nagel) using an Agilent 1100 series HPLC system (Agilent Technologies) with a flow rate of 1.0 mL $min^{-1}$ at 25°C. Compound detection and quantification were accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Brucker Daltronics) after diverting column flow-through in a ratio of 4:1. The mass spectrometer was operated as follows: skimmer voltage, 60 V; capillary voltage, 4,200 V; nebulizer pressure, 35 pounds per square inch; drying gas, 11 L $min^{-1}$; gas temperature, 330°C.

Phenolic compounds from spruce and enzyme assay products were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0 to 1 min, 100% A; 1 to 15 min, 0% to 65% B; 15 to 18 min, 100% B; 18 to 22 min, 100% A. The ESI-MS apparatus was operated in negative mode scanning mass-to-charge ratio (*m/z*) between 50 and 1,600 with an optimal target mass of 405 *m/z*. Capillary exit potential was kept at −121 V.

Aromatic CoA esters to be used as enzyme substrates were separated using 20 mM ammonium acetate (pH 4) and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0 to 20 min, 5% to 45% B; 20 to 21 min, 45% to 90% B; 21 to 23 min, 90% B; 23 to 28 min, 5% B. The ESI-MS apparatus was operated in alternating mode scanning between *m/z* 50 and 1,600 with an optimal target mass of 250 *m/z*. Capillary exit potential was kept at −109.8 V.

## Compound Identification and Quantification

Several substances were identified by direct comparison with authentic standards, including resveratrol (purchased from Merck) and piceatannol (from Alexis Biosciences). An astringin **(10)** standard was extracted and purified from spruce bark following a previous protocol (Li et al., 2008), and the identity and purity of this compound were verified by NMR spectroscopy (Supplemental Fig. S3). Standards were unavailable for the other compounds detected, (*E*)- and (*Z*)-isorhapontin **(11)**, (*E*)- and (*Z*)-6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone **(13),** 6-(3 methoxy,4-hydroxystyryl)-4-hydroxy-2-pyrone **(14)**, as well as (*E*)- and (*Z*)-4-hydroxy-6-(4-hydroxystyryl)-2-pyrone **(12),** and these were identified by mass spectra obtained by negative ionization comparing second- and third-order losses with those displayed by the available standards or previously described in the literature. For example, isorhapontin **(11)** [retention times in Supplemental Table S4] was characterized by a neutral loss of 162, just as seen for astringin **(10),** yielding a fragment with the same *m/z* value as the respective deprotonated aglucone (Supplemental Table S4; Supplemental Figs. S4 and S5). Fragmentation of resveratrol **(6)**, piceatannol **(7)**, as well as isorhapontin **(11)** treated with $\beta$-glucosidase prior to analysis was characterized by the appearance of two dominant product ions that represented the sequential neutral loss of two 42-D molecules (Supplemental Table S4; Supplemental Fig. S4). Similar fragmentation spectra have been previously described for resveratrol and piceatannol (Buiarelli et al., 2007; Lo et al., 2007), and the two 42-D molecules lost during fragmentation of these compounds have been identified as ketene molecules involving positions 3, 4, 5, and 6 on the stilbene skeleton (Supplemental Fig. S5). Fragmentation of the styrylpyrones **[(12)**, **(13)**, and **(14)]**, first described by Yamaguchi et al. (1999) as derailment products in PKS enzyme assays, were characterized by the neutral loss of two fragments of 44 and 42 D. Fragments arising from styrylpyrones have the same *m/z* as those observed during the fragmentation of stilbenes (Supplemental Fig. S4) and therefore represent the sequential loss of a carbon dioxide and a ketene molecule involving positions 2, 3, and 4 on the pyrone ring (Supplemental Fig. S5).

For quantification, compounds were detected in full-scan mode using negative ionization. Ions were extracted from total ion chromatograms using Brucker Daltronics Quant Analysis version 3.4 software employing 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. External calibration curves for catechin (Sigma) and astringin were created by linear regression. Flavan-3-ol concentrations were determined relative to the catechin calibration curve and stilbene glycosides relative to astringin. All results were normalized relative to the internal standard.

## Treatment of *P. glauca* Saplings with *Ceratocystis polonica*

A *C. polonica* isolate (CMW 7749) provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (University of Pretoria) was grown on 3% (w/v) malt extract agar (Carl Roth) for 12 d at 25°C.

*P. glauca* saplings originating from embryonic tissue (Pg653) were grown in a walk-in growth chamber for 2 years in 2-L pots under a light/temperature regime alternating between 4 months of exposure to 16 h of light at 25°C and 4 months of exposure to 8 h of light at 16°C. Inoculations with *C. polonica* were performed after saplings were acclimatized for 6 weeks to the higher light/temperature regime and had completed their "spring" flush.

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

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

## Determination of in Vitro Fungal Growth on Stilbene-Containing Medium

To obtain sufficient stilbene-containing extract for fungal bioassays, extractions were performed on the organs containing the highest stilbene content, the roots. Root tissue from the STS-overexpressing line 5 and the empty vector control was extracted with water (25 mL $g^{-1}$ tissue) at 4°C overnight and centrifuged. The supernatant was retained, and the insoluble debris was reextracted for 5 h with water (10 mL $g^{-1}$ tissue). Extracts were combined and sterilized by filtration through a membrane (pore size of 0.2 $\mu$m). Solid fungal growth medium was prepared by steam sterilizing 5% (w/v) agar in water (Carl Roth) and adding root extracts at a ratio of 1:1 to the hot medium. Medium (15 mL) was dispensed in petri dishes (8.4 cm in diameter).

For assays testing fungal growth on astringin, growth medium was prepared by steam sterilizing water agar (4%, w/v) amended with carrot (*Daucus carota*) juice (2% volume$^{-1}$). Solutions of 4 and 0.4 mg $mL^{-1}$ astringin in water were prepared and sterilized by filtration through a membrane (pore size of 0.2 $\mu$m). Astringin solutions and the agar mixture were mixed at a ratio of 1:2, and 8 mL was dispensed in petri dishes (5.2 cm in diameter).

After the medium solidified, an agar plug (4 mm in diameter) from 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.

## Statistical Analysis

Graphical representations of results are presented as means ± SE.

Statistical significance of differences in *STS*-transformed lines was determined using a one-way ANOVA on log-transformed data. Significance of differences in the *C. polonica* inoculation trial was determined using a two-way ANOVA. Differences in means were calculated using Tukey's posthoc pairwise comparisons test at a 95% confidence level. Differences in fungal in vitro growth were calculated using linear models. Analyses were conducted using the open source software R version 2.81 (www.r-project.org) and the LAERCIO package for pairwise comparisons.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JN 400047 to JN 400078.

## Supplemental Data

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

**Supplemental Figure S1.** Amino acid sequence alignment of STS from *P. abies*, *P. sitchensis*, *P. glauca*, and *P. densiflora*.

**Supplemental Figure S2.** Relative *PaSTS* gene copy number in transgenic *PaSTS1* lines and vector control.

**Supplemental Figure S3.** The NMR spectrum measured for astringin isolated from *P. abies*.

**Supplemental Figure S4.** Representative mass fragmentation spectra of tetrahydroxylated stilbene compounds reported in this study.

**Supplemental Figure S5.** Hypothetical mass fragmentation reactions of stilbenes leading to the fragmentation spectra used in identification.

**Supplemental Table S1.** Forward and reverse attB primers for amplifying and cloning *P. abies STS* and *CHS* into pDONR207.

**Supplemental Table S2.** National Center for Biotechnology Information accession numbers of CHS and STS sequences used for phylogenetic analysis.

**Supplemental Table S3.** Forward and reverse primers for quantitative real-time PCR of *P. abies*.

**Supplemental Table S4.** Analytical data for stilbenes and derailment products.

**Supplemental Materials and Methods S1.** Quantitative genomic PCR for copy number determination.

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