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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.

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 [^{14}C]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.

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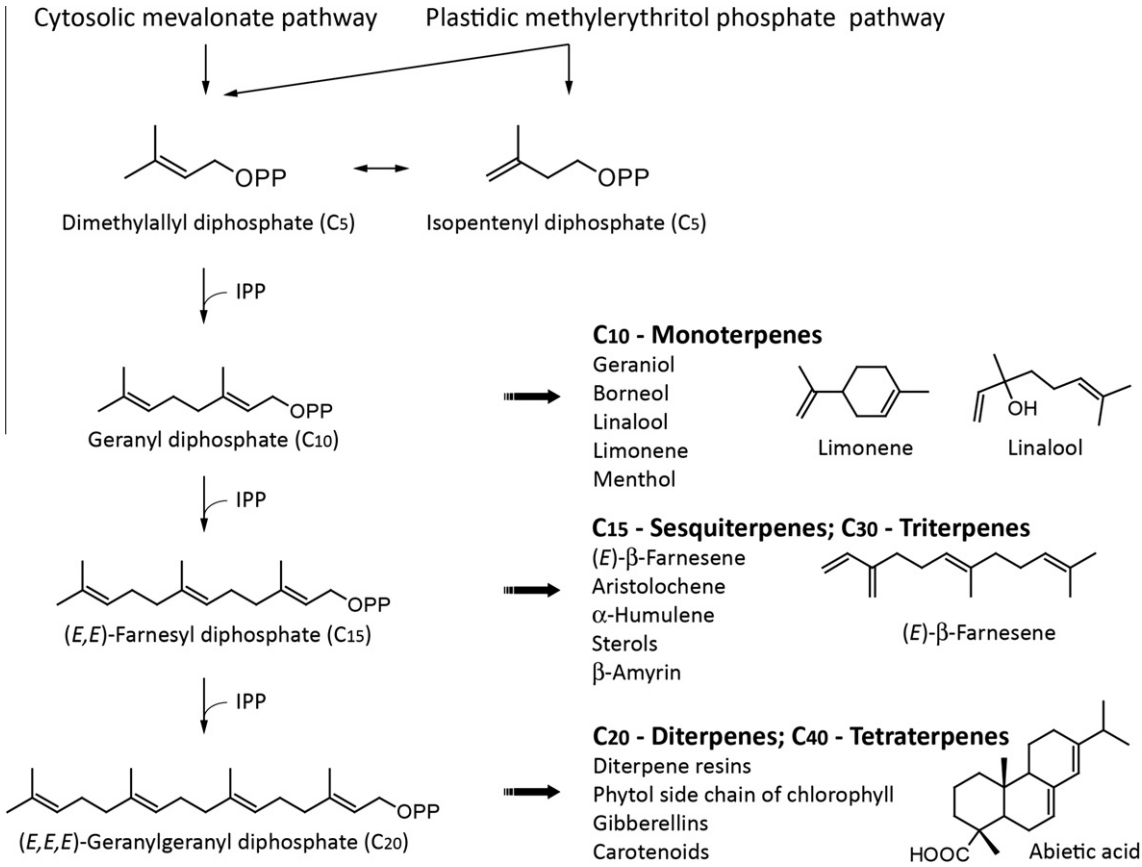

Fig.1. Outline of terpenoid biosynthesis. The schematic diagram depicts the reactions carried out by short-chain isoprenyl diphosphate synthases (IDSs). The five-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), produced either by the cytosolic mevalonate pathway 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