Acylsugars are insecticidal specialized metabolites produced in the glandular trichomes of plants in the Solanaceae family. In the tomato clade of the Solanum genus, acylsugars consist of aliphatic acids of different chain lengths esterified to sucrose, or less frequently to glucose. Through liquid chromatography-mass spectrometry screening of introgression lines, we previously identified a region of chromosome 8 in the Solanum pennellii LA0716 genome (IL8-1/8-1-1) that causes the cultivated tomato Solanum lycopersicum to shift from producing acylsucroses with abundant 3-methylbutanoic acid acyl chains derived from leucine metabolism to 2-methylpropanoic acid acyl chains derived from valine metabolism. We describe multiple lines of evidence implicating a trichome-expressed gene from this region as playing a role in this shift. S. lycopersicum M82 SlIPMS3 (Soly08g014230) encodes a functional end product inhibition-insensitive version of the committing enzyme of leucine biosynthesis, isopropylmalate synthase, missing the carboxyl-terminal 160 amino acids. In contrast, the S. pennellii LA0716 IPMS3 allele found in IL8-1/8-1-1 encodes a nonfunctional truncated IPMS protein. M82 transformed with an SlIPMS3 RNA interference construct exhibited an acylsugar profile similar to that of IL8-1-1, whereas the expression of SlIPMS3 in IL8-1-1 partially restored the M82 acylsugar phenotype. These IPMS3 alleles are polymorphic in 14 S. pennellii accessions spread throughout the geographical range of occurrence for this species and are associated with acylsugars containing varying amounts of 2-methylpropanoic acid and 3-methylbutanoic acid acyl chains.

Plants make large numbers of metabolites with great structural diversity, differences in cell and tissue type distribution and concentrations, and physiological roles. Beyond evolutionarily ancient and highly conserved central metabolic processes such as photosynthetic carbon fixation and amino acid metabolism, plants produce specialized metabolites (historically referred to as secondary metabolites) that typically are found in a subset of taxa (Milo and Last, 2012). These specialized biosynthetic pathways use building block metabolites derived from more ancient central metabolism (Modolo et al., 2009; de Kraker and Gershenzon, 2011).

Gene duplications and mutations have been documented to modify the activity, expression, or functional regulation of enzymes involved in central metabolism to yield catalysts for specialized metabolism. An especially elegant example is found in grass species, which have evolved a novel form of the Trp synthase a-subunit (indole synthase or indole-3-glycerolphosphate lyase) that produces indole in the first committing step of defensive cyclic hydroxamic acid biosynthesis (Frey et al., 1997; Melanson et al., 1997; Gierl and Frey, 2001). This evolutionary protein engineering created an enzyme that diverts indole away from amino acid production and toward specialized metabolism.

Other examples center upon the key theme of evolutionary modification of the regulation of the expression or activity of a committing enzyme of primary metabolism to produce an enzyme that is involved in specialized metabolism (Moghe and Last, 2015). For example,
elicitor-treated cell cultures of *Ruta graveolens* express a variant anthranilate synthase that is insensitive to allosteric inhibition by the end product Trp (Bohllmann et al., 1995, 1996). These changes in gene expression and allosteric regulation allow the variant enzyme, which is not subject to feedback inhibition by Trp, to be expressed under conditions in which anthranilate-derived alkaloid production is high. Another example of protein engineering is evident in the case of METHYLTHIOALKYLMALATE SYNTHASE (MAM). This evolutionary variant of ISOPROPYLMALATE SYNTHASE (IPMS), the enzyme that catalyzes the committing step in Leu biosynthesis in bacteria, fungi, and plants, catalyzes the first step in chain elongation in Met-derived glucosinolate biosynthesis in Arabidopsis (*Arabidopsis thaliana*) and other crucifers (de Kraker and Gershenzon, 2011). The MAM enzymes have evolved two novel functions. First, point mutations cause altered substrate specificity, which allows them to use methylthio derivatives of the more typical IPMS substrate 2-oxoisovalerate (OIV). Second, these enzymes lack the C-terminal protein domain that is responsible for Leu end product inhibition in IPMS enzymes. These three examples illustrate how gene duplication and neofunctionalization can lead to the conversion of amino acid biosynthetic activities to specialized metabolic enzymes by changing genetic and biochemical regulation as well as substrate preference (Milo and Last, 2012; Moghe and Last, 2015).

Many species within the Solanaceae (nightshade family) accumulate a diverse group of acylsugar specialized metabolites in their glandular trichomes (Fobes et al., 1985; McDowell et al., 2011; Schilmiller et al., 2012). The acylsugars identified thus far consist of short to medium (C2–C12) chain length aliphatic acids in normal (straight chain), iso (branched at the penultimate carbon), or anteiso (branched at the antepenultimate carbon) configuration esterified to Suc or Glc (Fig. 1A; Severson et al., 1985; Burke et al., 1987; King et al., 1990; Walters and Steffens, 1990). Acylsugars contribute to direct defense against a wide variety of insect herbivores (Rodriguez et al., 1993; Juvik et al., 1994; Chortyk et al., 1997; Puterka et al., 2003), and substitutions in either the sugar or acyl chain component alter their insecticidal activity (Puterka et al., 2003). Acylsugars also function in multitrrophic defense: young *Manduca sexta* (tobacco hornworm) larvae metabolize *Nicotiana attenuata* acylsugars and release volatile short-chain organic acids that attract predatory ants (Weinhold and Baldwin, 2011). Their activity in plant protection led to interest in breeding for altered acylsugar quantities and composition in *Solanum lycopersicum* (cultivated tomato). Because acylsugars are especially abundant and structurally varied in wild tomato species (Shapiro et al., 1994; Schilmiller et al., 2010, 2015; Kim et al., 2012; Ghosh et al., 2014), these relatives provide an attractive source of variation for improvement in *S. lycopersicum* pest tolerance (Maluf et al., 2010; Leckie et al., 2012, 2014).

We have been investigating acylsucrose biosynthesis in *S. lycopersicum* and wild relatives to understand the biochemical basis of the intraspecific and interspecific acylsugar diversity (Kim et al., 2012; Schilmiller et al., 2015) and to develop foundations for engineering insect tolerance. Two acyltransferases of the BAHD (for benzyl alcohol O-acetyltransferase [BEAT], anthocyanin O-hydroxycinnamoyltransferase [AHCT], N-hydroxycinnamoyl/benzoyltransferase [HCBT], deacet- tylvinolide 4-O-acetyltransferase [DAT]) superfamily involved in acylsugar biosynthesis were recently described: ACYLSUCROSE ACYLTRANSFERASE3 (SiasAT3) and SiasAT4 (formerly SIAT2) catalyze the third and fourth steps, respectively, of tetraacylsucrose biosynthesis in *S. lycopersicum* (Schilmiller et al., 2012, 2015). Allelic variation in these enzymes leads to diversity in the types of acylsugars present in accessions of the wild tomato *Solanum habrochaites* (Kim et al., 2012; Schilmiller et al., 2015).

Less is known about the genes and enzymes responsible for the diversity of acyl-CoA ester donor substrates of acylsugar biosynthesis across the Solanaceae. Feeding studies with 14C- and 3H-labeled amino acids showed that short, branched acyl chains (2-methylpropanoic acid [iC4], 3-methylbutanoic acid [iC5], and 2-methylbutanoic acid) on acylsugars are derived from branched-chain amino acids (BCAAs; Val, Leu, and Ile) metabolism (Fig. 1B; Kandra et al., 1990; Kandra and Wagner, 1990; Walters and Steffens, 1990). The branched acyl chains of C6 to C12 are derived from BCAAs by elongation through a fatty acid synthase-mediated mechanism or α-keto acid elongation pathway (van der Hoeven and Steffens, 2000; Kroumova and Wagner, 2003). In addition, Slocombe et al. (2008) used virus-induced gene silencing to reduce expression of the BCAA catabolic enzyme BRANCHED-CHAIN KETO ACID DEHYDROGENASE (BCKDH) and KETOACYL-ACYL CARRIER PROTEIN SYNTHETASE I genes, associated with BCAA degradation and fatty acid synthase, respectively. Their results are consistent with the model that acyl-CoA production from BCAAs is involved in acylsugar biosynthesis.

We seek to identify enzymes that control the range of acyl-CoA substrates used for acylsugar biosynthesis. Screening of *S. lycopersicum* M82 × *Solanum pennellii* LA0716 chromosome introgression lines identified a region of *S. pennellii* chromosome 8 that caused M82 to shift dramatically from acylsugars with abundant iC5 chains to iC4 acyl chains (Schilmiller et al., 2010). This change led to the hypothesis that there is an *S. pennellii* allele in this region that shifts BCAA metabolic flux from Leu (the source of iC5) to Val (the source for iC4; Fig. 1B; Schilmiller et al., 2010). Here, we report the identification of *S. lycopersicum* Solyc08g014230 (SIIPMS3), which encodes a type I/IV trichome-expressed IPMS. This enzyme differs from other plant and microbial IPMSs that function in Leu biosynthesis in that it has a C-terminal deletion that renders the protein insensitive to feedback inhibition by Leu without causing the loss of enzymatic activity. The *S. pennellii* LA0716 IPMS3 protein has a larger C-terminal deletion that eliminates in vitro IPMS activity and causes the protein to be defective in the yeast two-hybrid (Y2H) protein-protein interaction assay. This enzymatically inactive allele was found to be homozygous in LA0716 and other *S. pennellii* accessions that
accumulate large amounts of iC4 acyl chains in their acylsugars. In contrast, *S. pennellii* with higher iC5 acyl chains contain at least one copy of the enzymatically active IPMS3 allele. These results indicate that a relatively simple change in IPMS structure and function can cause dramatic changes in the types of acylsugar acyl chains that accumulate in cultivated and wild tomato species. These findings have implications for the understanding of insect defense in natural populations and approaches to traditional breeding and transgenic manipulation of insect resistance in crop plants.

**RESULTS**

**Identification of a Novel Trichome-Expressed IPMS Gene**

Leaf surface metabolites were profiled previously from *S. lycopersicum* M82 × *S. pennellii* LA0716 introgression lines (Eshed and Zamir, 1995), revealing several loci that influence trichome metabolite accumulation (Schilmiller et al., 2009, 2010, 2012, 2015; Kim et al., 2014). The introduction of *S. pennellii* LA0716 chromosomal DNA from region 8-1-1 at the top of chromosome 8 caused a dramatic increase in the levels of metabolites containing iC4 acyl chains, which are normally of low abundance in *S. lycopersicum* M82, at the expense of the typically abundant iC5 acyl chains (Schilmiller et al., 2010).

The IL8-1-1 region is predicted to encode approximately 700 genes based on the International Tomato Annotation Group 2.4 annotation (Mueller et al., 2005; Tomato Genome Consortium, 2012). This region was narrowed to 43 genes using selected lines from the 10K SOLCAP SNP-genotyped backcross inbred lines (BILs) that have recombination break points throughout this region (see "Materials and Methods"; Supplemental Table S1; Supplemental Fig. S1). To identify trichome-expressed genes in this interval, RNA sequencing (RNA-Seq) was performed using RNA isolated from stems, trichomes isolated from stems, or stems with the trichomes removed (Supplemental Data Set S1; where TS refers to stems with trichomes, T refers to isolated trichomes, and S refers to shaved stems). Two genes in the region of interest showed more than 2-fold differential expression between isolated trichomes and shaved stems (Supplemental Table S2; significance of $P < 0.05$; see yellow highlighted rows). These are Solyc08g014190,
annotated as a cytochrome P450, and Solyc08g014230, which is one of a cluster of three genes in this region annotated as encoding the first committing enzyme of Leu biosynthesis, IPMS (Fig. 1C). In addition, a fourth IPMS gene is found on chromosome 6 (Tomato Genome Consortium, 2012). We renamed these genes as follows: Solyc06g053400, SIIIPMS1; Solyc08g014130, SIIIPMS2; Solyc08g014230, SIIIPMS3; and Solyc08g014240, SIIIPMS4. The RNA-Seq analysis revealed that SIIIPMS3 was expressed more than 350-fold higher in isolated stem trichomes compared with shaved stems. In contrast, SIIIPMS2 was not differentially expressed, and expression of SIIIPMS4 was not detected in any of the tissues we analyzed (Supplemental Table S3) or in complementary DNA (cDNA) sequencing data for other tomato tissues in the Sol Genomics Network (http://solgenomics.net/). These expression results were cross validated by quantitative PCR (qPCR) of cDNA for SIIIPMS2 and SIIIPMS3 (Supplemental Fig. S2).

Acylsugars accumulate in the long and slender type I/IV long trichome (Fobes et al., 1985; McDowell et al., 2011). Recent work with two BAHD acylsucrose acyltransferases (SIASAT3 and SIASAT4) showed that these genes are specifically expressed in the apical cells of these long trichomes (Schlimiller et al., 2012, 2015), strongly suggesting that the biosynthetic pathway is located in this cell type. To look at the cell type-specific expression of SIIIPMS3, transgenic plants that express GFP-GUS under the control of the SIIIPMS3 promoter (ProSIIIPMS3:GFP-GUS) were generated. Examination of GFP fluorescence in three independent ProSIIIPMS3:GFP-GUS lines revealed fluorescence specifically within the apical cells of type I/IV trichomes (Fig. 1D). This expression pattern is indistinguishable from that seen for SIASAT3 and SIASAT4 and corroborates and extends the RNA-Seq and qPCR data.

Sequence Comparison of the S. lycopersicum and S. pennellii LA0716 IPMS Genes

In addition to the trichome apical cell expression described above, the S. lycopersicum Heinz 1706 IPMS gene model predicts that it encodes an unusual variant missing approximately 160 amino acids at the C terminus of the protein compared with the chromosome 6 SIIIPMS1. This would result in deletion of the Leu-binding allosteric regulatory domain described in Arabidopsis (de Kraker and Gershenzon, 2011) and microbes (Soper et al., 1976; Koon et al., 2004; de Carvalho et al., 2005). Resequencing confirmed that the S. lycopersicum M82 SIIIPMS3 also contains this truncation, indicating that this is a bona fide change rather than a sequence assembly error. Because introgression of the S. pennellii LA0716 region containing this gene has a major impact on acyl chain composition, we attempted to identify the orthologous gene from this accession using available sequence data (Bombarely et al., 2012; Tomato Genome Consortium, 2012; Bolger et al., 2014a). The SIIIPMS3 (Solyc08g014230) and S. pennellii (Sopen08g005140) genes are reciprocal best hits, with 97% identity within a large syntenic region comprising 361 collinear genes (see “Materials and Methods”). In addition, a phylogenetic reconstruction of IPMS sequences in the S. lycopersicum, S. pennellii, and Nicotiana benthamiana genomes suggested that Sopen08g005140 and SIIIPMS3 are orthologs (Fig. 2). Thus, we refer to the S. pennellii LA0716 allele Sopen08g005140 as SpIPMS3.

To determine whether the sequence of SpIPMS3 from the chromosome 8 introgression lines is the same as that in the S. pennellii LA0716 sequence, IPMS3 cDNA and genomic DNA were sequenced from the S. lycopersicum M82 and S. pennellii LA0716 mapping parents as well as IL8-1-1 and IL8-1. cDNA amplification of IPMS3 from M82 yielded a product of identical size and sequence to that predicted from the S. lycopersicum Heinz 1706 annotation. In contrast, sequence analysis of SpIPMS3 cDNA revealed a 109-bp deletion at the 3′ end of the sequence.

Figure 2. Phylogenetic relationships between IPMS and MAM sequences from different species. Sequences were aligned using MUSCLE (Edgar, 2004) and used for phylogenetic reconstruction using the method noted by Benderoth et al. (2009). Specifically, the tree was constructed with Mr. Bayes version 3.2.1 using the wag model (Huelsenbeck and Ronquist, 2001). The Markov Chain Monte Carlo simulation was run with four chains each for 20,000 iterations with a sample frequency of 200 and a burn-in fraction of 0.05. The average potential scale reduction factor was 0.996, and the average of the split frequencies was 0.01, indicating convergence of the algorithm. The values on the branches represent the posterior probabilities obtained via the Bayesian simulation. Only the values for the internal branches are shown. The tree was rooted using the Ostreococcus tauri (O1) IPMS sequence, based on previous knowledge from Benderoth et al. (2009). At, Arabidopsis; Bd, Boechera divaricarpa; Bi, Brassica insularis; Bo, Brassica oleracea; Ca, Capsicum annuum; Nb, N. benthamiana; Os, Oryza sativa; Pt, Populus trichocarpa; Sl, S. lycopersicum; Sopen, S. pennellii; Zm, Zea mays. The sequences that start with PG are for Solanum tuberosum, and the sequences that start with PG are for S. lycopersicum.
coding region, as was expected based on the *S. pennellii* LA0716 genome sequence annotation. This deletion is predicted to cause a frame-shift mutation and premature stop codon at amino acid 395, which would remove the entire subdomain II as well as the Leu-binding domain (Fig. 3, A and D). To determine whether the deletion is found in the genome, DNA from *S. lycopersicum* M82 and *S. pennellii* LA0716 were amplified by using PCR primers flanking the deletion break points. The result showed that the SpIPMS3 genomic DNA PCR product is 1,029 bp shorter than SlIPMS3 (Fig. 3B). Seventeen single-nucleotide polymorphisms were detected between M82 and LA0716 across the full-length SpIPMS3 cDNA (Supplemental Fig. S3), 10 of which are predicted to affect the amino acid sequence (Supplemental Fig. S4). Thus, SpIPMS3 from LA0716 is predicted to encode an enzyme of 395 amino acids, which would produce a protein missing 51 amino acids from the C terminus relative to the full-length SlIPMS3.

**SlIPMS3 Encodes a Feedback-Insensitive Enzyme, and SpIPMS3 Is Nonfunctional in Vitro**

IPMS from plants and bacteria contains two core domains, an N-terminal catalytic region and a C-terminal allosteric regulatory domain, which is documented to mediate Leu feedback inhibition in microbes (Soper et al., 1976; Koon et al., 2004; de Carvalho et al., 2005) and Arabidopsis (de Kraker and Gershenzon, 2011). These two domains are separated by two smaller subdomains (I and II; Koon et al., 2004). In the IPMS gene cluster on chromosome 8, **SlIPMS3** and **SlIPMS4** (64% amino acid identity with Arabidopsis IPMS; Supplemental Fig. S4) are predicted to lack approximately 160 amino acids at the C terminus, which include the Leu-binding allosteric regulatory domain. Lack of the C-terminal allosteric domain led us to hypothesize that **SlIPMS3** encodes a trichome-expressed IPMS protein that is not subject to Leu feedback inhibition.

To test this hypothesis, **SlIPMS1** and **SlIPMS3** were expressed in *Escherichia coli* as fusion proteins containing an N-terminal His tag. These proteins were purified with a nickel-nitrilotriacetic acid agarose column and assayed for IPMS activity using the IPMS substrate OIV. Both proteins used this substrate with kinetic properties similar to published Arabidopsis IPMS enzymes (Table I; de Kraker and Gershenzon, 2011). Two lines of reasoning led us to test the activity of **SlIPMS3** with the MAM substrate 4-methylthio-2-oxobutyrate (MTOB). First, the **SlIPMS3** enzyme has strong sequence similarity to the Met-derived glucosinolate biosynthetic enzymes AtMAM1 (AT5G23010) and AtMAM3 (AT5G23020); 56% and 58% amino acid identity, respectively; Fig. 2; Supplemental Figs. S4 and S5; Kroymann et al., 2001; Textor et al., 2004, 2007). Second, removal of the C terminus of AtIPMS2 increased its activity with MTOB substrate compared with the full-length AtIPMS2.

**Figure 3.** An internal deletion in **SpIPMS3** genomic DNA results in the loss of subdomain II. A, Short cDNA transcripts of **IPMS3** were detected by reverse transcription-PCR from *S. pennellii* LA0716 trichome cDNA. **SlIPMS3**, 1,338 bp; **SpIPMS3**, 1,229 bp. B, Genomic DNA deletion of **IPMS3** was detected by PCR from *S. pennellii* LA0716, which is consistent with the results of reverse transcription-PCR in A. **SlIPMS3**, 1,354 bp; **SpIPMS3**, 325 bp. C, Gene structure of **SlIPMS3**. Exons, introns, and untranslated regions are indicated with black boxes, black lines, and white boxes, respectively. The thin horizontal black line indicates the location of the 1,029-bp deletion in *S. pennellii* LA0716. **IPMS3**. Positions of the primers used for PCR in B are indicated by the arrows. D, Predicted protein structures of **SlIPMS3** and **SpIPMS3**. A 109-bp deletion in the **SpIPMS3** cDNA sequence, indicated by the dashed line, results in a premature translational termination (indicated with the asterisk) that is predicted to delete the sequence encoding subdomain II. Positions of the primers used for reverse transcription-PCR in A are indicated by the arrows.
While SlIPMS3 used MTOB as a substrate, the catalytic efficiency ($k_{cat}/K_m$) of SlIPMS3, which lacks the Leu allosteric domain, was 14-fold higher for the classic IPMS substrate OIV than for the MAM substrate MTOB (Table I). In contrast, the full-length SlIPMS1 did not exhibit detectable activity with MTOB.

We also tested the hypothesis that the absence of the C-terminal domain abolishes Leu-mediated inhibition of SlIPMS3 enzyme activity. As shown in Figure 4, while the activity of SlIPMS1 declined to 50% at 200 μM Leu and was reduced to 30% at higher concentrations, SlIPMS3 activity was insensitive to Leu concentrations up to the highest concentration measured (10 mM). These results are consistent with the hypothesis that SlIPMS3 encodes an IPMS enzyme that is not subject to Leu inhibition.

The shift from high iC5- to iC4-containing acylsugars in IL8-1-1 suggested that the S. pennellii LA0716 allele has reduced production of iC5-CoA due to a defect in Leu biosynthesis (Schilmiller et al., 2010). We asked whether the longer predicted C-terminal deletion in SpIPMS3, including the loss of subdomain II (Figs. 3 and 5A), impaired IPMS activity, as recently reported for Leptospira biflexa IPMS (Zhang et al., 2014). Recombinant SpIPMS3 protein was generated with a C-terminal glutathione S-transferase (GST) tag, and its activity was compared with the GST-tagged SlIPMS3, which retains subdomain II. In contrast to the enzymatically active SlIPMS3 C-terminal GST fusion, no IPMS activity was seen with SpIPMS3 fusion protein (Table I). These results are consistent with the hypothesis that SpIPMS3 is compromised in IPMS activity.

Arabidopsis IPMS behaves as a dimer or tetramer both in vivo and in vitro (Textor et al., 2004; de Kraker and Gershenzon, 2011). The lack of detectable in vitro IPMS activity led us to ask whether SpIPMS3 has altered ability to dimerize in the Y2H system. Full-length IPMS3 coding sequences were fused to the GAL4 DNA-binding domain or the GAL4 activation domain and coexpressed in Saccharomyces cerevisiae. As shown in Figure 5B, SlIPMS3 gave a positive result, demonstrating homodimerization. In contrast, no evidence was obtained for SpIPMS3 homodimerization or for interaction between SlIPMS3 and SpIPMS3 (Fig. 5B). The positive control SlIPMS1 and negative control AtMAM3 showed the expected results in the Y2H system. Taken together, the Y2H results suggested that the SpIPMS3 variant was unable to form oligomers.

We tested the hypothesis that the trichome-expressed SlIPMS3 does not function in Leu biosynthesis by measuring free BCAA levels in trichomes. No differences in Ile, Leu, or Val were detected in M82 (with an enzymatically active SlIPMS3) compared with IL8-1-1 (with the short form SpIPMS3). Taken together with the in vitro assay and Y2H data, these results are consistent with the hypothesis that SlIPMS3 functions in acylsugar biosynthesis and does not directly impact BCAAs (Supplemental Table S4).

**Genetic Manipulation of IPMS3 Alters Acylsugar Profiles in S. lycopersicum in Unexpected Ways**

To test the hypothesis that SlIPMS3 influences acylsugar composition in vivo, an IPMS3 gene-specific fragment was used for RNA interference (RNAi) expression in M82 transgenic plants, and the resulting lines were screened for acylsugar profiles. SlIPMS3 RNAi lines were recovered that exhibited

### Table I. Kinetic parameters for SlIPMS1, SlIPMS3, and SpIPMS3

All values are means ± sd with three replicates. ND, No detectable activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol s$^{-1} \times 10^6$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (μmol$^{-1}$ s$^{-1} \times 10^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SlIPMS1</td>
<td>OIV</td>
<td>143 ± 4</td>
<td>10.3 ± 0.8</td>
<td>1.08 ± 0.08</td>
<td>7.50 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>MTOB</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SlIPMS3</td>
<td>OIV</td>
<td>215 ± 11</td>
<td>5.2 ± 0.5</td>
<td>0.49 ± 0.01</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>MTOB</td>
<td>734 ± 25</td>
<td>1.2 ± 0.1</td>
<td>0.12 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>SpIPMS3</td>
<td>OIV</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td>MTOB</td>
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Figure 4. SlIPMS3 activity is insensitive to Leu. SlIPMS1 and SlIPMS3 activities are presented as percentages of the enzyme activity in the absence of Leu. Data points represent means ± se (n = 3).


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substantial reduction in levels of iC5 acyl chain-containing acylsugars and increased iC4 acyl chain-containing acylsugars compared with M82 (Fig. 6A). Notably, transgenic plants accumulated two acylsugars, S3:20 (4,4,12) and S4:22 (2,4,4,12), that were detected in IL8-1-1 but were near the detection limit in M82 (Fig. 6B; Supplemental Fig. S6 shows the mass spectra of peaks labeled in Fig. 6). This change from predominantly iC5-containing acylsugars to those with increased iC4 acyl chains argues that the accumulation of acylsugars with high iC5 requires active SlIPMS3. Taken together with the lack of in vitro SpIPMS3 activity, this result predicts that the SlIPMS3 allele is dominant to SpIPMS3.

A contradictory result was obtained when acylsugars were analyzed from M82 × IL8-1-1 F1 heterozygous plants, which contain one copy of SlIPMS3 and one copy of SpIPMS3: instead of complete reversion to the M82 phenotype of high iC5 and low iC4 expected for a recessive loss-of-function mutation, a partial conversion was observed (Fig. 7A, left). Peak areas of iC4 acyl chain-containing acylsugars were approximately 3-fold higher in the F1 plants than in M82, but no changes in iC5 acyl chain-containing acylsugars were observed. This result suggests that expression of the SpIPMS3 protein, which is inactive in vitro, influences acyl chain composition even in the presence of active SlIPMS3 enzyme.

To test this hypothesis, IL8-1-1 was transformed with SlIPMS3 driven by the type I/IV apical cell-specific SIASAT4 promoter (referred to as SIAT2 in Schilmiller et al., 2012; ProSIASAT4:SlIPMS3). We predicted that this transgene would increase iC5 acyl chain-containing acylsugars and decrease iC4 acyl chain-containing products. As predicted, 12 of the 16 independent T0 primary transgenic lines had total iC5 acyl chain-containing acylsugar levels modestly higher than measured in IL8-1-1. However, the expected decrease in iC4 acyl chain-containing acylsugars was not observed in these 12 lines (Fig. 7B; Supplemental Fig. S7). Thus, expression of the enzymatically active SlIPMS3 enzyme did not fully complement the SpIPMS3 genotype.

This lack of full complementation could be due to poor expression of the transgene or a negative effect of the SpIPMS3 protein on SlIPMS3 activity. Evidence for the latter hypothesis was obtained from transformation of S. lycopersicum M82 with the SpIPMS3 cDNA driven by the SIASAT4 promoter (ProSIASAT4:SpIPMS3). Transgenic lines were obtained with increased iC4-containing acylsugars compared with the M82 parent (Fig. 7A, right). This phenotype is reminiscent of that of the M82 × IL8-1-1 F1 plants. The combination of phenotypic results from the M82 × IL8-1-1 F1 and transgenic plants is consistent with the hypothesis that the SpIPMS3 allele influences the acylsugar phenotype in the presence of the enzymatically active SlIPMS3 by a currently unknown mechanism.
The Two IPMS3 Alleles Correlate with iC4/iC5 Acyl Chain Variation in S. pennellii Accessions

Acylsugar acyl chain composition varies among different accessions of wild tomato species (Shapiro et al., 1994; Kim et al., 2012). For example, some southern S. pennellii accessions, including LA0716, contained predominantly iC4 acyl chains, while other accessions had higher levels of iC5 (Shapiro et al., 1994). To test whether the two IPMS3 alleles found in IL8-1-1 and M82 are associated with the iC4/iC5 acyl chain variation in these native plants, acylsugar phenotypes and IPMS3 alleles were analyzed for 14 S. pennellii accessions collected across Peru (Fig. 8; Supplemental Fig. S8; Supplemental Table S5). In addition to LA0716, three other accessions (LA2963, LA1941, and LA1674) were found to be homozygous for the SpIPMS3-like short-form allele (labeled S/S in Fig. 8). Each of these accessions exhibited high levels of acylsugars containing iC4 acyl chains. In contrast, four accessions that were homozygous for the SlIPMS3-like long-form genotype (LA2560, LA2567, LA2565, and LA1649; labeled L/L in Fig. 8) accumulated predominantly iC5 acyl chains. The remaining six accessions (LA1356, LA1376, LA1282, LA1367, LA1911, and LA1946) were heterozygous SlIPMS3-like/SiIPMS3-like (labeled L/S in Fig. 8). Five of the heterozygotes showed a high iC5/iC4 ratio similar to that of the SlIPMS3-like homozygous plants. One of the heterozygous accessions (LA1946) had increased iC5 compared with SpIPMS3-like/SiIPMS3-like homozygotes, but the iC5/iC4 ratio was intermediate to that of the homozygotes. These results show that the natural IPMS3 allelic pattern was correlated with iC5/iC4 acyl chain variation and related to a specific geographical distribution.

DISCUSSION

BCAA metabolism has a central role in producing a wide variety of alkyl-acylated plant specialized metabolites (Hirosawa et al., 1995; Pérez et al., 2002; Karppinen et al., 2007; Wang et al., 2008; Clark et al., 2013; Zabalza et al., 2013), making it of interest to understand the various regulatory mechanisms that control flux from amino acid metabolism into specialized metabolite production. We describe the characterization of SlIPMS3, a gene encoding a variant IPMS enzyme that is expressed in the apical cells of type I/IV trichomes in the cultivated tomato S. lycopersicum. The gene produces an IPMS enzyme that is active in vitro and exhibits insensitivity to Leu-mediated allosteric inhibition due to an approximately 160-amino acid deletion at the C terminus. In contrast, the S. pennellii LA0716 gene SpIPMS3 encodes a protein that has a
51-amino acid longer C-terminal truncation, which renders the protein unable to use OIV and acetyl-CoA as substrates when assayed in vitro. The SpIPMS3 allele in the homozygous state (IL8-1-1 and several BILs) changes the pattern of acylsucroses from that typical of S. lycopersicum M82, with abundant iC5 acyl chains derived from Leu metabolism, to iC4 acyl chains derived from Val metabolism (Schilmiller et al., 2010). Of particular note is that the IPMS3 genotypes found in S. pennellii accessions from throughout the species range in Peru show strong association with the relative abundance of iC5 and iC4 acylsugar acyl chains (Fig. 8). These results indicate that variation in IPMS3 structure and function plays an important role in the observed acyl chain diversity in S. pennellii. This study also provides indirect evidence that CoA ester availability in flower influences the types of acyl chains found on acylsugar products, presumably because both iC4 and iC5 CoA esters can be used by acylsugar acyltransferases (see Supplemental Figures 7C and 7D in Schilmiller et al., 2015).

**SIIPMS3 Has Altered Feedback Regulation of Enzyme Activity and Gene Expression**

IPMS3 was identified by screening S. lycopersicum M82 × S. pennellii LA0716 chromosome introgression
 lines; this is the same approach that led to the identification of SIASAT3 and SIASAT4 enzymes, which catalyze the third and fourth steps of acylsucrose biosynthesis in *S. lycopersicum* (Schilmiller et al., 2012, 2015). Our results revealed that the type I/IV trichome apical cell-expressed SIIPMS3 enzyme catalyzes the first committing step of Leu biosynthesis using OIV and acetyl-CoA as substrates (Table I). In this regard, SIIPMS3 resembles previously identified IPMS enzymes from Arabidopsis, fungi, and bacteria (Roeder and Kohlhaw, 1980; Wiegel, 1981; de Kraker et al., 2007).

However, the SIIPMS3 protein has characteristics reminiscent of the MAM enzymes of Arabidopsis (Textor et al., 2004, 2007), which catalyze the committing step of Met-derived glucosinolate biosynthesis. First, both are missing the C-terminal allosteric regulatory domain and as a result are insensitive to Leu inhibition (Fig. 4). Second, as reported previously for C-terminal deletion alleles of both full-length Arabidopsis IPMS enzymes (de Kraker and Gershenzon, 2011), the naturally occurring SIIPMS3 truncated enzyme exhibits a change in substrate specificity, having acquired detectable MAM activity not seen in the full-length SIIPMS1 enzyme (Table I). Finally, the SplIPMS3 variant enzyme, which is inactive in vitro and fails to give a positive result in the Y2H assay (Fig. 5B), leads to a dramatic shift in acylsugar acyl chain composition in *S. pennelli* accessions (Supplemental Table S5). This is analogous to the situation with MAM alleles, including loss-of-function variants, which influence the types of Met-derived glucosinolates found in Arabidopsis accessions (Kroymann et al., 2003; Benderoth et al., 2009).

The evolutionary recruitment from a Leu biosynthetic enzyme to one that diverts OIV toward the production of iC5-CoAs for iC5 acylsugar synthesis also involves changes in the developmental regulation of mRNA accumulation. The most compelling evidence for cell type-specific gene expression comes from the analysis of transgenic plants containing the ProSIIPMS3: GFP-GUS transgene (Fig. 1D). These plants exhibit GFP expression in the apical cells of type I/IV trichomes, the long trichomes on stems, petioles, and leaves that are visible to the naked eye (Fig. 1D). That reporter gene expression faithfully represents the expression of the gene in trichomes is corroborated by RNA-Seq (Supplemental Table S2) and qPCR data (Supplemental Fig. S2), which show strong enrichment of the SIIPMS3 transcript in trichome RNA compared with shaved stems. This is in contrast to the other IPMS sequences in M82 cultivated tomato, whose gene expression is not enriched in stem and petiole trichome preparations (Supplemental Table S3). The observed selectivity of expression in type I/IV apical cells matches that seen for the *S. lycopersicum* ASAT3 and ASAT4. These results strongly suggest that the apical cells are metabolic factories that contain the enzymatic machinery needed to produce acylsucroses from simple products of central metabolism, including acetyl-CoA, Suc, and amino acids.

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**Evidence That BCAAs Are Precursors to Acylsugar Acyl Chains**

The involvement of a variant Leu biosynthetic enzyme in producing acylsugar iC5 side chains provides strong evidence for the proposed role of acyl-CoA in vivo fluxes as determinants of acylsugar composition (Slocombe et al., 2008; Schilmiller et al., 2012, 2015). RNA-Seq data with *S. lycopersicum* total trichome RNA (Supplemental Table S3) provide additional evidence for this hypothesis (Fig. 1B). Trichome-enriched expression was detected for the entire known pathway for the production of iC5-CoAs from the Leu biosynthetic pathway: IPMI (catalyzing the step after IPMS) and three subunits of the BCKDH complex, which produces iC5-CoA from the product of IPMI (Fig. 1B). These results are consistent with previously published findings indicating the involvement of BCAA metabolism in the production of branched acyl chains in acylsugar biosynthesis (Kandra et al., 1990; Kandra and Wagner, 1990; Walters and Steffens, 1990). For example, virus-induced gene silencing of the E1 β-subunit of the BCKDH complex caused the reduction of acylsugar accumulation in *N. benthamiana* (Slocombe et al., 2008).
Finally, we recently demonstrated the involvement of BAHD acyltransferases and acyl-CoA esters, including iC5-CoA, in acylsucrose biosynthesis (Schilmiller et al., 2012, 2015).

The Influence of IPMS on Acyl Chain Composition in Cultivated and Wild Tomato

One or more copies of the IPMS3 short form originally identified in IL8-1-1 and S. pennellii LA0716 was found in the majority of S. pennellii accessions that we analyzed (labeled S in Fig. 8). The short allele was found exclusively in a total of four accessions, including LA0716, and these were collected from the southern part of the species distribution. In contrast, only the longer SIIPMS3-like allele was identified in four other accessions (labeled L in Fig. 8), with three from the northern part of the distribution and one from the south. Finally, both long and short IPMS3-like sequences were isolated from six accessions collected from the large central range of the species. This pattern of contrasting homozygous genotypes at the geographical extremes of the species, with polymorphic genotypes in the center, is reminiscent of isozyme diversity data published more than 30 years ago by Charles Rick and colleagues for S. pennellii (Rick and Tanksley, 1981) as well as for S. pimpinellifolium (formerly Lycopersicon pimpinellifolium) and S. habrochaites (formerly Lycopersicon hirsutum; Rick et al., 1979). In those studies, polymorphic isozyme markers were identified that showed evidence for contrasting genotypes in the north and south of the geographic range and were more highly polymorphic in the central regions.

The IPMS3 genotypes are well correlated with iC5 and iC4 acylsugar acyl chain phenotypic patterns in wild and cultivated tomato. We found high iC5 and low iC4 acyl chains in trichome extracts of S. pennellii accessions for which only SIIPMS3-like long alleles were recovered (presumably homozygotes) and all but one accession, LA1946, that contains both long and short alleles (putative heterozygotes; Fig. 8). In contrast, iC4 acyl chains were abundant in acylsugars of the four accessions from which only short alleles were recovered (presumptive homozygotes). Taken together, these results support the hypothesis that this locus plays a major role in determining the types of acylsugars found in S. pennellii. Our phenotypic results show good agreement with the work of Shapiro et al. (1994), who reported that the high iC4 phenotype was found for more southerly accessions, including LA0716, LA1674, LA1946, and LA1941. They also found high iC5 acylsugars for the LA1376, LA2560, and LA1809, which we identified as having iC5-dominant acylsugar acyl chains associated with the presence of at least one SIIPMS3-like long-form allele in the genome. The influence of this region of S. pennellii chromosome 8 on S. lycopersicum iC4 versus iC5 composition is also consistent with published quantitative trait locus mapping and breeding studies from Martha Mutschler and colleagues (Blauth et al., 1999; Leckie et al., 2014).

While our results support the hypothesis that IPMS3 influences iC4 and iC5 acylsugar acyl chain accumulation, SpIPMS3 does not behave as a simple loss-of-function mutation in S. lycopersicum. The SIIPMS3-like/SpIPMS3-like heterozygote plants created by crossing IL8-1-1 with M82 have peak areas of iC4 acyl chain-containing acylsugars approximately 3-fold higher than in M82 (Fig. 7A, left). In addition, transgenic introduction of the IL8-1-1 SpIPMS3 (from S. pennellii LA0716) under the control of the type I/IV apical cell-specific SIAAT4 promoter (ProSIAAT4: SpIPMS3) resulted in increased iC4 acylsugar acyl chains (Fig. 7A, right). These genetic results are consistent with the hypothesis that the SpIPMS3 protein can exert a dominant inhibitory effect on SIIPMS3 enzyme activity in vivo. None of the short-form IPMS3 alleles from S. pennellii have accumulated other mutations that would cause the synthesis of more highly truncated forms of the enzyme (Supplemental Fig. S8), suggesting that the short-form SpIPMS3-like protein(s) may have some function. However, we have no data indicating what, if any, biochemical role is played by SpIPMS3 protein. For example, the Y2H assay revealed no evidence for SIIPMS3-SpIPMS3 interaction in the nucleus of yeast (Fig. 5B), and mixing E. coli-expressed extracts of His-SliIPMS3 and SpIPMS3-GST proteins did not result in the inhibition of IPMS enzyme activity. Another possible mechanism to explain the influence of SpIPMS3 is that the enzyme utilizes OIV to make an alternative product, indirectly reducing the production of iC5-CoA esters.

Implications of Multiple Instances of IPMS Recruitment for Plant Specialized Metabolism

Protein sequence comparisons strongly suggest that independent duplication and diversification events led to the evolution of the lineage-specific IPMS variants in the Brassicaceae (MAMs) and Solanaceae (the IPMS3 orthologous group). Our phylogenetic analysis of plant IPMS homologs (Figure 2; Supplemental Fig. S5) supports the hypothesis that the Brassicaceae IPMS gave rise to MAM, as proposed by de Kraker and Gershenzon (2011). Inclusion of the full-length and shorter variants from the Solanaceae in the tree reveals that the C-terminal truncated Solanaceae IPMS3 genes were derived from an IPMS after the last common ancestor of these two families (Supplemental Fig. S2). Multiple instances of IPMS recruitment within eudicots are not surprising, given that loss of the C terminus renders the enzyme free from Leu inhibition and allows it to function independent of amino acid biosynthesis and accumulation. This simple mechanism, combined with the large number of plant specialized metabolites derived from BCAA metabolism, leads us to predict that more examples of independent IPMS recruitment to specialized metabolic pathways will be found.

The rapid diversification of trichome-specialized metabolic enzymes within the genus Solanum (Sallaud et al.,
MATERIALS AND METHODS

BIL Generation and Genotyping

The BIL population was constructed from a cross between the cultivated tomato *Solanum lycopersicum* M82 and the wild species *Solanum pennellii* LA0716. Seedlings were grown in Jiffy peat pots (Hummert International) in a controlled growth chamber maintained for 16 h in the light (300 μmol m⁻² s⁻¹; mixed cool-white and incandescent bulbs) at 28°C and 8 h in the dark at 20°C.

Plant tissues were used for RNA isolation and quality control. Total RNA was isolated from leaf tissue, and the population was genotyped with 10K SOLCAP SNP markers. BILs were then crossed to the cultivated parent for two to three generations. The BILs were then grown in mixed cool-white and incandescent bulbs at 28°C and 8 h in the dark at 20°C. Plant tissues were used for RNA isolation and quality control.

RNA Extraction and Sequencing

Total RNA was extracted from the stems with trichomes, shaved stems, and isolated trichomes of 3-week-old *S. lycopersicum* M82 plants using the RNAeasy Plant Mini kit (Qiagen). Six samples (three tissues × two replicates) of total RNA were submitted for sequencing in a single lane. Sequencing libraries were prepared using the Illumina TrueSeq Stranded mRNA Library Preparation Kit. After quality control and quantitation, libraries were pooled and sequenced on the Illumina HiSeq 2500 Rapid Run flow cell (version 1) using Rapid sequencing by synthesis reagents according to the manufacturer’s specifications. Sequencing was performed in a 2 × 100-bp paired-end (PE100) format. Base calling was performed using Illumina Real Time Analysis version 1.17.21.3. The output of Illumina Real Time Analysis was demultiplexed and converted to FastQ files with Illumina Bcl2Fastq version 1.8.4. The RNA-Seq reads were deposited in the National Center for Biotechnology Information Sequence Read Archive under the Bioproject PRJNA271503.

Differential Expression Analyses

Approximately 22 to 30 million 100-bp paired-end read clusters were obtained for each submitted RNA sample. The paired-end reads were filtered using Trimmomatic version 0.32 (Bolger et al., 2014b) with the following settings: ILLUMINACLIP, TruSeq3-PE:2:2:30:10; LEADING, 20; TRAILING, 20; SLIDINGWINDOW, 4:20; MINLEN, 50. Approximately 88% of the reads in each data set passed the quality filter. The trimmed and filtered reads were then mapped to the *S. lycopersicum* genome version 2.3 with TopHat version 1.4.1 using the following parameters: --library-type, fr-firststrand; -I, 2000; -g, 1; –segment-length, 25; –mate-inner-dist, 200; –mate-std-dev, 50. Seventy-four percent to 88% of the filtered reads were found to map to the genome successfully. For differential expression analyses, the TopHat-mapped reads were counted for each gene using HTSeq version 0.6.1 (Anders et al., 2015), and the counts were used to calculate the differential expression for each tissue pair using edgeR (Robinson et al., 2015). Genes were called differentially expressed if log2 fold change was greater than 2 and P, corrected for multiple testing, was less than 0.05 (Storey, 2002).

Determination of Orthology

We determined orthology between the SlIPMS3 and SpIPMS3 copies using reciprocal best match, synteny, and phylogenetic analysis. For reciprocal best match, we performed an all-versus-all BLAST with all annotated *S. lycopersicum* and *S. pennellii* proteins (Bolger et al., 2014a) and selected pairs that were reciprocal best hits of each other at E < 1e-10. We determined genome-wide synteny between reciprocal best hits using MCScanX (Wang et al., 2013) using the default parameters. For phylogenetic reconstruction of IPMS and MAM sequences, we combined information from two sources. First, we obtained the MAM and IPMS sequences used in a previous study (Benderoth et al., 2009). Second, we obtained homologous sequences in *Nicotiana benthamiana*, *Capsicum annuum*, and *Solanum tuberosum* using a BLAST search with E < 1e-10. All amino acid sequences were aligned using MUSCLE (Edgar, 2004) and used for phylogenetic reconstruction using a Bayesian approach as implemented in Mr. Bayes version 3.2.1 (Huelsenbeck and Ronquist, 2001) using the procedure described by Benderoth et al. (2009). To determine the robustness of the tree inference, we also generated a maximum likelihood tree using RaxML version 8.0.6 (Stamatakis, 2014) using the approach noted in Supplemental Figure S5.

Analysis of IPMS Gene Expression in Trichomes

Total RNA was isolated from the stems with trichomes, shaved stems, and isolated trichomes as described above. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) following the manufacturer’s recommendation. qPCR was performed on the 7500 Fast real-time PCR system (Applied Biosystems) using Fast SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer’s protocol. The cultivated tomato *Etiola* gene was used as the endogenous control. The comparative threshold cycle method was used to evaluate the quantitative variation of replicates examined. The gene-specific primers are listed in Supplemental Table S6.

IPMS Sequence Analysis

Total RNAs from the leaves of *S. lycopersicum* M82 and *S. pennellii* LA0716, IL8-1-1, and IL8-1 were extracted with the RNAeasy Plant Mini Kit (Qiagen). cDNA was synthesized as described above. The mRNA coding sequence of IPMS3 was amplified from these four plants using primers IPMS3 FL F and IPMS3 FL R (Supplemental Table S6). Genomic DNAs from the leaves of *S. lycopersicum* M82 and *S. pennellii* LA0716, IL8-1-1, and IL8-1 were obtained using the DNeasy Plant Mini Kit (Qiagen). The SpIPMS3 deletion was confirmed through sequencing the PCR products amplified from *S. pennellii* LA0716, IL8-1-1, and IL8-1 genomic DNA using primer pair IPMS3 DEL F and IPMS3 DEL R (Supplemental Table S6). The same fragment from *S. lycopersicum* M82 was amplified and sequenced as well. All these PCRs were performed using high-fidelity Phusion polymerase (New England Biolabs), and samples were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. The DNA sequence was determined using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).
Isopropylmalate Synthase in Acylsugar Biosynthesis

strains Bacillus subtilis 168 (Clontech) were cultured and transformed as recommended by the manufacturer using standard synthetic dropout medium (Clontech) as indicated. HisS3 reporter assays were performed according to the manufacturer’s instructions (Clontech). Interactions were selected on synthetic dropout/-Leu-Trp-His medium supplemented with 1.5 μM 3-amino-1,2,4-triazole (Sigma-Aldrich) after 48 h of growth at 30°C.

Protein Expression and Enzyme Assay

To generate His-tagged recombinant proteins, the full-length open reading frames (ORFs) for SlIPMS3, SpIPMS3, and SlIPMS51 were cloned into BamHI and NofI sites of pET28b (Novagen). Because soluble His-tagged SpIPMS3 was not obtained from Escherichia coli, recombinant SpIPMS3 proteins were generated with N-terminal or C-terminal GST tags and compared with the activity of the GST-tagged SlIPMS53. A summary of the protein expression vectors is presented in Supplementary Table S7. To generate N-terminal GST-tagged recombinant proteins, the full-length ORFs for SlIPMS3 and SpIPMS3 were cloned into pGEX-4T-1 (GE) using BamHI and NofI sites. The forward primer used for this construct contains a sequence encoding a 6× His tag. To generate C-terminal GST-tagged recombinant GST, amplification was obtained from pGEX-4T-1 using the primers GST NOT1 F and GST NOT1 R, subsequently digested with NotI, and cloned into the His-tagged SlIPMS3 and His-tagged SpIPMS3 mentioned above.

The hexane layer was removed and extracted using saturated aqueous sodium carbonate solution. The reaction was allowed to proceed for 30 min in a fume hood with gentle rocking for 2 min. Dry weights of the extracted leaf samples were measured following drying at 50°C. The profiling of acylsugar was performed using liquid chromatography-time of flight-mass spectrometry on a Waters Xevo G2-S QTof instrument as described before (Schilmiller et al., 2015). Nonselective collision-induced dissociation mass spectrometry in negative ionization mode provided information about the number and length of acyl chains present on each acylsugar based on the masses of carbohydrate fragment ions (Schilmiller et al., 2010; Ghosh et al., 2014).

Metabolite Extraction and Analyses

Leaflets from the next-to-youngest leaf of 3-week-old plants were used for chemical analysis of acylsugar. One leaflet was dipped in 1 mL of isopropanol: acetone:nitritetrahydroxyl:water (3:2:1, v/v/v) containing 0.1% formic acid by volume and 10 μM propyl-4-hydroxybenzoate that served as an internal standard, with gentle rocking for 2 min. Dry weights of the extracted leaf samples were measured following drying at 50°C. The profiling of acylsugar was performed using liquid chromatography-time of flight-mass spectrometry on a Waters Xevo G2-S QTof instrument as described before (Schilmiller et al., 2015). Nonselective collision-induced dissociation mass spectrometry in negative ionization mode provided information about the number and length of acyl chains present on each acylsugar based on the masses of carbohydrate fragment ions (Schilmiller et al., 2010; Ghosh et al., 2014).

To analyze acyl chains on acylsugars, a leaflet was dipped in 1 mL of acetonitrile:water (1:2:1, v/v/v) containing 0.1% formic acid by volume and 10 μM propyl-4-hydroxybenzoate that served as an internal standard, with gentle rocking for 2 min. Dry weights of the extracted leaf samples were measured following drying at 50°C. The profiling of acylsugar was performed using liquid chromatography-time of flight-mass spectrometry on a Waters Xevo G2-S QTof instrument as described before (Schilmiller et al., 2015). Nonselective collision-induced dissociation mass spectrometry in negative ionization mode provided information about the number and length of acyl chains present on each acylsugar based on the masses of carbohydrate fragment ions (Schilmiller et al., 2010; Ghosh et al., 2014).

Sequence data from this article can be found in the GenBank database under the following accession numbers: SlIPMS53 (KP8853470), SpIPMS3 (KP8853471), SlIPMS52 (KP8853472), SlIPMS51 (KP8858649), SpIPMS3-LA1232-L (KP8858646), SpIPMS3-LA1232-S (KP8858656), SpIPMS3-LA1356-L (KP8858647), SpIPMS3-LA1356-L (KP8858654), SpIPMS3-LA1367-S (KP8858657), SpIPMS3-LA1367-L (KP8858658), SpIPMS3-LA1376-S (KP8858649), SpIPMS3-LA1376-L (KP8858649), SpIPMS3-LA1367-S (KP8858658), SpIPMS3-LA1649-L (KP8858659), SpIPMS3-LA1674-S (KP8858659), SpIPMS3-LA1809-L (KP8858651), SpIPMS3-LA1911-L (KP8858652), SpIPMS3-LA1941-S (KP8858660), SpIPMS3-LA1941-L (KP8858660), SpIPMS3-LA1941-S (KP8858663), SpIPMS3-LA2280-L (KP8858661), SpIPMS3-LA2280-L (KP8858664), and SpIPMS3-LA2963-S (KP8858662).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Schematic representation of chromosome 8 BILs.
Supplemental Figure S2. Relative expression of SlIPMS2, SlIPMS3, and type I/IV trichome apical cell-specific SIASAT4 measured by qPCR in different tissues.

Supplemental Figure S3. mRNA coding sequence differences between SlIPMS3 and SlIPMS3.

Supplemental Figure S4. Amino acid sequence alignment of IPMS and MAM sequences from different species.

Supplemental Figure S5. Phylogenetic relationships between IPMS and MAM sequences from different species.

Supplemental Figure S6. Negative ionization mode mass spectra of the peaks labeled in Figure 5B.

Supplemental Figure S7. Quantification of acylsugar peak areas in lines derived by transformation of ILB-1-1 with ProSIASAT4:SlIPMS3.

Supplemental Figure S8. Amino acid sequence alignment of IPMS3 from different S. pennelli accessions.

Supplemental Table S1. Phenotype and chromosome 8 introgression boundaries for S. pennelli LA0716 BILs used in this study.

Supplemental Table S2. Summary of the expression of 43 genes on chromosome 8 from Supplemental Data Set S1.

Supplemental Table S3. Summary of the expression levels of S. lycopersicum BCAA genes in Supplemental Data Set S1.

Supplemental Table S4. Results of BCAA analysis from M82 and ILB-1-1 trichomes.

Supplemental Table S5. Percentage peak area composition of acyl chains in trichome acylsugars from different S. pennelli accessions.

Supplemental Table S6. Primers used in this study.

Supplemental Table S7. Summary of protein expression vectors in this study.

Supplemental Data Set S1. Expression levels of all annotated transcripts in S. lycopersicum.

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