Nonradioactive assay for detecting isoprenyl diphosphate synthase activity in crude plant extracts using liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

Terpenoids form the largest class of plant metabolites involved in primary and secondary metabolism. Isoprenyl diphosphate synthases (IDSs) catalyze the condensation of the C_5 terpenoid building blocks, isopentenyl diphosphate and dimethylallyl diphosphate, to form geranyl diphosphate (C_{10}), farnesyl diphosphate (C_{15}), and geranylgeranyl diphosphate (C_{20}). These branch point reactions control the flow of metabolites that act as precursors to each of the major terpene classes—monoterpenes, sequiterpenes, and diterpenes, respectively. Thus accurate and easily performed assays of IDS enzyme activity are critical to increase our knowledge about the regulation of terpene biosynthesis. Here we describe a new and sensitive nonradioactive method for carrying out IDS assays using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) to detect the short-chain prenyl diphosphate products directly without dephosphorylation. Furthermore, we were able to separate *cisoid* and *transoid* isomers of both C_{10} enzyme products (geranyl diphosphate and neryl diphosphate) and three C_{15} products [(*E,E*)-, (*Z,E*)-, and (*Z,Z*)-farnesyl diphosphate]. By applying the method to crude protein extracts from various organs of *Arabidopsis thaliana*, *Nicotiana attenuata*, *Populus trichocarpa*, and *Picea abies*, we could determine their IDS activity in a reproducible fashion.

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More than 55.000 terpenes or isoprenoids form the largest single class of lower molecular weight plant metabolites and function in many processes in primary and secondary metabolism [1]. The biosynthesis of all terpenoids is initiated by the synthesis of isopentenyl diphosphate (IPP)[1] via the mevalonic acid or the methylerythritol phosphate pathway [2,3]. IPP and its isomer, dimethylallyl diphosphate (DMAPP), are the five-carbon building blocks that undergo sequential condensation reactions to form the prenyl diphosphates, geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (GGPP, C_{20}) as precursors for monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), respectively, as well as for other compounds such as sterols, carotenoids, and gibberellins. The enzymes catalyzing these sequential condensation reactions are prenyltransferases referred to collectively as isoprenyl diphosphate synthases (IDSs). Each of the short-chain IDS products (GPP, FPP, and GGPP) is formed by a specific enzyme named for its product: GPP synthase (GPPS), FPP synthase (FPPS), and GGPP synthase (GGPPS) (Fig. 1).

Until now, a fast and easy method to determine the products and rate of activity of plant IDSs has not been described. The established assay for measuring IDS enzyme activity *in vitro* in crude extracts of plants or in *Escherichia coli* extracts after heterologous expression of IDSs involves the use of radioactively labeled [14C]IPP as substrate followed by acid or alkaline hydrolysis. The corresponding alcohols are measured by radio-gas chromatography (radio-GC), radio-high-performance liquid chromatography (radio-HPLC), thin layer chromatography (TLC), or liquid scintillation counting (LSC) [4–14]. However, because of the precautions for using radioactive substrates and the complication of hydrolyzing the products, assays are rather laborious, time-consuming, and frequently imprecise. Hydroxylation is carried out either in strongly acidic conditions or by incubation with an alkaline phosphatase. However, both methods have serious disadvantages. Acid hydrolysis may generate multiple products from one prenyl diphosphate such as linalool, α-terpineol, and nerol from neryl diphosphate (NPP, C_{10}) [15]. Thus, it may be difficult to identify

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[1] *Abbreviations used:* IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IDS, isoprenyl diphosphate synthase; radio-GC, radio-gas chromatography; radio-HPLC, radio-high-performance liquid chromatography; TLC, thin layer chromatography; LSC, liquid scintillation counting; NPP, neryl diphosphate; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; PAR: photosynthetically active radiation; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid.
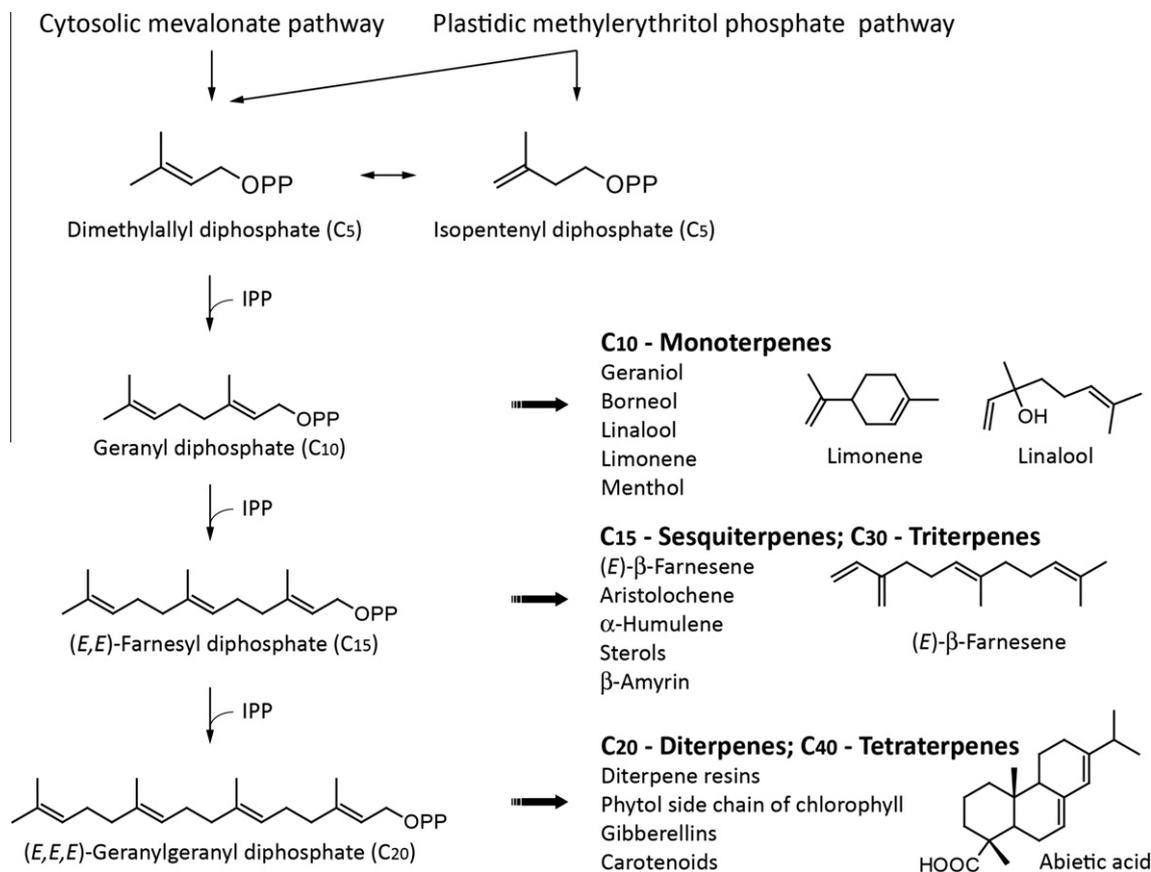
Fig.1. Outline of terpenoid biosynthesis. The schematic diagram depicts the reactions carried out by short-chain isoprenyl diphosphate synthases (IDSs). The five-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), produced either by the cytosolic mevalonate pathway or the plastidial methylerythritol phosphate (MEP) pathway, are the substrates for IDSs. Sequential condensations of DMAPP with 1 to 3 molecules of IPP mediated by IDSs form geranyl diphosphate (GPP), farnesyl diphosphate (FPP), or geranylgeranyl diphosphate (GGPP). The catalysts are referred to as GPP synthases, FPP synthases, or GGPP synthases. The different prenyl diphosphates are then converted into representatives of the different terpene classes—GPP to monoterpenes, FPP to sesqui- and triterpenes, and GGPP to di- and tetraterpenes.

the actual enzyme product. Alkaline phosphatases do not usually cause such rearrangements, but a shift of the pH optimum is needed for the reaction to occur and a long incubation time is required for complete cleavage of all prenylated diphosphates. Under these conditions, IDSs can still be active, making this method unsuitable for determining accurate rates of enzyme activity. Moreover, prenyl diphosphates are often poor substrates for commercially available phosphatases [16].

Recently, two unexpected new IDS reaction products were reported with *cisoid* structures instead of *transoid* structures that serve as substrates for new types of terpene synthases, the enzymes converting GPP, FPP, or GGPP to specific terpene skeletons [15,17,18]. The possible involvement of NPP and (*Z,Z*)-FPP, the *cisoid* isomers of GPP and (*E,E*)-FPP, respectively, in other reactions of plant terpenoid biosynthesis now makes it even more crucial to carefully identify IDS products. However, none of the previously described IDS assays is able to differentiate between these *cisoid* and *transoid* isomers without hydrolysis.

Here we describe a new method using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) to determine the products and rate of IDS enzyme reaction in crude protein extracts of plants without the need for radioactive substrates or a dephosphorylation step. The protocol can be used to detect IDS enzyme activity in a fast and reliable way in a broad range of plant protein extracts from angiosperms such as *Arabidopsis thaliana*, poplar (*Populus trichocarpa*), and tobacco (*Nicotiana attenuata*) as well as from gymnosperms such as Norway spruce (*Picea abies*).

The method can also distinguish between the different *transoid* and *cisoid* isoforms of GPP and FPP as products.

Materials and methods

Chemicals/materials

IPP, DMAPP, GPP, FPP, and GGPP standards, chemical reagents, and LC–MS-grade ammonium bicarbonate were purchased from Sigma–Aldrich (Munich, Germany). GC-grade chloroform was purchased from Roth (Karlsruhe, Germany), and HPLC-grade acetonitrile was purchased from VWR (Darmstadt, Germany). NPP and (*Z,Z*)-FPP were purchased from Echelon Bioscience (Salt Lake City, UT, USA). [1-^{14}C]IPP (50 Ci mol^{-1}) was purchased from Hartmann Analytic (Braunschweig, Germany).

Plant material

A. thaliana (Col-0) was grown from seeds in a climate chamber (22 °C, 55% relative humidity, and 100 μmol m^{-2} s^{-1} photosynthetically active radiation [PAR]) under long-day conditions (16/8-h light/dark) until flowers developed. Flowers and leaves were cut with scissors. *P. abies* tissue was harvested from clone 3369-Schongau (Samenklenge and Pflanzgarten Laufen) planted out originally as 1-year-old seedlings at Jena, Germany, in 2003. Bark and needles from a side branch were harvested in mid-April. *N. attenuata* was

cultivated as described by Baldwin and coworkers [19] and harvested at the rosette stage. *P. trichocarpa* was propagated stem cuttings of clone 606 (NW-FVA, Hann. Münden, Germany) grown in a climate chamber (24 °C, 60% relative humidity, 100 μmol m^{-2} s^{-1} PAR and 16/8-h light/dark). Plants were harvested after reaching 1 m of height. All plant material was immediately frozen in liquid nitrogen after harvest and stored at −80 °C.

Protein extraction

Plant tissues were ground with mortar and pestle under liquid nitrogen by hand to a fine powder. Cooled extraction buffer was added to frozen tissue in a ratio of 1:5 (tissue [g]/buffer [ml]). Extraction buffer was modified after Ref. [5] and contained 250 mM Mopso (pH 6.8), 5 mM ascorbic acid, 5 mM sodium bisulfite, 5 mM dithiothreitol (DTT), 10 mM MgCl$_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone (PVP, M_r = 10,000), 4% (w/v) polyvinylpolypyrrolidone (PVPP), 4% [w/v] Amberlite XAD-4, and 0.1% (v/v) Tween 20. Extraction was carried out in Eppendorf Protein LoBind tubes with an Eppendorf ThermoShaker at 4 °C and 1400 rpm for 30 min. After tubes were centrifuged for 20 min at 4 °C and 20,000g, the supernatant was passed through a 2-ml Zeba Spin desalting column with a 7-kDa molecular weight cutoff (MWCO, Thermo Scientific, Rockford, IL, USA) to exchange the buffer to 25 mM Mopso (pH 7.2), 10 mM MgCl$_2$, and 10% (v/v) glycerol according to the manufacturer's protocol. Protein concentration was determined using the Protein Assay Dye (Bradford) Reagent Concentrate (5×) (Bio-Rad, Munich, Germany) and bovine serum albumin as standard (Bio-Rad).

Prenyltransferase assay

Assays were carried out in a total volume of 200 μl with 50 μM IPP and 50 μM DMAPP and a total protein range between 16 and 50 μg per assay. Control assays were done without IPP and DMAPP or were boiled before adding the substrates. Assays were incubated at 30 °C for 2 h, stopped by freezing in liquid nitrogen, and stored for up to 1 week before analysis. Proteins were denatured by adding 500 μl of chloroform and vortexing followed by centrifugation at 3000g for 5 min. For LC–MS/MS analysis, the water phase was transferred to a glass vial. Assays for radio-HPLC analysis were performed in the same way except that 50 μM [1-^{14}C]IPP was used as substrate.

LC–MS/MS analysis

Analysis of isoprenoid pyrophosphates was performed after a modified method of Miriyala and coworkers [20] on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). For separation, a ZORBAX Extended C-18 column (1.8 μm, 50 × 4.6 mm, Agilent Technologies, Santa Clara, CA, USA) was used. The mobile phase consisted of 5 mM ammonium bicarbonate in water as solvent A and acetonitrile as solvent B, with the flow rate set at 0.8 ml min^{-1} and the column temperature kept at 20 °C. Separation was achieved by using a gradient starting at 0% B, increasing to 10% B in 2 min, 64% B in 12 min, and 100% B in 2 min (1-min hold), followed by a change to 0% B in 1 min (5-min hold) before the next injection. The injection volume for samples and standards was 20 μl. The mass spectrometer was used in negative electrospray ionization mode. Optimal settings were determined using standards. Ion source gas 1 and gas 2 were set at 60 and 70 psi, respectively, with a temperature of 700 °C. Curtain gas was set at 30 psi, and collision gas was set at 7 psi, with all gases being nitrogen. Ion spray voltage

was maintained at −4200 V. Multiple reaction monitoring (MRM) transitions were *m/z* 312.9/79 for GPP, *m/z* 380.9/79 for (*E,E*)-FPP, and *m/z* 449/79 for (*E,E,E*)-GGPP. The same transitions were used for the *cisoid* isomers NPP, (*Z,E*)-FPP, and (*Z,Z*)-FPP, respectively. For further details of settings see supplementary material (Table S1). Data analysis was performed using Analyst 1.5 build 3385 software (Applied Biosystems).

Radio-HPLC analysis

Analysis was carried on an Agilent 1100 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to a Radiomatic 500TR series Flow Scintillation Analyzer (Canberra, Rüsselsheim, Germany), having a volume of 0.5 ml for the liquid flow cell. The same separation conditions were used as described under LC–MS/MS analysis. Scintillation fluid (Ultima-Flo AP, PerkinElmer, Rodgau, Germany) was provided in a 4:1 ratio to the column eluent.

Results and discussion

We developed a sensitive, accurate, and reproducible LC–MS/MS method for the detection and quantification of C$_{10}$, C$_{15}$, and C$_{20}$ prenyl diphosphates after *in vitro* assays of IDS activity in plant protein extracts. The products were detectable in a linear range from 2 to 270 pmol (GPP), 2 to 230 pmol [(*E,E*)-FPP], and 4 to 100 pmol [(*E,E,E*)-GGPP] per injection (Fig. 2). The limits of detection for GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP were 0.9, 1.1, and 1.9 pmol, respectively, determined at a signal-to-noise ratio of 1:3. The sensitivity of this nonradioactive method is in the same range as the radio-GC detection used by Martin and coworkers [5] but had a much lower degree of variation. The method is two orders of magnitude more sensitive than analysis by radio-HPLC (see Fig. 1 in supplementary material) and presumably is an even greater improvement over analysis using radio-TLC detection. Technical reproducibility was high, with repeated injections showing ⩽5% variation.

The new IDS assay was first tested on various organs of *A. thaliana* because there is already considerable knowledge on the terpene metabolism of this species. For volatile terpenes, flowers are described to be higher emitters of mono- and sesquiterpenes than leaves [21], suggesting that the levels of endogenous
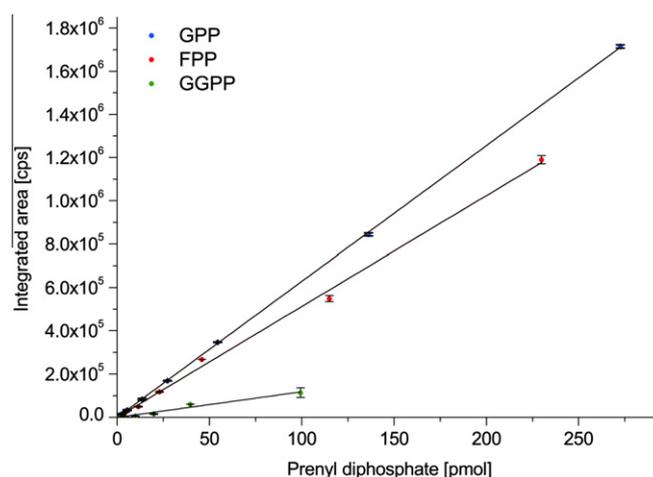

Fig.2. Calibration curves for quantification of GPP, FPP, and GGPP measured by LC–MS/MS under the conditions described. Each point was measured twice. R^2 for linear regression was 0.99 for GPP and FPP and was 0.96 for GGPP. At least three individual dilution series with two injections each were analyzed, and the variation was below 5%. Means ± standard deviations of at least three replicates are depicted.
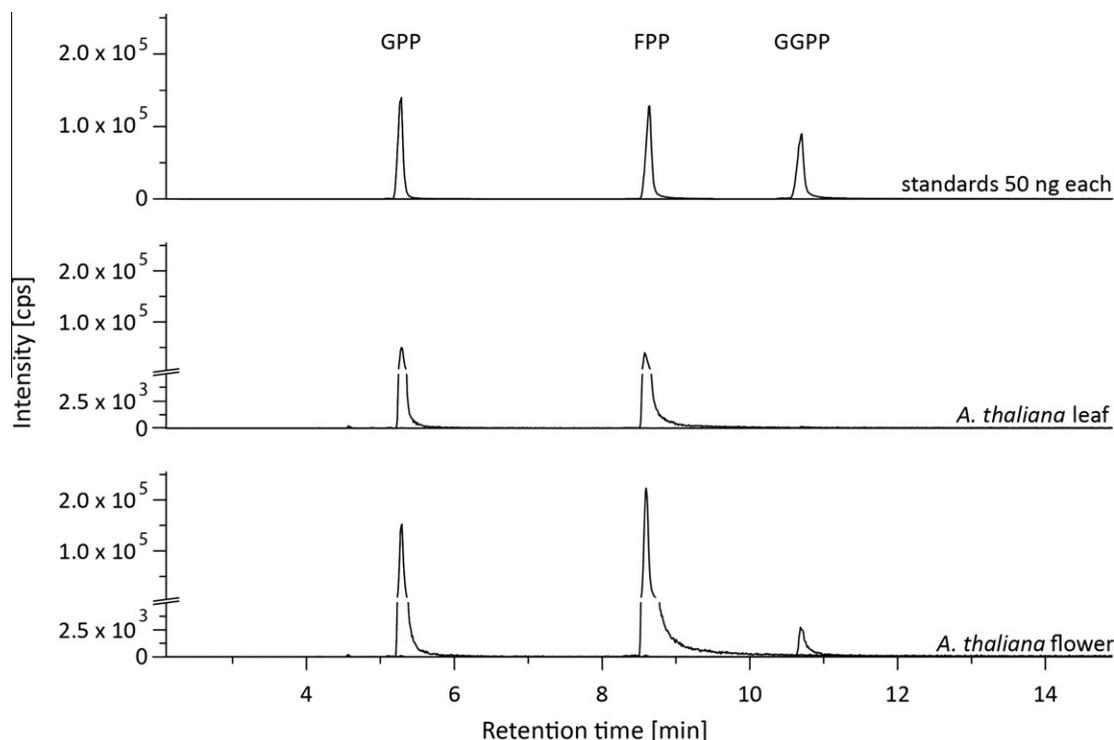
Fig.3. LC–MS/MS analysis of the products of IDS enzyme assays performed with crude protein extracts of *A. thaliana* leaves (middle panel) or flowers (lower panel). Products were identified by comparison with standards of 50 ng each of GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP (top panel). At least four different biological and two technical replicates of each sample were analyzed, and the standard deviations were below 6% for GPP and FPP and below 10% for GGPP.

Table 1
IDS activity determined in various plant species and organs.

Species	Organ	Activity (pmol h^{-1} µg total protein^{-1})					
		GPP		FPP		GGPP	
		Mean	SD	Mean	SD	Mean	SD
Arabidopsis thaliana	Leaf	3.55	0.07	4.62	0.22	Traces	
Arabidopsis thaliana	Flower	6.00	0.19	19.86	0.82	0.47	0.04
Populus trichocarpa	Leaf	2.75	0.15	9.94	0.58	0.94	0.02
Nicotiana attenuata	Leaf	2.19	0.08	4.05	0.11	Traces	
Picea abies	Needle	5.47	0.14	3.73	0.06	1.50	0.12
Picea abies	Bark	28.93	0.28	26.72	0.31	6.81	0.53

Note. SD, standard deviation.

terpenes and IDS activity might be higher in flowers. Using our methods, IDS activity was detectable for GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP in both organs. Flower extracts showed 4.3- and 1.6-fold higher levels of (*E,E*)-FPP and GPP formation, respectively, than those found in leaf extracts. (*E,E,E*)-GGPP was also easily detectable in assays of flower extracts, but only traces were found in assays of leaves (Fig. 3 and Table 1). The rate of (*E,E*)-FPP production determined for *A. thaliana* leaf extracts (4.62 pmol h^{-1} µg total protein^{-1}) is very similar to the rate of IDS activity previously found for these extracts by Manzano and coworkers (∼6 pmol h^{-1} µg total protein^{-1}) [4], but these authors measured enzyme activity by scintillation counting and so did not separate and identify the actual assay products. Control assays using boiled protein extracts or without any addition of the substrates showed no detectable products (data not shown).

To demonstrate that the method is not only useful for *A. thaliana*, the same protocol was used to assay IDS activity in extracts of other herbaceous and woody plant species, including both angiosperms and gymnosperms. Tissues investigated were leaves from a wild tobacco species (*N. attenuata*) and the black cottonwood (*P. trichocar-*

pa) as well as needles and bark of Norway spruce (*P. abies*) (Table 1). IDS activity was found for all samples. The bark of *P. abies* showed the highest activity for formation of GPP and (*E,E*)-FPP (28.9 and 26.7 pmol h^{-1} µg total protein^{-1}, respectively), whereas much lower rates were found for (*E,E,E*)-GGPP (6.8 pmol h^{-1} µg total protein^{-1}). The measured IDS activities in *P. abies* described by Martin and coworkers [5] were comparable at approximately 15, 10, and 13 pmol h^{-1} µg total protein^{-1} for GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP, respectively, although these authors used a different plant genotype and radio-GC for product detection. The much higher amounts of IDS activity compared with *A. thaliana* can be attributed to the storage of substantial amounts of monoterpene and diterpene products in the bark of *P. abies* as resin [5,22,23], a phenomenon not found in *A. thaliana*. In *N. attenuata*, the highest IDS activity was measured for GPP and (*E,E*)-FPP formation (2 and 4 pmol h^{-1} µg total protein^{-1}, respectively), whereas only traces were detected for (*E,E,E*)-GGPP, confirming the radio-GC-measured pattern found by Jassbi and coworkers that also gave the highest levels for FPP formation [24]. In *P. trichocarpa*, IDS activity levels were 3, 10, and 1 pmol h^{-1} µg total protein^{-1} [for GPP, (*E,E*)-FPP,
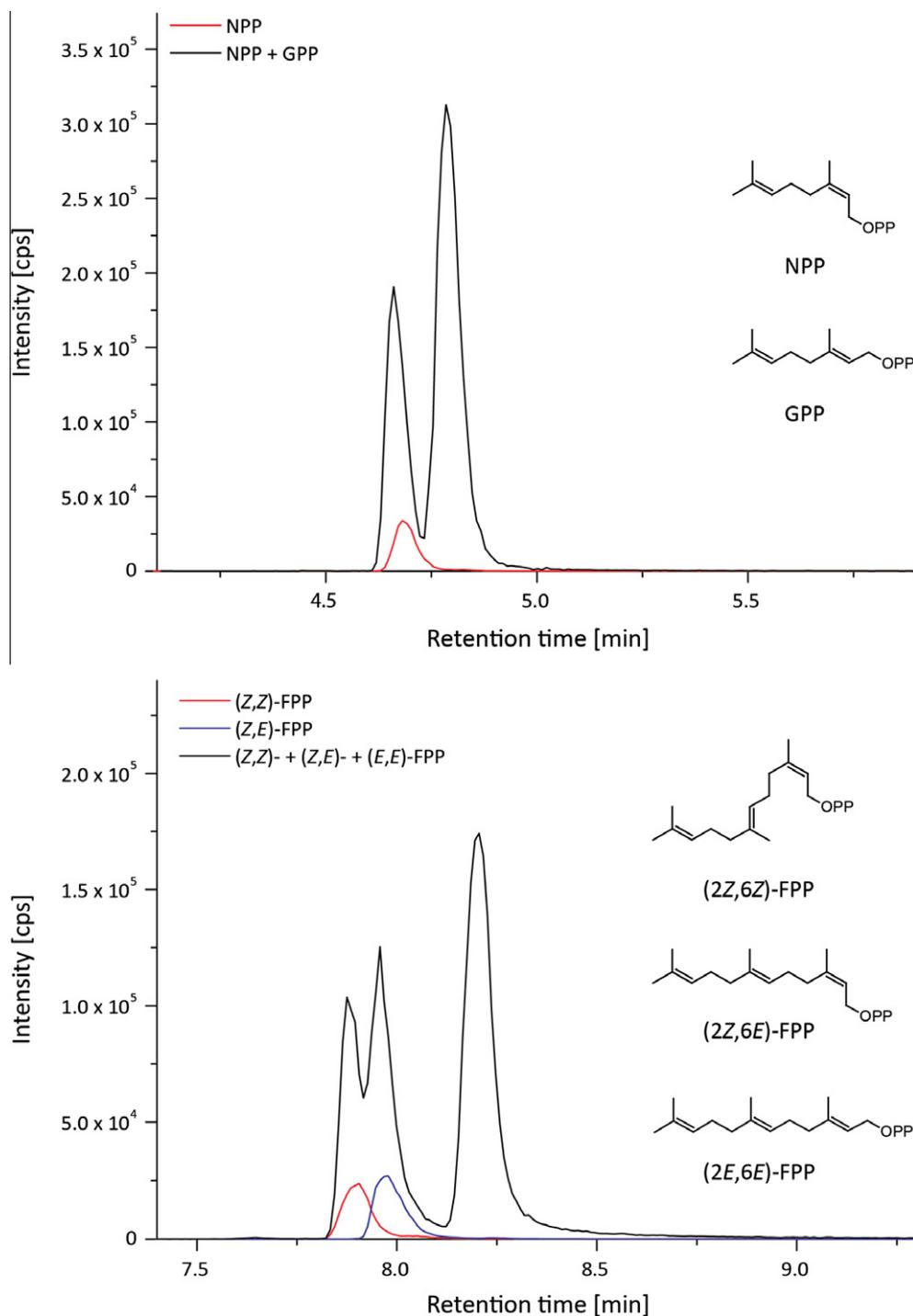
Fig.4. LC–MS/MS separation of short-chain *cisoid* and *transoid* prenyl diphosphate isomers. Separation among the C_{10} compounds, neryl diphosphate and geranyl diphosphate (top panel), and the C_{15} compounds, (*Z,Z*)-, (*Z,E*)- and (*E,E*)-farnesyl diphosphate (lower panel), are depicted. Chromatograms were obtained by coinjection of all isomers together or by individual injection of the *cisoid* isomers.

and (*E,E,E*)-GGPP, respectively] below the levels found in spruce but above tobacco. To our knowledge, this is the first time IDS has been measured in any poplar species. Although the emission rate of different terpene classes is known for poplar, these data cannot be compared with IDS enzyme activity due to different sampling techniques and variable measurement units [25,26].

The same protocol for measuring IDS activity was also successfully used for functional characterization of putative plant GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP synthases after heterologous expression in *E. coli* (data not shown). Although the C_{10}, C_{15}, and C_{20} pre-

nyl diphosphate products formed in these assays all were readily quantified, the C_5 substrates, IPP and DMAPP, eluted in a region of the chromatogram where ion suppression by buffer components prevented precise quantification. This limits the method to detection of C_{10}, C_{15}, and C_{20} prenyl diphosphates and excludes the C_5 prenyl diphosphates.

Given the recent discoveries of *cisoid* IDS products in plants, a new IDS assay will not gain wide acceptance unless it is able to distinguish between *cisoid* and *transoid* isomers among the various size classes of prenyl diphosphates. Under the measurement

conditions used, injections of mixtures of NPP and GPP as well as (Z,E)-, (Z,Z)-, and (E,E)-FPP in combination showed a clear, albeit not baseline, separation of these isomers (Fig. 4). Modifications, including smaller injection volumes, altered buffer composition, and a less steep solvent gradient did not improve separation of (Z,Z)- and (Z,E)-FPP. Nevertheless, the method can be effectively used to differentiate among the possible C_{10} and C_{15} prenyl diphosphate isomers occurring as IDS products.

Researchers of plant terpenoid metabolism have begun to devote increased attention to IDS enzymes because of their role in directing and modulating flux among the various branches of terpenoid biosynthesis. Our new, nonradioactive, LC–MS-based method of measuring IDS activity obviates the need for dephosphorylation of the assay products prior to analysis and, thus, should increase the speed, precision, and reliability of activity measurements without sacrificing the sensitivity of radio-GC detection. LC–MS detection of prenyl diphosphates could also be valuable in quantifying these biosynthetic intermediates *in vivo* to determine how flux is regulated among the different branches of terpene biosynthesis.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.12.037.

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