Analytical Biochemistry xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase

Changfang Zhou [a,b], Ziru Li [b], Amy E. Wiberley-Bradford [b,c], Sean E. Weise [b], Thomas D. Sharkey [b,*]

[a] School of Life Science, Nanjing University, Nanjing 210093, China
[b] Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA
[c] USDA–ARS–Vegetable Crops Research Unit, University of Wisconsin–Madison, Madison, WI 53706, USA

ARTICLE INFO

Article history:
Received 5 May 2013
Received in revised form 24 May 2013
Accepted 25 May 2013
Available online xxxx

Keywords:
Dimethylallyl diphosphate (DMADP)
Isomerase equilibrium
Isopentenyl diphosphate (IDP)
Isoprenoids
Recombinant enzyme

ABSTRACT

Isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP) are building units for all isoprenoids; thus, intracellular pool sizes of IDP and DMADP play important roles in living organisms. Several methods have been used to quantify the amount of DMADP or the combined amount of IDP plus DMADP, but measuring the DMADP/IDP ratio has been difficult. In this study, a method was developed to measure the ratio of DMADP/IDP. Catalyzed by a recombinant IDP isomerase (IDI) together with a recombinant isoprene synthase (IspS), IDP was converted to isoprene, which was then detected by chemiluminescence. With this method, the in vitro equilibrium ratio of DMADP/IDP was found to be 2.11:1. IDP and DMADP pools were significantly increased in *Escherichia coli* transformed with methylerythritol 4-phosphate pathway genes; the ratio of DMADP/IDP was 3.85. An *E. coli* strain transformed with IspS but no additional IDI had a lower DMADP level and a DMADP/IDP ratio of 1.05. Approximately 90% of the IDP and DMADP pools in light-adapted kudzu leaves were light dependent and so presumably were located in the chloroplasts; the DMADP/IDP ratios in chloroplasts and cytosol were the same as the in vitro ratio (2.04 in the light and 2.32 in the dark).

© 2013 Elsevier Inc. All rights reserved.

Isoprenoids (also called terpenoids) are the most functionally and structurally varied group of plant metabolites [1–3]. Based on numbers of the five-carbon units, the isoprenoid family is classified into hemiterpenes (1), monoterpenes (2), sesquiterpenes (3), diterpenes (4), triterpenes (6), tetraterpenes (8), and polyterpenes (>8) [1]. The simplest isoprenoid, isoprene, contributes one-third of the annual global emissions of volatile organic compounds, with more than 90% from terrestrial plant foliage and the rest from microbes, animals (including humans), and aquatic organisms [4,5].

Isopentenyl diphosphate (IDP)[1] and its isomer dimethylallyl diphosphate (DMADP) are the basic building blocks for all of the isoprenoids in living organisms [1,6] (Fig. 1). The mevalonic acid (MVA) pathway in a few bacteria, yeast, animals, and the cytosol of plants uses acetyl coenzyme A to synthesize IDP, which is then isomerized to DMADP. Common products of the MVA pathway include cholesterol, sesquiterpenes, and plant cytokinins [7,8]. The methylerythritol 4-phosphate (MEP) pathway in most bacteria, green algae, and plant plastids synthesizes IDP and DMADP simultaneously from hydroxymethyl-butenyl 4-diphosphate (HMBDP). Common products of the MEP pathway include monoterpenes and carotenoids [9–12]. The most abundant product of the MEP pathway is isoprene produced by many (but not all) plants. Isoprene production can be correlated with isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2), and so the interconversion of IDP and DMADP is important and potentially limiting, especially for isoprene synthesis [13,14].

Isoprenoid synthesis makes use of allylic diphosphate chemistry. Removal of the diphosphate results in a highly reactive carbocation that leads to a variety of reactions [15]. Isoprene is made directly from DMADP, whereas higher isoprenoids are made by adding IDP to DMADP, resulting in a new allylic diphosphate (e.g., geranyl diphosphate). The ratio of DMADP/IDP required for synthesis of isoprenoids varies from 100% DMADP (isoprene) to nearly all IDP (e.g., rubber) [1,6,16]. Despite the importance of DMADP and IDP, little is known about their concentrations and ratios in living organisms. In plants, the situation is particularly complex because the MEP pathway and the MVA pathway are in separate cellular compartments, the plastids and cytosol. The IDP and DMADP produced by the MEP and MVA pathways have sepa-

* Corresponding author. Fax: +1 517 353 9334.
E-mail address: tsharkey@msu.edu (T.D. Sharkey).

[1] *Abbreviations used:* IDP, isopentenyl diphosphate; DMADP, dimethylallyl diphosphate; MVA, mevalonic acid; MEP, methylerythritol 4-phosphate; HMBDP, hydroxymethyl-butenyl 4-diphosphate; IDI, isopentenyl diphosphate isomerase; LC–MS/MS, liquid chromatography followed by tandem mass spectrometry; IspS, isoprene synthase; GGDP, geranylgeranyl diphosphate; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; Ni-NTA, nickel–nitrilotriacetic acid; GC, gas chromatography.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.05.028

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

UNCORRECTED PROOF

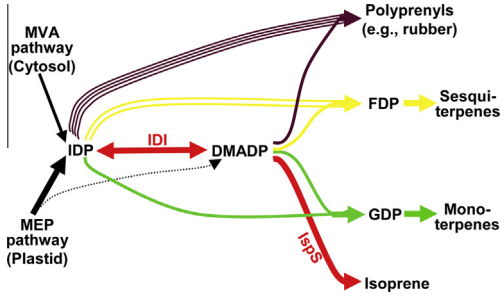

Fig.1. Isomerization of IDP and DMADP and their central positions in isoprenoid metabolism. The path from the MEP pathway to isoprene and the role of IDI are emphasized. (Redrawn from Ref. [16]).

rate functions, and so it is not only the pool sizes of IDP and DMADP but also their locations in cells and relative ratios that need to be known.

Several methods have been developed to measure DMADP levels in organisms, including isotope labeling [17], hydrolysis with alkaline phosphatase or acid [18–21], measuring with liquid chromatography followed by tandem mass spectrometry (LC–MS/MS) [22], and indirect methods based on isoprene emission patterns [23]. Our research group has also reported a method to detect DMADP by using a recombinant isoprene synthase (IspS, EC 4.2.3.27) to quantitatively convert DMADP to isoprene and then measuring the isoprene using a highly sensitive chemiluminescent detector (Fast Isoprene Sensor) [16]. However, measurement of IDP is more difficult. The LC–MS/MS method measures the total amount of DMADP and IDP but cannot distinguish between them [22]. Recently, Tong and coworkers described a method to quantify IDP in mammalian cells [24] by catalyzing the addition of an IDP to farnesyl diphosphate to make geranylgeranyl diphosphate (GGDP) and then conjugating the GGDP with a fluorescence-labeled peptide and quantifying by high-performance liquid chromatography (HPLC) with a fluorescence detector. This method does not measure DMADP.

In this study, we developed a new method to measure the IDP levels and the ratio of DMADP/IDP in bacteria and plant tissues. The amount of DMADP was measured by conversion to isoprene using IspS, and the total amount of DMADP plus IDP was measured by including IDI in the assay mixture. IDP was then calculated as the difference between the total measurement and the DMADP measurement. *Escherichia coli* cultures and leaves of *Pueraria montana* var. *lobata* [Willd.] Maesen and S. Almeida (kudzu) were used to test our methods in biological tissue. Four strains of *E. coli* (derived from FM5) with heterologous MEP pathway genes (including *idi*) and *IspS* genes were used. Kudzu is an isoprene-emitting plant that has been long used in our lab for isoprene-relevant research [25–27]. Kudzu contains both a light-dependent MEP pathway in the chloroplast and a light-independent cytosolic MVA pathway [23,28]. The DMADP and IDP pools in the cytosol and chloroplasts in kudzu leaves were separated by analyzing the differences on the pools in light- and dark-adapted leaves. Because flash freezing in liquid nitrogen and subsequent extraction in acetonitrile/isopropanol/100 mM NH_4HCO_3 (3:1:1) has been proven to be efficient in quenching metabolism [16], it was assumed that the IDP and DMADP pools we measured represent in vivo levels of living organisms. These ratios were also compared with the ratio we found in vitro.

Materials and methods

Expression and purification of recombinant IDI

A recombinant His-tagged IDI based on the sequence in *Populus trichocarpa* was expressed in *E. coli* BL21(DE3) pLysS with vector pRSETA. The *E. coli* was grown at room temperature until the OD_{600} was higher than 0.6, and then 20 μM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce gene expression. The culture was grown for another 24 h before cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C. After storage at −80 °C overnight, the pellet was thawed on ice, resuspended, and lysed with a Qiagen lysis kit according to the manufacturer's directions. Briefly, one complete mini ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany) was added to every 10 ml of lysis buffer (also to the wash and elution buffers). Here, 4 ml of lysis buffer, 40 μl of lysozyme, and 4 μl of benzonase were used per gram wet weight of cells. The mixture was transferred into a 50-ml Falcon tube and then incubated on ice for 30 min. Using a six-step sonication with a Branson Sonifer 250 and a 3-mm ultra high-intensity tapered micro-tip (Branson Ultrasonics, Danbury, CT, USA), lysis was carried out by a six-step sonication of 15 pulses each followed by cooling for 30 s with ice water. Output was set at 1 for the first four steps and at 2 for the last two steps, with the duty cycle set to 50%. His-tagged protein was then isolated using Qiagen nickel–nitrilotriacetic acid (Ni-NTA) agarose and a Bio-Rad Poly-Prep chromatography column. The lysate–Ni-NTA mixture was incubated for 1 h at 4 °C. After washing twice with wash buffer containing protease inhibitor, the protein was eluted with elution buffer containing protease inhibitor. High levels of protein were found in all elution fractions as tested using a modified Lowry Protein Assay (Thermo Scientific, http://www.thermofisher.com), and IDI in fractions 3 to 5 had a purity higher than 95% as verified by Coomassie blue staining of protein gels (Fig. 2).

IspS was prepared according to procedures described by Weise and coworkers [16]. Briefly, a *Eucalyptus* IspS with a 6× His tag was attached to the vector pJexpress 401 and expressed in *E. coli* FM5 [16]. The cells were grown under the conditions described above but induced with 400 μM IPTG. The proteins were then purified using the same protocols as described for IDI.

Activity assay of IDI

IspS purified in our lab was tested first for catalyzing DMADP conversion to isoprene. Activity of IDI was determined by examining isoprene emission from an IDP standard in the presence of IspS. A series of positive and negative control reactions were designed as in Fig. 3. The Hepes reaction buffer contained 50 mM (final concentration) Hepes (pH 7.8), 20 mM KCl, 10 mM $MgCl_2$, and 5% (v/v) glycerol. In a 2-ml Supelco vial, 4 μl (~0.45 nmol) of IDP or DMADP (both from Echelon Biosciences, Salt Lake City, UT, USA) and 5 μg of IDI and/or IspS were added to some of the reactions as described.

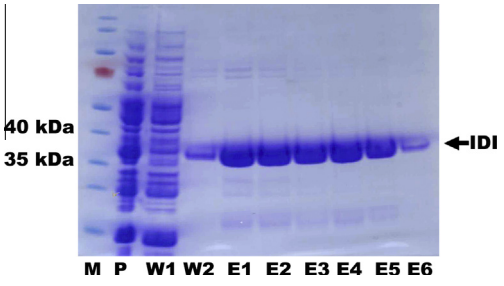

Fig.2. Recombinant IDI examined by Coomassie staining. Electrophoresis was carried out using a 4 to 12% NuPAGE Bis–Tris Mini Gel (Novex). M, PageRuler prestained protein ladder (Thermo Scientific); P, supernatant of cell lysate; W1 and W2, first buffer wash and last buffer wash, respectively; E1–E6, elution buffer from beginning (1) to end (6). The lanes were loaded with equal volumes of sample. Elutions E3 to E5 were used in this study.

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

UNCORRECTED PROOF

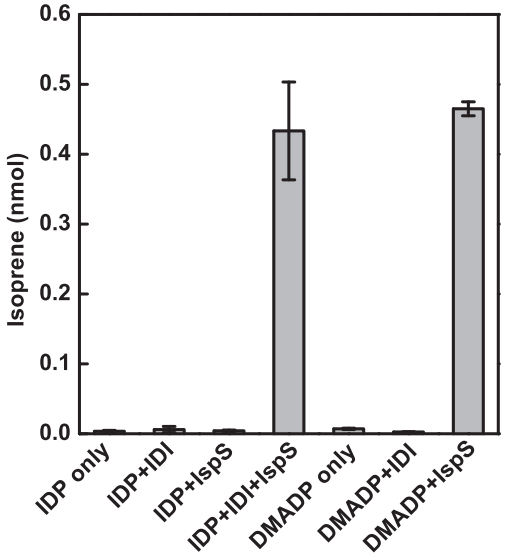

Fig.3. Isoprene produced by negative and positive control reactions. IDP or DMADP and IDI and/or IspS were added to the reactions as described. The reactions were incubated at 37 °C for 1 h in sealed Supelco vials, and then the headspace gas was sampled to test isoprene produced with a Fast Isoprene Sensor. Bars represent standard errors ($n = 3$).

The total reaction volume was adjusted to 150 µl with 2 mM NH_4HCO_3. The vial was sealed immediately after all chemicals were added and was incubated at 37 °C for 1 h. Then, 1 ml of headspace gas was sampled using a syringe, and the isoprene produced was quantified with a Fast Isoprene Sensor (Hills Scientific, Boulder, CO, USA), which uses chemiluminescence to measure the amount of isoprene. To avoid a vacuum while removing headspace gas, an equal volume of cold water was injected into the vial simultaneously. The total amount of isoprene in the vial was calculated by summing the amount of isoprene in the gas phase with the amount of isoprene in the reaction buffer assuming a Henry's constant of $H_{pc} = 7780$ Pa m^3 mol^{-1} at 25 °C [16].

In vitro equilibrium ratio between DMADP and IDP

To test the in vitro equilibrium ratio between DMADP and IDP in the presence of IDI, 8 µl of IDP or DMADP (~0.9 nmol) was first incubated with 20 µg of IDI in a total reaction volume of 292 µl in a 0.5-ml microtube for 1 h, and then IDI in the mixture was filtered away using a 3-kDa Amicon Ultra centrifugal filter (Millipore, Billerica, MA, USA). In a 2-ml Supelco vial, 146 µl of the filtrate and 4 µl (5 µg) of IspS were sealed, incubated for another 1 h, and measured for isoprene produced. The same volume of IDP or DMADP plus both IDI and IspS was added in new vials simultaneously, incubated for 2 h without filtration, and measured directly to quantify the amount of IDP or DMADP that was added for each reaction. The equilibrium was tested at both 37 and 15 °C.

DMADP and IDP pools and their ratio in E. coli

Four *E. coli* strains—FM5 untransformed (strain P) and transformed with the MEP pathway genes (strain Q), the IspS gene (strain R), and both (strain S)—were tested in this study. The MEP pathway genes were deoxyxylulose 5-phosphate synthase (DXS, EC 2.2.1.7) and deoxyxylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267) with promoter L, MEP cytidylyltransferase (MCT 2.7.7.60) and 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (CMK, EC 2.7.1.148) with promoter proC, and IDI with promoter 15 [29]. The IspS gene used IPTG-inducible pTrc as promoter. Seed cultures were grown on an orbital shaker at room temperature in minimal medium containing 5 g L^{-1} yeast extract and the basic nutrients (100 mM 4-morpholinepropanesulfonic acid, 38 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 2.9 mM KH_2PO_4, 7.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 475 µM citric acid, 109 µM $MnSO_4$, 18 µM $FeSO_4$, 7.1 µM $ZnSO_4$, 2 µM $CuSO_4$, 2.1 µM Na_2MoO_4, 2.1 µM $CoCl_2$, 2 mg L^{-1} thiamine, and 5 g L^{-1} glucose). For measurement, *E. coli* cultures were diluted and grown in minimal medium containing the same basic nutrients but 1 g L^{-1} yeast extract. To decide the time needed for IspS expression induced by IPTG, 30 ml of diluted *E. coli* strain S was first incubated with 400 µM IPTG (final concentration) for 24 h in a 250-ml stoppered flask.

Newly diluted ($OD_{600} = 0.6$) *E. coli* strains were equally stoppered and incubated for 3 h (with strains R and S fed with IPTG). Afterward, a headspace gas sample was taken from each flask to measure isoprene, and the cultures were measured for OD_{600} again and harvested by centrifugation at 5000*g* at 4 °C for 20 min. Pellets were washed with phosphate-buffered saline (pH 7.4) containing 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 2.7 mM KCl and were stored at −80 °C overnight. The following day, 1.5 ml of ice-cold extraction buffer (acetonitrile/isopropanol/100 mM NH_4HCO_3 = 3:1:1) was added to the frozen pellet. After a brief vortex, the pellet was broken up by a three-step sonication with duty cycle being 50%, output being 2, and 15 pulses in each step. The mixture was centrifuged at 20,000*g* at 4 °C for 10 min, and then the supernatant was divided equally into two Supelco vials and dried using a Savant AES 1010 SpeedVac. After redissolving with basic reaction buffer, 5 µg of IDI and 5 µg of IspS were added in one of the vials to let both IDP and DMADP convert to isoprene, whereas only IspS was added in another vial to let DMADP react. The amount of IDP was extrapolated by the difference of isoprene produced in the two vials, and then the ratio between DMADP and IDP was calculated.

To calculate the recovery rates of DMADP and IDP with our extraction and measuring methods for *E. coli,* additional DMADP and IDP were used as internal markers in separate samples. In this case, another 500-ml culture of *E. coli* strain P was grown in a 1000-ml flask with minimal medium containing basic nutrients and 5 g L^{-1} yeast extract. The cultures were then equally divided into nine tubes the next day, and cells were harvested. The first three samples were directly stored at −80 °C, 4 µl of DMADP was added to the second three samples, and 4 µl of IDP was added to the last three samples. The samples were then extracted and detected for DMADP and IDP with the same procedure as above. Recovery ratios were 64.3% for DMADP and 59.0% for IDP.

DMADP and IDP pools and their ratio in kudzu

Kudzu plants were grown in a Conviron growth chamber with a 12:12-h light regime, with a light level of 400 µmol photons m^{-2} s^{-1} and 24 °C in daytime and 20 °C at night. Light-adapted leaves at the top of the canopy were sampled with a lab-designed freeze clamp after the whole plant had been exposed to light for approximately 3 h. The leaves were harvested by clamping in between two pressurized copper blocks cooled in liquid nitrogen and were then transferred into liquid nitrogen immediately after clamping. Each leaf sample was divided into two halves and ground directly into 2-ml tubes using a Retsch Mill three times at a frequency of 30 Hz for 30 s each time. The powder was then extracted with ice-cold extraction buffer (acetonitrile/isopropanol/100 mM NH_4HCO_3 = 3:1:1) and centrifuged at 4 °C for 15 min. Supernatants were transferred into Supelco vials for drying, and DMADP and IDP were measured as above. For the test of recovery, 4 µl of DMADP and IDP was added to the other half of the leaf sample as internal standards and followed with the same extraction and testing procedure as described above for bacterial samples.

UNCORRECTED PROOF

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

Because DMADP and IDP levels in dark-adapted leaves were very low, each whole leaf was treated as one sample. Dark-adapted leaves were analyzed with the same procedure as for the light-adapted samples.

Results

Activity of IDI

To test the activity of the recombinant IDI, a series of positive and negative control reactions were carried out (Fig. 3). When IDP was incubated with no enzyme, with IDI alone, or with IspS alone, only negligible amounts of isoprene were detected. When IDP was incubated with both IDI and IspS, there was conversion of IDP to the expected amount of isoprene. Incubation of DMADP with no enzyme or with IDI alone resulted in negligible amounts of isoprene. Incubation of DMADP with IspS resulted in the expected conversion of DMADP to isoprene.

In vitro equilibrium between IDP and DMADP

Regardless of whether IDP or DMADP is the starting material, the ratio of DMADP to IDP should be the same once equilibrium is reached given adequate enzyme and time. Equilibrium was first tested at 37 °C. Regardless of whether the reaction was started with IDP and IDI to let part of the IDP turn into DMADP or with DMADP and IDI to let part of the DMADP turn into IDP, the ratio of DMADP to IDP was exactly the same, that is, 2.11 (Fig. 4). Equilibrium was also tested at 15 °C, and the same ratio of DMADP to IDP was found (data not shown).

IDP and DMADP in untransformed and transformed E. coli

In addition to the MEP pathway genes that were constitutively overexpressed in *E. coli* strains Q and S, *IspS* in *E. coli* strains R and S was designed to be induced by IPTG. However, it was only the *E. coli* strain S that contained both MEP pathway genes (including *idi*) and IspS that emitted massive amounts of isoprene. Using stoppered flasks, isoprene in the headspace was monitored with a fixed time schedule. Significant accumulation of isoprene in headspace was detected 2 h after the IPTG was added to *E. coli* strain S, and the accumulation rate was nearly linear for 24 h (Fig. 5). We concluded that 3 h was enough time for IspS induction.

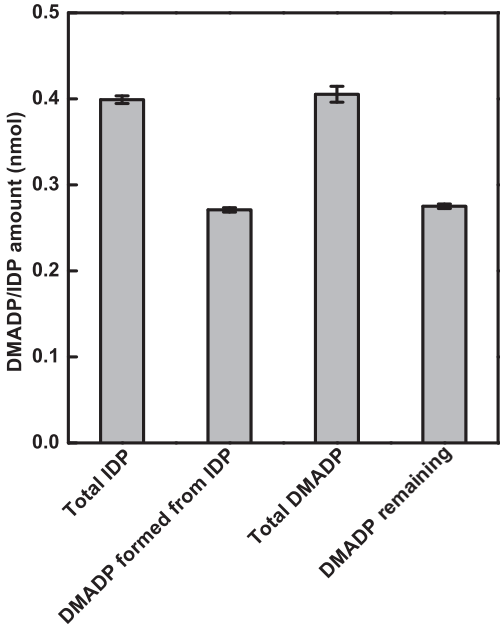

Fig.4. In vitro equilibrium between IDP and DMADP catalyzed by IDI at 37 °C. For equilibrium tests (second and fourth reactions), IDP or DMADP and IDI were incubated for 1 h before IDI was removed by a 3-kDa filter, and then IspS was added into the filtrate and incubated for another 1 h to let DMADP convert to isoprene. To quantify the total amount of IDP or DMADP used (first and third reactions), the step of filtration was skipped. Bars represent the means ± standard errors ($n = 6$ for DMADP formed or remaining after equilibrium; $n = 3$ for total IDP and DMADP).

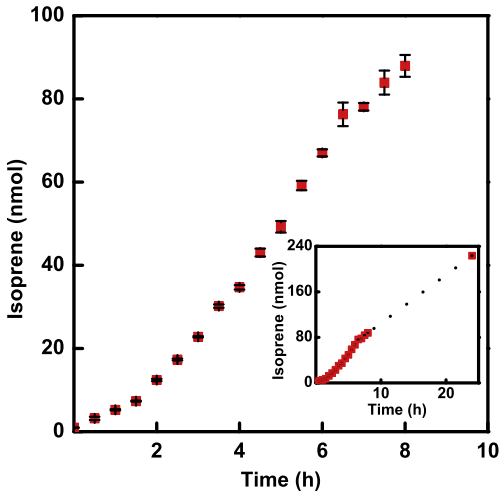

Fig.5. Isoprene emission from *E. coli* (strain S) transformed with MEP pathway and IspS. Beginning with an OD_{600} of 0.6, 30 µl of cells was induced by 400 µM IPTG and incubated in a 250-ml flask at room temperature. Data shown were isoprene accumulated in headspace of the flask tested using a Fast Isoprene Sensor. Bars represent the means ± standard errors ($n = 3$).

In comparing the four *E. coli* strains after incubation for 3 h (with IPTG for strains S and R), the isoprene emission rate from strain S was 18 to 32 times the rates from the rest of the strains (data not shown). The isoprene emission rate from *E. coli* strain R (having only *IspS*) was also a little higher than the rates from untransformed strain P ($P < 0.05$) and transformed strain Q with simply the MEP pathway ($P > 0.05$).

The *E. coli* cells were also harvested and analyzed for DMADP and IDP after incubation for 3 h. As expected, significantly higher amounts of DMADP and IDP were found in *E. coli* strains Q and S that contained the transformed MEP pathway (Fig. 6). DMADP in *E. coli* strains Q and S were 9 and 8 times that in untransformed strain P ($P < 0.001$), and IDP in *E. coli* strains Q and S were 7 and 5 times that in strain P ($P < 0.05$). DMADP in *E. coli* strain R transformed with *IspS* was a little lower than that in strain P, and IDP in strain R was a little higher; however, such differences were not statistically significant ($P > 0.05$).

The in vivo ratio of DMADP to IDP in *E. coli* FM5 was found to be 2.80, which was 33% higher than the ratio found in vitro (Fig. 6). Such ratios in strains Q and S overexpressing the MEP pathway were even higher, that is, 83 and 123% times higher, respectively, than the ratio in vitro. On the other hand, the ratio in strain R, with *IspS* but no additional *idi*, was visibly decreased at only 50% of the ratio in vitro.

IDP and DMADP in light- and dark-adapted kudzu leaves

After plants were acclimated in darkness overnight, DMADP and IDP levels in kudzu leaves decreased significantly (Fig. 7) to only 11 and 8%, respectively, of the levels found in light-adapted leaves ($P < 0.001$). In other words, the plastidic DMADP and IDP from the MEP pathway contributed to approximately 90% of the total pools in kudzu leaves, whereas only approximately 10% of the

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

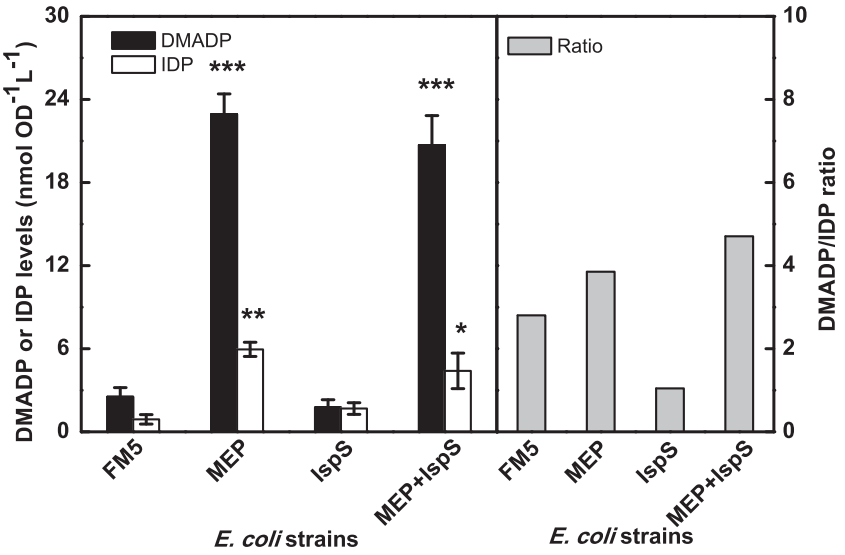

Fig.6. DMADP and IDP levels and their ratio in untransformed and transformed *E. coli* strains after being grown in minimal medium with 1 g L^{-1} yeast extract for 3 h. *E. coli* contained IspS induced by IPTG. IDP and DMADP extracted from cells were converted to isoprene by IDI and/or IspS and quantified using a Fast Isoprene Sensor. Bars represent the means ± standard errors ($n = 3$). Compared with data of untransformed FM5: $^{***}P < 0.001$; $^{**}P < 0.0$; $^{*}P < 0.05$ (analyzed with one-way analysis of variance). Ratios of the mean values of DMADP and IDP are listed.

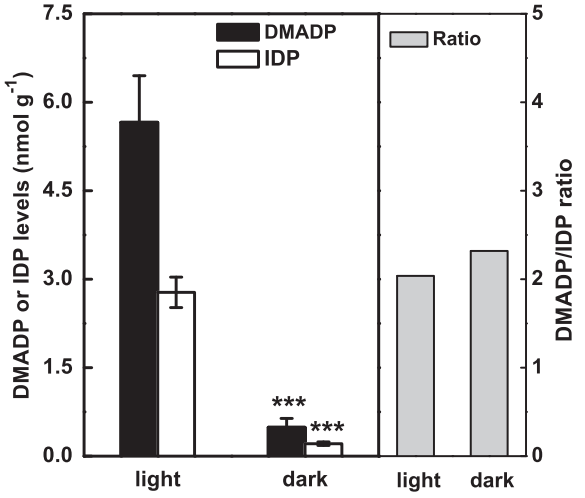

Fig. 7. DMADP and IDP levels and their ratios in light- and dark-adapted kudzu leaves. Light-adapted leaves were sampled after plants were acclimated in light for approximately 3 h. Dark-adapted leaves were sampled early in the morning before light was on. IDP and DMADP extracted from leaves were converted to isoprene with IDI and/or IspS and quantified using a Fast Isoprene Sensor. Bars represent the means ± standard errors ($n = 6$). Compared between light- and dark-adapted samples: $^{***}P < 0.001$ (analyzed with one-way analysis of variance). Ratios of the mean values of DMADP and IDP are listed.

pools were from the MVA pathway in cytosol. The in vivo ratios of DMADP/IDP in light- and dark-adapted kudzu leaves were 2.04 and 2.32, respectively, which were very close to the ratio found in vitro.

Discussion

The isomerization between IDP and DMADP catalyzed by IDI is important for production of the building units of all isoprenoids in living organisms [1,30]. For example, isoprene emission from oak trees and engineering of carotenoid synthesis in *E. coli* require high levels of IDI [13,14]. Purification and characterization of IDI has been widely reported from sources of plants as well as *E. coli*, yeast, and animals [30–33]. Here, we succeeded in expressing and purifying a poplar IDI in *E. coli* BL21(DE3) pLysS. The recombinant IDI had high activity and was highly specific in the isomerization of IDP and DMADP in our experiment.

A wide range of equilibrium ratios of DMADP/IDP have been reported. Earlier records include 13.3 reported by Agranoff and coworkers in 1960, tested by labeling IDP with ^{14}C and measuring the remaining radioactivity after DMADP formed was destroyed by adding acid [34]. A value of 3 was reported by Koyama and coworkers in 1983. IDP was isomerized in deuterium oxide, and after treatment with alkaline phosphatase, IDP and DMADP were analyzed by gas chromatography (GC)–MS [19]. Another value of 3.1 was reported by Lützow and Beyer in 1988, tested by treatment with alkaline phosphatase and analysis of the liberated alcohols with GC [20]. In this study, we found an in vitro ratio of DMADP/IDP as 2.11. The equilibrium was established using a recombinant IDI, and DMADP and IDP in the mixture were quantified by analyzing the enzymatically derived isoprene with chemiluminescence. The ratio we found is a little lower compared with what has been reported before. The isoprene measurement by chemiluminescence is highly reliable and has a very large dynamic range of detection. Our limit of quantitation (defined as the signal at which the signal-to-noise ratio was 10 or higher) was 10 pmol. The efficiency of conversion of DMADP to isoprene by our recombinant IspS has been tested in our previous work [16]. In this work, we showed that without IDI, the auto-isomerization between IDP and DMADP is negligible over 1 h in our reaction condition given that almost no isoprene was detected directly from an IDP source. Previous studies of DMADP and the DMADP/IDP ratio often relied on acid hydrolysis of DMADP [19,20,34], which requires complete destruction of DMADP. Conversion of DMADP to isoprene by acid hydrolysis is not quantitative [16], although it may be that DMADP is completely destroyed.

There are also studies of the initial ratios of DMADP/IDP in direct reduction products from HMBDP in the MEP pathway. A mutant form of the hydroxymethylbutenyl diphosphate reductase (also known as ispH or lytB) may produce only DMADP [35], but for the wild-type enzyme the in vitro ratio of DMADP/IDP is reported to be 1:5 or 1:6, tested by acid hydrolysis and measuring the corresponding alcohols [17], isotope labeling, and nuclear magnetic resonance spectroscopy [36,37] or anion exchange HPLC [38].

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

The in vivo ratio of DMADP/IDP tested by isotope labeling is reported to be 15:85 [39]. Because large differences in estimates of DMADP/IDP ratios exist between initial products and an equilibrium mixture, IDI is considered to play an important role in maintaining the equilibrium of the two compounds. The method reported here could be used to get additional estimates of the ratio of DMADP to IDP produced in this reaction.

As expected, IDP and DMADP pools in transformed *E. coli* strains Q and S with additional MEP pathway genes were significantly higher than those in the untransformed *E. coli* stain P, indicating that the additional MEP pathway genes are functional. Because *E. coli* strain S also contained a transformed IspS, a very large emission of isoprene was found. However, such emission showed little effect on the IDP and DMADP pool sizes. This is consistent with negative feedback on the MEP pathway [40], such that the amount of DMADP is reasonably constant despite a wide range of fluxes. The in vivo ratio of DMADP/IDP in untransformed *E. coli* strain P was found to be closer to the value obtained in vitro, but the ratios of the transformed *E. coli* strains Q and S were higher. It might be that the equilibrium between IDP and DMADP is different in the internal environment of *E. coli,* perhaps because of ionic concentration or protein concentration making DMADP more stable. The *E. coli* strain R, which contained *IspS* but no additional *idi,* had a lower DMADP/IDP ratio than was found in vitro, which is reasonable because part of DMADP was converted into isoprene and the DMADP pool was reduced.

Plants have the MEP pathway in plastids and the MVA pathway in their cytosol, both of which produce IDP and DMADP. Because the MEP pathway in chloroplasts is light dependent, whereas the MVA pathway in cytosol is light independent [23,28], the two pools can be distinguished by measuring light-adapted leaves for the total amounts and measuring dark-adapted leaves for only cytosolic pools and calculating the difference of the two for the plastidic pools. With this method, we found that approximately 90% of the total IDP and DMADP in light-adapted kudzu leaves comes from the MEP pathway in chloroplasts; such results are in accord with what was reported by Li and Sharkey [22].

Similar diurnal and nocturnal differences of DMADP pools in other isoprene-emitting and non-emitting plants have also been reported [41,42]. The in vivo ratios of DAMDP/IDP were detected to be 2.04 and 2.32 in light- and dark-adapted kudzu leaves, respectively, being quite similar to what was found in vitro, indicating that DAMDP and IDP are in isomerase equilibrium in both chloroplasts and cytosol.

In conclusion, a new method for measuring the DMADP/IDP ratio was reported, and the in vitro ratio was found to be 2.11 regardless of whether IDP conversion to DMADP or DMADP conversion to IDP was followed. The method is effective in measuring DMADP, IDP, and their ratio in biological material, which can be of benefit to studies of isoprene emission from plants and engineering organisms for production of isoprenoids.

Acknowledgments

The plasmid containing MEP pathway genes was designed by Hal Alper in consultation with ZuvaChem. This material is based on work supported by the National Science Foundation under grants IOS-0950574 and IOS-0640853. Any opinions, findings, conclusions, and recommendations expressed in this article are those of the authors and do not necessarily reflect the views of the National Science Foundation. Additional support was provided by ZuvaChem. Changfang Zhou was supported by Chinese Fundamental Research Funds for the Central Universities (1105020807) and a scholarship from Jiangsu Overseas Research & Training Program for University Prominent Young & Middle-Aged Teachers and Presidents.

References

[1] M. Rodríguez-Concepción, A. Boronat, Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids: a metabolic milestone achieved through genomics, Plant Physiol. 130 (2002) 1079–1089.
[2] M.A. Phillips, P. León, A. Boronat, M. Rodríguez-Concepción, The plastidial MEP pathway: unified nomenclature and resources, Trends Plant Sci. 13 (2008) 619–623.
[3] G. Mongélard, M. Seemann, A.-M. Boisson, M. Rohmer, R. Bligny, C. Rivasseau, Measurement of carbon flux through the MEP pathway for isoprenoid synthesis by ^{31}P-NMR spectroscopy after specific inhibition of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate reductase: effect of light and temperature, Plant Cell Environ. 34 (2011) 1241–1247.
[4] A. Guenther, T. Karl, P. Harley, C. Wiedinmyer, P.I. Palmer, C. Geron, Estimates of global terrestrial isoprene emissions using MEGAN (model of emissions of gases and serosols from nature), Atmos. Chem. Phys. Discuss. 6 (2006) 107–173.
[5] S.P. Harrison, C. Morfopoulos, K.G.S. Dani, I.C. Prentice, A. Arneth, B.J. Atwell, M.P. Barkley, M.R. Leishman, F. Loreto, B.E. Medlyn, U. Niinemets, M. Possell, J. Penuelas, I.J. Wright, Volatile isoprenoid emissions from plastid to planet, New Phytol. 197 (2013) 49–57.
[6] F. Bouvier, A. Rahier, B. Camara, Biogenesis, molecular regulation, and function of plant isoprenoids, Prog. Lipid Res. 44 (2005) 357–429.
[7] S. Heuston, M. Begley, C.G.M. Gahan, C. Hill, Isoprenoid biosynthesis in bacterial pathogens, Microbiology 158 (2012) 1389–1401.
[8] I. Buhaescu, H. Izzedine, Mevalonate pathway: a review of clinical and therapeutical implications, Clin. Biochem. 40 (2007) 575–584.
[9] J.-F. Hoeffler, A. Hemmerlin, C. Grosdemange-Billiard, T.J. Bach, M. Rohmer, Isoprenoid biosynthesis in higher plants and in *Escherichia coli:* on the branching in the methylerythritol phosphate pathway and the independent biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate, Biochem. J. 366 (2002) 573–583.
[10] M. Rohmer, M. Knani, P. Simonin, B. Sutter, H. Sahm, Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate, Biochem. J. 295 (1993) 517–524.
[11] B.M. Lange, R. Croteau, Isopentenyl diphosphate biosynthesis via a mevalonate-independent pathway: isopentenyl monophosphate kinase catalyzes the terminal enzymatic step, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 13714–13719.
[12] M. Lohr, J. Schwender, J.E.W. Polle, Isoprenoid biosynthesis in eukaryotic phototrophs: a spotlight on algae, Plant Sci. 185 (2012) 9–22.
[13] N. Brüggemann, J.P. Schnitzler, Relationship of isopentenyl diphosphate (IDP) isomerase activity to isoprene emission of oak leaves, Tree Physiol. 22 (2002) 1011–1018.
[14] M. Albrecht, N. Misawa, G. Sandmann, Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin, Biotechnol. Lett. 21 (1999) 791–795.
[15] D.J. Tantillo, Biosynthesis via carbocations: theoretical studies on terpene formation, Nat. Prod. Rep. 28 (2011) 1035–1053.
[16] S.E. Weise, Z. Li, A.E. Sutter, A. Corrion, A. Banerjee, T.D. Sharkey, Measuring dimethylallyl diphosphate available for isoprene synthesis, Anal. Biochem. 435 (2013) 27–34.
[17] P. Adam, S. Hecht, W.G. Eisenreich, J. Kaiser, T. Gräwert, D. Arigoni, A. Bacher, F. Rohdich, Biosynthesis of terpenes: Studies on 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 12108–12113.
[18] A.J. Fisher, T.N. Rosenstiel, M.C. Shirk, R. Fall, Nonradioactive assay for cellular dimethylallyl diphosphate, Anal. Biochem. 292 (2001) 272–279.
[19] T. Koyama, Y. Katsuki, K. Ogura, Studies on isopentenyl pyrophosphate isomerase with artificial substrates: Z-E isomerization of Z-3-methyl-3-pentenyl pyrophosphate, Bioorg. Chem. 12 (1983) 58–70.
[20] M. Lützow, P. Beyer, The isopentenyl-diphosphate delta-isomerase and its relation to the phytoene synthase complex in daffodil chromoplasts, Biochim. Biophys. Acta 959 (1988) 118–126.
[21] M. Raschke, M. Fellermeier, M.H. Zenk, A high-performance liquid chromatography method for the analysis of intermediates of the deoxyxylulose phosphate pathway, Anal. Biochem. 335 (2004) 235–243.
[22] Z. Li, T.D. Sharkey, Metabolic profiling of the methylerythritol phosphate pathway reveals the source of post-illumination isoprene burst from leaves, Plant Cell Environ. 36 (2013) 429–437.
[23] B. Rasulov, L. Copolovici, A. Laisk, Ü. Niinemets, Postillumination isoprene emission: in vivo measurements of dimethylallyldiphosphate pool size and isoprene synthase kinetics in aspen leaves, Plant Physiol. 149 (2009) 1609–1618.
[24] H. Tong, C.H. Kuder, B.M. Wasko, R.J. Hohl, Quantitative determination of isopentenyl diphosphate in cultured mammalian cells, Anal. Biochem. 433 (2013) 36–42.
[25] T.D. Sharkey, F. Loreto, Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves, Oecologia 95 (1993) 328–333.

Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028

UNCORRECTED PROOF

[26] T.D. Sharkey, S. Yeh, A.E. Wiberley, T.G. Falbel, D. Gong, D.E. Fernandez, Evolution of the isoprene biosynthetic pathway in kudzu, Plant Physiol. 137 (2005) 700–712.
[27] A.E. Wiberley, A.R. Linskey, T.G. Falbel, T.D. Sharkey, Development of the capacity for isoprene emission in kudzu, Plant Cell Environ. 28 (2005) 898–905.
[28] Z. Li, E.A. Ratliff, T.D. Sharkey, Effect of temperature on post-illumination isoprene emission in oak and poplar, Plant Physiol. 155 (2011) 1037–1046.
[29] H. Alper, C. Fischer, E. Nevoigt, G. Stephanopoulos, Tuning genetic control through promoter engineering, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 12678–12683.
[30] A.C. Ramos-Valdivia, R. Heijden, R. Verpoorte, Isopentenyl diphosphate isomerase: a core enzyme in isoprenoid biosynthesis—A review of its biochemistry and function, Nat. Prod. Rep. 14 (1997) 591–603.
[31] K. Berthelot, Y. Estevez, A. Deffieux, F. Peruch, Isopentenyl diphosphate isomerase: a checkpoint to isoprenoid biosynthesis, Biochimie 94 (2012) 1621–1634.
[32] K. Kaneda, T. Kuzuyama, M. Takagi, Y. Hayakawa, H. Seto, An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 932–937.
[33] N.A. Heaps, C.D. Poulter, Type-2 isopentenyl diphosphate isomerase: evidence for a stepwise mechanism, J. Am. Chem. Soc. 133 (2011) 19017–19019.
[34] B.W. Agranoff, H. Eggerer, U. Henning, F. Lynen, Biosynthesis of terpenes: VII. Isopentenyl pyrophosphate isomerase, J. Biol. Chem. 235 (1960) 326–332.
[35] K.-J. Puan, H. Wang, T. Dairi, T. Kuzuyama, C.T. Morita, *FldA* is an essential gene required in the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis, FEBS Lett. 579 (2005) 3802–3806.
[36] F. Rohdich, S. Hecht, K. Gärtner, P. Adam, C. Krieger, S. Amslinger, D. Arigoni, A. Bacher, W. Eisenreich, Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 1158–1163.
[37] F. Rohdich, F. Zepeck, P. Adam, S. Hecht, J. Kaiser, R. Laupitz, T. Gräwert, S. Amslinger, W. Eisenreich, A. Bacher, D. Arigoni, The deoxyxylulose phosphate pathway of isoprenoid biosynthesis: studies on the mechanisms of the reactions catalyzed by IspG and IspH protein, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 1586–1591.
[38] B. Altincicek, E.C. Duin, A. Reichenberg, R. Hedderich, A.-K. Kollas, M. Hintz, S. Wagner, J. Wiesner, E. Beck, H. Jomaa, LytB protein catalyzes the terminal step of the 2-*C*-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis, FEBS Lett. 532 (2002) 437–440.
[39] D. Tritsch, A. Hemmerlin, T.J. Bach, M. Rohmer, Plant isoprenoid biosynthesis via the MEP pathway: In vivo IPP/DMAPP ratio produced by (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase in tobacco BY-2 cell cultures, FEBS Lett. 584 (2010) 129–134.
[40] A. Banerjee, Y. Wu, R. Banerjee, Y. Li, H. Yan, T.D. Sharkey, Feedback inhibition of deoxy-D-xylulose 5-phosphate synthase regulates the methyl erythritol 4-phosphate pathway, J. Biol. Chem. 288 (2013) 16926–16936.
[41] T.N. Rosenstiel, A.J. Fisher, R. Fall, R.K. Monson, Differential accumulation of dimethylallyl diphosphate in leaves and needles of isoprene- and methylbutenol-emitting and nonemitting species, Plant Physiol. 129 (2002) 1276–1284.
[42] I. Nogués, F. Brilli, F. Loreto, Dimethylallyl diphosphate and geranyl diphosphate pools of plant species characterized by different isoprenoid emissions, Plant Physiol. 141 (2006) 721–730.

UNCORRECTED PROOF

Q1 Please cite this article in press as: C. Zhou et al., Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.05.028