Overexpression of an Isoprenyl Diphosphate Synthase in Spruce Leads to Unexpected Terpene Diversion Products That Function in Plant Defense

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Spruce (Picea spp.) and other conifers employ terpenoid-based oleoresin as part of their defense against herbivores and pathogens. The short-chain isoprenyl diphosphate synthases (IDS) are situated at critical branch points in terpene biosynthesis, producing the precursors of the different terpenoid classes. To determine the role of IDS and to create altered terpene phenotypes for assessing the defensive role of terpenoids, we overexpressed a bifunctional spruce IDS, a geranyl diphosphate and geranylgeranyl diphosphate synthase in white spruce (Picea glauca) saplings. While transcript level (350-fold), enzyme activity level (7-fold), and in planta geranyl diphosphate and geranylgeranyl diphosphate levels (4- to 8-fold) were significantly increased in the needles of transgenic plants, there was no increase in the major monoterpene and diterpene acids of the resin and no change in primary isoprenoids, such as sterols, chlorophylls, and carotenoids. Instead, large amounts of geranylgeranyl fatty acid esters, known from various gymnosperm and angiosperm plant species, accumulated in needles and were shown to act defensively in reducing the performance of larvae of the nun moth (Lymantria monacha), a conifer pest in Eurasia. These results show the impact of overexpression of an IDS and the defensive role of an unexpected accumulation product of terpenoid biosynthesis with the potential for a broader function in plant protection.

Terpenes are a structurally very diverse class of metabolites (Köksal et al., 2011) that have major roles in primary and secondary metabolism (Buchanan et al., 2000; Gershenzon and Dudareva, 2007). The basic pathways of terpene biosynthesis have been well studied. Two separate pathways, the mevalonate (MVA) pathway in the cytosol and peroxisomes and the methylerythritol phosphate (MEP) pathway in plastids, produce the universal C5 precursors for terpene biosynthesis, dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP). Next, sequential condensations of DMADP with one to three molecules of IDP yield the elongated C10, C15, and C20 intermediates, geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP), respectively (Arigoni et al., 1997; Rodríguez-Concepción, 2006; Hammerlin et al., 2012).

The enzymes catalyzing these condensation reactions are designated short-chain isoprenyl diphosphate synthases (IDS) and belong to the large family of prenyltransferases (Wang and Ohnuma, 2000; Liang et al., 2002; Fig. 1). The enzymes are named after their main products as GDP synthase, FDP synthase, and GGDP synthase and usually belong to the group of trans-IDS enzymes based on the stereochemistry of the double bond of the reaction product (Kharel and Koyama, 2003; Liang, 2009), although short chain cis-IDS enzymes are known (Sallaud et al., 2009; Schlimminger et al., 2009; Akhtar et al., 2013) The short-chain prenyl diphosphates produced are the substrates for terpene synthases (Chen et al., 2011) that form the huge variety of terpene skeletons, which in turn may be further modified, such as by cytochrome P450 oxidoreductases (Bohlmann and Keeling, 2008) or other oxidative, reductive, and conjugation processes. Thus, the IDS enzymes function at an important branch point in terpene biosynthesis where the pathway splits into routes to the major classes, such as monoterpene (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). However, little is known about how IDS control flux into the different branches. A few recent publications have addressed the importance of FDP synthases (Chen et al., 2000; Masferrer et al., 2002; Manzano et al., 2004, 2006; Han et al., 2006; Banyai et al., 2010; Closa et al., 2010; Keim et al., 2012), but only one focuses on a GDP synthase (Lange et al., 2011) and one on a GGDP synthase (Kai et al., 2011).

Among the best known terpenes functioning in antiherbivore defense are the oleoresin components of...
conifers such as spruce (*Picea* spp.) that are stored in specialized resin ducts and deter insects such as bark beetles and their associated fungi (Erbilgin et al., 2006; Keeling and Bohlmann, 2006a, 2006b; Zhao et al., 2011; Schiebe et al., 2012). The oleoresin is composed mainly of monoterpenes (C10) and diterpene acids (C20), with very minor amounts of sesquiterpenes (Martin et al., 2002). This composition is congruent with the product profile of an unusual short-chain IDS of *Picea abies* (PaIDS1) that produces both GDP (C10) and GGDP (C20). When bark beetle attack is simulated by methyl jasmonate application to the bark, the expression of the *IDS1* gene is increased and the translated IDS1 protein can be found in cambial cells lining the newly formed traumatic resin ducts that fill them with terpenes (Schmidt et al., 2010, 2011). In contrast to bark, less is known about the regulation of terpenoid biosynthesis and defenses in spruce needles. Treatment with methyl jasmonate increases the amount of stored and emitted needle terpenes (Martin et al., 2003). Similar to the devastating effects of the spruce budworm in North America, the nun moth (*Lymantria monacha*) of Eurasia is also a damaging pest of spruce foliage (Wellenstein, 1942; Klimetzek and Vite, 1989; Keena et al., 2010). The introduction of the nun moth to North America from imported Eurasian timber poses a threat for native North American spruce, since survival and development are possible on species such as *Picea glauca* (Keena, 2003). This suggests that North American spruce should be tested for defenses against the nun moth. Testing herbivores on conifer lines from a common genetic background in which terpene resin formation has been genetically manipulated should be a good approach to investigating the defensive role of these compounds.

In this investigation, we overexpressed *IDS1* in spruce saplings to study its role in terpenoid formation and to generate altered terpene phenotypes.

**RESULTS**

Characterization of Transgenic *IDS1*-Overexpressing Lines

To learn more about the potential of IDS to regulate the direction and flux through terpene biosynthesis, transgenic *P. glauca* lines were created overexpressing a *P. abies* IDS gene (PaIDS1) whose encoded protein produces GDP and GGDP. The protein sequence is more than 99% identical to that of the *P. glauca* IDS1 (PgIDS1; Supplemental Fig. S1) that was deposited in GenBank under accession number KF840686. Transgenic 2-year-old saplings were characterized from three independent *IDS1*-overexpressing lines and three independent lines transformed with an empty vector. *IDS1*-overexpressing saplings had 500-fold higher *IDS1* transcript abundance in needles compared with the empty vector control saplings, but there were no *IDS1* transcript differences in bark. At the metabolite level, no significant differences were observed between *IDS1*-overexpressing lines and controls in monoterpenes, sesquiterpenes, diterpene resin acids, sterols, carotenoids, and chlorophylls (Supplemental Fig. S2).
Overexpression of *IDS1* in Needles Increases *IDS1* Transcript, *IDS1* Protein, *IDS1* Enzyme Activity, and Isoprenyl Diphosphate Intermediate Levels in Vivo, But There Was No Change in Monoterpene and Diterpene Resin Acid Contents

A single *IDS1*-overexpressing line (line 3) and a single empty vector control line (line 1), both 3 years old, were selected for detailed characterization to compare tissue from different growing seasons as well as different genetic backgrounds. There were no noticeable morphological differences between the two lines. The size, the number of side branches, and the coloration of the needles were all similar (Supplemental Fig. S3). After the spring flush of growth, bark and needles were harvested from both lines and divided by age. Most needles of the first year growth had already abscised. In needles, *IDS1*-overexpressing saplings had 300- to 700-fold higher *IDS1* transcript levels than the vector control saplings, with third year needles of the overexpressing line having a 2.3-fold higher abundance of *IDS1* transcript than the second year needles (Fig. 2B). The *IDS1* protein was also much more abundant in needles of the *IDS1*-overexpressing saplings than the controls, as shown by western blots probed with specific antibodies for *IDS1* (Fig. 2D). Total IDS enzyme activity as measured by in vitro assay increased substantially for GDP (C₁₀) when comparing the needles of the *IDS1*-overexpressing versus empty vector control lines, about 7-fold (from 8 to 57 pmol h⁻¹ μg⁻¹ total protein) for second year needles and 17-fold (from 5 to 84 pmol h⁻¹ μg⁻¹ total protein) for third year needles. However, FDP (C₁₅) production decreased 23-fold in *IDS1*-overexpressing lines (from 7 to 0.3 pmol h⁻¹ μg⁻¹ total protein) for second year needles and 10-fold (from 3 to 0.3 pmol h⁻¹ μg⁻¹ total protein) for third year needles compared with controls. On the other hand, GGDP (C₂₀) was only produced...

![Figure 2](image-url)
in enzyme assays of IDS1-overexpressing saplings, with values of 10 pmol h\(^{-1}\) \(\mu\)g\(^{-1}\) total protein in second and third year needles (Fig. 2C). Despite the increased enzyme activity, individual and total amounts of monoterpenes (0.8–1.3 \(\mu\)g \(\mu\)g\(^{-1}\) fresh weight) and diterpene resin acids (1.9–2.7 \(\mu\)g \(\mu\)g\(^{-1}\) fresh weight) were not significantly changed in IDS1-overexpressing saplings versus empty vector controls; sesquiterpenes were only detected in traces and, therefore, were not quantified (Fig. 2, E and F).

Overexpression of IDS1 Increases the in Vivo Levels of Prenyl Diphosphate Intermediates

The substantial increases in IDS1 transcript, IDS1 protein, and in vitro IDS1 enzyme activity upon overexpression in needles were inconsistent with the lack of change in terpene content. To try to reconcile this discrepancy, we also measured the amounts of GDP, FDP, and GGDP as intermediates in planta using a liquid chromatography (LC)-tandem mass spectrometry (MS/MS) method. Since large amounts of plant material were needed for this analysis, we used side branch needles not separated by age instead of stem needles. Needle of side branches have a comparable level of transcript elevation and IDS1 enzyme activity to the stem needles (Supplemental Fig. S4). In needles of IDS1-overexpressing saplings, GDP was increased from 0.07 to 0.56 nmol g\(^{-1}\) fresh weight (8-fold) compared with vector controls, and GGDP was increased from 1.23 to 5.45 nmol g\(^{-1}\) fresh weight (4.5-fold). However, FDP levels (0.07 nmol g\(^{-1}\) fresh weight) were not changed (Fig. 3).

Overexpression of IDS1 Had No Major Effects on Bark Tissue

In bark tissue, IDS1 transcripts were unchanged or had 2 to 3 times higher levels in IDS1-overexpressing saplings than controls for the first, second, and third years of growth (Fig. 4A). These increases were much less than those observed in IDS1-overexpressing needles, and there was no significant change in total IDS1 enzyme activity when comparing IDS1-overexpressing saplings and vector controls in the bark of different years of growth (GDP as product, 40 pmol h\(^{-1}\) \(\mu\)g\(^{-1}\) total protein; FDP, 5 pmol h\(^{-1}\) \(\mu\)g\(^{-1}\) total protein; GGDP, 6 pmol h\(^{-1}\) \(\mu\)g\(^{-1}\) total protein). Moreover, the levels of most prenyl diphosphates in vivo in bark were not significantly altered by IDS1 overexpression. The amounts of GDP (0.1 nmol g\(^{-1}\) fresh weight) and FDP (0.16 nmol g\(^{-1}\) fresh weight) did not differ between IDS1-overexpressing saplings and vector controls independent of the age. GGDP levels increased 1.6- and 1.9-fold with IDS1 overexpression for the first and second year growth, respectively, but these increases were much less than in the needles (Fig. 3). As for the needles, no significant differences in monoterpenes (4.5–7.5 mg g\(^{-1}\) fresh weight), sesquiterpene (75–185 \(\mu\)g g\(^{-1}\) fresh weight), and diterpene resin acid (8.5–12.5 mg g\(^{-1}\) fresh weight) amounts were observed between IDS1 overexpression and control lines (Fig. 4, B–F; Supplemental Fig. S5).

Overexpression of IDS1 Did Not Affect Terpenoids of Primary Metabolism

The effects of IDS1 overexpression were also investigated on selected terpenes of primary metabolism, such as sterols, carotenoids, and chlorophylls. For carotenoids and chlorophylls, the first and second years of growth were used, and for sterol analysis, the third year of growth was used. Relative amounts of all three metabolites did not change between vector control and IDS1-overexpressing saplings in either the bark or the needles (Fig. 5).

Overexpression of IDS1 Leads to the Accumulation of Geranylgeranyl Fatty Acid Esters

Instead of increasing the content of monoterpenes and diterpene resin acids, IDS1 overexpression in P. glauca was found to dramatically increase the amounts of esters of geranylgeraniol with fatty acids in comparison with empty vector controls. Palmitic acid (C16:0), anteiso-heptadecanoic acid (14-methylhexadecanoic acid [aC17:0]), linoleic acid (C18:2), oleic acid (C18:1), and stearic acid (C18:0) were identified as the major components of the acid moieties of the esters (Fig. 6). The occurrence of these esters was initially indicated by the presence of geranylgeraniol in saponified needle extracts of IDS1-overexpressing saplings prepared for sterol quantification. This compound was not found in saponified needle extracts of vector control saplings (Supplemental Fig. S6) or after alkaline phosphatase treatment (Supplemental Fig. S7). In addition, high amounts of fatty acids were detected in IDS1-overexpressing saplings during a diterpenoid extraction procedure that included methylation with the reagent trimethylsulfonium hydroxide (TMSH), which catalyzes transesterifications (Butte, 1983), but not after treatment with diazomethane, which methylates only free acids (Christie, 2010). Mass spectrometry (MS) and NMR measurements in comparison with those of synthesized standards of geranylgeranyl heptadecanoate and geranylgeranyl octadecanoate confirmed the presence of these and related geranylgeranyl fatty acid esters (Fig. 6B; Supplemental Fig. S8). Final confirmation of the structure of the fatty acid moiety required purification of the geranylgeranyl esters followed by transesterification with TMSH to form fatty acid methyl esters, which were compared with commercially available fatty acid methyl ester standards.

The geranylgeranyl fatty acid esters were major constituents of needles of IDS1-overexpressing saplings,
with 12.2 mg g⁻¹ in the second year of growth, 7.76 mg g⁻¹ in the third year of growth, and 6.19 mg g⁻¹ in needles from side branches not subdivided according to their age. Their composition was dominated by esters with either C18:1 or C18:2 and C16:0 and lesser amounts of esters with aiC17:0 and C18:0 (Table I). In bark of IDS1-overexpressing saplings or in bark and needles of empty vector control saplings, these esters were not detectable in amounts greater than 0.5 mg g⁻¹ (Table I; Supplemental Fig. S9). To determine if the accumulation of these prenyl esters is solely a consequence of transformation in the genetic background of *P. glauca*, *IDS1*-overexpressing saplings of *P. abies* were also produced. Needles of 1-year-old *P. abies* saplings had a total amount of geranylgeranyl esters of 8.35 mg g⁻¹, similar to quantities present in transformed *P. glauca*.

Geranylgeranyl Fatty Acid Esters Decrease the Growth and Survival of a Spruce Insect Herbivore

Geranylgeranyl fatty acid esters have previously been reported at low levels in *P. abies* (Ekman, 1980). Their occurrence in large quantities in the needles of our *IDS1*-overexpressing line made it possible to test whether, like many other terpenes, they might have a role in plant defense. For this purpose, larvae of the nun moth, an insect that feeds on the foliage of conifers and other
trees, were offered foliage from *IDS1*-overexpressing, empty vector control, and wild-type saplings. Larval survival and weight 21 d after hatching were significantly lower on *IDS1*-overexpressing plants than on either the empty vector or wild-type controls (*P*, 0.005, log-rank test; *P*, 0.02, Student’s *t* test; Fig. 7, A and B). Developmental time, time from hatching to pupation, and time from pupation until adulthood did not vary between the treatments (Supplemental Fig. S10). Sex ratios of emerged adults were also the same, with an almost 50:50 ratio for larvae feeding on *IDS1*-overexpressing and control foliage (Supplemental Fig. S11).

To confirm that the higher mortality from feeding on *IDS1*-overexpressing plants is actually caused by the occurrence of geranylgeranyl fatty acid esters, branches from wild-type *P. glauca* saplings were treated with...

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**Figure 4.** Detailed characterization of bark from 3-year-old *P. glauca IDS1* in comparison with an empty vector control (vc) with respect to bark from first-, second-, and third-year growth transgenic saplings. A, Relative expression of *IDS1* as determined by quantitative PCR. B, Total IDS enzyme activity measured in vitro forming GDP (C_{10}), FDP (C_{15}), and GGDP (C_{20}) as determined by LC-MS/MS. C and D, Monoterpene (C) and diterpene resin acid (D) contents as quantified with GC-FID. The minor monoterpenes are additionally shown separately in Supplemental Figure S5. Data are means ± SD of measurements from five plants of each line. FW, Fresh weight.
geranylgeranyl stearate dissolved in hexane or hexane as a control. Larvae fed on foliage amended with this purified geranylgeranyl fatty acid ester had a significantly lower survival rate than when fed on controls ($P < 0.001$, log-rank test; Fig. 7C).

**DISCUSSION**

IDS functions at branch points of terpenoid biosynthesis, catalyzing the formation of intermediates of varying chain length, such as GDP (C$_{10}$), FDP (C$_{15}$), and GGDP (C$_{20}$). However, only scattered information is available about the role of IDS in controlling direction and flux through these pathways. IDS1 isolated from *P. abies* catalyzes the condensation of the C$_{5}$ units IDP and DMADP into GDP and GGDP, but not FDP (Schmidt et al., 2010). The C$_{10}$ and C$_{20}$ intermediates, GDP and GGDP, are precursors for the monoterpene hydrocarbons and diterpene resin acids, respectively, the two major classes of terpenes in conifer oleoresin. *P. abies* IDS1 has been suggested to have a major role in conifer oleoresin formation, because of the strong correlations of transcript and protein with the synthesis of resin terpenes in traumatic ducts after methyl jasmonate application (Schmidt et al., 2010, 2011). Here, transgenic spruce lines overexpressing IDS1 were used to test how increasing enzyme activity would affect terpene amount and composition.

**Overexpression of IDS1 Does Not Increase the Content of Typical Resin Monoterpenes and Diterpenes**

Overexpression of *IDS1* under the control of the maize (*Zea mays*) ubiquitin promoter led to a massive (up to 700-fold) increase in *IDS1* transcript level in needles but only a 2- to 3-fold increase in bark. Previous measurements on wild-type saplings had shown that *IDS1* transcripts were about 50-fold higher in bark, the site of extensive terpene resin synthesis and storage, than in needles (Schmidt et al., 2010). The strength of the ubiquitin promoter might not be sufficient to increase transcription much further in bark, but in needles, where basal transcription is very low, this promoter could enhance transcription quite significantly. For successful overexpression in bark, another promoter should be chosen, or better, an as-yet-unidentified promoter specific for oleoresin production in ducts.

The elevated transcript levels in needles increased the amount of IDS1 protein, leading to a higher IDS1 enzyme activity and higher in planta levels of the products GDP and GGDP. However, FDP did not

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**Figure 5.** Quantification of other selected isoprenoids in *P. glauca* IDS1 compared with an empty vector control line (vc) with respect to first-, second-, and third-year needles and bark. A and B, Relative content of β-sitosterol, a predominant plant sterol, measured by GC-FID. The resulting peak areas, calibrated using the internal standard ergosterol, were expressed relative to the area of the vector control, set to 1. C and D, Relative content of major carotenoids was determined by HPLC with diode-array detector at 455 nm. For each organ and year, the integrated peak areas are expressed relative to that of the vector control, set to 1. E and F, Relative chlorophyll content was determined by HPLC with diode-array detector at 650 nm. For each organ and year, the integrated peak areas are expressed relative to that of the vector control, set to 1. Data are means ± so of measurements from five plants of each line.
increase in planta in the needles. The reduction of FDP in the in vitro enzyme assays can be attributed to the ability of IDS1 to use FDP as a substrate for the production of GGDP (Schmidt et al., 2010). Despite the increase in IDS1 protein activity and GDP and GGDP, no significant changes were detected in terpene content, with the exception of a dramatic increase in geranylgeranyl fatty acid esters. The quantities of resin monoterpenes, sesquiterpenes, and diterpene resin acids did not increase in *P. glauca* and are in the same range as already reported for *P. abies* (Martin et al., 2002, 2003). In addition, there were no changes in the levels of the primary isoprenoids measured, including sterols, chlorophylls, and carotenoids. While overexpression of IDS1 was able to significantly increase the prenyl diphosphate intermediates GDP and GGDP, the lack of increased flux to the resin monoterpenes and diterpenes, respectively, may be due to the low activity of the next step of terpene biosynthesis, catalyzed by terpene synthases.

Figure 6. Evidence for the accumulation of geranylgeranyl fatty acid esters in IDS1-overexpressing *P. glauca* saplings. A, Structure of geranylgeranyl stearate (geranylgeranyl-C18:0). B, GC-MS chromatogram of a needle extract overlaid with a chromatogram of synthetic geranylgeranyl-C18:0. C, GC-MS chromatogram showing fatty acid methyl esters derived from the hydrolysis of isolated geranylgeranyl fatty acid esters. The peaks just to the left of C18:2 are impurities generated by derivatization with N-methyl-N-(trimethylsilyl)trifluoroacetamide and not fatty acid esters. The abbreviation “aiC17:0” stands for 14-methylhexadecanoate, an anteiso fatty acid that is gymnosperm specific.
Another limitation to terpene accumulation in these plant lines may be the broad targeting of IDS1 overexpression under the control of the maize ubiquitin promoter. In wild-type spruce, this *P. abies* gene and genes encoding later enzymes of terpene resin biosynthesis in spruce are localized in bark cells surrounding resin ducts (Abbott et al., 2010; Schmidt et al., 2010). Here, the resin components are formed and then secreted into the ducts. Resin terpenes in spruce needles are stored in resin ducts (Weng and Jackson, 2000) and thus are likely also localized in bark cells surrounding resin ducts (Abbott et al., 2006). In *Artemisia annua*, overexpression increased the amount of the diterpene tanshinone (Kai et al., 2011), while overexpression of Arabidopsis FDPS1, because this particular GDP synthase was, in fact, subsequently shown to produce medium/long-chain prenyl diphosphates instead of GDP (Hsieh et al., 2011). In this work, overexpression of the *IDS1* gene, which has a plastid-targeting signal peptide (Schmidt et al., 2010), had no impact on the content of resin monoterpene and diterpenes, but flux was diverted to prenyl esters. Thus, constitutive overexpression of an IDS does not seem to be an appropriate way to increase the amount of resin terpenes. An RNA interference approach is currently under way to further investigate the role of IDS in terpene biosynthesis.

To the best of our knowledge, this work provides the first in planta quantification of GDP, FDP, and GGDP together. GDP has been previously quantified in two publications on spruce and other woody plants, giving concentrations 200-fold higher than what we obtained (Nogués et al., 2006; Ghirardo et al., 2010). These previous values were obtained by acid hydrolysis of the diphosphate moiety to form geraniol, which rearranges to linalool, quantified by proton-transfer reaction MS. The authors themselves (Ghirardo et al., 2010) recognized that their values might be overestimates of in planta GDP levels, since linalool might be formed from other substances under acidic conditions. For GGDP, 10-fold higher amounts in etiolated oat (*Avena sativa*) seedlings were reported (Benz et al., 1983). Large amounts of GGDP might be present in etiolated seedlings to form chlorophyll and initiate photosynthesis rapidly on illumination. The concentration of DMADP reported (Nogués et al., 2006; Ghirardo et al., 2010) is over 100- to 1,000-fold greater than our measurements of GDP, FDP, and GGDP. If this disparity is correct, it indicates a metabolic bottleneck in terpenoid metabolism after the MEP or MVA pathway and prior to the action of IDS enzymes. In some isoprene-emitting woody plant species, including spruce, a large pool of DMADP may be critical in supplying substrate for isoprene biosynthesis (Monson et al., 2013).

To make terpenes larger than C10, more IDP than monoterpenes is needed, and it is assumed that the ratio between IDP and DMADP is regulated by isopentenyl diphosphate synthases in spruce.
diphosphate isomerase (Berthelot et al., 2012). In kudzu (Pueraria lobata) leaves, which make high levels of isoprene (C5), this ratio was determined to be about 0.5 (Zhou et al., 2013), but it might be higher in plants that make high levels of terpenes with 10 or more carbon atoms. The greater amount of GGDP in our measurements, especially in needles, in comparison with that of GDP and FDP may be a result of GGDP’s role as a precursor for GAs, carotenoids, and the phytol side chain of chlorophyll in photosynthetically active tissue. No detailed data are yet available about the regulatory interactions between the IDS enzymes and isopentenyl diphosphate isomerase and isoprene synthase. However, it was recently demonstrated that IDP and DMADP have inhibitory effects on the initial step of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate synthase (Banerjee et al., 2013). Thus, future efforts should be made to determine whether GDP, FDP, or GGDP are also inhibitory to this enzyme or other MEP or MVA pathway enzymes as well as to determine how metabolic flux is controlled among the IDS-catalyzed steps of terpene biosynthesis.

Elevated Levels of GGDP Are Diverted to Form Geranylgeranyl Fatty Acid Esters

IDS1-overexpressing spruce saplings showed no significant changes in their content of typical P. glauca resin monoterpenes and diterpene acids; instead, they exhibited a dramatic increase in a diversion product, esters of geranylgeraniol with various fatty acids. Given a typical concentration of 2.5 to 3.0 mg g⁻¹ fresh weight diterpene acids in spruce needles, the amount of GGDP diverted to the esters in IDS1-overexpressing plants is about twice the amount of GGDP employed for diterpene acid formation (assuming about half of the mass of geranylgeranyl fatty acid esters, 4–6 mg g⁻¹ fresh weight, results from geranylgeraniol). Thus, there has been at least a 3-fold increase in GGDP production in the transgenic plants, which is in broad agreement with the 4.5-fold increase in GGDP measured in vivo.

Despite elevated levels of isoprenoid pathway intermediates, such as GGDP, we did not detect any increase in other primary terpenoids, such as phytol and carotenoids, also formed from GGDP (Fig. 8), indicating that formation of these products is tightly regulated (Cazzonelli and Pogson, 2010). Although we did not measure the levels of the GAs, another group of GGDP metabolites, an increase in these hormones also seems unlikely. First, no obvious morphological differences were found between the transgenic saplings and the controls (Supplemental Fig. S3). Second, GA biosynthesis is known to be regulated mainly by transcriptional changes of transcripts of the early pathway enzymes, ent-copalyl diphosphate synthase and ent-kaurene synthase, rather than the level of GGDP (Silverstone et al., 1997; Hedden and Thomas, 2012).

The pathway for the formation of geranylgeranyl fatty acid esters likely proceeds from GGDP to geranylgeraniol...
via phosphatase cleavage (Fig. 8). A prenyl diphosphate phosphatase from *Croton stellatopilosus* was recently identified (but not biochemically characterized) that is part of a family of related phosphatases from eukaryotic as well as prokaryotic sources (Nualkaew et al., 2013). The resulting free geranylgeraniol is then esterified. In Arabidopsis, two enzymes were described as being responsible for the synthesis of esters using the more saturated C₂₀ isoprenoid alcohol, phytol, instead of geranylgeraniol (Fig. 8). These are named PHYTYL ESTER SYNTHASE1 and PHYTYL ESTER SYNTHASE2 and are members of the esterase/lipase/thioesterase family of acyltransferases (Lippold et al., 2012). Since both are localized to the plastids, this suggests that the entire sequence for the formation of geranylgeranyl esters via IDS1, phosphatase, and then the ester synthase is localized in plastids. For phytol fatty acid esters, localization in the plastoglobules was shown (Gaude et al., 2007). An identical localization of geranylgeranyl fatty acid esters can thus be assumed, because of the close structural similarities between these compounds.

### Geranylgeranyl Fatty Acid Esters Are Common in Plants and Function in Defense

Esters of geranylgeraniol with fatty acids were already described in *P. abies* wood (Ekman, 1980), in mosses (Liljenberg and Karunen, 1978; Karunen et al., 1980), and in other plants (Lütke-Brinkhaus et al., 1985; McKibben et al., 1985; Jondiko and Pattenden, 1989; Reiter and Lorbeer, 2001; Biedermann et al., 2008). Esters with geraniol instead of geranylgeraniol are also known in rose (*Rosa* spp.) petals (Dunphy, 2006). However, no esters with geraniol were found in our transgenic *P. glauca*, although IDS1 is known to produce GDP and GGDP in vitro (Schmidt et al., 2010) and both intermediates were elevated in planta also, with the GGDP concentration being 12 times higher than that of GDP (Fig. 5A). The occurrence of phytol esters is also common in plants, as mentioned above (Csupor, 1970; Liljenberg, 1977; Anderson et al., 1984; Pereira et al., 2002; Gaude et al., 2007), indicating that esterification of prenyl alcohols to fatty acid esters is a widespread phenomenon. The unusual antiseiso fatty acid found as a component of these esters, antiseisoheptadecanoic acid (14-methylhexadecanoic acid), has also already been reported from other conifers of the Pinaceae (Wolff et al., 1997, 2001).

In contrast to phytol esters, which are known to be involved in chlorophyll *a* and *b* synthesis and in senescence (Ischebeck et al., 2006), the role of geranylgeranyl fatty acid esters had not yet been investigated. Here, we found these compounds to reduce the growth and survival of the conifer needle-feeding larvae of the nun moth, suggesting that they function in plant defense against herbivores (Fig. 7). Their toxicity may be a consequence of hydrolysis in the insect. Gut extracts of the closely related species *Lymnantria dispar* possess high esterase activity (Kapin and Ahmad, 1980) that could cause cleavage of the ester bond and the release of geranylgeraniol, which has been described to exert toxic effects on membranes in vitro and against *Staphylococcus aureus* (Funari et al., 2005; Inoue et al., 2005). Interestingly, another group of plant defense compounds, the hydroxygeranylinalool glycosides found in *N. attenuata* (Jassbi et al., 2008; Heiling et al., 2010; Dinh et al., 2013), share a similar terpene moiety and also can be readily cleaved to release an acyclic diterpene alcohol. The toxicity of geranylinalool by itself has been reported for ants and termites (Lemaire et al., 1990). In both cases, conjugation of the diterpene moiety with a sugar or fatty acid could function as a strategy for storing these toxins to prevent damage to membranes or other plant components (Fig. 8). In fact, the overaccumulation of geranylgeraniol moieties as fatty acid esters in *IDS1*-overexpressing plants may be essential to avoid auto-toxicity. Further experiments are planned to investigate the occurrence of geranylgeranyl fatty acid esters in spruce and their role in defense against other herbivores and pathogens.
MATERIALS AND METHODS

Chemicals

Geranylgeraniol, octadecanoyl chloride, pentadecanoyl chloride, 4-(dimethylamino) pyridine, para-cymene, iodine, alkaline bovine phosphatase, Supelco 37 Component FAME Mix, chloroform, dichloromethane, isopentenyl diphasphate, dimethylallyl diphasphate, ammonium bicarbonate, tert-butyl methyl ether (TBME), and acetonitrile (LC-MS grade) were purchased from Sigma-Aldrich. N-Methyl-N-(trimethylsilyl) trifluoroacetamide and TMSH were offered from Macherey-Nagel. Methyl 14-methylhexadecanoate was purchased from Larodan Fine Chemicals, and tetradroxyacidic was from Wako Pure Chemical Industries.

Plant Material

Agrobacterium tumefaciens-mediated transformation of a Picea glauca embryonic cell culture overexpressing IDS1 from Picea abies (Schmidt et al., 2010) was carried out as described previously by Klimaszewska et al. (2001) and Hammerbacher et al. (2011), with the gene under the control of a maize (Zea mays) ubiquitin promoter. Somatic embryogenesis and plant regeneration of transgenic saplings were carried out, and plants were transferred to soil. For the analysis of geranylgeranyl fatty acid ester accumulation in P. abies, an identical transformation protocol was used. In the case of P. glauca, three independent transformed lines plus three empty vector control lines were characterized initially, using four plants per line. For later in-depth characterization, one line each from transfectors and controls were chosen using five plants per line.

Monoterpene, Sesquiterpene, and Diterpene Analysis

The protocol was adapted from Lewinsohn et al. (1993). In brief, 100 mg of frozen plant material was ground in 1 mL of TBME with 37 μg mL−1 p-cymene and 46 μg mL−1 tetradroxyacidic as internal standards and extracted under continuous shaking for 24 h. The extract was removed, washed with 0.3 mL of 0.1 M (NH4)2CO3, pH 8.0, and dried by using a Pasteur tip filled with 100 mg of Na2SO4. The Na2SO4 column was further washed with 1 mL of TBME. For 0.4 mL of extract, 50 μL of TMSH was added for methylation of diterpene resin acids, which were subsequently analyzed by gas chromatography (GC)-MS and GC-flame ionization detection (FID). The rest was used for monoterpene and sesquiterpene analysis. GC conditions were as described by Schmidt et al. (2011). All terpenes were quantified on a fresh weight basis; the ratio of fresh weight to dry weight is about 3.5 for needles and 4.0 for bark tissues.

Isolation, Purification, and MS Analysis of Geranylgeranyl Fatty Acid Esters

Plant material was extracted with hexane:diethyl ether (9:1, v/v) in a ratio of 100 mg of tissue to 4 mL of solvent for 24 h under continuous shaking. The solvent was evaporated to dryness under a stream of nitrogen, and the sample was dissolved in 2 mL of 2 M KOH in ethanol:water (3:1, v/v) followed by an incubation for 2 h at 60°C. After 2 mL of water was added, extraction was done three times with 2 mL of diethyl ether. The organic phases were combined and evaporated to dryness, and 100 mL of tetrahydrofuran and 100 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide were added. An Agilent 6890 series GC device with an Agilent 5973 mass spectrometer and a Zebron ZB-MSi column (30 m × 0.25 m × 0.25 μm; Phenomenex) was used for analysis.

NMR Analysis of Geranylgeranyl Fatty Acid Esters

All NMR spectra were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin), operating at 500.13 MHz for 1H and 125.75 MHz for 13C. A triple resonance inverse cryoprobe (5 mm) was used to measure spectra at a probe temperature of 300 K. Spectra are referenced to tetramethylsilane at δ 0 ppm. For data processing and spectrometer control, TOFSPIN version 2.1 was used. The all-trans-configuration of the geranylgeranyl moiety could be deduced from chemical shifts in accordance with previously published data and from selective rotating frame nuclear Overhauser effect correlation spectroscopy experiments (Tanaka et al., 1982; Tanaka and Hirasawa, 1989).

Synthesis of Geranylgeranyl Stearate as a Standard in Geranylgeranyl Fatty Acid Ester Analysis

The synthesis was modified after a published method (Vassio et al., 2007). Quantities of 100 mg of geranylgeraniol (purity, 85%) and 30 mg of 4-(dimethylamino)-pyridine were dissolved in 25 mL of dichloromethane on an ice bath, and 100 mg of either octadecanoyl or heptadecanoyl chloride was added. The solution was stirred further for 24 h at room temperature. To stop the reaction, 20 mL of a saturated NaCl solution were added. The organic phase was dried with Na2SO4 and concentrated using a rotary evaporator. The reaction products were dissolved in 1 mL of hexane:diethyl ether (91:1, v/v) and purified using a SPE cartridge (CHROMABOND SiOH; Macherey-Nagel) equilibrated and eluted with hexane:diethyl ether (91:1, v/v). The eluate was fractionated and tested for purity by applying 10 μL of each fraction to a silica gel thin-layer chromatography plate, which was developed in hexane:diethyl ether (91:1, v/v) and stained with iodine vapor.

Sterol Analysis

One hundred milligrams of plant material was extracted with 4 mL of dichloromethane:methanol (2:1, v/v), including 60 μg mL−1 ergosterol as an internal standard, for 2 h at room temperature under continuous shaking. The solvent was evaporated under a stream of nitrogen, and the sample was dissolved in 2 mL of 2 M KOH in ethanol:water (3:1, v/v) followed by an incubation for 2 h at 60°C. After 2 mL of water was added, extraction was done three times with 2 mL of diethyl ether. The organic phases were combined and evaporated to dryness, and 100 mL of tetrahydrofuran and 100 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide were added. An Agilent 6890 series GC device with an Agilent 5973 mass spectrometer and a Zebron ZB-MSi column (30 m × 0.25 m × 0.25 μm; Phenomenex) was used for analysis. One microlitr of sample was injected in split mode 1:20 with an injector temperature of 280°C and a flow rate of 1 mL min−1 helium. Initial oven temperature was 200°C, held for 2 min, then raised by 15°C min−1 to 280°C and by 2°C min−1 to 300°C, and held there for 4 min. ChemStation G1701 was used for data analysis and integration of the peak areas.

Carotenoid and Chlorophyll Analysis

Extraction of 100 mg of plant material was carried out with 2 mL of acetone in brown glass vials at 4°C for 24 h under continuous shaking. An 800-μL portion of the extract was diluted with 200 μL of water, and 50 μL was injected on an Agilent 1100 HPLC instrument using a Supelcosil LC-18 column (7.5 cm × 4.6 mm × 3 μm; Sigma-Aldrich). Column temperature was kept at 20°C with a flow rate of 1.5 mL min−1. Buffer A was 1 mM NaHCO3, and buffer B was acetone. Starting conditions were 65% B, held for 4 min, followed by an increase to 90% B in 8 min and to 100% B in 8 min, held for 2 min. Carotenoids were detected at 455 nm and chlorophyll at 650 nm. For data analysis and integration of peak areas, ChemStation for LC 3D was used.

SDS-PAGE and Western Blot

Plant material was extracted as described (Nagel et al., 2012), and 34 μg of total protein was separated on a 12% SDS-PAGE gel under reducing conditions and transferred to an Immobilon-P polyvinylidene difluoride membrane (Merck Millipore). The membrane was blocked with 5% skim milk powder in PBS-T (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2.0 mM KH2PO4, pH 7.4, and 0.05% [v/v] Tween 20). Thereafter, the membrane was incubated with an IDR5 specific polyclonal antibody serum (Schmidt et al., 2010) diluted 1:500 in blocking solution. The secondary antibody was an anti-rabbit horseradish peroxidase (Sigma-Aldrich) diluted 1:5,000 in TBS without skim milk powder. Washes between the incubation steps were done with PBS-T. Signal detection was achieved by using West Pico Chemiluminescent Substrate (Thermo Scientific) and Amersham Hyperfilm ECL (GE Healthcare).
**Protein Extraction and Quantification of IDS Enzyme Activity**

Protein extraction, enzyme assays, and analysis were carried out as described by Nagel et al. (2012). Data analysis was performed using Analyst Software 1.6 Build 3773 (AB Sciex Instruments).

**Quantification of Short-Chain Prenyl Diphosphates in Planta**

A 0.75-g portion of plant material was extracted three times with 5 mL of methanol/water (7:3, v/v), including a total of 0.3 μg of geranyl S-thiodiphosphate and farnesyl S-thiodiphosphate each (Echelon Biosciences). Extracts were combined, and 5 mL of water was added. Extracts were purified using 150 mg (6 mL) of CHROMABOND HR-XA columns (Macherey-Nagel), conditioned with 5 mL of methanol and 5 mL of water. After application of extract, the column was washed with 4 mL of water followed by 5 mL of methanol. Prenyl diphosphates were eluted with 3 mL of 1 M ammonium formate in methanol, evaporated under a stream of nitrogen to dryness, and dissolved in 250 μL of water:methanol (1:1). Quantification was done using an Agilent 1260 HPLC system (Agilent Technologies) coupled to an API 5000 triple-quadrupole mass spectrometer (AB Sciex Instruments). For separation, a ZORBAX Extended C-18 column (1.8 μm, 50 mm × 4.6 mm; Agilent Technologies) was used. The mobile phase consisted of 5% ammonium bicarbonate in water as solvent A and acetonitrile as solvent B, with the flow rate set at 1.2 mL min⁻¹, and the column temperature was kept at 20°C. Separation was achieved by using a gradient starting at 5% B, increasing to 7% B in 5 min and 100% B in 1 min (0.5-min hold), followed by a change to 0% B in 1.5 min (1-min hold) before the next injection. The injection volume for samples and standards was 2 μL; autosampler temperature was 4°C. The mass spectrometer was used in the negative electrospray ionization mode. Optimal settings were determined using standards. Levels of ion source gases 1 and 2 were set at 60 and 70 pounds per square inch (1 p.s.i.), respectively, with a temperature of 700°C. Curtain gas was set at 30 p.s.i., and collision gas was set at 7 p.s.i., with all gases being nitrogen. Ion spray voltage was maintained at ~4.200 V. Multiple reaction monitoring was used to monitor analyte parent ion-to-product ion formation: m/z 312/79/9 for GDP, m/z 380/91/79 for FDP, m/z 449/79 for CGDP, m/z 329/79 and 329/159 for geranyl S-thiodiphosphate, and m/z 379/79 and 379/159 for farnesyl S-thiodiphosphate. Data analysis was performed using Analyst Software 1.6 Build 3773 (AB Sciex Instruments).

**Quantitative Real-Time PCR and Quantitative Genomic PCR**

RNA isolation, complementary DNA synthesis, and quantitative PCR from needle tissue were done as described by Schmidt et al. (2011). Quantitative genomic PCR was done from needle tissue as described by Schmidt et al. (2010).

**Cloning of PgIDS1 and Alignment with PaIDS1**

RNA was isolated from bark tissue of *P. glauca*, and the PgIDS1 sequence was amplified with the primers and conditions described by Schmidt et al. (2010). The resulting fragment was cloned into pCR 4-TOPO (Invitrogen) and transformed into *Escherichia coli* strain TOP10F (Invitrogen) according to the manufacturer's instructions. Positive clones were selected, and sequence analysis was carried out using an ABI 3100 automatic sequencer (Applied Biosystems). The DNAStar Lasergene program version 9.0 (MegAlign) was used to align PgIDS1 (GenBank accession no. KF840686) with PaIDS1 (GenBank accession no. GQ369788).

**Insect Assays**

Eggs of nun moth (*Lymnaea monacha*) were collected in fall 2011 near Herzberg/Elster, Germany, and kept at ~8°C for 2 months plus 2 weeks at +4°C and then at room temperature until hatching. Hatched larvae were selected randomly for the different treatments and placed in groups of 50 larvae in Steri Vent Containers (Duchefa). Cut branches of wild-type, vector control IDS1-overexpressing spruce saplings were inserted in a water-filled 2-mL tube through a hole in the lid of each container and changed every third day. Larvae were counted once per week, and numbers were equalized every second week in each treatment group. Larval weight was determined separately for each individual after 21 d. To determine the time needed for pupation and the sex of the emerging adult, containers were checked for pupae every day after the first pupation event occurred, and pupae were placed in separate containers. In total, 300 larvae were fed on vector control and IDS1-overexpressing saplings, and 150 larvae were fed on wild-type plants.

For the feeding assay with geranylgeranyl stearate standard applied to branches from wild-type spruce saplings, ends of cut branches were placed in water-filled 2-mL tubes through a hole in the lid and swirled in hexane including 2 mg mL⁻¹ geranylgeranyl stearate or pure hexane for 30 s. Newly treated branches were placed in a container with nun moths every day, and larvae were counted on days 3, 5, 7, 9, and 12. One hundred larvae were used for each treatment.

**Statistical Analysis**

The statistical significance of nun moth weight gain was tested by pairwise comparison using Student's *t* test. Survival curves were compared using a log-rank test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number KF840686 (*PgIDS1*).

**Supplemental Data**

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

- Supplemental Figure S1. Amino acid alignment of PgIDS1 and PaIDS1.
- Supplemental Figure S2. Initial characterization of three independent IDS1 transgenic lines.
- Supplemental Figure S3. General morphology of vector control and IDS1-overexpressing *Picea glauca* saplings.
- Supplemental Figure S4. *IDS1* expression and total IDS enzyme activity of side branch needles.
- Supplemental Figure S5. Content of sesquiterpenes and minor monoterpines in bark.
- Supplemental Figure S6. Identification of geranylgeraniol in saponified plant material.
- Supplemental Figure S7. Determination of the origin of geranylgeraniol in needles of IDS1-overexpressing saplings.
- Supplemental Figure S8. NMR analysis of geranylgeranyl fatty acid esters in needles.
- Supplemental Figure S9. Abundance of geranylgeranyl fatty acid esters in bark and needles of IDS1-overexpressing saplings.
- Supplemental Figure S10. Developmental times of *Lymnaea monacha* fed on IDS1-overexpressing foliage versus controls.
- Supplemental Figure S11. Sex ratio of *Lymnaea monacha* fed on IDS1-overexpressing foliage versus controls.
- Supplemental Figure S12. GC-MS spectra of geranylgeranyl fatty acid esters.

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**LITERATURE CITED**


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