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Analysis of the isoprenoid pathway intermediates, dimethylallyl diphosphate and isopentenyl diphosphate, from crude plant extracts by liquid chromatography tandem mass spectrometry

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Abstract

Objective: We sought to develop a sensitive and accurate analytical method for the detection and quantification of IDP and DMADP as well as their monophosphate derivatives in crude plant extracts.

Methods: A liquid chromatography method coupled to tandem mass spectrometry (LC–MS/MS) with multiple reaction monitoring (MRM) was established to measure the amounts of IDP and DMADP down to low picogram levels, which was linear over at least three orders of magnitude. Extracts were enriched using an anion exchanger, and chromatographic separation was achieved using a β-cyclodextrin column. A S-thiolodiphosphate analog of DMADP was employed as an internal standard.

Results: Dilution series of authentic compounds were used to determine the limits of detection and quantification for IDP, DMADP and their corresponding monophosphates. A survey of plant species producing varying amounts of isoprenoids showed a corresponding variation in IDP and DMADP with the ratio of DMADP/IDP ranging from 4:1 to 2:1. Trace levels of isopentenyl monophosphate (IP) and dimethylallyl monophosphate (DMAP) were also detected.

Conclusion: The LC–MS/MS method described enables absolute quantification of *in planta* levels of IDP and DMADP for the first time. The method is also suitable for analysing bacterial and animal samples as well as enzyme assays.

KEYWORDS

4-hydroxy-3-methylbut-2-enyl diphosphate reductase, dimethylallyl diphosphate, isopentenyl diphosphate, isopentenyl diphosphate isomerase, isoprenoids, methyl-D-erythritol 4-phosphate pathway, terpenes

1 | INTRODUCTION

Isoprenoids are the most functionally and structurally diverse group of natural products with more than 65000 different compounds described to date from all forms of life and originate from the head-to-tail condensation of the five-carbon precursors, isopentenyl diphosphate (IDP) and its allylic isomer dimethylallyl diphosphate (DMADP).[1–5] Depending on the number of C_5-building blocks they contain, isoprenoids are classified as hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}) or polyterpenoids (C_n).[6] Hemiterpenoids are formed from IDP or DMADP

directly, like isoprene or 2-methyl-but-3-en-2-ol (2,3,2-MBO).[7,8] Dephosphorylation of IDP and DMADP to their corresponding alcohols can be catalysed by different phosphatases, leading to isoprenol (3-methyl-3-butenyl-1-ol) and prenol(3-methyl-2-butenyl-1-ol), respectively.[9] Dephosphorylation to the corresponding monophosphates, isopentenyl monophosphate (IP) and dimethylallyl monophosphate (DMAP), also occurs catalysed by Nudix hydrolases.[10] In combination with rephosphorylation to IDP and DMADP by isopentenyl phosphate kinases (IPKs; EC: 2.7.4.26), this may regulate the supply of isoprenoid building blocks.[11,12]

Condensation of IDP and DMADP gives geranyl diphosphate (GDP), and further addition of IDP moieties to GDP leads to farnesyl diphosphate (FDP), geranylgeranyl diphosphate (GGDP) and geranylfarnesyl diphosphate (GFDP).[13] These intermediates can be converted to different classes of sesquiterpenes, diterpenes, triterpenes and polyterpenoids like phytol, gibberellins, sterols, brassinosteroids, and dolichols (Figure 1).

IDP and DMADP in plants are synthesised by two distinctive pathways, the mevalonate (MEV) pathway found in the cytosol, and the methylerythritol 4-phosphate (MEP) pathway found in the plastids.[14] Whereas the MEV pathway only produces IDP, the MEP pathway synthesises both IDP and DMADP catalysed by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR; EC: 1.17.7.4).[15–19] IDP and DMADP can be interconverted by isopentenyl diphosphate isomerase (IDI; EC: 5.3.3.2).[20] Although both pathways are spatially separated in plants, a bidirectional transport mechanism has been described that supplies the cytosol with plastid derived IDP and vice versa.[21] Because of their importance for the production of so many different isoprenoids, the intracellular concentrations of IDP and DMADP are likely to be carefully regulated. Weise *et al.* demonstrated, that plastidial DMADP pools change according to circadian rhythm and are reduced in the dark.[22] IDP concentrations are also likely to vary upon different stimuli, but this has never been reported to date.

Several analytical methods using radioactive labelled precursors have been described for determining the amounts of IDP and DMADP in plant extracts or enzyme preparations. Detection and quantification of individual compounds has been carried out by using either thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) coupled to a radio detector, but chromatographic separation or absolute quantification has never been demonstrated.[23 24]

Also, other methods have been developed to derivatise IDP and DMADP for analysis. DMADP but not IDP can be degraded by strong acids to isoprene which can then be quantified by using gas chromatography coupled to a reduction gas detector (GC–MS).[25–27] This

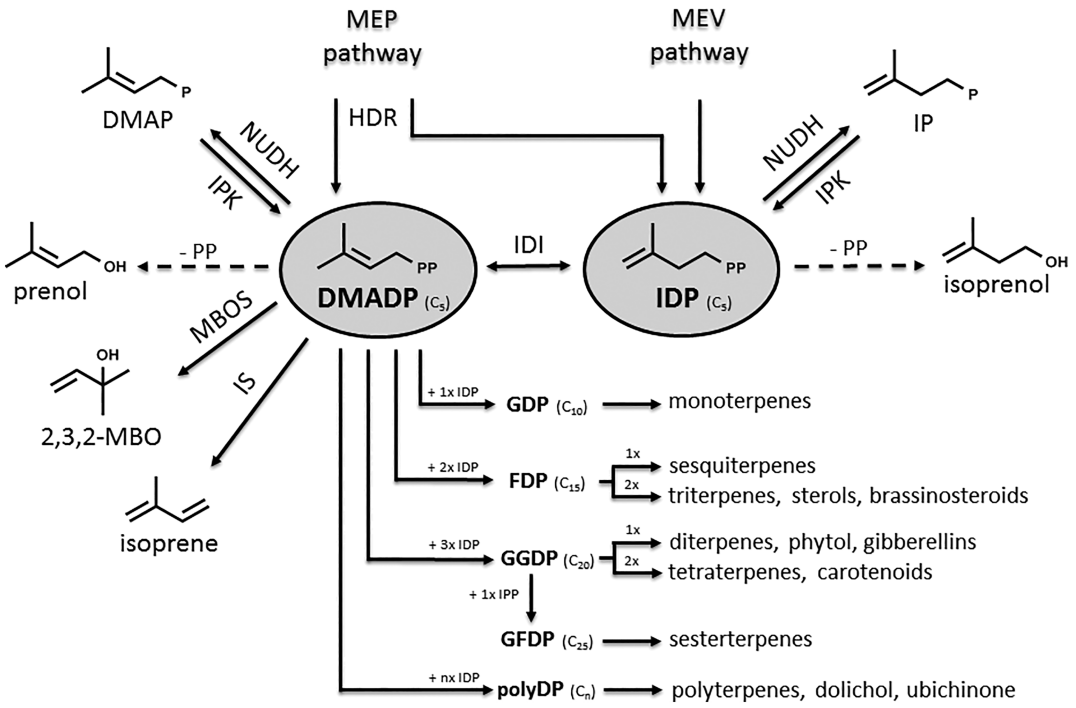

FIGURE 1 The central roles of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) in plant isoprenoid biosynthesis. The mevalonate (MEV) pathway produces IDP, while the methylerythritol 4-phosphate (MEP) pathway produces both IDP and DMADP, which then can be interconverted by isopentenyl diphosphate isomerase (IDI; EC: 5.3.3.2). The diphosphates can be further transformed to the monophosphates dimethylallyl monophosphate (DMAP) and isopentenyl monophosphate (IP), catalysed by Nudix hydrolases (NUDH; EC: 3.6.1.-), which can then be rephosphorylated back to the diphosphates via isopentenyl phosphate kinases (IPKs; EC: 2.7.4.26). The diphosphates can also be hydrolysed to the corresponding alcohols (dashed arrow). DMADP can be transformed into 2-methyl-3-buten-2-ol (2,3,2-MBO) via 2,3,2-MBO synthase or isoprene via isoprene synthase (EC: 4.2.3.27). The C_{10} intermediate geranyl diphosphate (GDP), the C_{15} farnesyl diphosphate (FDP), and the C_{20} geranylgeranyl disphosphate (GGDP) are produced by condensing one molecule of DMADP with up to three units of IDP. Larger molecules are summarised as polyprenyl disphosphates (polyDP). These diphosphates serve as precursors for many complex terpenoid structures

procedure was further improved by using recombinant isoprene synthase to replace the acidification, but IDP remained unquantifiable.[28] Another derivatisation method exploits the ability to convert IDP and DMADP to their corresponding alcohols isoprenol and prenol by dephosphorylation using alkaline phosphatase.[9] These alcohols can be further identified by GC–MS techniques. Unfortunately, this method has never been established for quantitative analysis.[29] A method for quantifying the plastidic DMADP pool involves measuring 'postillumination isoprene emission'.[30] This technique exploits the fact that upon moving plants into the dark, IDP and DMADP production ceases, but isoprene is still emitted in proportion to the size of the plastidial DMADP pool. However, the total pools of DMADP as well as IDP are not measured.

Liquid chromatography tandem mass spectrometry (LC–MS/MS) methods have already been applied to the measurement of several isoprenoid diphosphates in plants, such as GDP, FDP, and GGDP using reversed phase separation.[22,31–33] Unfortunately, quantification of IDP and DMADP has not yet been possible due to the overlap in their retention times on standard C_{18} columns. About 20 years ago, Lange *et al*. were able to separate IDP and DMADP extracted from the glandular trichome cells of peppermint (*Mentha* × *piperita*), by using β-cyclodextrin as the stationary phase.[34] Application of various metabolic inhibitors demonstrated changes in the DMADP/IDP ratios, but absolute quantification was not attempted. In addition, these authors measured the signals of the extracted $[M\text{-}H]^-$ molecular ions according to the technological standard of that time, whereas now MS/MS detection in multiple reaction monitoring (MRM) mode would greatly increase the sensitivity of this method.

In this study, we established an improved method for quantifying the levels of IDP and DMADP from plant tissue with LC–MS/MS. The use of a cyclodextrin column gave baseline resolution of these two isomers, while MRM modes provided much higher sensitivity and better selectivity compared to previous work. Absolute quantification of IDP and DMADP from material of several plant species and organs is presented to demonstrate the robustness of the method. Additionally, the monophosphates IP and DMAP were quantified because of their newly suggested roles as isoprenoid intermediates.[11] The ability to rapidly quantify IDP and DMADP in parallel should accelerate progress in studying how isoprenoid biosynthesis is regulated *in vivo* and how these pathways can be manipulated.

2 | EXPERIMENTAL

2.1 | Chemicals

IP and IDP where purchased as lithium salts and DMAP and DMADP as ammonium salts from Sigma-Aldrich (Munich, Germany). Dimethylallyl *S*-thiolodiphosphate (DMASDP) triammonium salt standard was from Echelon Biosciences Inc. (Salt Lake City, UT, USA), ammonium acetate from Carl Roth (Karlsruhe, Germany) and HPLC-grade methanol and acetonitrile from VWR (Darmstadt, Germany).

2.2 | Plant material

Arabidopsis thaliana (Col-0) plants were grown from seeds in a climate chamber [22°C, 55% relative humidity, and 100 μmol/m^2/s photosynthetically active radiation (PAR)] for two weeks under long day conditions (16 h:8 h light/dark) until flower formation. *Populus canescens* (clone INRA 717) and *Populus trichocarpa* [clone 606 (NW-FVA, Hann. Münden, Germany)] were propagated from stem cuttings and were grown in a climate chamber (24°C, 60% relative humidity, 100 μmol/m^2/s PAR, and 16 h:8 h light/dark) till they reached a height of 1 m. *Nicotiana attenuata* leaves were a kind gift of Dr Klaus Gase (MPI for Chemical Ecology, Jena, Germany). Leaves of the plants mentioned earlier were detached, immediately frozen in liquid nitrogen, grounded to a fine powder by using mortar and pestle and stored at −80°C. *Picea abies* tissue was harvested from clone 3369-Schongau (Samenklenge und Pflanzgarten, Laufen, Germany) planted out originally as 1-year-old seedlings in Jena, Germany, in 2003. Needles and young stem tissue were separated in September 2018 and stored at −80°C after grinding.

2.3 | Extraction of IDP/DMADP and IP/DMAP

Based on the protocol of Nagel *et al*.[35] ground plant material (leaves, needles or stem tissue; 100–500 mg) including DMASDP (0.5 μg) as an internal standard was extracted three times with methanol/water (5 mL, 7:3, *v*/*v*) by vortexing (30 min, 4°C) followed by centrifugation (15 min, 4°C, 4000 × *g*, Beckman Avanti J-20XP) (Beckman, Krefeld, Germany). Extracts were combined, and water (5 mL) was added to reach a methanol/water ratio of 1:1 (*v*/*v*). Extracts were purified by solid-phase extraction (SPE) using Chromabond HR-XA SPE cartridges (150 mg, 6 mL, Macherey-Nagel, Düren, Germany), conditioned with methanol (5 mL) followed by water (5 mL). After application of extracts, columns were washed with water (4 mL) followed by methanol (5 mL). The monophosphates and diphosphates were eluted with 1 M ammonium acetate in methanol (3 mL), evaporated under a stream of nitrogen to dryness, and dissolved in methanol/water 1:1 (100 μL, *v*/*v*). Due to the high salt content, the final volume varied between 150 and 250 μL. Therefore, the exact volume was determined with a 500 μL Hamilton® syringe for proper calculation of analyte concentration (V_a; see equations 3 and (4)). After initial extraction of plant material (100 mg), *Populus trichocarpa* needed to be extracted with 300 mg and *A. thaliana* with 500 mg to obtain adequate amounts of the targeted compounds for accurate quantification.

2.4 | LC–MS/MS analysis

Quantification was done on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 5000 triple-quadrupole mass spectrometer (AB Sciex Instruments, Darmstadt, Germany). For separation, an Astec® Cyclobond® I 2000 column (4.6 mm × 250 mm, 5 μm; Supelco, Bellefonte, PA, USA) was used.

The mobile phase consisted of 50 mM ammonium acetate in water (pH 6.5; solvent A) and acetonitrile (solvent B), with the flow rate set at 1.0 mL/min, and the column temperature kept at 20°C. The column was conditioned according to the manufacturer's protocol prior to use. Separation was achieved by using a gradient starting at 20% A held for 15 min, increasing to 60% A in 15 min, held for 7 min, and a change back to 20% A, held for 10 min. The injection volume for samples and standards was 5 μL; autosampler temperature was 4°C. The mass spectrometer was used in the negative ionisation 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 (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 −4200 V. DMASDP was used as an internal standard. MRM was used to monitor analyte parent-ion-to-product-ion formation. The following parameters were used: IDP/DMADP: *m/z* (Q_1): 245; *m/z* (Q_3): 79; declustering potential (DP): −45.0 V; collision energy (CE): −24.0 V. IP/DMAP: *m/z* (Q_1): 165; *m/z* (Q_3): 79; DP: −45.0 V; CE: −24.0 V. DMASDP: *m/z* (Q_1): 261; *m/z* (Q_3): 79; DP: −60.0 V; CE: −36.0 V. Retention times (RTs) were 24.4 min (DMAP), 25.6 min (IP), 30.9 min (DMADP), 32.5 min (IDP) and 32.6 min (DMASDP) (Figure 2). Data analysis was performed using Analyst Software 1.6.3 Build 1569 (AB Sciex Instruments, Framingham, MA, USA).

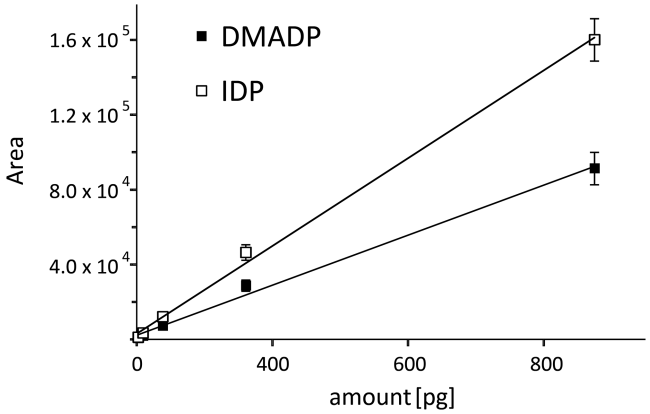

FIGURE 3 Calibration curves for the quantification of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) over a range of 4 to 1350 pg. Linear regression resulted in the following equations: IDP: $y = 117.2x + 3123.1$ ($R^2 = 0.998$), DMADP: $y = 66.8x + 2301.5$ ($R^2 = 0.994$). Values are given as mean ± standard deviation of three independent dilution series measured by LC–MS/MS

3 | RESULTS AND DISCUSSION

3.1 | Method development

We first optimised the LC–MS/MS analysis of IDP and DMADP by establishing suitable MRM modes, and then evaluated the sensitivity of detection by making serial dilutions of pure standards. A linear range of 4 to 1350 pg was measured in three independent dilution series (Figure 3). The limit of detection (LOD) was found to be 3.9 pg and 3.6 pg for IDP and DMADP, respectively, determined at a signal-to-noise ratio of 1:3.3 using equation 1:

$$\text{LOD} = 3.3 \cdot \frac{\text{standard deviation of the blank}}{\text{the slope of the regression line}} \quad (1)$$

The limit of quantification (LOQ) was further determined at a signal-to-noise ratio of 1:10 using equation 2:

$$\text{LOQ} = 10 \cdot \frac{\text{standard deviation of the blank}}{\text{the slope of the regression line}} \quad (2)$$

For quantification, linear regression equations of the respective standard curves were used for IDP (equation (3)) and for DMADP (equation (4)), which are shown in Figure 3:

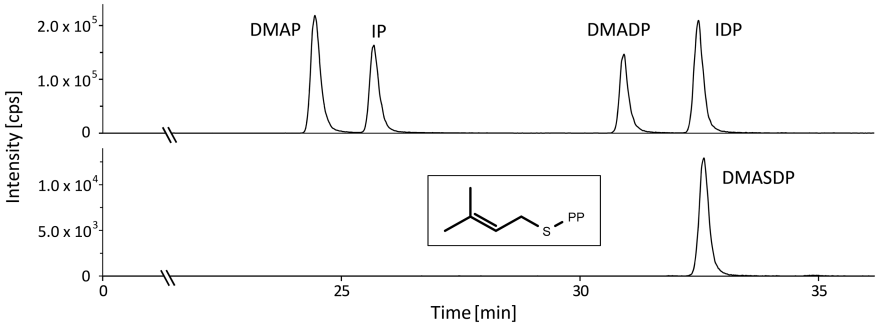

FIGURE 2 LC–MS/MS chromatograms showing separation and quantification of C_5 prenylated monophosphates and diphosphates. Depicted are multiple reaction monitoring (MRM) modes with *m/z* 165 79 for dimethylallyl monophosphate (DMAP) and isopentenyl monophosphate (IP), and *m/z* 245 79 for isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (upper panel) and the MRM of *m/z* 261 79 for the thiodiphosphate internal standard (lower panel). Injected quantities were 5 ng of dimethylallyl *S*-thiolodiphosphate (DMASDP), IP and DMAP and 2.5 ng of IDP and DMADP. Elution was at 24.5 min (DMAP), 25.7 min (IP), 30.9 min (DMADP), 32.5 min (IP), and 32.6 min (DMASDP)

$$c_{\mathrm{IDP}} = \frac{(A_p - 3123.1) \cdot V_a}{117.2 \cdot m_p \cdot V_i} \quad (3)$$

and

$$c_{\mathrm{DMADP}} = \frac{(A_p - 2301.5) \cdot V_a}{66.8 \cdot m_p \cdot V_i} \quad (4)$$

where c_{IDP} is the concentration of IDP [in pg/mg fw (fresh weight)], c_{DMADP} is the concentration of DMADP [in pg/mg fw (fresh weight)], A_{p} the peak area (in units), the volume after extraction (in microlitres), V_{i} the volume injected into the HPLC–MS/MS (in microlitres) and m_{p} the mass of plant material used for extraction (in milligrams).

Next, extraction efficiency was examined, which showed that at least two extractions with the methanol/water (7:3, *v*/*v*) mixture were necessary to collect 90 ± 11% of the plant pools of IDP and DMADP while a third extraction increased recovery to 96 ± 13%. Six more extractions were assumed to yield the full pool (Supporting Information Figure S1A). Spiking plant material with IDP and DMADP (1 μg each) showed the same recovery, except that more of the spiked metabolites were obtained from the first extraction (Figure S1B). The accuracy resulting from varying amounts of plant material was addressed by analysing plant extracts in the range from 1 to 9 μL. Linear correlations of R^2 = 0.989 and = 0.997 for IDP and DMADP, respectively, were observed, indicating the independence of the method from varying sample weights or amounts of extracts in this range. Reproducibility and precision of the measurements were tested by multiple injections of standards (2.5 ng IDP or DMADP each) in inter-day and intra-day experiments, resulting in a standard deviation of less than 3%.

The internal standard DMASDP overlapped chromatographically with IDP (RT 32.6 min), but was separated by its individual MRM trace in the mass spectrometer. Therefore, DMASDP was a suitable control for the analytical separation and detection. Figure 4 shows an exemplary chromatogram from *Picea abies* needle material. One additional plant-derived compound shows the same MRM as IDP and DMADP and elutes at 20.5 min. It also appeared in the other plant species analysed, but never interfered with IDP and DMADP quantification.

3.2 | Species survey

Several plant species were chosen to investigate the potential of this method, since these may differ in the sizes of their IDP and DMADP pools. The model species thale cress (*A. thaliana*) and coyote tobacco (*N. attenuata*) are known to have a low abundance of terpenoid natural products,[36,37] while the western balsam-poplar *Populus trichocarpa* and the grey poplar *Populus canescens*, produce high amounts of the hemiterpene isoprene.[38] Additionally, we included the conifer Norway spruce *Picea abies*, which produces an abundant mixture of monoterpenes, sesquiterpenes and diterpenes as a resin.[39]

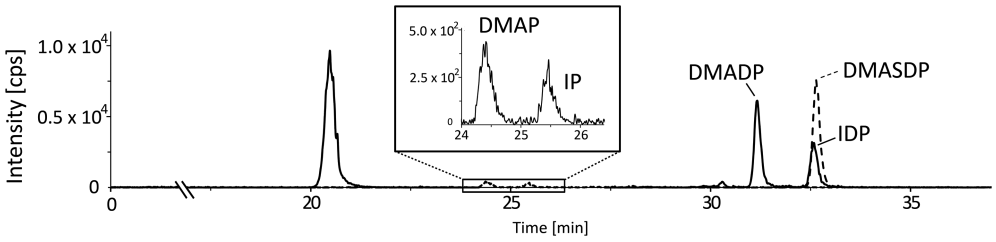

FIGURE 4 LC–MS/MS chromatogram of C_5 isoprenylated monophosphates and diphosphates extracted from *Picea abies* needles. Dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) show retention times of 30.9 min and 32.5 min, respectively, with the solid line showing the multiple reaction monitoring (MRM) mode of *m*/*z* 245 79. The internal standard dimethylallyl *S*-thiolodiphosphate (DMASDP), eluting at 32.6 min, overlaps with IDP (dashed line depicts, MRM mode of *m*/*z* 261 79). The corresponding monophosphates eluted at 24.5 min (DMAP) and 25.7 min (IP); depicted are the MRM mode of *m*/*z* 165 79 (enlarged in the inset). These compounds were detectable in *Picea abies* needles (though below the limit of quantification), but not in the other samples analysed. A plant-derived compound with the same MRM as the diphosphates appeared at 20.5 min

TABLE 1 Content of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) in different plant species and organs sampled.

		Content (pg/mg fresh weight)				Ratio (DMADP/IDP)	
		DMADP		IDP			
Species	Tissue	Mean	SD	Mean	SD	Mean	SD
Arabidopsis thaliana	Leaf	3.3	3.0	1.1	0.3	3.0	0.9
Populus canescens	Leaf	113.8	3.4	33.2	1.5	3.4	0.1
Populus trichocarpa	Leaf	21.7	1.3	5.2	0.7	4.2	0.4
Nicotiana attenuata	Leaf	109.5	3.0	32.2	1.2	3.4	0.2
Picea abies	Needle	612.8	8.7	303.7	9.6	2.1	0.1
Picea abies	Stem	50.1	7.5	16.2	4.5	3.2	0.3

Note: SD, standard deviation; $n = 3$.

The lowest abundance of DMADP and IDP of all tested species was in *A. thaliana* with 3.3 pg/mg fw DMADP and 1.1 pg/mg fw IDP, the latter value below the quantification limit (LOQ) and thus of uncertain precision (Table 1). Next came *Populus trichocarpa* with 21.7 pg/mg fw DMADP and 5.2 pg/mg fw IDP. *Nicotiana attenuata* and *Populus canescens* show nearly similar levels of both compounds with 109.5 pg/mg fw DMADP and 32.2 pg/mg fw IDP and 113.8 pg/mg fw DMADP and 33.2 pg/mg fw IDP, respectively. Needles of *Picea abies* accumulated 612.8 pg/mg fw of DMADP and 303.7 pg/mg fw of IDP. Stem tissue showed a lower amount with 50.1 pg/mg fw of DMADP and 16.2 pg/mg fw of IDP. Among all species the ratio of DMADP/IDP differed between 4.2:1 and 2.1:1, but always showed an excess of DMADP.

Previous measurements of plant DMADP/IDP ratios were only made for heterologously expressed enzymes assayed *in vitro*. IDI upon reaching substrate-product-equilibrium, showed excess DMADP giving DMADP/IDP ratios ranging from 2:1 to 7:1,[40–42] but since substrate was added for these assays, they are not likely to represent natural concentrations. The DMADP/IDP ratio is also influenced by HDR, the last enzyme of the MEP pathway. For this enzyme, *in vitro* DMADP/IDP ratios of 1:5 to 1:7 have been reported,[43–46] while an *in vivo* measurement of 15:85 was obtained for tobacco BY2 cells.[47] Since, *in vivo* and *in vitro* measurements were consistent in the ratio of DMADP/IDP, protein concentration in *in vitro* enzyme assays did not affect concentrations of the prenyl diphosphates produced. Nevertheless, the correlation of the ratios of *in vivo* DMADP and IDP pools and those obtained from *in vitro* enzyme assays and metabolic processes is not always high and deserves more attention in the future. Using the method we developed, we were able to show that *in planta* the levels of DMADP are clearly elevated compared to those of IDP, suggesting that IDI, in contrast to the HDR, has more influence on the actual ratio between these two prenyl diphosphate intermediates in the plant.

Plants were additionally screened for IP and DMAP, since IP has recently been identified as a metabolite of IDP associated with the MVA pathway,[11] and IP might be more readily involved in intracellular transport than its diphosphate analogue. Depending on the conversion rate between IP and IDP, their pool sizes could differ in a considerable manner. Using standard curves, the LOD and LOQ for IP and DMAP was determined to be 7 and 20 pg, respectively. In the samples used in this experimental work, IP and DMAP were detected only in *Picea abies* needles (Figure 4), but were below the LOQ. To our knowledge, this is the first time that these metabolites have been detected in extracts of plant material. DMAP appeared to be present in an amount twice as high as IP (Figure 4, inset). The fact that spruce needles showed the highest content of the corresponding diphosphates is consistent with this being the only sample in which monophosphates were detected, and suggests that the monophosphates are usually present in concentrations several orders of magnitude less than the diphosphates. That monophosphates are direct degradation products of the diphosphates catalysed by phosphatases is supported by the ratios detected for DMADP/IDP and DMAP/IP, which are both 2:1.

The separation of IDP and DMADP using β-cyclodextrin as the stationary phase and ammonium acetate in water/acetonitrile as mobile phase, and detection via HPLC/MS–MS offers significant improvement over the analytical methods used in the past. For the first time, absolute quantification of these metabolites is possible from plant samples with the LOD shown to be below 4 pg for both compounds. The use of *S*-thiolodiphosphate DMADP-analogue (DMASDP) as an internal standard enabled comparisons among different plant tissues and sample types. Since all organisms biosynthesise terpenoids, the method should find wide application in microbiology, medicine, ecology, and biochemistry. The quantification of IDP and DMADP may shed new light on disease processes and the response of organisms to biotic and abiotic stresses.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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