Evolution of a flipped pathway creates metabolic innovation in tomato trichomes through BAHD enzyme promiscuity

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Plants produce hundreds of thousands of structurally diverse specialized metabolites via multistep biosynthetic networks, including compounds of ecological and therapeutic importance. These pathways are restricted to specific plant groups, and are excellent systems for understanding metabolic evolution. Tomato and other plants in the nightshade family synthesize protective acylated sugars in the tip cells of glandular trichomes on stems and leaves. We describe a metabolic innovation in wild tomato species that contributes to acylsucrose structural diversity. A small number of amino acid changes in two acylsucrose acyltransferases alter their acyl acceptor preferences, resulting in reversal of their order of reaction and increased product diversity. This study demonstrates how small numbers of amino acid changes in multiple pathway enzymes can lead to diversification of specialized metabolites in plants. It also highlights the power of a combined genetic, genomic and in vitro biochemical approach to identify the evolutionary mechanisms leading to metabolic novelty.
Plants produce large numbers of structurally diverse specialized metabolites1, with individual classes typically restricted both taxonomically and spatiotemporally2–3. The key families of enzymes involved in producing these metabolites, e.g., cytochrome P450-dependent monoxygenases, terpene synthases, and BAHD acyltransferases, show strong signs of diversification over the course of land plant evolution4, giving rise to hundreds of thousands of structurally diverse plant metabolites with roles ranging from pollinator and symbiotic interaction to pathogen and herbivore defense5–7. These enzymes exhibit signs of rapid evolution compared with those of core metabolism8, and a variety of factors are associated with this liability. For example, specialized metabolic enzymes are encoded by multigene families, and genetic redundancy creates the potential for evolution of novel regulation or enzymatic activities with reduced negative impacts on fitness9. In addition, the cell and tissue specificity of specialized metabolic pathways is conducive to changes in enzyme activities without causing adverse fitness effects. Finally, enzyme promiscuity—the ability to utilize multiple structurally related substrates—is associated with the ability of specialized metabolic enzymes to evolve rapidly10–13. Changes in one enzymatic activity influence its ability to use substrates produced by other enzymes, and in turn generate products that are substrates for other enzymes. Understanding the evolution of pairs or sets of enzymes can contribute to our knowledge of catalysis and inform novel metabolic engineering strategies.

Several characteristics make the acylsucrose biosynthetic network in cultivated tomato (Solanum lycopersicum) and its wild relatives an exemplary system for understanding biosynthetic pathway evolution. Acsylsugars are of interest as natural plant pest control agents, with documented direct protection from fungal pathogen14 and herbivore14–17 attack, and indirect protection through tri-trophic interactions18,19. The core network in cultivated tomato is relatively simple, with four glandular trichome-expressed BAHD (BEAT, AHCT, HCBT, DAT20,21) class acylsucrose acyltransferases (ASATs) catalyzing consecutive reactions22. In recent studies, ASATs from Solanaceae species distantly related to the tomato group of Solanum—Petunia axillaris and Salpiglossis sinuata—were characterized, and these create acylsucroses with structures different from those of wild and cultivated tomatoes23,24. In cultivated tomato, the entire acylsugar biosynthetic pathway can function in a single test tube with addition of sucrose and acyl-CoA substrates to four enzymes, permitting facile characterization22,25. Despite the compact pathway structure, these enzymes produce diverse tri- and tetra-acylated products in cultivated tomato, due in part to the acyl-CoA substrate promiscuity of the second and third enzymes in the pathway22,26–28. Study of wild tomato relatives in the Solanum revealed examples of enzyme and product diversification compared with the domesticated species25–30.

For example, populations of S. habrochaites from northern Ecuador lack activity of ASAT4, the last enzyme of the pathway, resulting in loss of pyranose ring acetylation.31 The lack of ASAT4 activity eliminates the potential for the biosynthesis of acylsucroses with acylations on the furanose and pyranose rings. Hence, the possibility for the generation of natural structurally diverse acylsucroses with a minimum of three acyl chains is reduced. S. habrochaites lacks acyl-CoA acyltransferase activity, which is associated with the ability of specialized metabolic enzymes to evolve rapidly10–13. Changes in one enzymatic activity influence its ability to use substrates produced by other enzymes, and in turn generate products that are substrates for other enzymes. Understanding the evolution of pairs or sets of enzymes can contribute to our knowledge of catalysis and inform novel metabolic engineering strategies.

We found that these species produce ASAT3-P enzyme, which is a variant that uses a monoaacylated acylsucrose acceptor substrate, rather than the diacylated substrates used by S. lycopersicum type SI-ASAT3 activity.32 However, this work left open the question of what enzyme converts the diacylated ASAT3-P product to the final triacylated P-type acylsucrose, which has all three substitutions on the pyranose ring.

In the current study, we address this question using a combination of genetic and transgenic plant approaches along with site-
directed mutagenesis and in vitro biochemistry. We identify a cultivated tomato line that produces P-type acylsucrases and found that the concerted action of two regions of the S. pennellii LA0716 genome causes this novel phenotype. These regions harbor the Sp-ASAT2 and Sp-ASAT3 genetic loci, and P-type acylsucrose accumulation results from epistasis between these loci. The combination of these alleles is necessary and sufficient to produce the pyranose ring acylated products in vivo. Biochemical analysis demonstrates that the P-type acylsucrases result from reaction of Sp-ASAT3 followed by Sp-ASAT2, which is the reverse order of action compared with that of the cultivated tomato enzymes. In vitro mutagenesis reveals that a small number of amino acid changes in each enzyme is sufficient to switch the activities between the F- and P-acyl sucroses pathways. Investigation of these pathways in other tomato species allows inference of the evolutionary events giving rise to divergence of F- and P-type acylsucrases. This work demonstrates multiple evolutionary mechanisms in biochemical pathway evolution, including gene duplication and loss, amino acid substitution, and how emergence of pathway epistatic interactions restructured a specialized metabolic pathway and led to metabolic product innovation.

Results
Two S. pennellii loci influence acylsucrose acylations. Single chromosomal introgressions from S. pennellii LA0716 cause dramatic changes in the types of specialized metabolites that accumulate in cultivated tomato trichomes22,26,27,30 and other tissues.33 The availability of Backcrossed Inbred Lines (BILs), containing multiple S. pennellii chromosomal regions in individual S. lycopersicum lines33,35,36 provides an opportunity to identify epistatic interactions that influence the types of metabolites that accumulate. We analyzed trichome acylsugar metabolites of 257 BILs by liquid chromatography-time of flight mass spectrometry (LC-ToF MS) to look for phenotypes not observed in single chromosomal introgression lines.

One BIL line—referred to as 6180 in this report—was found to produce an acylsugar profile absent from S. lycopersicum M82 or single introgression lines (ILs) previously screened32 (Fig. 1b). BIL6180 exclusively accumulated detectable triacylsucrases with all three acylchains on one ring, as revealed by positive mass spectrometry (MS) (Supplementary Fig. 1a). These resemble the acylsugars produced in S. pennellii and S. habrochaites27 and the minor acylsugar peaks produced in IL4-S.22 NMR analysis of two of the major BIL6180 triacylsucrose peaks, namely S3:15 (5, 5, 5) and S3:22 (5, 5, 12) (note that “S” refers to a sucrose backbone, “3:15” indicates three acyl chains with total fifteen carbons and the length of each acyl chain is shown in the parentheses), showed that they were acylated exclusively on the six-membered pyranose ring at the R2, R3, and R4 positions (Supplementary Fig. 1b). This pattern is in contrast to that of the parental S. lycopersicum, which accumulates F-type acylsucrases with a single acyl chain at the R3 position of the five-membered furanose ring (Fig. 1a). However, it resembles the acylation pattern of the S. pennellii LA0716 acylsucrose S3:19-P (5R2, 10R3, 4R4) previously resolved by NMR.30 Another similarity between the BIL6180 and S. pennellii LA0716 acylsucrases is that both S3:22-P (5R2, 12R3, 4R4) and S3:19-P (5R2, 10R3, 4R4) have the long acyl chain—C12 in 6180 and C10 in S. pennellii LA0716—at the R3 position, with C4 or C5 chains at R2 and R4 (Supplementary Fig. 1b). Furthermore, each detected BIL6180 acylsucrose peak was composed of multiple isomers differing by branched acyl chain types. Liquid chromatography-based separation resulted in the co-elution of a mixture of S3:15 (5,5,5) structural isomers (Supplementary Fig. 1b), and their NMR spectra revealed either iC5 or aiC5 at each of the three acylation positions. In addition, two co-eluting S3:22 (5, 5, 12) isomers (Supplementary Fig. 1b) differed by the presence of iC5 or aiC5 acyl chains at the R3 position. Taken together, these results indicate that BIL6180 accumulates pyranose ring acylated triacylsucrases lacking furanose ring acylation, which are similar to those found in the S. pennellii LA0716 parent (Fig. 1b). The similarity between the acylsucrases from BIL6180 and S. pennellii LA0716 led us to hypothesize that BIL6180 could be utilized to characterize the S. pennellii acylsucrose biosynthetic pathway.

Sp-ASAT2 and Sp-ASAT3 make P-type acylsucrases. BIL6180 contains three S. pennellii introgressions, including regions on chromosome 4 and 11 that contain Sp-ASAT2 and Sp-ASAT3, respectively (Fig. 1c). These are orthologs of the S. lycopersicum ASAT genes SI-ASAT2 and SI-ASAT3, which encode enzymes catalyzing the second and third steps of the cultivated tomato acylsucrose biosynthetic pathway.22,27. This led to the hypothesis that Sp-ASAT2 and Sp-ASAT3 contribute to biosynthesis of P-type acylsucrases in S. pennellii LA0716. To determine whether the S. pennellii loci that led to P-type acylsucrose accumulation in BIL6180 are dominant or recessive, we crossed homozygous BIL6180 with the parent S. lycopersicum M82 and analyzed the leaf acylsugar profiles of the F1 progeny by LC-ToF MS (Supplementary Fig. 2). Both F- and P-type acylsucrases accumulated in the F1 plants, indicating that the loci contributing to F- and P-type acylsucrases are co-dominant, which led us to design transgenic experiments to test our hypothesis.

We engineered S. lycopersicum M82 plants by simultaneously expressing the Sp-ASAT2 and Sp-ASAT3 coding regions driven by the type I/IV trichome specific promoters22,29 of SI-ASAT2 and SI-ASAT3, respectively (Fig. 2a). As predicted, the P-type acylsucrases S3:15 (5, 5, 5)-P and S3:22 (5, 5, 12)-P were produced in the regenerated transgenic T0 plants, but not in the parental M82 (Fig. 2b and Supplementary Fig. 3). Similar to the F1 double heterozygotes (Supplementary Fig. 2), the F-type acylsucrases were also detected (Fig. 2b and Supplementary Fig. 3). This result provides strong evidence that Sp-ASAT2 and Sp-ASAT3 are sufficient for P-type acylsucrose production in S. pennellii and BIL 6180, and led us to characterize their enzymatic activities.

Reversed reaction order leads to P-type acylsucrases. To test their role in P-type acylsucrose biosynthesis, we performed in vitro assays using purified Sp-ASAT2 and Sp-ASAT3 proteins. Published work demonstrated that SI-ASAT2 acylates the monocyclonosucrose S1:5 (iC5R4) pyranose ring R3 position to produce a diacylsucrose.22 In contrast, Sp-ASAT2 does not use the monocyclonosucrose as a substrate with any of the acyl-CoA donors.22 Surprisingly, the S. pennellii LA0716 ortholog of the third enzyme in the cultivated tomato pathway—Sp-ASAT3—utilized monocyclonosucrose and iC5-CoA donor substrates to produce a diacylated product with the second chain on the pyranose ring.27 NMR analysis of the purified diacylsucrose S2:10 (iC5, iC5R4) produced by the combination of SI-ASAT1 and Sp-ASAT3 revealed that the second chain is acylated at the R3 position of the pyranose ring (Supplementary Fig. 4). Finally, HPLC analysis revealed that the diacylsucrose S2:10 (iC5R2, iC5R4) made by Sp-ASAT3 did not co-elute with the SI-ASAT2 product S2:10 (aiC5R3, iC5R4) (Fig. 3a), supporting the hypothesis that the two diacylsucrases made by Sp-ASAT3 and SI-ASAT2 are different compounds.

The production of S2:10 (iC5R2, iC5R4) by Sp-ASAT3 led us to hypothesize that the S. pennellii LA0716 ASAT ortholog uses this diacylsucrose as an acceptor substrate to produce the P-type
Amino acids that contribute to F- and P-type ASATs divergence. We used a phylogeny-guided approach to identify amino acid substitutions responsible for the differences in activities of the orthologous ASAT2 and ASAT3 enzymes in cultivated tomato and S. pennellii LA0716. This approach benefited from the observation that both Sp-ASAT2 and Sp-ASAT3 share more than 92% identity at the amino acid level with their orthologs in cultivated tomato. In addition, ASAT2 orthologs exist in wild tomato species that fall within the Solanum tomato clade that includes cultivated tomato and S. pennellii. We tested the activities of ASAT2 variants from these wild tomato species using S1:5 (iC5R4) and S2:10 (iC5R2, iC5R4) as acyl acceptor substrates. As shown in Fig. 4a, the presence of Gly at position 304 correlates with the ability of ASAT2 to use S2:10 (iC5R2, iC5R4) as a substrate. In contrast, Gln135 and Tyr136 are found in ASAT2 enzymes that use S1:5 (iC5R4) as a substrate (Fig. 4a). In vitro mutagenesis of C304G in SL-ASAT2 led to the ability to convert S2:10 (iC5R2, iC5R4) to the P-type triacylsucrose S3:22 (iC5R2, nC12R3, iC5R4) (Fig. 4b). This single amino acid substitution made the enzyme more promiscuous because it retained the SL-ASAT2 ability to use S1:5 (iC5R4) as a substrate (Supplementary Fig. 6). The predicted change in substrate specificity was seen following in vitro mutagenesis of ASAT2 to generate the mutant with both substitutions H135Q and C136Y: this mutant enzyme gained the ability to utilize S1:5 (iC5R4) as the acceptor, producing the diacylsucrose S2:10 (aiC5R3, iC5R4), as did wild-type SL-ASAT2 (Fig. 4c). Mutation of both amino acids (135 and 136) was required for complete conversion: both single mutants of Sl-ASAT2 H135Q and C136Y showed a residual ability to use S1:5 (iC5R4) as a substrate (Supplementary Fig. 7).

To assess whether the residues associated with ASAT2 F- or P-type activity have a similar function in other tomato relatives, the F-type ASAT2 from S. arcanum LA2172 and the P-type ASAT2 from S. pennellii LA1926 were cloned and subjected to in vitro mutagenesis. Indeed, S. arcanum LA2172-ASAT2 with substitution C304G gained the P-type ability to acylate S2:10 (iC5R2, iC5R4) (Supplementary Fig. 8a). Similar to the S. pennellii LA0716 Sp-ASAT2 mutant with both residue changes H135Q and C136Y, substitution of P135Q and C136Y in the SL-ASAT2 enzyme to catalyze the F-type activity by acylating S1:5 (iC5R4) (Supplementary Fig. 8b). These results corroborated those obtained by mutagenesis of SL-ASAT2 and Sp-ASAT2.

We employed a similar approach to identify amino acid residues that shape ASAT3 enzyme activities. A previous study documented 35 ASAT3 variants from S. habrochaites that belong to two clades—F- and P-type—which catalyze furanose and pyranose ring acylation similar to SI-ASAT3 and Sp-ASAT3, respectively. A survey of amino acids from six Sh-ASAT3 orthologs allowed the identification of amino acids that contribute to F- and P-type ASAT activity. We tested the importance of these amino acids by making the SI-ASAT3 triple mutant with Y161H, C162S and T289V: this engineered protein gained the ability to acylate S1:5 (iC5R4), as seen in P-type ASAT3 enzymes (Fig. 5b). This mutant was promiscuous for acceptor substrate, maintaining the ability to catalyze the furanose ring acylation of S2:10 (aiC5R3, iC5R4) (Supplementary Fig. 6b). Interestingly, each of the three SI-ASAT3 single-residue mutants gained a partial ability to acylate S1:5 (iC5R4), though less than the triple mutant (Supplementary Fig. 9a). The Sp-ASAT3 triple mutant—with the reciprocal changes H161Y, S162C, and V289T—lost the ability to acylate S1:5 (iC5R4) without gaining F-type ASAT3 activity. Further sequence comparison of the twelve ShASAT3 variants revealed that the residues at positions 354, 381, and 382 correlate with enzyme group differences (Fig. 5a). In SI-ASAT3, these residues are Leu354, His381, and Pro382, whereas in Sp-ASAT3 acylsucroses found in this species and in BIL6180 (Fig. 1b). Indeed, Sp-ASAT2 produced triacylsucroses (Fig. 3b) with the same positive-ion mode MS fragmentation ions (Supplementary Fig. 1a) and chromatographic retention times (Fig. 3b) as the BIL6180 acylsucroses. These results are consistent with the hypothesis that the in vitro products and in vivo metabolites have the same structures. Based on these results we deduced that Sp-ASAT2 adds the third acyl chain to the pyranose ring R3 position, as shown in those obtained by mutagenesis of Sl-ASAT2 and Sp-ASAT2.

We tested this ‘flipped pathway’ hypothesis by transgenic RNA interference (RNAi) silencing of Sp-ASAT3 in BIL6180. We expected greatly reduced acylsugar accumulation in these silenced lines if Sp-ASAT3 catalyzes the second step in the S. pennellii LA0716 pathway. This hypothesis is based on past work that showed that monooacylated sucrose S1:5 (iC5R4) does not accumulate to detectable levels in SI-ASAT2 silenced S. lycopersicum M82 (22). Indeed, most of the BIL6180 T0 transgenic lines had reduced total acylsugar levels (Supplementary Fig. 5), with diacylsucroses undetected in the silenced lines. These transgenic plant results reinforce the in vitro enzyme assays showing that Sp-ASAT3 catalyzes the second biosynthetic step of acylsucroses in BIL6180 and S. pennellii LA0716 (Fig. 3c).

Fig. 2 Transgenic expression of Sp-ASAT2 and Sp-ASAT3 causes trichomes of cultivated tomato S. lycopersicum M82 to produce P-type acylsucroses. a Schematic representation of the transgenic locus used to express Sp-ASAT2 and Sp-ASAT3, driven by the SI-ASAT2 and SI-ASAT3 promoters, respectively. A 1kb spacer separates these genes. b T0 first generation transgenic plants accumulate the F-type acylsucroses S3:15-F and S3:22-F, which are produced in M82, as well as the P-type acylsucroses S3:15-P and S3:22-P found in BIL6180. The Y-axes represent abundance of each peak relative to the most abundant ion intensity in each LC-MS run.
they are Val\(^{353}\) and Ser\(^{380}\), with one amino acid deletion adjacent to position 380 compared with Sl-ASAT3. To test whether these residues are associated with the ability of ASAT3 to acylate the furanose ring of S2:10 (aiC5R3, iC5R4), Sp-ASAT3 was mutagenized to generate the following changes: V353L and S380HP. Indeed, the mutagenized enzyme gained the F-type ability to acylate S2:10 (aiC5R3, iC5R4) in vitro (Fig. 5c). Meanwhile, we detected a small amount of F-type activity for Sp-ASAT3 mutants with the single substitutions V353L or S380HP (Supplementary Fig. 9b). While these amino acid substitutions correlate with—and predict—enzymatic activity in the twelve ASAT3 proteins documented in Fig. 5, there are 10 P-type ASAT3 enzymes that...

**Fig. 3** Sp-ASAT2 and Sp-ASAT3 produce P-type acylsucroses via a “flipped pathway”. a S. pennellii LA0716 wild tomato Sp-ASAT3 acylates S1:5 (iC5R4) and produces a diacylsucrose product (peak 2) with a retention time distinct from that made by cultivated tomato Sl-ASAT2 (peak 3). b Sp-ASAT2 converts the S2:10 (iC5R2, iC5R4) diacylsucrose produced by Sp-ASAT3 (peak 2) to triacylsucroses with the same retention times as those produced by BIL6180 (peaks 4 and 5). “iC5-CoA” and “nC12-CoA” refer to the two different acyl donor substrates used in the two enzyme reactions. c The “flipped” acylsucrose biosynthetic pathways in S. lycopersicum and S. pennellii LA0716. The numbers on top of each compound refer to the chromatographic peaks in a and b.
do not follow the Val^{253} and Ser^{380} pattern (gray circles in Supplementary Fig. 12).

Taken together, this combined comparative biochemical and mutagenesis analysis revealed multiple ASAT2 and ASAT3 residues that contribute to the divergence of ASAT2 and ASAT3 enzyme activities, which in turn result in F- or P-type acylsucrases. As expected for amino acids that influence substrate specificity, homology modeling with the trichothecene 3-O-acetyltransferase-acyl CoA complex structure (PDB code 3B2S), predicted that these residues surround the reaction center (Supplementary Fig. 10).

**Acylsucrose pathway divergence in the Solanum tomato clade.**

We previously characterized the distribution pattern of F- or P-type acylsucrases in the tomato group. The results described above led to the hypothesis that the distribution of F- and P-type acylsucrases across this group reflects the divergence of the acylsucrose pathway in the tomato clade driven by ASAT2 and ASAT3. To test this hypothesis, we sequenced the ASAT2 and ASAT3 genes of species across this group and determined whether the plant acylsucrose phenotypes can be inferred using ASAT2 and ASAT3 genotypes.

In the tomato subclade ranging from *S. lycopersicum* through *S. cornelomulleri*, all characterized ASAT2 proteins in this tomato subclade possessed only F-type enzymatic activities (Fig. 4a). In addition, sequenced ASAT3 orthologs from five species in this subclade all have the F-type activity-associated residues Leu^{553} and His^{381} Pro^{382} (see bottom of Supplementary Fig. 12), which predicts that all of these ASAT3 orthologs have F-type enzymatic activities. The in vitro enzyme activities were tested for three ASAT3 from *S. arcanum* (LA2172-ASAT3_1), *S. huaylasense*...
Fig. 5 Identification of residues that confer P-type activity to Sl-ASAT3 or F-type activity to Sp-ASAT3. a Amino acid sequences of ASAT3-P or -F alleles from different S. habrochaites accessions revealed residues that correlate with each enzyme type, and these are color coded. Blue amino acids are associated with F-type and green residues with P-type activity. b The results of in vitro assays performed with the two parental enzymes and the triple mutant created by site-directed mutagenesis of Sl-ASAT3 enzyme. The Sl-ASAT3 protein with three residue substitutions—Y161H, C162S, and T289V—produced the diacylsucrose product that has the same chromatographic retention time with the product of Sp-ASAT3 using S1:5 (iC5R5) as the substrate. c The double amino acid position mutant Sp-ASAT3 with V353L and S380HP—produced the triacylsucrose product that has the same chromatographic retention time with the product of SI-ASAT3 using S2:10 (aiC5R3, iC5R4) as the substrate. d Interpretation of the results of parts b and c, showing the key amino acids associated with F- and P-type ASAT3 enzyme activities.

Indeed, they have the F-type activity, acylating S2:10 (aiC5R3, iC5R4) (Supplementary Fig. 13, left panel). The combination of ASAT2-F and ASAT3-F alleles predicts that species in this group would only have the F-type acylsucrose biosynthetic pathway (Fig. 6b), matching the previously described F-type trichome acylsucroses of S. arcuatum, S. neorickii, S. huylasense, S. peruvianum, and S. cornelionmulleri27. Interestingly, LA1364-ASAT3 and LA1278-ASAT3_1 have residues His161 and Ser162, respectively; each was associated with the P-type activity from the S. habrochaites and S. pennellii analysis. Consistent with this observation, these two ASAT3 enzymes are promiscuous, showing a minor P-type activity to acylate the R3 position of S1:5 (iC5R5) (Supplementary Fig. 13, right panel).

In contrast to other tomato species, only P-type acylsuccesors were detected in S. pennellii accessions27, suggesting that the ‘LA0716 biosynthetic pathway’ alone is present in these accessions. Indeed, all ASAT3 isoforms isolated from ten S. pennellii accessions only have the residues His<sup>161</sup>Ser<sup>162</sup> and Val<sup>289</sup>, which are associated with P-type activity (Fig. 6a and Supplementary Fig. 12). This suggests that the uniform P-type ASAT3 activity would direct the pathway to produce P-type acylsuccesors (Fig. 6b).

In addition, ASAT2 cloned from five S. pennellii accessions originating from south Peru (Supplementary Fig. 14) possess the residue Gly<sup>304</sup>, which is associated with P-type activity (Supplementary Fig. 11). However, S. pennellii ASAT2 cloned from northern Peruvian accessions (Supplementary Fig. 11) have both F- and P-type activity-associated residues (Supplementary Fig. 14), which predicts that they have hybrid F- and P-type enzyme activities: this combination is reminiscent of S. habrochaites LA1777-ASAT2 (Fig. 4a). Although this ASAT2 F-type activity would enable the pathway to branch and make both F- and P-type acylsuccesors, the F-type diacylsucrose intermediates are not expected to be acylated further due to a lack of ASAT3 F-type activity (Fig. 6b). This presumably is why no F-type acylsuccesors were found in these northern Peruvian accessions27.

S. habrochaites is the only species in the tomato group with accessions demonstrated to accumulate a mixture of F- and P-type acylsuccesors27, providing information regarding the evolutionary relationship of the F- and P-type acylsuccesors pathways. Our previous study identified 13S. habrochaites accessions with both the F and P alleles of ASAT327. To extend this study, we further cloned sequences for ASAT2 enzymes from six of the S. habrochaites accessions that harbored both ASAT3-F
and ASAT3-P, including accessions LA1772, LA1978, LA2156, LA2650, LA2722, and LA2861. We identified at least two ASAT2 alleles from each of the selected S. habrochaites accessions; in each case, one ASAT2 harbors both F- and P-type residues and the other one only possesses the F-type residues (Supplementary Fig. 11). The combination of ASAT2 and ASAT3 with both F- and P-type residues suggests the co-existence of F- and P-type acylsucrose biosynthetic pathway in these plants (Fig. 6b), which is consistent with S. habrochaites species having the most varied acylsucrose phenotypes across the tomato group described to date.

Discussion

Metabolic innovation events can endow an organism with a greater ability to survive in a challenging environment. The mixture of specialized metabolites produced in a plant species or group results from assembly of enzymes into metabolic pathways or networks, and evolution of novel products occurs one step at a time. In this process, change in the activity of a single enzyme to produce a new product can lead to a variety of consequences. The simplest productive outcome is that the next enzyme in the pathway is promiscuous and uses the new product as a substrate, or networks, and evolution of novel products occurs one step at a time.

Supplementary Figs. 11 and 12 for detailed gene phylogenies. The plant trichome F- or P- acylsucrose phenotypes are assigned as F- or P- protein types based on residues identified by in vitro mutagenesis. Amino acids associated with F- or P-type activities are in blue and green, respectively. See Supplementary Figs. 11 and 12 for detailed gene phylogenies. The plant trichome F- or P- acylsucrose phenotypes are assigned as F- or P- protein types based on residues identified by in vitro mutagenesis. Amino acids associated with F- or P-type activities are in blue and green, respectively.
Evidence that these acylsucrose acyltransferases are sufficient for a BIL6180 × wild-type M82 heterozygote (Supplementary Fig. 11) and Sp-ASAT2 next converts this product into P-type triacylsucroses. The combination of in vivo and in vitro results demonstrate that the ‘flipped pathway’ is at the center of divergence of the F- and P-type acylsucrose pathways (Fig. 3c).

Comparative biochemical analysis revealed a relatively small number of ASAT2 and ASAT3 amino acid differences that mediate the change in pathway order. ASAT2 neofunctionalization from F- to P-type enzymatic activity is associated with three amino acid substitutions (Fig. 4). S. pennellii and S. habrochaites accessions contain the ASAT2 change C304G, which is sufficient to promote ASAT2 P-type enzyme activity. In contrast, the phylogenetically more restricted Gln135Tyr136 to His135Cys136 change (Supplementary Figs. 11 and 14) abolishes the ASAT2 F-type activity in S. pennellii accessions from southern Peru (including LA0716, the S. pennellii parent of the IL and BIL). The differences between extant P- and F-type ASAT3 isoforms are mediated by changes in multiple amino acids, a theme common in enzyme evolution. At least three residue substitutions—Y161H, C162S and T289V—are needed for the modern F-type Sl-ASAT3 to acquire P-type enzyme activity (Fig. 5b). Interestingly, comparison of all amino acids involved in flipping the pathway modeled the Sl-ASAT2 C304 and Sl-ASAT3 T289 to be at the same position in the predicted 3-D structures, near the acyl-CoA binding site as a candidate for future efforts to engineer BAHD acyltransferases for synthetic biology, and for understanding the evolution of form and function of related enzymes.

These results add to past work documenting how inter- and intra-specific variation in ASAT acyl donor promiscuity influence diversification of Solanum trichome acylsucrose products. For example, F-type ASAT2 enzymes from different tomato species specifically vary in preference for iC5-CoA as substrate, without affecting aiC5-CoA or nC12-CoA, thus influencing acyl chain types at the sucrose R1 position. In addition, all S. habrochaites F-type ASAT3 isoforms tested utilized iC5-CoA, but only a subset also accepted the long chain nC12-CoA donor. Taken together with the results described above, we conclude that ASAT2 and ASAT3 acyl donor and acceptor promiscuity played roles in the evolution of these BAHD acyltransferases and diversification of trichome metabolite accumulation.

Despite the involvement of two enzymes, the divergence of F- and P-type acylsucrose appears to have occurred relatively recently—over the past 2.5 million years—which was a period of rapid diversification within the Solanum tomato clade. We hypothesize that this innovation occurred following divergence leading to the S. habrochaites/S. pennellii group and away from the lineage containing the cultivated tomato S. lycopersicum and its closer relatives. Discovery of the enzymes of P-type acylsucrose biosynthesis was facilitated by LC/ToF-MS screening of BILs, each of which contains multiple regions of the S. pennellii LA0716 genome in an S. lycopersicum genetic background. The P-type acylsucrose producer (BIL1680) has three S. pennellii chromosomal regions, including those containing Sp-ASAT2 and Sp-ASAT3 (Fig. 1c).

Evidence that these acylsucrose acyltransferases are sufficient for the P-type acylsucrose phenotype came from S. lycopersicum M82 transgenic lines expressing both Sp-ASAT2 and Sp-ASAT3 in type I/IV trichomes. These accumulated both F- and P-type acylsucroses (Fig. 2), as predicted based on the phenotype of a BIL6180 × wild-type M82 heterozygote (Supplementary Fig. 2). Results of in vitro enzyme analysis confirmed that these enzymes produce P-type products (Fig. 3). Sp-ASAT3 uses monoacylsucrose S1:5 (iC5R4) as an acyl acceptor substrate—as previously reported for Sl-ASAT2—to produce a diacylsucrose product S2:10 (iC5R2, iC5R4) that is distinct from any in the previously identified F-type acylsucrose pathway (Fig. 3c). Sp-ASAT2 next converts this product into P-type triacylsucroses.

**Fig. 7** Summary of results suggesting that P-type ASAT2 and ASAT3 enzyme evolution occurred after the last common ancestor of the tomato clade of Solanum. Ancestral ASAT2 and ASAT3 genotypes and the origin of ASAT2 P-type activity occurred before the speciation of S. habrochaites and S. pennellii (the red-colored branch) and away from the lineage containing the cultivated tomato S. lycopersicum and its closer relatives.
sequences from wild tomato species other than *S. habrochaites* reinforce the hypothesis that ASAT3 duplication occurred after the last common ancestor of the extant tomato group and before the last common ancestor of the extant tomato group and before *S. habrochaites* and *S. pennelli* speciation.27 (red line in Fig. 7).

While we have insufficient resolution to reconstruct the order of events leading to evolution of the P-type acylsucrose pathway, our results led to a general model for P-type pathway evolution, where promiscuity features prominently. For example, the C304G mutation is associated with P-type activity: mutagenesis of Sl-ASAT2 causes acquisition of P-type activity, while retaining the original F-type activity (Fig. 4b and Supplementary Fig. 6). This is not a merely a laboratory anomaly as there are ASAT2 natural variants that have both F- and P-type activities. For example, LA1777-ASAT2 cloned from *S. habrochaites* acylates both S1:5 (iC5R4) and S2:10 (aiC5R3, iC5R4) (Fig. 4a). Similarly, substitution of Sl-ASAT3 with three amino acids associated with ASAT3-P activity (Y161H, C162S, T289Y), led to S1:5 (iC5R4) acylation on the pyranose ring, while retaining F-type activity (Supplementary Fig. 6). In fact, single substitution of each of these amino acids led to low detectable amounts of Sp-ASAT3 activity (Supplementary Fig. 9a, compare top three chromatograms with the bottom one). The promiscuity of such single or multiple mutants could have potentiated evolution of the ancestral enzyme, leading to production of small amounts of P-type diacylsucrases. In this scenario, accumulation of other mutations to the potentiated enzymes would eventually lead to improved catalytic activity, and a shift to the “purer” P-type activity seen in *S. pennelli* and *S. habrochaites*. ASAT2-P refinement by losing F-type activity presumably involved changes at positions 135 and 136 of Sl-ASAT2: Gln135 (His or Pro)135 and Tyr136 to Cys136, based on exact sequences shown in Supplementary Fig. 11.

The results of this study leave interesting questions unanswered. For example, when did acylsugar degrading acylhydrolases30 evolve and did these enzymes influence emergence or refinement of P-type enzymes? Did emergence of the ASAT3-P activity, which acylates pyranose at the same R2 position as does the F-type pathway ASAT4 acetyltransferase, contribute to the ASAT4 gene inactivation events in ancestors of northern Ecuadorian *S. habrochaites* accessions29 and *S. pennelli* LA071626? Do differences in sucrose acylation patterns influence accumulation of acylglucoses in southern versus northern *S. pennelli* accessions45? Thus, this work on variation in two acyltransferases opens up opportunities to study evolution of epistatic interactions between three or more enzymes in tomato and more broadly in the Solanaceae.

## Methods

### Plant growth and trichome acylsugar extraction

Tomato wild species seeds were obtained from the C.M. Rick Tomato Genetic Resource Center (http://tgrc.ucdavis.edu) and *S. pennelli* BIL6180 seeds were from Dr. Dani Zamir (Hebrew University of Jerusalem). The tomato seeds were germinated on damp filter paper for one week and then transferred to petri pots. The plants were maintained in a growth chamber for another two to three weeks, with the growth condition as follows: 28 °C for 16 h in the light (30 μmol m⁻² s⁻¹) and 20 °C for 8 h in the dark. Trichome metabolites were extracted by submerging the youngest fully developed leaf for 2 min with gentle agitation in 1 mL of extraction solution, which contained acetonitrile/isopropanol/water (3:3:2) with 0.1% formic acid and 10 μM propyl-4-hydroxybenzoate as internal standard. Dry weight measurement was measured after the extracted tissue was dried in an oven at 60 °C. We consider each individual plant as a biological replicate. At least three plants from the same line were used for the trichome acylsugar extraction and LC/MS analysis.

### Tomato transformation

To express Sp-ASAT2 and Sp-ASAT3 in *S. lycopersicum* M82 trichomes, four fragments—a 1.6 kb region upstream of Sl-ASAT2; the Sp-ASAT2 gene with a 1 kb region downstream; 1.7 kb region upstream of Sl-ASAT3, and the Sp-ASAT3 gene—were amplified and cloned into the vector pENTR/D-TOPO. The resultant plasmid was used to transform BIL6180 plants. Tomato transformation was performed using *A. tumefaciens* strain AGL049.

Trichome metabolite extractions were performed with the independent T0 primary transformants and T1 plants resulted from self-crossing of T0 lines. The primers used for genotyping of the T1 lines are described in Supplementary Table 1.

### Analysis of Sp-ASAT3 and Sp-ASAT2 gene expressions

To analyze the mRNAs from Sp-ASAT2 or Sp-ASAT3 in T1 RNAi suppression lines targeting Sp-ASAT3 in BIL6180, the leaves with the same developmental stages as those used for acylsugar extractions were collected. RNA extraction was performed using the Plant RNeasy kit (QIAGEN), and treated with DNase I. The first-strand cDNA synthesis by SuperScript II (Invitrogen) used total RNA as templates. Five plants from each independent T1 lines were used as biological replicates. The Quantitative real-time PCR was performed to analyze the gene expression using the elongation factor gene (*Soyl6g005060*) as an internal control. Primers are listed in Supplementary Table 1. QuantStudio 7 Real-TimePCR System with Fast SYBR Green Master was used to evaluate the relative transcripts levels.

### LC/MS analysis of acylsugars

Both trichome acylsugar extracts and enzyme assay samples were analyzed using a Waters Acquity UPLC system coupled to a Waters Xevo G2-QToF LC-MS. For all sample injections, ten microliters were used for reverse-phase separation in a fused core Ascentis Express C18 column (2.1 mm × 10 cm, 2.7 μm particle size) with the following temperature profile: 40 °C. The LC starting conditions were 95% solvent A (0.15% formic acid in water) and 5% solvent B (acetonitrile) with a flow rate of 0.3 mL/min. The 7 min and 14 min elution gradients – used for separating plant trichome acylsugars and enzyme assay samples—were as follows: for the 7 min elution gradient: ramp to 40% B at 1 min, then to 100% B at 5 min, hold at 100% B to 6 min, return to 95% A at 6.01 min and hold until 7 min. For the 14 min elution gradient: ramp to 35% B at 1 min, then to 85% B at 12 min, ramp to 100% B at 12.01 min, hold until 13 min, return to 5% B at 13.01 min and hold until 14 min. For the MS settings: 2.14 kV capillary voltage, 90 °C source temperature, 550 °C desolvation temperature, 660 liters h⁻¹ desolvation nitrogen gas flow rate, 10 V cone voltage, and 100° C mass taper. A Shimadzu LC-20AD HPLC system connected to a Waters LCT Premier ToF-MS system was used for screening trichrome acylsugars of the BILs. Ten microliters trichome metabolite samples were used for reverse-phase separation in a fused core Ascentis Express C18 column (2.1 mm × 10 cm, 2.7 μm particle size) with the following temperature profile: 40 °C. The LC starting conditions were 95% solvent A (0.15% formic acid in water) and 5% solvent B (acetonitrile) with a flow rate of 0.4 mL/min. The 7 min elution gradient used to separate the trichrome metabolites: ramp to 40% B at 1 min, then to 100% B at 5 min, hold at 100% B to 6 min, return to 95% A at 6.01 min and hold until 7 min. The 14 min elution gradient used to separate the BIL6180 acylsugars in Supplementary Fig. 1 employed the following linear steps: starting with 95% solvent A and 5% solvent B, ramp from 5% B to 10% B at 3 min, then to 34% B at 5 min, ramp to 35% B at 20 min, then to 38% B at 21 min, ramp to 59% B at 36 min, then to 100% B at 37 min and hold until 40 min. For the MS settings: 2.5 kV capillary voltage, 100 °C source temperature, 350 °C desolvation temperature, 350 liters h⁻¹ desolvation nitrogen gas flow rate, 10 V cone voltage, and 30–1500 m/z mass range. Three separate acquisition functions were set up to generate spectra at different collision energies (5, 25, and 60 eV).

### Purification of acylsugars for NMR analysis

Thirty BIL6180 plants were used to extract trichrome acylsugars for NMR analysis. All plant leaves including the petiole tissues were harvested into a 5-liter beaker that contained 2 L of 100% methanol. After gentle agitation for 2 min, the solvents were transferred into two 1-liter glass bottles and the extraction solvent dried using a rotary evaporator under vacuum and then dissolved in 3 mL of 80% acetonitrile with sonication for 10 min. The concentrated solutions were centrifuged at 5000×g for 10 min at 25 °C to remove any insoluble debris and 200 μL aliquots were transferred to HPLC vials with glass inserts, and acylsugars were purified as described below. The 2D-1H (iC5, iC5) was used for NMR analysis was made through sequential enzymatic reactions. In brief, the first enzymatic step was performed by incubating

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isopropanol/formic acid (1:1:0.001), and dried using a vacuum concentrator under vacuum. NMR spectra were recorded in solutions in Shigemi solvent—used to introduce mutations to ASAT3 and 100 μL of M acylsucrose acceptor at 25 °C, the supernatant was aliquoted into ten glass inserts in HPLC vials. The acylsugars were purified using a Waters 2595 HPLC system coupled with an LKB BROMMA 221 Supercarr fraction collector, using a 200 μL injection volume. A Thermo Scientific Acclaim 120 C18 HPLC column (4.6 x 150 mm, 5 μm particle size) was used for all compound separations with the column temperature set at 40 °C. Solvent A (0.15% formic acid in water) and solvent B (acetonitrile) were used for all compound separations with the column temperature set at 40 °C. Solvent A (0.1% formic acid in water) and solvent B (acetonitrile) was used for LC/MS analysis. All enzyme reactions were performed at least three times. The representative LC/MS chromatograms from one of the assays are shown in the figures. The acyl-CoA substrates—iC5-CoA and nC12-CoA—were purchased from Sigma-Aldrich. Chemical synthesis of aC5-CoA was as described.

ASAT orthologs amplification and phylogenetic analysis. ASAT orthologs cloned from S. typhimurium through S. corneliaeisi that were used to build the phylogenetic tree in Supplementary Fig. 11 are from a previous study[22]. We amplified additional ASAT ortholog sequences from additional accessions of S. habrochactis and S. pennelli genomic DNA using the primers listed in Supplementary Table 1. In the previous report, multiple ASAT3-F and ASAT3-P type alleles were amplified from different accessions of S. habrochactis using the F- and P-type primer sets[23]. In the current report, the primer sets were used to amplify F- and P-type ASAT3 alleles in multiple wild tomato species. cdNA was used as templates to amplify ASAT3 from S. pinnatifidus, S. galapagense, S. neorickii, S. arcanum, S. chilense, S. peruvianum, and S. corneliaeisi for S. pennelli accessions, genomic DNA was used to amplify ASAT3. The amplified products were cloned into pET28b using the restriction cloning method and the ASAT2 or ASAT3 sequences obtained by Sanger sequencing using plasmids extracted from individual clones. The sequences are GenBank with the accession numbers listed below.

For each ASAT ortholog, the full coding sequences were used to build the phylogenetic tree. The regions introduced by F- or P- forward primers—24 bp in the 5’ end of the ASAT3 sequences—were eliminated from the phylogenetic analysis to avoid hybrid coding sequences. The nucleotide sequences were aligned using the default MUSCLE algorithm with the MEGA7 software[24]. For both ASAT2 and ASAT3, the Tamura 3-parameter Maximum Likelihood model was chosen for phylogenetic tree construction from 24 different nucleotide substitution models based on the lowest Bayesian Information Criterion. The bootstrap values were obtained after 1000 replicates. The phylogenetic trees in Fig. 4a, Supplementary Figs. 11 and 12 were all made using the above method.

Homology structural modeling of SI-ASAT2 and SI-ASAT3. The Phyre2 web portal[25] was used to predict the tertiary structures of SI-ASAT2 and SI-ASAT3. The Open Source PyMOL software (The PyMOL Molecular Graphics System, LLC) was applied to overlay the SI-ASAT2 and SI-ASAT3 modeled structures with the trichothecene-3-O-acetylenesulfate structure (PDB code 3BO8) [26].

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. The NMR raw data for the four purified compounds S2:10, S3:15, S52:2a, and s3:22[b] are deposited in the public data repository Open Science Framework under the link https://osf.io/ to support the findings of this study are available as GenBank/EMBL data libraries under these accession numbers. ASAT2: SI-ASAT2 (KT359567), Sp-ASAT2 (KT359557), LA1578-ASAT2 (KT359558), LA1278-ASAT2 (KT359559), LA2133-ASAT2 (KT359560), LA1777-ASAT2 (KT359561), LA1642-ASAT2 (KT359562), LA1007-ASAT2 (KT359563), LA2098-ASAT2 (KT359557), LA1541-ASAT2 (KT359556), SI-ASAT3 (KM516150), LA1718-ShASAT2_1 (KY962560), LA1737-ShASAT2_2 (KY962561), LA1737-ShASAT2_3 (KY962562), LA1772-ShASAT2_1 (KY962563), LA1772-ShASAT2_2 (KY962564), LA1978-ShASAT2_1 (KY962565), LA2156-ShASAT2_1 (KY962567), LA2156-ShASAT2_2 (KY962571), LA2156-ShASAT2_3 (KY962572), LA2196-ShASAT2_1 (KY962573), LA2196-ShASAT2_2 (KY962574), LA2630-ShASAT2_1 (KY962575), LA2630-ShASAT2_2 (KY962576), LA2722-ShASAT2_1 (KY962577), LA2722-ShASAT2_2 (KY962578), LA2838-ShASAT2_1 (KY962579), LA2838-ShASAT2_2 (KY962580), LA2975-ShASAT2_1 (KY962581), LA2975-ShASAT2_2 (KY962582), LA2975-ShASAT2_3 (KY962583), LA1282-SpASAT2 (KY962585), LA1356-
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Author contributions
P.F. and R.L.L. designed research; P.F. and A.M.M., performed experiments; X.L. performed NMR analyses; P.F., A.M.M., X.L., A.D.J., and R.L.L. analyzed data; P.F. and R.L.L. wrote the manuscript. All authors read and edited the manuscript.

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