Two Arabidopsis Genes (IPMS1 and IPMS2) Encode Isopropylmalate Synthase, the Branchpoint Step in the Biosynthesis of Leucine1[W][OA]

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Heterologous expression of the Arabidopsis (Arabidopsis thaliana) IPMS1 (At1g18500) and IPMS2 (At1g74040) cDNAs in Escherichia coli yields isopropylmalate synthases (IPMSs; EC 2.3.3.13). These enzymes catalyze the first dedicated step in leucine (Leu) biosynthesis, an aldol-type condensation of acetyl-coenzyme A (CoA) and 2-oxoisovalerate yielding isopropylmalate. Most biochemical properties of IPMS1 and IPMS2 are similar: broad pH optimum around pH 8.5, Mg2+ as cofactor, feedback inhibition by Leu, Km for 2-oxoisovalerate of approximately 300 μM, and a Vmax of approximately 2 × 104 μmol min−1 g−1. However, IPMS1 and IPMS2 differ in their Km for acetyl-CoA (45 μM and 16 μM, respectively) and apparent quaternary structure (dimer and tetramer, respectively). A knockout insertion mutant for IPMS1 showed an increase in valine content but no changes in Leu content; two insertion mutants for IPMS2 did not show any changes in soluble amino acid content. Apparently, in planta each gene can adequately compensate for the absence of the other, consistent with available microarray and reverse transcription-polymerase chain reaction data that show that both genes are expressed in all organs at all developmental stages. Both encoded proteins accept 2-oxo acid substrates in vitro ranging in length from glyoxylate to 2-oxohexanoate, and catalyze at a low rate the condensation of acetyl-CoA and 4-methylthio-2-oxobutrate, i.e. a reaction involved in glucosinolate chain elongation normally catalyzed by methylthioalkylmalate synthases. The evolutionary relationship between IPMS and methylthioalkylmalate synthase enzymes is discussed in view of their amino acid sequence identity (60%) and overlap in substrate specificity.

Isopropylmalate synthase (IPMS; EC 2.3.3.13) catalyzes the first dedicated step in Leu biosynthesis, an aldol-type condensation between acetyl-CoA and 2-oxoisovalerate yielding 2-isopropylmalate (Fig. 1). The absence of IPMS and other enzymes of branched-chain amino acid biosynthesis (Leu, Ile, and Val) in monogastric animals has been an important stimulus for the development of herbicides that specifically inhibit the synthesis of branched-chain amino acids in plants with minimal toxicity to animals. Nonetheless, in contrast to Val and Ile, the biosynthesis of Leu in plants is largely unexplored. The available evidence indicates that plants use the pathway depicted in Figure 1, which is the same one found in bacteria and yeast (Singh and Shaner, 1995; Singh, 1999; Coruzzi and Last, 2000).

IPMS activities of plants have been described from crude extracts of maize (Zea mays) embryos (Oaks, 1965), thylakoid fractions of spinach (Spinacia oleracea) chloroplasts (Hagelstein and Schultz, 1993), and soluble chloroplast-enriched preparations of nasturtium (Tropaeolum majus), Diplotaxis tenuifolia, Eruca sativa, and Arabidopsis (Arabidopsis thaliana), all members of the Brassicaceae (Falk et al., 2004). The next step in Leu biosynthesis is isomerization of the IPMS product 2-isopropylmalate to 3-isopropylmalate (Fig. 1), but an isopropylmalate isomerase has until now not been described from any plant source. More is known about the penultimate step in Leu biosynthesis since Wittenbach et al. (1994) partially purified an isopropylmalate dehydrogenase from pea (Pisum sativum) and showed the inhibition of this enzyme by an herbicide. In addition, a cDNA clone encoding an enzyme that dehydrogenates and decarboxylates 3-isopropylmalate to 4-methyl-2-oxovalerate was isolated from both oil seed rape (Brassica napus; Ellerstrom et al., 1992) and potato (Solanum tuberosum; Jackson et al., 1993). Transamination of the resulting 2-oxo acid yields the final product Leu (Singh, 1999).

A gene unambiguously encoding IPMS activity has not yet been identified from any plant source, despite the considerable attention devoted to four genes of Arabidopsis (Columbia [Col]-0) that show similarity to IPMS sequences of other organisms (e.g. Kroymann et al., 2001; Junk and Mourad, 2002; Field et al., 2004; Textor et al., 2004). A principal reason for this attention is the potential role of these genes in glucosinolate
biosynthesis. Two of these IPMS-like sequences (At5g23010 and At5g23020) are located at the GS-Elong locus on chromosome V, which regulates the side-chain length of the aliphatic glucosinolates in Arabidopsis (Campos de Quiros et al., 2000; Kroymann et al., 2001, 2003).

Glucosinolates are amino acid-derived plant secondary metabolites (formed mainly from Met, Trp, and Phe in Arabidopsis) that consist of a β-thio-Glc moiety, a sulfonated oxime, and a variable side chain formed from the parent amino acid. In the case of the Met-derived glucosinolates, the side chain of the amino acid is elongated by up to six methylene groups prior to biosynthesis of the glucosinolate backbone (Fig. 2), resulting in the formation of products with C3 to C8 side chains (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006). In the iterative, three-step elongation process demonstrated in in vivo feeding studies, transaminated Met (4-methylthio-2-oxobutyrate) is subject to the same type of reactions (Fig. 2) that convert 2-oxoisovalerate into Leu (Fig. 1; Chisholm and Wetter, 1964; Matsuo and Yamazaki, 1968; Serif and Schmotzer, 1968; Graser et al., 2000). Each cycle of elongation is initiated by an aldol-type condensation between 2-oxoisovalerate and acetyl-CoA leading to formation of a ω-methylthioalkylmalate.

Figure 1. The biosynthesis of Leu and Val from pyruvate. The action of acetohydroxyacid synthase (AHAS), ketoacid reductoisomerase (KARI), and dihydroxyacid dehydratase (DHAD) yields 2-oxoisovalerate that is either transaminated to Val or subjected to additional reactions specific for Leu biosynthesis. The dedicated step in Leu biosynthesis is the aldol-type condensation between 2-oxoisovalerate and acetyl-CoA that results in formation of 2-isopropylmalate. Isomerization and oxidative decarboxylation by isopropylmalate isomerase (IPMI) and isopropylmalate dehydrogenase (IPMDH) yield 4-methyl-2-oxovalerate that is transaminated to Leu. The enzymes that catalyze the reactions from pyruvate to 2-oxoisovalerate are also involved in biosynthesis of Ile, using 4-oxobutyrate (product of Thr dehydratase) as an initial substrate, but for simplicity have not been depicted. AHAS and IPMS are subject to feedback inhibition as shown with dashed lines.
genes, specifically, MAM1 and MAM3 (also known as MAM-L). A third MAM gene, MAM2, has been described from other Arabidopsis ecotypes (Kroymann et al., 2003). Analysis by Field et al. (2004) of a MAM3 knockout line from Arabidopsis showed the absence of long-chain glucosinolates (C6, C7, and C8), confirming the role of MAM3 in glucosinolate biosynthesis.

The function of the two other IPMS-like sequences, located at opposite ends of chromosome I (At1g18500 and At1g74040), has not yet been clearly determined, though cluster analysis of the deduced amino acid sequences revealed that they are more closely related to one another and to the two reported IPMS sequences from wild tomato (Lycopersicon pennellii; GenBank accession nos. AAB61598 and AAB61599) than to the MAM1 or MAM3 sequences, suggesting they probably encode true IPMSs (Kroymann et al., 2001). Just recently, a MAM sequence (GenBank accession no. DQ143886) was reported from Brassica atlantica that shares a high amino acid identity (90% and 86%) with the predicted IPMS homologs of Arabidopsis and is able to restore the growth of an IPMS-null Escherichia coli mutant in the absence of Leu (Field et al., 2006), and thus was called an IPMS (BatIMS). Such a rescue of an IPMS-null E. coli mutant was also described for At1g74040 and furthermore for both MAM-encoding genes, MAM1 and MAM3 (Junk and Mourad, 2002; S. Textor, unpublished data). An overview of these confusing, and in part contradictory, results (Table I) makes the point that it is problematic to definitely assign the in planta function of these genes based solely on complementation of an IPMS-null E. coli mutant strain. Analyses of a knockout mutant in At1g18500 also gave little information about the function of this IPMS gene.
since neither a significant change in Leu nor in glucosinolate content was detected, and only (pleiotropic?) alterations in Asn, Gln, His, and Val content were observed (Field et al., 2004).

At least one of the two predicted IPMS genes at chromosome I of Arabidopsis, named IPMS1 (At1g18500) and IPMS2 (At1g74040) in this article, should encode an active IPMS because such an enzyme activity is absolutely essential to the plant for its synthesis of Leu. In this study, IPMS1 and IPMS2 were cloned and heterologously expressed in E. coli. Determination of the substrate specificity of the purified proteins in conjunction with analysis of knockout mutant lines reveals that IPMS1 and IPMS2 both encode bona fide IPMSs involved in Leu biosynthesis and do not participate in the chain elongation of glucosinolates.

RESULTS

IPMS1 and IPMS2 Have IPMS Activity

The open reading frames (ORFs) of IPMS1 (At1g18500) and IPMS2 (At1g74040) from Arabidopsis were separately cloned without the predicted N-terminal targeting sequence (ChloroP; Emanuelsson et al., 1999) into an expression vector containing a polyhistidine coding domain (His-tag) and expressed in E. coli. The recombinant protein was purified over an Ni-NTA agarose affinity column and showed on SDS-PAGE a protein band at the expected size of around 65 kD that was 90% to 95% pure (data not shown); about 4.5 mg of protein was obtained from a 100-mL bacterial culture.

Incubation of 0.5 mM [14C]acetyl-CoA and 3 mM 2-oxoisovalerate with 50 μg of partially purified IPMS protein for 1 h gave a complete incorporation of the 14C label into a product that yielded a single peak in the radiodetector trace of the HPLC (Fig. 3A). This product peak coeluted with a synthetic standard of 2-isopropylmalate (Fig. 3C). In these measurements, no difference was detected in enzyme activity between IPMS1 and IPMS2. In the absence of 2-oxo acid substrate, [14C]acetyl-CoA was partially hydrolyzed to [14C]acetate and CoA (Fig. 3B), probably due to the pH of the enzyme assay and a minor acetyl-CoA hydrolyzing activity of the enzyme itself (see also Table III).

Boiled enzyme incubated with 2-oxoisovalerate and [14C]acetyl-CoA yielded a similar chromatogram (data not shown). To check for MAM activity associated with formation of chain-elongated, Met-derived glucosinolates, the purified IPMS proteins were incubated with 4-methylthio-2-oxobutyrate and [14C]acetyl-CoA under the same conditions. However, a much smaller amount of [14C]-2-(2′-methylthio)ethylmalate was measured compared to the amount of 2-isopropylmalate formed from 2-oxoisovalerate, and most of the [14C]acetyl-CoA remained (Fig. 3D). These data suggest that the IPMS1 and IPMS2 genes are much more likely to serve as IPMSs in Leu biosynthesis than in the formation of methylthioalkylmalate compounds for glucosinolate biosynthesis.

Additional support for the function of the IPMS2-encoded protein comes from the observation that it is able to complement the IPMS-null E. coli strain CV512(DE3). This bacterium was not able to grow on a minimal medium without supplemented amino acids, unless it had been transformed with a construct carrying either the E. coli IPMS gene leuA or the IPMS2 gene from Arabidopsis. Transcription of the gene construct was induced with isopropyl-β-galactoside (IPTG), and both the E. coli leuA transformant (positive control) and the IPMS2 transformant grew at 30°C within 3 d, whereas no bacterial growth was observed for CV512(DE3) alone (data not shown). Growth of the IPMS2 transformant was less at 37°C. In contrast to this experiment, our attempts to complement CV512(DE3) with the IPMS1 construct were unsuccessful.

IPMS1 and IPMS2 Have Similar But Not Identical Biochemical Characteristics

IPMS1 and IPMS2 activity not only depended upon the presence of a 2-oxo acid substrate and acetyl-CoA, but also required Mg2+ in millimolar concentrations (Fig. 4A). Hence, 4 mM Mg2+ was added routinely to the incubations. Quantitative measurements using an endpoint assay with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; see “Materials and Methods”) showed a 75% to 95% loss of enzyme activity when the His-tag purified protein was desalted into a buffer without Mg2+ and then incubated in the absence of Mg2+. Enzyme activity was completely lost in the presence of 10 mM EDTA. The residual enzyme activity in the absence of Mg2+ (monitored with the radio-HPLC assay)

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Table 1. Complementation of IPMS-null E. coli mutant by IPMS-like genes of Arabidopsis

<table>
<thead>
<tr>
<th>AGI Code No.</th>
<th>Name</th>
<th>Junk and Mourad (2002)a</th>
<th>Field et al. (2004)b</th>
<th>S. Textor (Unpublished Data)c</th>
<th>This Articled</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g18500</td>
<td>IPMS1</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>At1g74040</td>
<td>IPMS2</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>At5g23010</td>
<td>MAM1</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>At5g23020</td>
<td>MAM3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Conditions not described. bOvernight at 37°C. cThree days at 28°C. dThree days at either 30°C or 37°C.
was also completely lost if the incubation assay was adjusted to 4 mM Ca\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), or Zn\(^{2+}\), whereas the residual activity was not inhibited by the presence of 4 mM Fe\(^{2+}\) or Co\(^{2+}\). While 4 mM K\(^{+}\) gave a slight stimulation of the residual enzyme activity and the addition of 4 mM Mn\(^{2+}\) restored about 50% of the initial activity, only the addition of 4 mM of Mg\(^{2+}\) brought the enzyme activity back to its full initial rate.

The pH curves of IPMS1 and IMPS2 are similar and display rather broad optima around pH 8.5 (Fig. 4B). The more flattened curve for IPMS2 with a sharp drop of enzyme activity above pH 9.5 was seen consistently in replicate experiments. A pH of 8.0 was routinely used in enzyme assays to minimize the spontaneous chemical hydrolysis of acetyl-CoA that increased noticeably above this value.

The molecular mass of the native recombinant protein was determined by gel filtration on a calibrated Superdex-200 column, assaying the activity of eluted 1-mL fractions by the DTNB method (Fig. 4C).

The main activity of IPMS1 eluted at a molecular mass of 124 kD, suggesting that the functional protein is a dimer, as the monomer size calculated from the amino acid sequence is 63.1 kD (including the additional 0.8 kD from the His-tag). On the other hand, the main activity of IPMS2 eluted at approximately 280 kD, indicating this enzyme is active as a tetramer (predicted monomer size 64.1 kD). Omitting the 150 mM of NaCl from the elution buffer or adding 2 mM MgCl\(_2\) to the elution buffer did not affect these results.

Enzyme kinetics were determined in a continuous spectrophotometric assay with N-ethylmaleimide (NEM; see “Materials and Methods”) and are presented in Table II. The main difference between IPMS1 and IPMS2 was the \(K_m\) obtained for acetyl-CoA: 45 \(\mu\)M and 16 \(\mu\)M, respectively. There was no significant difference observed in the \(K_m\) for 2-oxoisovalerate (304 \(\mu\)M and 279 \(\mu\)M), nor any difference in specific activity between IPMS1 and IPMS2.

IPMS activity in plants is generally considered to be feedback inhibited by Leu (Singh, 1999; Coruzzi and Last, 2000). To test this possibility, aliquots of the incubation mixture were adjusted to Leu concentrations between 0.025 mM and 10 mM. We detected a slight inhibitory effect of Leu on the activity of IPMS1 and IPMS2 (Fig. 5). Inhibition of both enzymes reached a maximum of 30% to 35% around 1 mM Leu, but declined to 15% at higher concentrations of Leu in the case of IMPS2. Changing the pH of the incubation mixture to 7.5 did not augment the inhibitory effect of Leu as had been observed for the IPMS of yeast (Ulm et al., 1972). To account for the possible influences of posttranscriptional modifications of the IPMS proteins in planta, the effect of Leu was also tested on the IPMS activity present in crude extracts of Arabidopsis. An extract prepared from leaf material (3.5 weeks old) according to the protocol of Textor et al. (2004), but leaving out the ammonium-sulfate precipitation step, was measured in the presence of an acetyl-CoA regenerating system. The inhibitory effect of Leu on the IPMS activity present in the crude extract was similar to those values measured for the heterologously expressed IPMSs: 14% at 0.5 mM Leu, 33% at 1.0 mM Leu, and 48% at 5.0 mM Leu. No inhibitory effects on the

Figure 3. Radio-HPLC analyses of the biochemical assay for IPMS2. Results for IPMS1 had a similar pattern. A, Incubation of IPMS2 with 500 mM \([^{14}C]\)acetyl-CoA and 3 mM 2-oxoisovalerate shows \([^{14}C]\)-2-isopropylmalate (IPM) as the only radioactive labeled product. B, In the absence of 2-oxo acid substrate, a small amount of \([^{14}C]\)acetate (Ac) is formed, whereas most of the \([^{14}C]\)acetyl-CoA (Ac-CoA) remains intact and is hardly retained in the HPLC column. C, UV trace (230 nm) of the HPLC showing the elution pattern of a standard solution containing 10 mM acetic acid (Ac) and 5 mM 2-isopropylmalate (IPM), and the injection peak at 15 min. D, Incubation of the IPMS2 gene product with 4-methylthio-2-oxobutyrate and \([^{14}C]\)acetyl-CoA yields a small amount of \([^{14}C]2\)-(2'-methylthioethyl)malate (MTEM), whereas most of the \([^{14}C]\)acetyl-CoA (Ac-CoA) remains.
crude IPMS enzyme were observed for Ile, Val, and Gly. In previous work, the IPMS of spinach was reported to be 100% inhibited by micromolar concentrations of Leu (Hagelstein and Schultz, 1993). However, our experiments on the IPMS activity present in a chloroplast-enriched crude extract of spinach prepared after Falk et al. (2004), without the gel-filtration step, did not show such a pronounced effect. The inhibition of IPMS activity was similar to that seen with the Arabidopsis crude extract: 32% at 0.5 mM Leu, 42% at 1 mM Leu, and 66% at 5 mM Leu.

**Both Enzymes Accept Other Substrates and Catalyze the First Condensation Reaction of Glucosinolate Chain Elongation at Low Rates**

The detectable conversion of 4-methylthio-2-oxobutyrate to its alkylmalate derivative by IPMS1

**Figure 4.** Properties of Arabidopsis IPMSs. A, Effect of MgCl₂ concentration on IPMS2 activity; 0 mM corresponds to a desalted enzyme preparation without addition of MgCl₂. A similar graph was obtained for IPMS1. B, pH Curves for enzyme activity of IPMS1 and IPMS2 using MES (■), BisTris-propane (▲), and 2-amino-2-methyl-1-propanol (●). Each data point corresponds to a duplicate enzyme activity measurement with DTNB that is corrected for chemical hydrolysis of acetyl-CoA at each particular pH value. C, Determination of the molecular mass of IPMS1 (□) and IPMS2 (○) by calibrated gel-filtration chromatography. The column was calibrated by measuring the elution volume (●) of β-amylase (200 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4 kD). The void volume of the column determined with Blue Dextran (2.0 × 10³ kD) was 44 mL.

**Table II.** Kinetic parameters for IPMS1 and IPMS2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>( K_m ) (μM) ± SE</th>
<th>( V_{max} ) (μmol min⁻¹ g⁻¹) ± SE</th>
<th>( k_{cat} ) (s⁻¹)</th>
<th>( k_{cat}/K_m ) (s⁻¹ μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMS1</td>
<td>2-Oxoisovalerate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>304 ± 68</td>
<td>2.3 × 10⁻³ ± 0.2 × 10⁻³</td>
<td>2.4</td>
<td>7.8 × 10⁴</td>
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<tr>
<td></td>
<td>Acetyl-CoA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 ± 10</td>
<td>2.1 × 10⁻³ ± 0.1 × 10⁻³</td>
<td>2.2</td>
<td>4.7 × 10⁴</td>
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<tr>
<td>IPMS2</td>
<td>2-Oxoisovalerate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>279 ± 51</td>
<td>2.2 × 10⁻³ ± 0.1 × 10⁻³</td>
<td>2.3</td>
<td>8.3 × 10³</td>
</tr>
<tr>
<td></td>
<td>Acetyl-CoA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16 ± 4</td>
<td>1.8 × 10⁻³ ± 0.1 × 10⁻³</td>
<td>1.9</td>
<td>1.2 × 10⁵</td>
</tr>
</tbody>
</table>

<sup>a</sup>Measured in the presence of 500 μM acetyl-CoA.  <sup>b</sup>Measured in the presence of 1 mM 2-oxoisovalerate.
and IPMS2 from Arabidopsis (Fig. 3) led us to test various 2-oxo acid substrates other than 2-oxoisovalerate. Potential substrates were incubated with [14C]acetyl-CoA at saturating concentrations and the reaction mixtures analyzed by radio-HPLC. The reaction products were identified by coelution of synthetic standards and/or liquid chromatography-mass spectrometry analyses (Kroymann et al., 2001; Textor et al., 2004; S. Textor, unpublished data). The enzymatic rates for converted substrates were determined with DTNB in a timed enzyme assay and are expressed relative to the conversion of 2-oxoisovalerate (Table III). The specific activities for IPMS determined in this way are comparable to those in Table II that were determined with NEM in the less-sensitive spectrophotometric assay.

There was no major difference between IPMS1 and IPMS2 with respect to their substrate specificities. Of the tested substrates, 2-oxobutyrate seems to perform as well or even better than the true substrate 2-oxoisovalerate (common name for 3-methyl-2-oxobutyrate), while pyruvate and 2-oxovalerate were also converted in ample yield. However, 3-methyl-2-oxovalerate and 4-methyl-2-oxovalerate, i.e. transaminated products of Ile and Leu, respectively, were converted only in trace amounts, while phenylpyruvate, the 2-oxo acid of Phe, was not converted at all. The reaction between 4-methylthio-2-oxobutyrate and acetyl-CoA occurred at a relatively low yield (consistent with the data in Fig. 3), but was 3 to 4 times more efficient than the enzymatic conversion of its methylene analog, 2-oxohexanoate. As mentioned in the introduction, formation of 2-(2'-methylthio)ethylmalate represents the aldol-type condensation reaction in the first cycle of Met side-chain elongation and yields a direct precursor of C₅-glucosinolate biosynthesis. However, this reaction proceeds at a far slower rate than the conversion of 2-oxoisovalerate to isopropylmalate. Estimations by the DTNB assay gave a $K_m$ value of at least 3 mM for 4-methylthio-2-oxobutyrate that results in a specificity constant ($k_{cat}/K_m$) of less than 13 M⁻¹ s⁻¹. The $K_m$ for pyruvate was in the millimolar range as well. Substrates having a carbon chain longer than 4-methylthio-2-oxobutyrate or 2-oxohexanoate were not accepted at all.

To shed further light on whether IPMS1 and IPMS2 participate in glucosinolate formation, we investigated whether or not the low activity of these enzymes with 4-methylthio-2-oxobutyrate is a general feature of IPMS enzymes. When the substrate specificity of the IPMS cloned from E. coli (the leuA gene) was measured by incubation of the purified His-tag protein with [14C]acetyl-CoA, the substrates 4-methylthio-2-oxobutyrate, pyruvate, and 2-oxovalerate were converted to their malate derivatives to the same extent relative to 2-oxoisovalerate as observed for the IPMSs of Arabidopsis (Supplemental Fig. S1).

**Plant Lines Mutated in IPMS1 and IPMS2 Still Had Wild-Type Levels of Leu**

To determine how the IPMS genes contribute to amino acid and glucosinolate biosynthesis, we characterized three mutant lines of Arabidopsis (Col-0), one with a T-DNA insertion in the IPMS1 gene (Salk_101771) and two with a T-DNA insertion in the IPMS2 gene (Salk_051060 and Salk_000074; Alonso et al., 2003). The genomic DNA of individuals from crosses segregating for each insert were tested for the presence of a T-DNA insert in the respective genes using oligonucleotide primer pairs derived from the T-DNA insert and the respective IPMS genes (Supplemental Table S1), and the product sizes and sequences obtained were consistent with the reported insertion sites (Supplemental Fig. S2). Individual plants were identified that were homozygous for the T-DNA insert, heterozygous, or lacking the T-DNA insert (outsegregants).

As shown in Figure 6, transcripts for IPMS1 and IPMS2 were readily detected in wild-type plants by reverse transcription (RT)-PCR, but no transcript of IPMS1 was detected in homozygotes for insertion in this gene (Salk_101771 line). In homozygotes for the IPMS2 insertions, no IPMS2 transcript was observed in the Salk_051060 line, and only a weak band for IPMS2 was detected in homozygotes for the Salk_000074 line. In the mutant lines for each IPMS gene, transcript of the other IPMS gene was present, but no compensatory effects were observed. The IPMS1 mutant line grew somewhat slower and had undulated leaves that tended to be slightly chlorotic as...
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Elongation Step</th>
<th>Relative Conversion (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyoxylate</td>
<td><img src="image" alt="Glyoxylate Structure" /></td>
<td></td>
<td>0.3 ± 0.0 0.2 ± 0.1</td>
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<td>Pyruvate</td>
<td><img src="image" alt="Pyruvate Structure" /></td>
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<td>12.7 ± 1.8 15.7 ± 1.2</td>
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<td>2-Oxobutyrate</td>
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<td>112.2 ± 2.3 97.5 ± 2.7</td>
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<td><strong>2-Oxoisovalerate</strong></td>
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<td>100.0 ± 5.6 100.0 ± 3.5</td>
</tr>
<tr>
<td>2-Oxovalerate</td>
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<td>27.2 ± 0.8 33.8 ± 2.0</td>
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<td>3-Methyl-2-oxovalerate</td>
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<td>2-Oxohexanoate</td>
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<tr>
<td>4-Methylthio-2-oxobutyrate</td>
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<td>C$_2$→C$_3$</td>
<td>1.3 ± 0.1 1.7 ± 0.1</td>
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<td>5-Methyl-2-oxohexanoate</td>
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<td>5-Methylthio-2-oxopentanoate</td>
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<td>C$_3$→C$_4$</td>
<td>– –</td>
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<tr>
<td>Absence of 2-oxo-substrate</td>
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<td>0.4 ± 0.0 0.6 ± 0.0</td>
</tr>
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</table>

*Incubations were done with 20 mM 2-oxo acid and 500 μM acetyl-CoA. Reaction of these substrates leads to glucosinolates with the indicated change in number of carbon atoms in the side chain. 100% Enzyme activity corresponds to a specific activity of 2.6 × 10$^3$ μmol$^{-1}$ min$^{-1}$ g$^{-1}$ for IPMS1 and 2.9 × 10$^3$ μmol$^{-1}$ min$^{-1}$ g$^{-1}$ for IPMS2. Represents the hydrolysis of acetyl-CoA due to the presence of enzyme without 2-oxo acid substrate; this value was taken as a blank for all measurements.
and S_051060.

Results for the leaf tissues of Salk T-DNA insertion lines, homozygous (mm) for the IPMS2 mutant line S_000074; B, results for the IPMS2 mutant lines S_000074 and S_051060.

![Figure 6. Semiquantitative RT-PCR analyses of IPMS transcript levels in leaf tissues of Salk T-DNA insertion lines, homozygous (mm) for the insertion or lacking the insertions (ww), and Col-0 wild type (WT). A, Results for the IPMS1 mutant line S_101771 compared to the IPMS1 mutant line S_000074; B, results for the IPMS2 mutant lines S_000074 and S_051060.](image)

Both Genes Are Expressed Constitutively throughout the Plant

Semiquantitative RT-PCR analysis revealed that both IPMS genes are expressed throughout the plant and mRNA transcript levels do not differ extensively between various organs (Supplemental Fig. S4). These results are consistent with data from the Arabidopsis ATH1 Genome Array (Affymetrix), posted on the Internet and retrieved with the GENEVESTIGATOR program (Zimmermann et al., 2004). The collected microarray data also show that both IPMS genes are similarly expressed among different developmental stages and after specific stress treatments (Supplemental Fig. S5). An exception is the level of IPMS1 transcript in the seeds during silique ripening, which is at least twice as high as in most other plant tissues.

DISCUSSION

IPMS1 and IPMS2 Function in Leu Formation But Not in Glucosinolate Biosynthesis

Heterologous expression of the Arabidopsis IPMS1 (At1g18500) and IPMS2 (At1g74040) cDNAs in E. coli and the study of the enzymatic properties of the protein products demonstrated that both genes encode IPMSs, which catalyze the aldol-type condensation reaction between acetyl-CoA and 2-oxoisovalerate in the biosynthesis of Leu. Nonetheless, both proteins were also able to catalyze the MAM reaction between acetyl-CoA and 4-methylthio-2-oxobutyrate to some extent. However this reaction occurs with such a low specificity constant ($k_{cat}/K_m < 13 M^{-1} s^{-1}$) in comparison with the previously measured value for MAM3 ($1.4 \times 10^3 M^{-1} s^{-1}$; S. Textor, unpublished data) that this MAM activity is probably of no significance in vivo. In addition, an insertion mutant for IPMS1 with undetectable transcript levels of IPMS1 (Salk_101771), an insertion mutant for IPMS2 with undetectable transcript levels of IPMS2 (Salk_051060), and an IPMS2 insertion mutant line with reduced IPMS2 transcript levels (Salk_000074) all did not display any changes in glucosinolate content (Supplemental Fig. S3). Hence, we conclude that IPMS1 and IPMS2 have a primary function in Leu biosynthesis and are not significantly involved in the Met side-chain elongation of glucosinolate biosynthesis.

In accordance with this conclusion, the IPMS2 gene in a prokaryotic expression construct rescued the IPMS-null E. coli mutant strain CV512(DE3), but curiously an IPMS1 construct did not. Rescue of the Leu-deficient E. coli strain by IPMS2, but not IPMS1, was also observed by Field et al. (2004); however, Junk and Mourad (2002) could not demonstrate any complementation with IPMS2, but did for MAM1 and MAM3 (see also Table I). The inconsistencies in demonstrating the ability of these plant genes to complement the Leu auxotrophy in E. coli provide a cautionary tale about the value of complementation assays as surrogates for a full characterization of in vitro enzyme activity.

Amino acid analysis of the IPMS1 and IPMS2 insertion mutants also gave equivocal results about in vivo function, since none of the insertion mutants showed any changes in soluble Leu content (Fig. 7). As plants require Leu for survival, a total lack of Leu accumulation was not expected in these viable mutant lines. Nevertheless, the absence of any detectable change in Leu content is noteworthy and must demonstrate that IPMS1 and IPMS2 compensate for each other’s absence very well, because it is unlikely that the MAM enzymes with their very low IPMS activity can contribute to Leu biosynthesis in an efficient way (S. Textor, unpublished data).
Despite the lack of a reduction in Leu, the IPMS1 insertion mutant has a clear phenotype in its elevated soluble Val content (Fig. 7). Such an increase in Val content was also observed in an independently generated En-1 insertion mutant of IPMS1 by Field et al. (2004), but was not discussed any further. This feature may be a consequence of the fact that the IPMS1 mutant—despite the presence of IPMS2—has less IPMS activity relative to Val-aminotransferase activity than found in wild-type plants. Since both enzymes use 2-oxoisovalerate as a substrate, such an imbalance will increase the amount of Val at the expense of Leu. This will reduce the feedback inhibition of acetohydroxyacid synthase (AHAS), because Val is a less potent inhibitor of AHAS activity than Leu and, moreover, it is actually the combination of Leu and Val that has the strongest inhibitory effect on AHAS (Miflin and Cave, 1972; Lee and Duggleby, 2001). Such a scenario may account for the maintenance of normal levels of Leu in the IPMS1 insertion mutant, despite lower quantities of IPMS, and an enhancement of Val content. A higher activity of AHAS could also raise the levels of Ile, but this does not occur probably due to feedback inhibition by Ile of Thr dehydratase—the enzyme that makes 2-oxobutyrate, the substrate of AHAS for Ile synthesis (Singh, 1999; Coruzzi and Last, 2000).

**Figure 7.** Analyses of the free amino acid content of homozygous T-DNA insertion lines for IPMS1 (A; Salk_101771 [mm]) and IPMS2 (B; Salk_051060 [mm] and Salk_000074 [mm]). The IMPS1 mutant shows a significant increase in Val content in comparison with the corresponding outsegregants (ww) and Col-0 wild type. Error bars indicate SD.

**IPMS1 and IPMS2 Differ Somewhat in Gene Expression Pattern and Properties of the Encoded Protein**

Based on RT-PCR analyses and publicly available microarray experiments assembled with the
GENEVESTIGATOR program (Supplemental Figs. S4 and S5), both IPMS genes are constitutively expressed throughout the plant and thus can complement each other, consistent with the normal Leu phenotype of the insertion mutant lines. However, the two genes are at least subfunctionalyzed at a regulatory level (Moore and Purugganan, 2005) since IPMS1 expression, but not that of IPMS2, is twice as high in seeds as in most other plant tissues. In the developing seed, Leu is one of the main free amino acids and may be specifically synthesized there since it is not abundant in the phloem (Baud et al., 2002). The particular role of IPMS1 during seed development might explain the 20% reduction in seed fecundity reported by Field et al. (2004) for an IPMS1 knockout line.

The major biochemical difference between IPMS1 and IPMS2 appears to be the $K_m$ value for acetyl-CoA, 45 μM and 16 μM, respectively, making IPMS2 the more efficient enzyme at comparable turnover numbers. Calibrated gel-filtration chromatography showed that IPMS1 is active as a dimer (124 kD), whereas IPMS2 is mainly active as a tetramer (approximately 280 kD; Fig. 4C); nonetheless, these data should be interpreted with care as both proteins contain a His-tag that might influence the formation of quaternary structure (Wu and Filutowicz, 1999). The IPMS enzymes previously isolated from microorganisms were characterized either as dimers (Saccharomyces cerevisiae, Corynebacterium glutamicum, Mycobacterium tuberculosis) or tetramers (Salmonella typhimurium; Leary and Kohlhaw, 1972; Roeder and Kohlhaw, 1980; Pátek et al., 1994; Koon et al., 2004). Gel filtration of the IPMS activity present in crude extracts of Arabidopsis showed a broad band of enzyme activity between 50 and 200 kD with a major peak at 95 to 120 kD (Textor et al., 2004). Assuming that IPMS1 is also present as a dimer in vivo and IPMS2 mostly as a tetramer, the main IPMS activity present in the plant crude extract is IPMS1.

Other IPMSs Have Different Kinetic Constants, Cation Preferences, and Sensitivity to Leu Feedback Inhibition

The $K_m$ of IPMS1 and IPMS2 for 2-oxoisovalerate are similar to each other (304 μM and 279 μM) and are much higher than the reported value of 75 μM for the IPMS present in spinach chloroplasts. The spinach IPMS also has a distinct $K_m$ value for acetyl-CoA, 5 μM versus 45 μM and 16 μM for IPMS1 and IPMS2, respectively (Hagelstein and Schultz, 1993).

The IPMS enzymes of Arabidopsis, just as the spinach IPMS, are dependent upon millimolar concentrations of Mg$^{2+}$ (Fig. 4) for optimal enzyme activity. This cofactor can only be replaced with Mn$^{2+}$, which yields about 50% of the initial enzyme activity. However, Mn$^{2+}$ is clearly preferred over Mg$^{2+}$ as a cofactor by the MAM enzymes (Falk et al., 2004; Textor et al., 2004). Some IPMS enzymes of fungi and bacteria also employ Mn$^{2+}$ or Zn$^{2+}$ as a cofactor (Stieglitz and Calvo, 1974; Wiegel, 1978; Roeder and Kohlhaw, 1980; Koon et al., 2004). The broad pH optima at 8.5 obtained for IPMS1 and IPMS2 is common for IPMSs, irrespective of their source, and the same pH dependence has also been observed for the MAMs (Falk et al., 2004; Textor et al., 2004). Generally, enzymes at critical branchpoints in plant amino acid biosynthesis are feedback inhibited by their end-product amino acids (Coruzzi and Last, 2000). Accordingly, Leu inhibits the enzyme activity of both heterologously expressed IPMSs. A maximum of 30% to 35% inhibition was reached around a concentration of 1 mM Leu (Fig. 5), and a similar effect was detected on the IPMS activity in the crude extract of Arabidopsis. A somewhat greater effect has been reported on the IPMS present in crude extracts of maize embryos (61% inhibition at 50 μM Leu, 84% inhibition at 5 mM Leu; Oaks, 1965), but the IPMS of spinach chloroplasts was reported to be 100% inhibited by micromolar concentrations of Leu (Hagelstein and Schultz, 1993). However, in a chloroplast-enriched crude extract from spinach, we only detected a 66% inhibition of IPMS at 5 mM Leu. A 100% inhibition of IPMS activity at 1 mM concentrations of Leu is common among bacteria (Stieglitz and Calvo, 1974). The production of 2-oxoisovalerate itself is regulated through feedback inhibition of AHAS by Leu, Val, and Ile (Fig. 1; Singh, 1999). Concentrations of 1 to 5 mM of these amino acids result in a 45% to 65% inhibition of AHAS enzyme activity in Arabidopsis (Lee and Duggleby, 2001) and other plants (Miflin and Cave, 1972), i.e. an effect of similar magnitude as that of 1 to 5 mM Leu on IPMS activity.

IPMSs Produce Other Products in Vitro and in Vivo

The substrate specificity of IPMSs is not very high. For example, the reported reaction rates for those 2-oxobutyrate are usually higher than those found for the true substrate 2-oxoisovalerate, despite the higher $K_m$ for 2-oxobutyrate (Webster and Gross, 1965; Strassman and Ceci, 1967; Rabin et al., 1968; Gross, 1970; Kohlhaw and Leary, 1970; Uml et al., 1972; Wiegel and Schlegel, 1977; Wiegel, 1981; Kohlhaw, 1988). This is also true for the Arabidopsis enzymes. In addition, most reported IPMSs and, to a lesser extent, the MAMs from Arabidopsis (S. Textor, unpublished data) can catalyze the condensation between pyruvate and acetyl-CoA. The product of this condensation reaction, citramalate, has been reported as a metabolite in Arabidopsis (Fiehn et al., 2000) and tomato (27.6 μmol g⁻¹ fresh weight in orange-colored fruits; Roessner-Tunali et al., 2003). The presence of citramalate has led to the suggestion that plants possess a tricarboxylic acid cycle bypass previously described in bacteria (Grant and Smith, 2000). Citramalate may also have a function in Ile biosynthesis by serving as a precursor of 2-oxobutyrate, a substrate for AHAS. This metabolic route has been demonstrated for a mutant of the microorganism Serratia marcescens (Kisumi et al., 1977), the halophilic archean Haloarcula hispanica (Hochuli et al., 1999), and in Leptospira intergogens (Xu et al., 2004).
Isopropylmalate Synthase Genes in Arabidopsis

Figure 8. Alignment of deduced amino acid sequences for IPMS1, IPMS2, MAM1, and MAM3 with IPMS sequences from wild tomato (GenBank accession nos. AAB61598 and AAB61599), *E. coli* (Swiss-Prot: P09151), and *M. tuberculosis* whose protein structure has been elucidated (PDB: 1SR9). Black shading indicates individual amino acids that are conserved within all sequences, dark gray shading individual amino acids that are identical in at least six out of eight sequences, and light gray
Compounds with a carbon chain length longer than 2-oxoisovalerate, like 2-oxovalerate, 4-methyl-2-oxovalerate, and 2-oxohexanoate, have usually been reported to be active-site inhibitors of IPMS (Webster and Gross, 1965; Gross, 1970; Kohlhaw and Leary, 1970; Ulm et al., 1972; Wiegel and Schlegel, 1977; Wiegel, 1981; Kohlhaw, 1988) rather than substrates, as described in this study. An exception is the paper of Rabin et al. (1968) that reports on the conversion of 2-oxovalerate, 4-methyl-2-oxovalerate, and 2-oxohexanoate by an IPMS activity from Pseudomonas aerugi-
osa at yields comparable to those reported there. Our results indicate that the IPMSs from Arabidopsis and E. coli can use 2-oxo acids ranging in length from glyoxylate to 2-oxohexanoate. However, the shortest and longest substrates react at rates that might have escaped detection in older work with less-sensitive methods.

IPMS and MAM Proteins Share Some But Not All Structural Features

The most striking difference between the amino acid sequences of the IPMS and MAM proteins from Arabi-
dopsis is the absence of about 150 amino acids at the C-terminal domain of the MAMs (Fig. 8). This domain contains a conserved allostERIC Leu binding site (Koon et al., 2004) whose absence is likely responsible for the lack of Leu inhibition of the E. sativa MAM syn-
thase (Falk et al., 2004). One can expect the MAMs of Arabidopsis to show similar behavior. The only pub-
ished crystal structure for an IPMS is that of the M. tuberculosis protein (Koon et al., 2004). Although Arabidopsis IPMS1 and IPMS2 share only about 25% amino acid identity with the M. tuberculosis protein (Fig. 8; ISR9), the amino acids of the hydrophobic Leu binding pocket of the M. tuberculosis protein are either conserved in the Arabidopsis proteins (Tyr-554, Ala-558, Ala-565, Ala-567) or replaced by comparable conserved in the Arabidopsis proteins (Tyr-554, Ala-
M. tuberculosis (Fig. 8; 1SR9), the amino acids of the hydrophobic Leu binding site that are marked with an ‘‘L’’ and the conserved motifs GxGERXG and HxH(D/N)D. Amino acid
shading amino acids that are identical in at least five out of eight sequences. The ChloroP-predicted cleavage sites are marked
with an undecor. Amino acid residues mentioned in the text are represented below the alignment, likewise amino acid residues of the Leu binding site that are marked with an ‘‘L’’ and the conserved motifs GxGERXG and HxH(D/N)D. Amino acid
positions are numbered relative to 1SR9.

Figure 8. (Continued.)

shading amino acids that are identical in at least five out of eight sequences. The ChloroP-predicted cleavage sites are marked with an underscore. Amino acid residues mentioned in the text are represented below the alignment, likewise amino acid residues of the Leu binding site that are marked with an ‘‘L’’ and the conserved motifs GxGERXG and HxH(D/N)D. Amino acid positions are numbered relative to 1SR9.
additional selection, most of the original activity for 2-oxoisovalerate has been lost and some of the MAMs have developed affinities for substrates even larger than 4-methylthio-2-oxobutyrate. In future research, we will seek evidence for this scenario and try to determine what structural changes in IPMS accompanied this process.

MATERIALS AND METHODS

Plants

Seeds of Arabidopsis (Arabidopsis thaliana L. Heynh), ecotype Col-0 (CS8079 Arabidopsis Biological Resource Center), were sown densely in or- dered rows with vermiculite (3:1). Plants were raised in a controlled growth chamber with a diurnal cycle of 10 h light and 14 h dark at 22°C. Illumination was from a mixture of Fluora (Osmar) and Cool White lamps at 230 μmol m⁻² s⁻¹. Seeds of the Salk mutant lines were obtained from the European Arabidopsis Stock Centre (Nottingham, UK).

RNA Isolation and cDNA Cloning

Total RNA was isolated from liquid nitrogen-frozen root tissue (IPSM1) or total leaf tissue (IPSM2) with Trizol reagent (Invitrogen) according to man- ufacturer’s instructions. First-strand cDNA was synthesized with 2 μg of total RNA, 200 units of MMLV reverse transcriptase (Promega), and 0.5 μg of gene-specific oligonucleotide primer using the reagents and instructions provided. IPMS2 was amplified from the first-strand cDNA product using the primer pair 1ipms2m/2ipms2n (for all primers used, see Supplemental Table S1), resulting in a truncated ORF lacking 138 nucleotides corresponding to a putative chloroplast transit peptide (ChloroP; Emanuelsson et al., 1999). The reaction product was gel purified using a Qiagen gel extraction kit (Qiagen) cloned directly into the pBAD-TOPO (Invitrogen) expression vector, and transformed into TOP10 cells (Invitrogen) according to the manufacturer’s instructions. The DNA of transformed colonies was purified by a miniprep (Invisorb spin plasmid mini kit; Invitek) and screened by restriction analyses (Savant). A portion of the concentrated supernatant (20 μL) was analyzed on the radio-HPLC according to Falk et al. (2004).

Enzyme Assays

Continuous Spectrophotometric Assay (NEM Assay)

The reaction mixture contained 10 to 500 μM acetyl-CoA, 50 to 2,000 μM 2-oxoisovalerate, 0.1 mM NEM (Sigma-Aldrich), 4 mM MgCl₂, and 200 μL Tris buffer, pH 8.0, and 5 μL of enzyme preparation in a total volume of 500 μL. The change in optical density over time at 30°C was followed against a solution of 1 mM NEM in a UV-2501PC spectrophotometer (Shimadzu) using micro quartz cuvettes (Hellma) with black walls. The loss of the thioester bond in acetyl-CoA was monitored by the appearance of acetyl-CoA and the loss of the thioester bond in acetyl-CoA and the loss of the double bond in NEM upon binding to CoA-SH result in an ∆OD₂₅₈ of -1.08 × 10⁻³ (1-cm light path) per mole of CoA-SH formed (Webster and Gross, 1965).

Spectrophotometric End-Point Assay (DTNB Assay)

The enzyme preparation (1–5 μL) was incubated for 10 min at 30°C with 10 mM 2-oxoisovalerate, 500 μM acetyl-CoA, 4 mM MgCl₂, and 100 mM Tris, pH 8.0, in a total volume of 150 μL. Incubations were synchronized by pipetting the enzyme solution in the lid of an Eppendorf tube containing the incubation mixture, after which the enzyme in all vessels was simultaneously spun down for a few seconds in a table-top centrifuge; reactions were stopped by freezing the vessels in liquid nitrogen. To the frozen reaction mixture, 200 μL of ethanol and 200 μL of a fresh 1 mM solution of DTNB (Sigma-Aldrich) in 100 mM Tris, pH 8.0, were added. The mixture was left at room temperature to allow the
free thiol group of CoA to react with DNTB, forming a yellow-colored, 3-carboxy-4-nitrophenol anion with an ε_{412} of 14,140 μM^{-1} cm^{-1} (Kohlbaw, 1988). When no further color developed, the mixture was centrifuged for 5 min at 16,000g and the absorbance measured against water at 412 nm. The enzyme assay was corrected for unspecific hydrolysis of acetyl-CoA by subtracting the absorbance of a blank incubation where no 2-oxo-acid substrate had been added. The assay was generally linear for the first 15 min with 2 μg of protein.

### Enzyme Characterization

#### Substrate Specificity

The various 2-oxo acid substrates tested in the radio-HPLC assay with 14C-acetyl-CoA were either obtained from Fluka or Sigma-Aldrich, or synthesized in our laboratories (Falk et al., 2004; S. Textor, unpublished data). Reaction rates of the different substrates were determined at 20 μmol (estimated to be a saturating concentration) with the DNTB assay; the amount of enzyme added was varied from 1 to 50 μL, depending on the substrate, to stay within detection limits.

### Enzyme Kinetics

The K_{m} and V_{max} values for acetyl-CoA and 2-oxo-isovalerate were determined with the NEM assay. They were calculated from the recorded initial linear reaction rates at different substrate concentrations of three to four independent experiments using the Enzyme Kinetic Module (Version 1.1) of SigmaPlot (Version 8.0).

### Other Enzyme Characteristics

The pH optima for IPMS1 and IPMS2 were determined in the pH range of 5.5 to 10.5 in 0.5 pH units using the DTNB assay in which Tris buffer was replaced by MES (pH 5.5–6.5), BisTris-propane (pH 6.5–9.5), or 2-amino-2-methyl-1-propanol (pH 9.5–10.5). The DTNB assay was also used to test the effect of Leu on enzyme activity in a range from 25 μM to 10 mM. Furthermore, the effect on enzyme activity of Mg^{2+} concentration, Mn^{2+}, and K^{+} was investigated with the DTNB assay taking an enzyme preparation after His-tag purification that had been desalted to Tris buffer lacking MgCl_{2}. The effect of other cations, i.e. Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mn^{2+}, and Zn^{2+}, on this enzyme preparation had to be tested with the qualitative radio-HPLC assay because these ions are strong oxidizers that react with the 3-carboxy-4-nitrophenol anion of the DTNB assay, causing a rapid loss of the yellow color. All cations were used as their chloride salts, except Fe^{3+} and Zn^{2+} that were used as their sulfate salts.

The molecular mass of the native recombinant protein was estimated by exclusion chromatography on a Superdex 200 column (Hiload 16/60; Pharmacia Biotech) that had been calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). The column was loaded with 200 μL of enzyme preparation and eluted at 1 mL min^{-1} with a buffer consisting of 50 mM Tris, pH 8.0, 10% glycerol, and 150 mM NaCl. Sixty fractions of 1 mL were collected after discarding the first 40 mL of eluent, and 138.5 μL of each fraction was tested in a modified DTNB assay (25-min incubation at room temperature) for enzyme activity.

### E. coli CV512 Complementation and Origin of E. coli IPMS

The IPMS1/PCR-T7/CT-TOPO and IPMS2/pBAD-TOPO cDNA constructs were transformed in E. coli CV512 (F'lexA737; Somers et al., 1973). The CV512 strain lacks functional IPMS and is able to grow on M9 minimal medium with Glc as a carbon source only when it is either supplemented with Casamino acids (Difco) or transformed with a construct containing a functional IPMS. To ensure expression from a T7 polymerase-driven construct, the IPTG inducible T7-polymerase gene was introduced into strain CV512 by a ADES 1 s 3 y s e n o g e n e s i s kit (Novagen) giving CV512(DE3). As a positive control, a pET28a (Novagen) construct harboring the E. coli DHFSa (Hanahan, 1983) IPMS gene leuA was used for complementation studies. The leuA gene was amplified by PCR from DHFSa with the primer pair IPMFEH/IMPMEv (Supplemental Table S1) and cloned into pET28a using the BamHI/XhoI restriction sites provided by the primers. Complementation efficiency was tested at two different incubation temperatures, 30°C and 37°C.

The pET28a/LeuA construct was also used as a source of E. coli IPMS that was tested in the radio-HPLC assay for its substrate specificity. BL21(DE3) E. coli cells (Invitrogen) with the LeuA construct were grown in 100 mL of Luria-Bertani medium containing 50 μg mL^{-1} kanamycin at 37°C until an OD_{600} of 0.5 was reached. Expression was induced with 1 mM IPTG, and incubation continued for 2.5 h. Cells were harvested and the expressed protein isolated and purified in the same way as described for the Arabidopsis IPMSs, taking advantage of the C-terminal His-tag present in the construct.

### Plant Mutant Lines

#### PCR of Genomic DNA

Genomic DNA was extracted from expanding leaves using an abbreviated protocol of Rogers and Bendich (1985). About 5 mg of young leaf tissue was collected in a 1.5-ml microtube and homogenized with 10 μL of 2× CTAB solution (2% cetyltrimethylammonium bromide [w/v], 100 mM Tris, pH 8.0, 14 mM NaCl, 1% polyvinylpyrrolidone [4,000 molecular weight]) using a micropestle. The sample was incubated at 65°C for 1 to 2 min, cooled briefly on ice, and extracted with 10 μL of chloroform/isoamyl alcohol (24:1, v/v). After 10 μL of water was added, the samples were subjected to centrifugation at 11,000g. The upper phase was recovered and 0.1 to 0.5 μL was used per PCR of 20-μL volume (for details, see below).

#### PCR of RNA Transcript

Total RNA was isolated from freshly harvested, freeze-dried whole rosettes of individual 3- to 4-week-old plants with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. A cDNA population was synthesized with 2 μg of RNA, either 0.5 μg of dT_{18} or 0.17 μg of gene-specific primers (Invitrogen) and 200 units of MMLV reverse transcriptase (Promega) using the reagents and instructions provided. From each of these reverse transcriptase reactions, a 20-μL PCR was prepared consisting of 1× PCR buffer (Promega), 0.2 mM dNTPs, 0.5 μM each primer (see Supplemental Table S1 for specific primer pairs for ACT8, IPMS1, and IPMS2), 0.5 units of Taq Polymerase (Promega), and an aliquot of reverse transcriptase reaction equivalent to 40 ng of template RNA. The reactions were subjected to an initial thermal denaturation of 94°C for 2 min, followed by 30 or 35 cycles at 94°C for 30 s, a primer-dependent annealing temperature for 30 s, 72°C for 2 min, and a final incubation at 72°C for 5 min. After electrophoresis and incubation in ethidium bromide, DNA fragments were analyzed and quantified (GeneGenius with GeneTools Analysis Software Version 3.02 [Synoptics]; or Gel Logic 200 with Kodak Digital Science) of individual 3- to 4-week-old plant material and to quantify the individual amino acids in a specific oligonucleotide primer pairs were done at least two times.

### Amino Acid Analyses

The procedure was optimized to extract total free amino acids from Arabidopsis plant material and to quantify the individual amino acids in a single HPLC run. The amino acids present in a crude plant extract were derivatized with mercaptobenzothiazole and O-phthalaldehyde yielding a fluorescent isocyanide (Roth, 1971; Sarwar and Botting, 1993), a method inappropriate for the detection of Cys (weakly fluorescent derivative) and Pro (contains no primary amino group for derivatization reaction). Rosettes of individual 3- to 4-week-old individual plants were harvested, and individually frozen in liquid nitrogen, lyophilized, and pulverized. Aliquots of 10 and 50 mg were transferred to 2-mL Eppendorf tubes and resuspended with 0.8 mL of 0.1 N HCl to rehydrate the tissue. After incubation for 15 min at room temperature, the samples were centrifuged at 16,000g and the supernatant transferred to a fresh tube. Samples for HPLC were prepared by adding 50 μL of the supernatant to a 350-μL glass vial insert containing 50 μL of a 1:1 (v/v) solution of 0.1 N HCl and 0.5 M potassium borate, after which the vials were capped. The autosampler of the HPLC (Agilent HPL1100 series) was programmed to mix the vial content with 30 μL of reagent consisting of 0.085 M O-phthalaldehyde (Fluka) and 1% (v/v) β-mercaptoethanol in a 0.5 mM sodium borate solution immediately before injection of 50 μL of sample onto the HPLC column (Supelcosil LC-18-DB [250 × 4.6 mm, 5-μm particle size]; Supelco). The column was run with a 0.02 μL citrate solution of pH 5.5 (solvent A) and methanol/acetonitrile (65:35, v/v; solvent B) at 28°C with a gradient as follows: 15% of solvent B at start, linear gradient to 38.3% of
Glucosinolate Analyses

Glucosinolates were extracted from 10 mg of lyophilized plant material and converted into their desulfoglucosinolate counterparts according to Brown et al. (2003). The desulfoglucosinolates were identified and quantified by HPLC analyses on a C18 reversed-phase column (LiChrospher RP-18, 250 × 4.6 mm i.d., 5-μm particle size; Chrompack) by comparison of retention times and UV spectra to those of purified standards and by measuring the A250 relative to an internal standard (Reichert et al., 2002; Brown et al., 2003).

Supplemental Data

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

Supplemental Figure S1. Radio-HPLC analyses of the biochemical assay for the IPMS of E. coli.

Supplemental Figure S2. Predicted positions of the T-DNA inserts in the Salk knockout lines for IPMS1 (Salk_101771) and IPMS2 (Salk_051060 and Salk_000074).

Supplemental Figure S3. Analyses of the glucosinolate content of homozygotic T-DNA insertion lines for IPMS1 (Salk_101771 [mm]) and IPMS2 (Salk_051060 [mm] and Salk_000074 [mm]).

Supplemental Figure S4. Semiquantitative RT-PCR analysis of IPMS1 and IPMS2 gene expression in various plant organs.

Supplemental Figure S5. Expression levels of IPMS1 and IPMS2 in different plant tissues and various growth stages according GENE-VESTIGATOR.

Supplemental Table S1. Oligonucleotide primers used in this study.

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