Plants make many biologically active, specialized metabolites, which vary in structure, biosynthesis, and the processes they influence. An increasing number of these compounds are documented to protect plants from insects, pathogens, or herbivores or to mediate interactions with beneficial organisms, including pollinators and nitrogen-fixing microbes. Acylsugars, one class of protective compounds, are made in glandular trichomes of plants across the Solanaceae family. While most described acylsugars are acylsucroses, published examples also include acylsugars with hexose cores. The South American fruit crop naranjilla (foló; Solanum quitoense) produces acylsugars containing a myoinositol core. We identified an enzyme that acetylates triacylinositols, a function homologous to the last step in the acylsucrose biosynthetic pathway of tomato (Solanum lycopersicum). Our analysis reveals parallels between S. lycopersicum acylsucrose and S. quitoense acylinositol biosynthesis, suggesting a common evolutionary origin.

Plants are master chemists and collectively produce an array of structurally diverse specialized metabolites (traditionally called secondary metabolites) from common building blocks (Bennett and Wallsgrove, 1994; Verpoorte and Alfermann, 2000; Wink, 2010). Examples include alkaloids derived from amino acids (Ziegler and Facchini, 2008), terpenes made from isoprenoids (Chappell, 1995; Pichersky and Raguso, 2018; Zhou and Pichersky, 2020), and acylsugars synthesized from acyl-CoAs and sugars (Fan et al., 2019). Most specialized metabolites are restricted in cell- or tissue-specific accumulation and found in phylogenetically restricted groups of plants (Pichersky and Lewinsohn, 2011). An increasing number have been shown to be beneficial; examples include deterring or killing microbes and herbivores, inhibiting germination or growth of competitor plants, or attracting beneficial insects and microbes (Bennett and Wallsgrove, 1994; Pichersky and Gershenzon, 2002; Howe and Jander, 2008; Leckie et al., 2016; Massalha et al., 2017). Humans have adapted specialized metabolites as medicines, including the analgesic morphine and the anticancer drugs paclitaxel and vinblastine (Fabricant and Farnsworth, 2001), as spices, including piperine in black pepper (Piper nigrum) and capsaicin in hot chilies (Capsicum annuum; Srinivasan, 2013), and as fragrances (e.g. limonene and linalool; Dudareva and Pichersky, 2006). As biologically active molecules, specialized metabolites can have adverse effects on the plant making them, and the production or storage of these compounds in specialized structures can mitigate negative effects (Schilmiller et al., 2012b; Schenck and Last, 2020). Epidermal glandular trichomes are an example of biochemical factories that store a wide variety of specialized metabolites across the Plantae kingdom (Fahn, 2000; Schuurink and Tissier, 2020). Classes of specialized metabolites produced in these glandular trichomes include terpenoids (Sallaud et al., 2009; Schilmiller et al., 2009; Brückner et al., 2014), flavonoids (Tattini et al., 2000; Schmidt et al., 2011; Kim et al., 2014), and acylsugars (Schilmiller et al., 2010, 2012a, 2015; Fan et al., 2016).

Acylsugars are specialized metabolites made from sugars and acyl-CoAs in species across the Solanaceae family (King et al., 1986; King and Calhoun, 1988;
Matsuzaki et al., 1991; Maldonado et al., 2006; Ghosh et al., 2014; Liu et al., 2017; Moghe et al., 2017). These compounds serve as microbial and herbivory defense agents (Goffreda et al., 1989; Leckie et al., 2016; Luu et al., 2017; Smeda et al., 2018) and contain a sugar core esterified to multiple acyl chains (Fan et al., 2019). The acyl chains are branched or straight and are proposed to be derived from amino acid or fatty acid metabolism, respectively (Walters and Steffens, 1990; Kroumova and Wagner, 2009; Ning et al., 2015). Acylsugar cores from Solanaceae species are primarily Suc or sometimes Glc (King et al., 1986; King and Calhoun, 1988; Maldonado et al., 2006; Ghosh et al., 2014; Liu et al., 2017; Moghe et al., 2017; Leong et al., 2019). Orangeberry nightshade (Solanum lanceolatum) is a published exception, accumulating acylated disaccharides that contain myoinositol bound to Glc or Xyl (Herrera-Salgado et al., 2005). Moghe et al. (2017) screened acylsugars in plants across the Solanaceae family. One species, the South American fruit crop naranjilla or lulo (Solanum quitoense), produced unusual acylsugars that yielded lower signals of fragment ions generated in a mass spectrometer than acylsuccrates or acylglucoses from other members of the Solanaceae family (Hurney, 2018).

Acylsugar acyltransferases (ASATs) are clade III BAHD (BEAT, AHCT, HCT, and DAT) acyltransferase family enzymes (D'Auria, 2006; Moghe et al., 2017) that catalyze the core acylation reactions of characterized acylsugar biosynthetic pathways (Schilmiller et al., 2012a, 2015; Fan et al., 2016, 2019; Moghe et al., 2017; Nadakuduti et al., 2017). ASATs sequentially transfer acyl chains from acyl-CoAs to generate acylsuccrates in plants as phylogenetically divergent as cultivated tomato (Solanum lycopersicum), Solanum pennellii, Petunia axillaris, and Salpiglossis sinuata (Schilmiller et al., 2012a, 2015; Fan et al., 2016, 2017; Moghe et al., 2017; Nadakuduti et al., 2017). The expression of characterized ASATs is enriched in glandular trichomes relative to non-acylsugar-producing tissues (Ning et al., 2015; Moghe et al., 2017; Nadakuduti et al., 2017; Leong et al., 2019; Mandal et al., 2020). Phylogenetic and functional analyses revealed that orthologs have similar, but distinct, substrate selectivity in acylsugar biosynthesis across the Solanaceae family (Moghe et al., 2017; Nadakuduti et al., 2017). The combination of tissue enrichment and phylogenetic relatedness is a powerful tool to identify enzymes in other acylsugar pathways.

Here, we report the structural characterization of monosaccharide acylsugars that are built on a myoinositol core from S. quitoense aerial tissues along with the demonstration of function of a triacylinositol acyltransferase (TAIAT) that catalyzes the fourth and final acylation step in S. quitoense acylsugar biosynthesis. Liquid chromatography-mass spectrometry (LC-MS) and NMR spectroscopy analysis revealed that these are myoacylinositols with three to four acyl chains of two, 10, and 12 carbon atoms as major acylsugars. We used RNA sequencing (RNA-seq) data and phylogenetic analysis to identify a protein related to SIASAT4, the enzyme that acetylates triacylated succrates in S. lycopersicum. A combination of virus-induced gene silencing (VIGS) and in vitro biochemistry demonstrated that this enzyme acetylates S. quitoense triacylinositols at the 4-position of myoinositol. This analysis reveals parallels between the S. quitoense acylinositol and S. lycopersicum acylsucrose biosynthetic pathways that suggest a common recent evolutionary origin.

RESULTS

NMR and LC-MS Analyses Reveal S. quitoense Acylinositol Structures

LC-MS analysis of S. quitoense leaf surface extracts revealed previously undescribed acylhexoses and acyldisaccharides (Fig. 1A). Subsequent generation of MS-MS product ions of [M+formate]− ions of the four major acylhexoses yielded spectra dominated by fragment ions assigned as fatty acid carboxylates of 10 (mass-to-charge ratio [m/z] 171.14) and 12 (m/z 199.17) carbons but few other fragments (Supplemental Fig. S1). Lack of intermediate sugar core-containing fragment ions derived from neutral losses of acyl groups is inconsistent with previous collision-induced dissociation mass spectra observed for acylsuccrates and acylglucoses analyzed (Leong et al., 2019). In contrast, MS-MS product ion mass spectra of [M+NH4]+ of the four S. quitoense acylhexoses yielded fragment ions consistent with neutral losses of two-, 10-, or 12-carbon aliphatic acids and ketenes (Supplemental Fig. S2). The fatty acid ions and neutral losses from these compounds indicated that these acylsugars have one to two carbon acyl chains and two longer chains of 10 or 12 carbons on hexose and disaccharide cores.

NMR analysis of the purified compounds, the four highest abundance S. quitoense acylsugars, confirmed the hypothesis that the sugar core differed from Glc. These sugar esters each contain three to four acyl chains, two unbranched and saturated C10 or C12 acyl chains and one or two acetyl (C2) groups, consistent with the LC-MS-MS analysis (Fig. 1B; Supplemental Fig. S2; Supplemental Table S1). Each metabolite contains a myoinositol core (Fig. 1B; Supplemental Table S1), which is a sugar alcohol acylsugar core previously reported in one species in the Solanaceae family (Herrera-Salgado et al., 2005). There are five less abundant acylinositol modified by glycosylation with Glc, Xyl, or GlcNAc (Hurney, 2018). Our study focused on the nonglycosylated acylinositols, representing more than 85% of the integrated LC-MS acylsugar peak areas (Hurney, 2018). The nomenclature used to describe these compounds is as follows: IXY (A,B,C,D), where I signifies the myoinositol core, X represents the total number of acyl esters, Y is the sum of carbons in those acyl chains, while A, B, C, and D represent the number of carbons in each acyl chain. Taken together, the NMR results revealed the four most abundant...
Identification of Acylinositol Acyltransferase Candidates in *S. quitoense*

With knowledge of acylinositol structures in hand, we sought candidates for their biosynthesis using several criteria to analyze RNA-seq data from Moghe et al. (2017). First, we selected candidate BAHD enzymes based on the presence of two conserved BAHD signature motifs (D’Auria, 2006): an HXXXD catalytic motif and a DFGWG structural motif, each present in a specific orientation. A second criterion was that candidates had to be between 400 and 500 amino acids in length, as found in characterized functional BAHDs. Together, these steps resulted in a list of 42 candidates. Previously characterized ASATs are expressed and enriched in glandular trichomes relative to non-acylsugar-accumulating tissues (Schilmiller et al., 2012a; Ning et al., 2015; Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017; Leong et al., 2019; Mandal et al., 2020). Thus, the 17 candidates with more than 500 reads in the trichome samples were selected for further analysis.

We hypothesized that the acylinositol pathway is evolutionarily related to acylsucrose biosynthesis (Fan et al., 2019), selecting the six putative BAHDs most closely related to previously characterized ASATs in clade III of BAHD acyltransferases (Fig. 2; Supplemental Fig. S3; Schilmiller et al., 2012a, 2015; Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017). Given the presence of C2 groups on the NMR-characterized acylinositols, the following candidates were of great interest: three transcripts, c38687_g1_i4, c38687_g1_i1, and c38687_g2_i1 (where c#####_g#_i# represents distinct transcripts in the RNA-seq assembly), formed a phylogenetic cluster with SlASAT4, which catalyzes acetylation of triacylsucroses in *S. lycopersicum* (Figs. 2 and 3A; Table 1; Supplemental Fig. S3). Our analysis focused on c38687_g1_i4 because it has the seventh largest number of reads of all transcripts in the trichome data set and is enriched in trichomes relative to stem tissue (Table 1).
Results of the in planta and biochemical analyses described below lead to the conclusion that this gene is a TAIAT.

**In Vivo Analysis of TAIAT Function**

The acylinositol NMR structures are consistent with the hypothesis that monoacetylated *S. quitoense* triacylinositols with a single acetyl ester are biosynthetic precursors of tetraacylinositols with two acetyl esters (Fig. 1; Supplemental Table S1). This predicts that acetylation of the 4-position hydroxyl is the final step in tetraacylated inositol biosynthesis, because both I3:22 (2,10,10)/I4:24 (2,2,10,10) and I3:24 (2,10,12)/I4:26 (2,2,10,12) differ by acetylation at this position (Figs. 1 and 3A).

VIGS was developed for *S. quitoense* (Fig. 3B) to test whether TAIAT has a role in acylinositol biosynthesis. As predicted for an enzyme catalyzing the second acetylation reaction, VIGS plants derived from two different TAIAT constructs showed strong and statistically significant increases in the ratio of monoacetylated I3:22 (2,10,10) to diacetylated I3:22 (2,10,10) relative to the controls (Fig. 3, C and D; Supplemental Table S2). A similar increase in the ratio of I3:24 (2,10,12) was observed relative to I4:26 (2,2,10,12; Fig. 3, C and D; Supplemental Table S2). Analysis by quantitative PCR (qPCR) revealed reductions in TAIAT expression in some of the target plants relative to the controls (Supplemental Fig. S4). The increased ratio of triacylated to tetraacylated inositols in TAIAT VIGS plants supports the hypothesis that I3:22 (2,10,10) and I3:24 (2,10,12) are direct precursors of the more acetylated products and that TAIAT catalyzes the acetylation of these triacylinositols.

**In Vitro Analysis of TAIAT**

We tested TAIAT in vitro activity to determine whether it can acetylate I3:22 (2,10,10) and I3:24 (2,10,12). His-tagged TAIAT was expressed in *Escherichia coli* and purified using nickel-nitrilotriacetic acid (Ni-NTA) resin. Acylinositol substrates were enriched from *S. quitoense* leaf surface extracts using semipreparative LC.

In vitro assays validated that TAIAT acetylates *S. quitoense* triacylinositols. TAIAT acetylated I3:22 (2,10,10) to form I4:24 (2,2,10,10; Fig. 4A) and also converted I3:24 (2,10,12) to I4:26 (2,2,10,12; Fig. 4B). In vitro products and in planta acylsugars coeluted with, and fragmented similarly to, each other (Supplemental Figs. S5 and S6). The two other ASAT4 homologs show minimal activity with triacylinositols (less than 1% of TAIAT product accumulation; Supplemental Figs. S7 and S8). These results indicate that TAIAT converts triacylinositols to tetraacylinositols in *S. quitoense*.

We took advantage of the ability of BAHs to catalyze reverse reactions when provided with its usual (Supplemental Fig. S4). The increased ratio of triacylated to tetraacylated inositols in TAIAT VIGS plants supports the hypothesis that I3:22 (2,10,10) and I3:24 (2,10,12) are direct precursors of the more acetylated products and that TAIAT catalyzes the acetylation of these triacylinositols.
product and CoA to ask whether TAIAT specifically catalyzes transfer of the acetyl group from position 4. Reactions were performed using extracts containing individual in planta acylinositols that had two acetyl groups. TAIAT deacetylated both I4:24 (2,2,10,10) and I4:26 (2,2,10,12) to generate single chromatographic peaks for I3:22 (2,10,10) and I3:24 (2,10,12), respectively, using two different LC columns (Supplemental Fig. S9). The reaction products coeluted with, and fragmented similarly to, S. quitoense triacylinositols (Supplemental Figs. S6 and S10). The combination of forward and reverse in vitro enzymatic activities and in planta VIGS phenotypes provides compelling evidence that TAIAT acetylates S. quitoense triacylinositols.

**DISCUSSION**

Acylsugars constitute a diverse group of specialized metabolites produced across the Solanaceae family
Variation in acyl chain length, branching pattern, or occasionally other constituents such as malonyl groups all contribute to this diversity (Fan et al., 2019). Other factors include differences in acylation position, acyl-CoA specificity, and sugar core diversity (Fan et al., 2016, 2017, 2019; Moghe et al., 2017). Most characterized acylsugar-producing species accumulate primarily acylsucroses, with isolated occurrences of acylglucoses in Nicotiana miersii, Datura metel, or S. pennellii (Burke et al., 1987; King and Calhoun, 1988; Matsuzaki et al., 1989; Ghosh et al., 2014; Liu et al., 2017; Moghe et al., 2017). Accumulation of acylsugars has only been explored in a small fraction of the approximately 2,700 known species in the Solanaceae. Our study focused on one such underexplored species, S. quitoense.

The structures of S. quitoense acylsugars we characterized are distinct from those previously reported in two ways. First, these metabolites represent an example of myoinositol in acylsugars: the other published case is S. lanceolatum (Herrera-Salgado et al., 2005), where inositol was a component of disaccharide cores. Acyl chain makeup is another differentiating element, with exclusively C10 or C12 unbranched acyl chains and acetyl groups. This contrasts with S. lycopersicum and S. sinuata, which produce acylsugars with branched acyl chains presumably derived from branched chain amino acid precursors (Walters and Steffens, 1990; Kroumova and Wagner, 2009; Ghosh et al., 2014; Ning et al., 2015; Liu et al., 2017; Moghe et al., 2017). The unusual sugar core and acyl chain structures led us to characterize acylinositol biosynthesis.

We characterized the BAHD acetyltransferase, TAIAT, as the enzyme that catalyzes a terminal step in S. quitoense tetraacylinositol biosynthesis. Two lines of evidence indicate that TAIAT acetylates S. quitoense triacylinositols. Use of a newly established S. quitoense VIGS protocol revealed that reduction in TAIAT expression caused increased ratios of triacylated to tetracylated inositols (Fig. 3). In vitro assays demonstrated that TAIAT can acetylate S. quitoense-produced I3:22 (2,10,10) and I3:24 (2,10,12) and, in the reverse reaction, can deacetylate I4:24 (2,2,10,10) and I4:26 (2,2,12,12; Fig. 4; Supplemental Fig. S9). The combined in planta and in vitro findings implicate TAIAT as the last acetylation step in tetraacylinositol biosynthesis.

TAIAT substrates are similar to SlASAT4. Our results show that TAIAT uses acetyl-CoA, the acyl donor substrate of several BAHD enzymes in clade III, including SlASAT4 (Fig. 4; D’Auria, 2006; Schilmiller et al., 2012a; Moghe et al., 2017). The acyl acceptor substrates are also similar: S. lycopersicum SlASAT4 acetylates triacylsucroses, whereas TAIAT acetylates triacylinositols in S. quitoense.

The combination of trichome-enriched expression and phylogenetic analysis is a powerful tool to identify uncharacterized BAHDs involved in acylsugar biosynthesis (Moghe et al., 2017; Nadakuduti et al., 2017). S. quitoense trichome RNA-seq data contain five putative ASAT homologs in addition to TAIAT (Fig. 2). It is
possible that these homologs play a role in one or more of the remaining biosynthetic steps based on their trichome expression and phylogenetic relationships.

Our study uncovered parallels that suggest a common recent evolutionary origin between acylinositol and acylscurcrose biosynthesis. The acetyl-CoA substrate used by SLASAT4 and TAIAT, their close phylogenetic relationship, and enrichment in trichomes are evidence for a link between these pathways. Phylogenetic analysis of acylsugar-producing species point to Suc as the ASAT ancestral acyl acceptor: it is the sugar acceptor in earlier diverging lineages leading to S. sinuata and P. axillaris (Moghe et al., 2017; Nadakuduti et al., 2017).

Furthermore, acylscurcrose-accumulating species are present throughout the Solanaceae family (Burke et al., 1987; King and Calhoun, 1988; Matsuizaki et al., 1991; Maldonado et al., 2006; Ghosh et al., 2014; Liu et al., 2017, Moghe et al., 2017), whereas reported acylinositol accumulators are S. quitoense and S. lanceolatum (Fig. 1; Herrera-Salgado et al., 2005). Both species are members of the Leptostemonum or Spiny Solanum clade. These limited data suggest that acylinositol biosynthesis more recently emerged from the acylscurcrose pathway; screening the Leptostemonum clade and the Solanum genus more broadly would provide insight into when acylinositol biosynthesis emerged. One or more factors behind the emergence of acylinositol biosynthesis is not clear. Acylinositol biosynthesis provides opportunities to better understand acylsugar diversification and broaden our knowledge of both pathway and enzyme evolution as well as the chemical ecology.

MATERIALS AND METHODS

Heterologous Protein Expression and Purification from Escherichia coli

Heterologous protein expression was achieved using pET28b+ (EMD Millipore), in which open reading frames for the enzymes were cloned into BamHI/XhoI doubly digested vector. Inserts were amplified by PCR using Q5 2x Hotstart mastermix (New England Biolabs) and purified by agarose gel purification and extraction (primers: TAIAT, c38687_g1_F/R, c38687_g1_i1, c38687_g1_F/c38687_g1_i1_R, c38687_g2_F/R). The doubly digested vectors were assembled with a single fragment containing the open reading frames containing 5′ and 3′ adapters for Gibson assembly using 2x NEB HiFi Mastermix (New England Biolabs). The finished constructs were transformed into BL21 Rosetta (DE3) cells (EMD Millipore), verified using colony PCR, and Sanger sequenced using 17 promoter and terminator primers. Primer sequences are listed in Supplemental Table S3.

Luria-Bertani (LB) overnight cultures with kanamycin (50 μg mL⁻¹) and chloramphenicol (35 μg mL⁻¹) were inoculated with a single colony of the bacterial strain containing the desired construct and incubated at 37°C, 225 rpm, overnight. Larger LB cultures (1 L) were inoculated 500:1 with the same antibiotics and incubated at the same temperature and speed. OD₆₀₀ of the cultures was monitored until between 0.5 and 0.8. Cultures were chilled on ice for 15 min, at which point isopropylthio-β-galactoside was added to a final concentration of 0.1 mM. Cultures were incubated at 16°C and 180 rpm for 16 h.

Note that all of the following steps were on ice or at 4°C. Cultures were centrifuged at 4,000g for 10 min to collect the cells and repeated until all the culture was processed (4°C). The cell pellets were resuspended in 25 mL of extraction buffer (50 mM NaPO₄, 300 mM NaCl, 20 mM imidazole, and 5 mM 2-mercaptoethanol, pH 8) by vortexing. The cell suspension was sonicated for eight cycles (30 s on, intensity 4, 30 s on ice). The cellular extracts were centrifuged at 30,000g for 10 min. The supernatant was transferred into another tube and centrifuged again at the same speed and duration. Ni-NTA resin (Qiagen) was centrifuged at 1,000g for 1 min and resuspended in 1 mL of extraction buffer. The slurry was centrifuged again at 1,000g for 1 min, and the supernant was decanted. The resin was resuspended using the centrifuged extract and incubated at 4°C, mutating for 1 h. The slurry was centrifuged at 3,200g for 5 min, and the supernatant was decanted. The resin was resuspended in 5 mL of extraction buffer and transferred to a gravity flow column (Bio-Rad). After loading, the resin was washed with 3 column volumes of extraction buffer (~30 mL). The resin was further washed with 1 column volume of wash buffer (extraction buffer with 40 mM imidazole). The remaining protein was eluted and collected using 2 mL of elution buffer after a 1-min incubation with the resin. The eluent was diluted into 15 mL of storage buffer (extraction buffer but no imidazole), concentrated using 10-kD centrifugal filter units (EMD Millipore), and repeated until diluted 1,000-fold. An equal volume of 80% (v/v) glycerol was added to the elution, mixed, and stored at −20°C. SDS-PAGE was used to analyze elution fractions, and the presence of enzymes was confirmed by immunoblot using the anti-His-6 antibody conjugated to peroxidase (BMC-His-1 monoclonal antibody; Roche).

Enzyme Assays

Assays were run in 100 mM sodium phosphate buffer at a total volume of 60 μL with pH 6 as the default unless otherwise stated. Acyl-CoA (or CoA) was added to a final concentration of 100 μM and obtained from Sigma-Aldrich. Acylasugar acceptors, such as 13,22, were dried down using a SpeedVac and dissolved in an ethanol:water mixture (1:1) with 1 μL added to the reaction. Six microliters of enzyme was added to each reaction (or water). The assays were incubated at 30°C for 30 min unless otherwise stated. After the incubation, 2 volumes of stop solution, composed of a 1:1 mixture of acetonitrile and isopropanol with 0.1% (v/v) formic acid and 1 μL telmesartan as an internal standard (Sigma-Aldrich), was added to the assays and mixed by pipetting. Reactions were stored in the −20°C freezer for 20 min and centrifuged at 17,000g for 5 min. The supernatant was transferred to LC-MS autosampler vials and stored at −20°C. For negative control assays in Supplemental Figures S7 and S8, an aliquot of the enzyme was incubated at 95°C for 10 min to inactivate it.

LC-MS Analysis

LC-MS analyses of products from enzyme assays and extracts of plant tissues were performed on a Waters Acquity UPLC coupled to a Waters Xevo G2-QToF mass spectrometer. Ten microliters of the extracts was injected into an Ascentis Express C18 HPLC column (10 cm × 2.1 mm, 2.7 μm) or an Ascentis Express F5 HPLC column (10 cm × 2.1 mm, 2.7 μm, Sigma-Aldrich) that was maintained at 40°C. The LC-MS methods used the following solvents: 10 mM aqueous ammonium formate, adjusted to pH 2.8 with formic acid as solvent A, and 100% acetonitrile as solvent B. A flow rate of 0.3 mL min⁻¹ was used unless otherwise specified. All LC-MS-MS chromatograms were acquired at 0.2 mL min⁻¹, 60% B at 1 min, 100% B at 5 min, held at 100% B until 6 min, 5% B at 6.01 min, and held at 5% until 7 min. A 21-min linear elution gradient consisted of 5% B at 0 min, 60% B at 3 min, 100% B at 15 min, held at 100% B until 18 min, 5% B at 18.01 min, and held at 5% until 21 min.

For ESI+ MS, settings were as follows: capillary voltage, 2 kV; source temperature, 100°C; desolvation temperature, 350°C; desolvation nitrogen gas flow rate, 600 L h⁻¹; cone voltage, 40 V; mass range, m/z 50 to 1,000 (with spectra accumulated at 0.1 s per function). Three acquisition functions were used to acquire spectra at different collision energy settings (0 and 10–60 V ramp). Lock mass correction was performed using Leu enkephalin [M+H]⁺ as the reference, and corrections were applied during data acquisition.

For ESI− MS, settings were as follows: capillary voltage, 3 kV; source temperature, 100°C; desolvation temperature, 350°C; desolvation nitrogen gas flow rate, 600 L h⁻¹; cone voltage, 35 V; mass range, m/z 50 to 1,000 (with spectra accumulated at 0.1 s per function). Two acquisition functions were used to acquire spectra at different collision energy settings (0 and 10–60 V ramp). Lock mass correction was performed using Leu enkephalin [M+H⁻]⁻ as the reference, and corrections were applied during data acquisition.

Data-dependent LC-MS-MS analyses were performed on an LC-20ADvp ternary pump (Shimadzu) coupled via an Ascentis Express C18 column (2.1 × 100 mm, 2.7 μm; Supelco) to a Xevo G2-XS mass spectrometer (Waters). Ion source parameters were as described above for LC-MS analyses. Column temperature was held at 50°C. Gradient elution employed solvent A (10 mM aqueous ammonium formate, adjusted to pH 3 with formic acid) and solvent B...
Acylsugar Purification for Enzyme Assays

Acylsugars were extracted from 20 leaves from 12-week old *Solanum quitoense* plants into 500 mL of methanol with 0.1% (v/v) formic acid with gentle agitation in a 1-L beaker. The methanol was transferred to a round-bottom flask and evaporated under reduced pressure using a rotary evaporator with a warm water bath (−40°C). The dried residue was dissolved in 2 mL of acetonitrile (0.1% (v/v) formic acid) and stored in an LC-MS vial at −20°C. A total of 500 μL of the solution was transferred into a microcentrifuge tube and evaporated to dryness using a SpeedVac. The residue was dissolved in 550 μL of 4.1 acetonitrile-water with 0.1% (v/v) formic acid. Five 100-μL injections were made onto a Waters 2795 HPLC device equipped with a C18 semipreparative column (Acclaim C18 5 μm, 120 Å, 4.6 × 150 mm) using a 63-min chromatographic method to separate the acylsugars with a flow rate of 1.5 mL min⁻¹ and column temperature of 30°C. Solvent A was water with 0.1% (v/v) formic acid and solvent B was acetonitrile.

The chromatographic gradient was as follows: 5% B at 0 min, 60% B at 1 min, 100% B at 50 min, hold at 100% B until 60 min, 5% B at 60.01 min, and hold at 5% B until 63 min. One-minute fractions were collected for a total of 63 fractions. Fractions were screened (LC-MS) for the presence of acylsugars, and fractions judged to have sufficient purity were pooled and evaporated to dryness using the SpeedVac. Each acylsugar was resuspended in acetonitrile with 0.1% (v/v) formic acid, transferred to LC-MS vials, and stored in the −20°C freezer. Aliquots of 200 μL of the acylsugars were transferred to microcentrifuge tubes with glass inserts and evaporated to dryness using a SpeedVac before dissolution in 20 μL of 1:1 ethanol/water mixture for use in enzyme assays.

Gene Identification and Phylogenetic Analysis

All transcript assemblies are from Moghe et al. (2017) and were analyzed using Geneious R8.1.9. Sequences were selected by HXXHD motifs, which were detected using the Search for Motif function (zero mismatches). Those sequences were further parsed to include only those that contain the DFGWG cation and Phylogenetic Analysis. Alignments were performed in Geneious R8.1.9 using the MUSCLE method, Jones-Taylor-Thornton (JTT) matrix, and Phylogenetic Analysis. One thousand nucleotide sequences were further aligned using MUSCLE under default parameters. A maximum likelihood method was used to generate the phylogenetic tree. Phylogenetic reconstructions were performed using MEGA X (Kumar et al., 2018) and several characterized ASATs (Schilmiller et al., 2012a, 2015; Fan et al., 2016; Moghe et al., 2017). Both transcript and expression data are available in the study by Moghe et al. (2017).

Plant Growth Conditions

Plants were grown at 24°C in a 16-h-light/8-h-dark cycle with a light intensity of 70 μmol m⁻² s⁻¹ photosynthetic photon flux density in Jiffy 7 peat pellets (jiffy). Plants were watered four times per week with deionized water and fertilized once per week with one-half-strength Hoagland solution. These were the plant growth conditions unless otherwise specified.

Acylsugar Analysis

The interactive protocol for acylsugar extracts is available at Protocols.io at https://dx.doi.org/10.17504/protocols.io.x2jkip. The acylsugar extraction protocol was described previously (Leong et al., 2019). LC-MS conditions used for acylsugar analysis are described in the LC-MS analysis section. Acylsugars were analyzed in ESI⁺ mode as formate adducts and in ESI⁻ mode as ammonium adducts.

VIGS Analysis

pTRV2-LIC as previously described was digested using *PstI*-HP to generate the linearized vector (Dong et al., 2007). Fragments were amplifed using PCR with adapters for ligation into pTRV2-LIC (*c38687_g1_p1_F/R*, *c38687_g1_p2_F/R*, or *SqPDS_VIGS_F/R*). Note that *c38687_g1_p1_R* is located in the 3' untranslated region. Both the linearized vector and PCR products were purified using a 1% (w/v) agarose gel and gel extracted using an Omega EZNA gel extraction kit. Both the PCR fragment and the linearized vector were incubated in separate 5-μL reactions using NEB 2.1 buffer with T4 DNA polymerase and 5 μm dATP or dTTP (PCR insert/vector). The reactions were incubated at 22°C for 30 min and subsequently incubated at 70°C for 20 min. The reactions were then stored on ice. One microliter of the pTRV2-LIC reaction and 2 μL of the PCR-LIC reaction were mixed by pipetting. Reactions were incubated at 65°C for 2 min and then 22°C for 10 min. After this the constructs were transformed into chemically competent *E. coli* DH5α cells. Primer sequences are available in Supplemental Table S3.

Constructs were tested for the presence of the insert using colony PCR and pTRV2-LIC_seq_F/R primers amplifying a 300-bp insert. Positive PCR products were miniprepped (Qiagen) and Sanger sequenced using the same primers. Sequence constructs and pTRV1 were transformed into *Agrobacterium tumefaciens* strain GV3101 using the protocol described previously except on LB plates with kanamycin (50 μg mL⁻¹), rifampicin (50 μg mL⁻¹), and gentamicin (10 μg mL⁻¹; Leong et al., 2019). Colonies were assayed for the presence of the insert using the colony PCR and pTRV2_LIC_seq_F/R primers previously described. The presence of the pTRV1 vector in *A. tumefaciens* strain GV3101 was assayed using colony PCR primers pTRV1_F/R. Primer sequences are available in Supplemental Table S3.

The VIGS protocol was adapted from Velásquez et al. (2009). Seeds were incubated in 10% bleach for 30 min, followed by five to six washes with water. Seeds were transferred to a petri dish with Whatman paper and water in the bottom of the dish. Seeds were stored in the dark at room temperature until hypocotyl emergence, at which point the hypocotyls were moved to a window sill. Once cotyledons emerged, seedlings were transferred to pot peat (jiffy) and grown for approximately 1 week under a 16-h/8-h day/night cycle at 24°C until inoculated. At 2 d preinoculation, LB cultures (kanamycin/rifampicin/gentamicin) were inoculated with the cultures used for leaf inoculation. The strains have constructs containing the gene of interest in pTRV2-LIC, an empty vector pTRV2-LIC, and pTRV1. Cultures were grown overnight at 30°C with shaking at 225 rpm. Larger cultures composed of induction medium (4.88 g of MES, 2.5 g of Glc, 0.12 g of sodium phosphate monobasic monohydrate in 500 mL pH 5.6, and 200 μM acetylsorbose) were inoculated using a 25:1 dilution of the overnight culture (50 mL total). The larger culture was incubated at 30°C, 225 rpm, overnight. Cells were harvested by centrifugation at 5,200g for 10 min. Cells were resuspended in 10 mL of 4:1 acetonitrile:water mixture for use in enzyme assays.
Acylinositol Biosynthesis in a Nonmodel Plant

Supplemental Figure S1. Negative-ion MS-MS spectra of product ions generated from [M + formate]− of S. quitoense acylinositol reveal C10 and C12 fatty acid carboxylate ions.

Supplemental Figure S2. Positive-ion MS-MS spectra of product ions generated from [M + Na]⁺ of S. quitoense acylinositols.

Supplemental Figure S3. Multiple sequence alignment of sequences used in phylogenetic analysis.

Supplemental Figure S4. Quantitative PCR analysis of TAIAT transcript abundance in VIGS plants.

Supplemental Figure S5. ESI+ mode nonmass selective fragmentation of forward enzyme assay products of TAIAT [m/z 14:24 (2,2,10,10) and 14:26 (2,2,10,12)] generated from incubations of triacylinositols 13:22 and 13:24 with acetyl-CoA.

Supplemental Figure S6. Coelution analysis of in vitro assay products of TAIAT using C18 and F5 chromatography.

Supplemental Figure S7. In vitro assays comparing different ASAT4 homolog activities with 13:22 (2,10,10).

Supplemental Figure S8. In vitro assays comparing different ASAT4 homolog activities with 13:24 (2,10,12).

Supplemental Figure S9. In vitro reverse reactions of TAIAT.

Supplemental Figure S10. Positive-ion mode mass spectra with ramped collision voltage ([M–60 V] of triacylinositol products generated from reverse enzyme assay products of TAIAT with 14:24 and 14:26 [products are 13:22 (2,10,10) and 13:24 (2,10,12)].

Supplemental Table S1. NMR chemical shifts for four acylinositols from S. quitoense.

Supplemental Table S2. Acylisugar analysis of S. quitoense VIGS plant samples.

Supplemental Table S3. Oligonucleotides used in this study.

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qPCR Analysis

RNA was extracted with the RNaseasy Plant Mini Kit including on-column DNA digestion (Qiagen), according to the manufacturer’s instructions. RNA was quantified with a Nanodrop 2000c instrument (Thermo Fisher Scientific). cDNA was synthesized using 1 μg of the isolated RNA and SuperScript II Reverse Transcriptase (Invitrogen). The cDNA samples were diluted 200-fold (10-fold initial dilution and 20-fold dilution into qPCRs). qPCRs (10 μL) were created with SYBR Green PCR Master Mix (Thermo Fisher Scientific), and primers were used at a final concentration of 200 nM. RT_TAIAT_F/R, RT_ACTIN_F/R, and RT_EF1a_F/R primers were used to detect TAIAT, ACTIN, and EF1a transcripts, respectively (Supplemental Table S3). Reactions were carried out with a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) by the Michigan State University RTSF Genomics Core. The following temperature cycling conditions were applied: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression of TAIAT was calculated with the ΔΔct method (Pfaffl, 2001) and normalized to the geometric mean of ACTIN and EF1a transcript levels. The mean expression values of the transcripts in the control plants were used for normalization. Three to four technical replicates were used for all the qPCRs.

Purification of Myoinositol Acylsugars and Analysis by NMR Spectroscopy

For metabolite profiling and purification, aerial tissues of 20 10-week-old S. quitoense plants (~0.25 m height) were extracted in 1.9 L of acetonitrile-isopropanol (1:1, v/v) for 10 min (plants were cut at the stems and stem junctions). Approximately 1 L of the S. quitoense bulk extract was concentrated to dryness under vacuum, redissolved in 5 mL of acetonitrile-isopropanol, and fractionated by repeated injection of 200-μL aliquots onto a Thermo Scientific Acclaim 120 C18 HPLC column (4.6 × 150 mm, 5-μm particles) with reverse-phase gradient elution (0.15% formic acid and acetonitrile) and automated fraction collection. HPLC fractions of sufficient purity of a single metabolite, as assessed by LC-MS, were combined and concentrated to dryness. Samples were dissolved in acetonitrile-d6 (99.96 atom % D) and transferred to solvent-matched Shimadzu tubes or Kontes tubes for NMR analysis. 1D (1H, 13C, and gCOSY) and 2D (gCOSY, gHSQC, gHMBC, J-resolved, TOCSY, and ROESY) NMR spectroscopic techniques served as the basis for structure elucidation of purified S. quitoense acylsugars. 1H, 13C, gCOSY, gHSQC, coupled-gHSQC, gHMBC, J-resolved, TOCSY, and ROESY NMR experiments were performed using a Bruker Avance 900-MHz spectrometer equipped with a TCI triple resonance probe or an Agilent DDR2 500-MHz spectrometer equipped with OneNMR probe (with Proteum accessory for hands-off tuning). 1D TOCSY transfer experiments were performed using a Varian Inova 600-MHz spectrometer equipped with a Naloric 5-mm PFG switchable probe (pretuned for 1H and 13C). All spectra were referenced to nondeuterated solvent signals: acetonitrile-d6 (s = 1.94 and d = 118.7 ppm). NMR spectra were processed using TopSpin 3.5p17 or MestReNova 12.0.0 software. Because these metabolites were identified without authentic standards or synthetic confirmation, their structures should be considered putative, meeting level 2 criteria of the Metabolomics Standards Initiative guidelines (Sumner et al., 2007). Further evidence for structural assignments described herein are outlined by Humey (2018). The basis for the clockwise carbon numbering system used for the assignment of myoinositols in this study traces from the biosynthetic conversion of α-Glc-6-P to 1-α-myoinositol-1-phosphate (Loewus and Murthy, 2000). Due to the plane of symmetry of the myoinositol ring system about the C2 and C5 positions, an enantiomeric assignment is possible by substituting the C1 and C3 positions (1-α-myoinositol), followed by counterclockwise numbering. Due to the scarcity of purified material (less than 3 mg), absolute stereochemical configurations were not determined.

Accession Numbers

ASAT4 homolog sequence data are available in GenBank as follows: TAIAT, MT024677; c38687_g1_i1, MT024678; and c38687_g2_i1, MT024679.

Supplemental Data

The following supplemental materials are available.
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