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Tip of the trichome: evolution of acylsugar metabolic diversity in Solanaceae

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Abstract

Acylsugars are insecticidal plant specialized metabolites produced in the Solanaceae (nightshade family). Despite having simple constituents, these compounds are unusually structurally diverse. Their structural variations in phylogenetically closely related species enable comparative biochemical approaches to understand acylsugar biosynthesis and pathway diversification. Thus far, varied enzyme classes contributing to their synthesis were characterized in cultivated and wild tomatoes, including from core metabolism – isopropylmalate synthase (Leu) and invertase (carbon) – and a group of evolutionarily related BAHD acyltransferases known as acylsucrose acyltransferases. Gene duplication and neofunctionalization of these enzymes drove acylsugar diversification both within and beyond tomato. The broad set of evolutionary mechanisms underlying acylsugar diversity in Solanaceae make this metabolic network an exemplar for detailed understanding of the evolution of metabolic form and function.

Introduction

Plants collectively produce hundreds of thousands of low molecular mass molecules known as specialized or secondary metabolites. In contrast to the smaller collection of broadly distributed core metabolites such as proteogenic amino acids and enzyme cofactors, specialized metabolites are restricted to specific taxonomic groups [1] and cell or tissue types [2]. The wide variety of specialized metabolite structures and functions presumably result from millions of years of mediating both positive and defensive plant-microbe and plant-animal interactions [3,4]. While their lineage-specificity limits the number of biosynthetic pathways that can be studied in any specific species [5], recent advances in next generation sequencing and mass spectrometry technologies reduce our reliance on model species and pave the way for unravelling and exploiting metabolic diversity in all plants.

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Acylsugars are structurally diverse protective metabolites sporadically detected in multiple plant families. For example, they are found in trichomes [6] of plants in the Solanaceae, Martyniaceae, Rosaceae, Geraniaceae, and Caryophyllaceae, and in roots of Brassicaceae plants [7]. Acylsugars from the Solanaceae family – including plants of the *Solanum*, *Physalis*, *Nicotiana*, *Petunia*, and *Salpiglossis* genera [8] – are extensively characterized. They are glycolipids consisting of a sugar core decorated by straight- and branched-chain acyl esters of lengths from C2 to C20 (Fig. 1), produced in glandular trichome tip cells. In cultivated tomatoes, they are produced in long type I/IV trichomes [9,10], but not in the type VI glands [10,11]. Acylsugars have documented insecticidal characteristics. For example, they mediate tritrophic interactions in plant defense by tagging the herbivore for predation through volatile acylsugar breakdown products [12] and also directly protect plants from fungal pathogen attacks [13]. In tomato, breeding efforts seek to manipulate acylsugar content and composition for better insect resistance and have generated improved breeding lines with increased trichome density [14], higher acylsugar amounts [14], varying proportions of sucrose or glucose based acylsugars [15,16], and different acyl chain profiles [15,17].

Acylsugar biosynthesis has characteristics that make it a compelling target for understanding the evolution of genotypic and phenotypic variation. First, trichome tip cell specific synthesis greatly facilitates gene discovery and has implications for pathway evolution. Second, structural variation of acylsugars within and across species and genera facilitate comparative biochemical approaches. Third, they are synthesized from simple components by soluble enzymes, facilitating *in vitro* analysis, including pathway reconstruction in a test tube. These, combined with functional genomics tools of transformation, CRISPR-Cas9 [18,19] and viral induced gene silencing (VIGS) [20] available in the Solanaceae, permit direct and detailed analysis of how genotypic variation affects phenotypic diversity.

Acylsugar structural diversity in the Solanaceae family and their biosynthesis in tomatoes

Acylsugars are remarkably structurally diverse, especially considering that they are built from simple components (Fig. 1) by a small number of core biosynthetic network enzymes (Fig. 2a). The disaccharide sucrose is the predominant sugar core and was identified in genera across the family, including *Solanum* [21], *Petunia* [22], and in *Salpiglossis sinuata* [8]. Acylsugars built on hexose cores were characterized in a small number of species dispersed across the family. For example, acylated inositols in *Solanum lanceolatum* [23] and hexoses of as yet uncharacterized structure *in Solanum nigrum* [8]. Glucose-based acylesters were demonstrated to be sporadically distributed in species of *Solanum* [24,25], *Nicotiana* [26], and *Petunia* [27]. Using sucrose as an example and taking all possible esterifications into consideration, the theoretical number of unique acylsugar structures approaches 815 million (13^8) – calculated based upon numbers of sucrose hydroxyl groups and different acyl moieties so far characterized from acylsugar producing species. This implies that even a single plant has the potential to generate vast acylsugar diversity. For example, *Petunia axillaris* leaf metabolites reveal numerous chromatographic peaks that are predicted as acylsugars based on LC/MS analyses [22], far beyond the smaller number of

abundant acylsugars that were structurally resolved through NMR characterization. This point is reinforced by work on *S. sinuata*, where NMR structures are established for 16 of >400 acylsucroses annotated by LC/MS of leaf surface extracts (Steven Hurney, PhD thesis, Michigan State University, 2018). One key to elucidating the structural complexity of acylsugars produced in a plant species is to study the biochemical and genetic basis for acylsugar biosynthesis.

Our understanding of acylsugar biosynthesis is most complete in the tomato clade of *Solanum*. Cultivated tomato (*Solanum lycopersicum*) makes tri- and tetra-acylated sucrose molecules, with acylations on both the sucrose pyranose and furanose rings (Fig. 2a, blue shaded, F-type acylsucroses). In contrast, trichomes of the wild tomato *Solanum pennellii* LA0716 produce both acylglucoses and pyranose acylated tri-acylsucroses (Fig. 2a, green shaded, P-type acylsucroses). Acylsugar biosynthetic enzyme gene discovery benefited enormously from two germplasm collections derived from crosses between these two species: isogenic introgression lines (ILs) [28] and backcross introgression lines (BILs) [29]. While these species are sexually compatible, their acylsugar metabolism diverge to the point that *S. pennellii* alleles act as loss of function mutations in cultivated tomato, which facilitated gene discovery through ‘forward’ genetic screening [30].

This strategy was used to identify three out of four of the core pathway acylsucrose acyltransferases (ASATs) [31–33], which belong to the rapidly evolving BAHD acyltransferase enzyme group [34,35]. In *S. lycopersicum*, four ASATs sequentially add acyl chains to the sucrose backbone to produce a small number of tri- and tetra-acylsucroses (Fig 2a) [31]. In addition to ASATs, allelic variation in the ILs led to discovery of a neofunctionalized enzyme of cultivated tomato Leu biosynthesis, which is responsible for production of isovaleryl coenzyme A (isoC5-CoA) (Fig. 2b) [36]. Although it catalyzes the same reaction leading to one carbon elongation as the canonical microbial and plant amino acid biosynthetic enzymes, isopropylmalate synthase like 3 (IPMS3) has unique regulation of enzymatic activity and gene expression, setting it apart. First, it lacks the inhibitory Leu-binding allosteric C-terminal domain, and thus is insensitive to Leu end-product feedback inhibition. Second, unlike the broad expression expected of amino acid biosynthetic enzymes, IPMS3 expression is limited to trichome secretory cells. In *S. pennellii* LA0716, the IPMS3 protein is further truncated at the C-terminus, losing detectable *in vitro* enzyme activity (Fig. 2b). This results in accumulation of acylsugars containing isobutyl (isoC4) chains in this and other *S. pennellii* accessions homozygous for this doubly deleted allele [36]. As more acylsugar biosynthetic genes were uncovered and pieced together to complete the biosynthetic pathways (Fig. 2a)[31–33,36,37], it became possible to study how gene duplication and enzyme variation contributed contribute to the evolution of a metabolic network and created acylsugar diversity.

Tomato acylsugar diversity via enzymatic promiscuity, flipping enzyme order and invertase ‘hijacking’

Acylsugars in the tomato clade show striking inter- and intraspecific structural diversity, which is associated with gene duplication and functional diversification of acylsugar

biosynthetic genes. Wild tomato species differ in the types and amounts of acylsugars in comparison to cultivated tomato, and we found examples where product structural differences are associated with evolution of ASATs, leading to enzymes that vary in substrate specificity and degree of promiscuity [31,32,37].

Key amino acid differences that cause specific phenotypic variations were identified using a comparative biochemical approach [31,37,38]. For example, green-fruited tomato relatives have an ASAT2 – the second enzyme in the *S. lycopersicum* pathway – which promiscuously uses both anteisoC5-CoA (aiC5-CoA; 2-methylbutyryl-CoA) and isoC5-CoA donors, leading to products with either chain at the sucrose R3 position. In contrast, SlASAT2 of cultivated tomato and its closest relatives fail to use isoC5-CoA as donor due to a single Val408 to Phe amino acid substitution, leading to a less diverse set of acylsucrose structures accumulating *in vivo* (Fig. 2c, top panel) [21,31]. Another example of a single amino acid change modulating promiscuity and metabolic diversity was found by comparing ASAT3 of *S. lycopersicum* to those of *Solanum habrochaites* accessions. In this case, a single Tyr41 to Cys amino acid substitution in the *S. lycopersicum* protein changes an enzyme that only uses shorter chain C5 CoA esters to a more promiscuous one, which adds either isoC5 or lauroyl (nC12) group to the furanose ring at R3' (Fig. 2c, middle panel) [31,32]. These *in vitro* activity changes correlate with both interspecific *in vivo* differences across the tomato group and intraspecific acylsugar variations in different *S. habrochaites* species [32]. Together with more global differences in C4 vs. C5 acyl chain abundance caused by allelic variation in *S. pennellii* IPMS3 (Fig. 2b) [36], ASAT acyl CoA substrate specificity and promiscuity influence tomato acylsugar acyl chain composition.

Differences in ASAT acyl acceptor substrate specificity have an even more profound influence on *in vivo* acylsugar product structural diversity in the *S. pennellii/S. habrochaites* tomato subclade [37]. Trichomes in this group produce 'P-type' triacylsucroses acylated only on the pyranose ring (Fig. 2a). This is the result of a reconfiguration of the biosynthetic network, where the ASAT3-P enzyme catalyzes the second step – utilizing monoacylated sucrose acceptor substrate – and ASAT2-P catalyzes the third step converting diacylsucroses to the final triacylated products. Detailed analysis of the amino acid differences responsible for this 'flipped pathway' revealed a small number of amino acids mediating the acceptor substrate specificity changes (Fig. 2a) [37]. In fact, a single Cys304 to Gly *in vitro* mutation in *S. lycopersicum* ASAT2, to an amino acid present in all tested *S. pennellii* ASAT2 enzymes, led to a promiscuous enzyme that accepts both mono- and di-acylsucrose substrates (Fig. 2c, bottom panel). Complete conversion to a P-type enzyme, without detectable F-type activity, requires an additional two amino acid changes. This strongly suggests that P-type ASAT2 activity evolved from an F-type enzyme through a promiscuous enzyme intermediate. In contrast, evolution of the seemingly ancestral ASAT3-F furanose ring acylating activity to *S. pennellii* ASAT3-P, which only acylates the pyranose ring, involved gene duplication, neofunctionalization, and gene loss (Fig. 2d) [32,37]. *S. habrochaites* appears to be an intermediate case, retaining both ASAT3-F and ASAT3-P paralogs, leading to accumulation of sucrose products of both the P- and F-types (Figs. 2a and 2d).

The *S. pennellii* LA0716 flipped pathway not only produces P-type triacylsucrose products, but also is associated with evolution of the unusual *S. pennellii* acylsucrose β-fructofuranosidase (SpASFF1), a trichome-specific invertase-like enzyme [24] (Fig. 2a). This enzyme specifically hydrolyzes P-type triacylsucroses, to produce the triacylglucoses found in abundance on the surface of *S. pennellii* LA0716, yet is inactive against furanose ring acylated tri- or tetraacylsucroses made by the cultivated tomato pathway. This evolutionary innovation is remarkable for several reasons. First, it was potentiated by the neofunctionalization of ASAT2 and ASAT3 that produces P-type triacylsucroses. Second, it involves modification of β-fructofuranosidase (invertase), an enzymatic activity long associated with core carbohydrate metabolism [39]. Finally, as with IPMS3 and ASAT enzymes, recruitment of this activity to specialized metabolism involved evolution of cell type specific expression.

Acylsugar diversity in a biogeographic context

Striking intraspecific acylsugar variation is seen among different accessions of *S. habrochaites* or *S. pennellii* species collected from habitats isolated by deep valleys or mountain ridges across the Andes (Kim et al., 2012; Schilmiller et al., 2015). With increasing understanding of acylsugar pathway diversification, these acylsugar biogeographical associations can be interpreted at the molecular level. For example, the mixed distribution of two IPMS3 alleles – one functional and the other non-functional due to two different C-terminal deletions (Fig. 2b) [36] – is associated with the varied ratio of acylsugar isoC4 and isoC5 acyl chains in different *S. pennellii* accessions from the northern to southern part of the range. Similarly, loss of ASAT4 R2 acetylation activity – through multiple presumably independent gene inactivation events – correlates with the lack of acylsugar acetylation in *S. habrochaites* populations from the northern part of the species range [40]. Different *S. habrochaites* ASAT3 alleles – varying by presence or absence of the ability to perform long chain furanose ring acylation ($n \geq 10$) – provide another instance of ASAT variation shaping plant acylsugar acylation patterns across native geographies [32,37]. The diversification of acylsugars in tomato accessions dispersed across Peru and Ecuador is reminiscent of a recent study documenting that species in the genus *Solanum* experienced explosive diversification in the Neotropic region, shaped by geographical long-distance dispersal and past climate changes [41]. The acylsugar biosynthetic network and the pathway diversification could serve as a microcosm for mechanistic understanding of the rise of broad phenotypic diversity in the Neotropics.

Evolution of the acylsugar biosynthetic network beyond tomato

The analysis of metabolic evolution in the *Solanum* tomato clade described above reveals that phenotypic diversification over the past seven million years involved multiple mechanisms within and across species. These include *ASAT3* gene duplication, varied examples of neofunctionalization of BAHD acyltransferase activities, ‘recruitment’ of an invertase and an isopropylmalate synthase from core metabolism, and ASAT4 gene function loss in accessions of *S. habrochaites*.

The steadily increasing genomics and metabolomics data across the Solanaceae provide opportunities to look deeper in time and develop hypotheses regarding the origin of this pathway and mechanisms of diversification across 50–100 million years [8]. The data suggest that the ASAT gene clade originated from an alkaloid biosynthetic BAHD ancestor, with the genes duplicating prior to Solanaceae and Convolvulaceae divergence (Fig. 3; phylogenetic tree on left) [8]. Comparison of tomato to *Salpiglosis sinuata* and *Petunia* – species representing earlier emerging lineages – revealed a major difference in the pathway: altered order of acylation catalyzed by non-orthologous ASATs. As schematized in Fig 3, ancestral ASAT1 (aASAT1) enzyme – catalyzing the first step of the ancestral pathway in *S. sinuata* and *Petunia* – acylates position R2 of the sucrose pyranose ring, with the next two acylations at R4 and R3 by aASAT2 and aASAT3, consecutively. This is in contrast to the 'modern' cultivated tomato pathway starting with sucrose R4 acylation by mASAT1, followed by mASAT2 R3 acylation, and then furanose ring R3' acylation by mASAT3 (Fig. 3) [8,37,42]. The rise of the modern pathway seen in tomato is associated with changes in aASAT2 and aASAT3 ortholog activities to perform the first and second step in the modern pathway (mASAT1 and mASAT2, respectively); this served to 'shift' the order of orthologous enzyme action, associated with loss of ancestral aASAT1 following the *Solanum-Capsicum* lineage split (Fig. 3) [8].

The different acylation orders to produce the P-type acylsucroses in *S. pennellii* and *P. axillaris* (Fig. 4, left panel, green ribbon) [37,42] and F-type acylsucroses in *S. lycopersicum and S. sinuata* (Fig. 4, left panel, blue ribbon) [8,31] are reminiscent of convergent evolution in caffeine biosynthesis (Fig. 4, right panel) [43]. Synthesis of caffeine requires three sequential methylations of a xanthine backbone, but the order of methylation varies across the four plant orders studied (Fig. 4, right panel). Although the convergent evolution of caffeine is achieved through different biosynthetic routes, the methyltransferases involved share the same evolutionary origin [44,45]. Similarly, ASATs appear to share a common ancestor involved in alkaloid biosynthesis [8]. For both caffeine and acylsugar biosynthesis, the promiscuity of ancestral enzymes primed pathway divergence and eventually led to similar but distinct derived pathways [37,45]. The factors that drove the multiple origins of P-type acylsucroses seem unclear, whereas they may have set the stage for phytochemical diversity – probably through ASFF hydrolysis activity [24] – that leads to mixed acylsucroses and acylglucoses accumulation in several Solanaceae species [25–27].

Conclusion – future directions

Acylsugars are a group of related but structurally diverse plant specialized metabolites, which were reported in six plant families so far [6,7]. The structures and biosynthesis of acylsugars are best characterized in trichomes of the Solanaceae, where they are synthesized from commonly available sucrose and acyl-CoA primary metabolites via short biosynthetic pathways. As biosynthesis of these compounds is analyzed in other families, it will be interesting to explore the details of their parallel evolution and to learn whether interactions between plants, microorganisms, and herbivores drove their evolution and structural diversification.

While our understanding of acylsugar biosynthesis increases as more enzymes in the biosynthetic network are characterized, rational modification of these biotic stress tolerance molecules will also improve with knowledge in areas not discussed in this review. Acylsugar acylhydrolases (ASHs), which remove acyl chains from specific acylsugar positions *in vitro* [46], may be involved in acylsugar degradation or editing *in vivo*. However, these enzymes appear to create a futile cycle by degrading ASAT acylsugar products. The enigmatic ASH activities suggest the possible existence of unidentified mechanisms for generating acylsugar diversity. For example, acylsugar structures could be remodeled by ASH activities, creating alternative ASAT acyl acceptor substrates. Given ASAT enzyme promiscuity, manipulation of trichome tip cell acyl CoA pools should be an effective way to modify chain types. Proof of concept for this approach was seen when the inactive SpIPMS enzyme replaced the catalytically active version in cultivated tomato IL8–1, leading to isoC4 acylsucrose accumulation instead of isoC5. Understanding the mechanisms by which longer straight- and branched-chain acyl CoA substrates are synthesized for acylsugar biosynthesis in the tip cells should lead to strategies for manipulating acylsugar acyl chain length. Such engineering could improve insect tolerance, as seen in the tomato breeding lines with different fatty acid ester profiles [47,48] or sugar cores [49], which show synergistic effects against insects. The ‘toolbox’ for manipulating diverse acylsugar structures will promote new strategies to improve plant insect defense through breeding or genetic engineering of desired plant acylsugar phenotypes.

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acylglucose content on chromosomes 3, 4, and 11, and identified them as necessary for high acylglucose accumulation. The authors also tested the efficacy of their multiple *S. pennellii*-introgressed breeding line against beet armyworm and western flower thrips, revealing decreased oviposition of the breeding line compared to leaf disks containing no acylsugars.

Highlights

- Solanaceae glandular trichomes produce structurally diverse insecticidal acylsugars
- Diversity is achieved by enzyme promiscuity and core metabolic enzyme ‘recruitment’
- *In vitro* pathway reconstruction facilitates genotype to phenotype analysis
- These pathways are amenable to evolutionary reconstruction over 10’s of millions of years

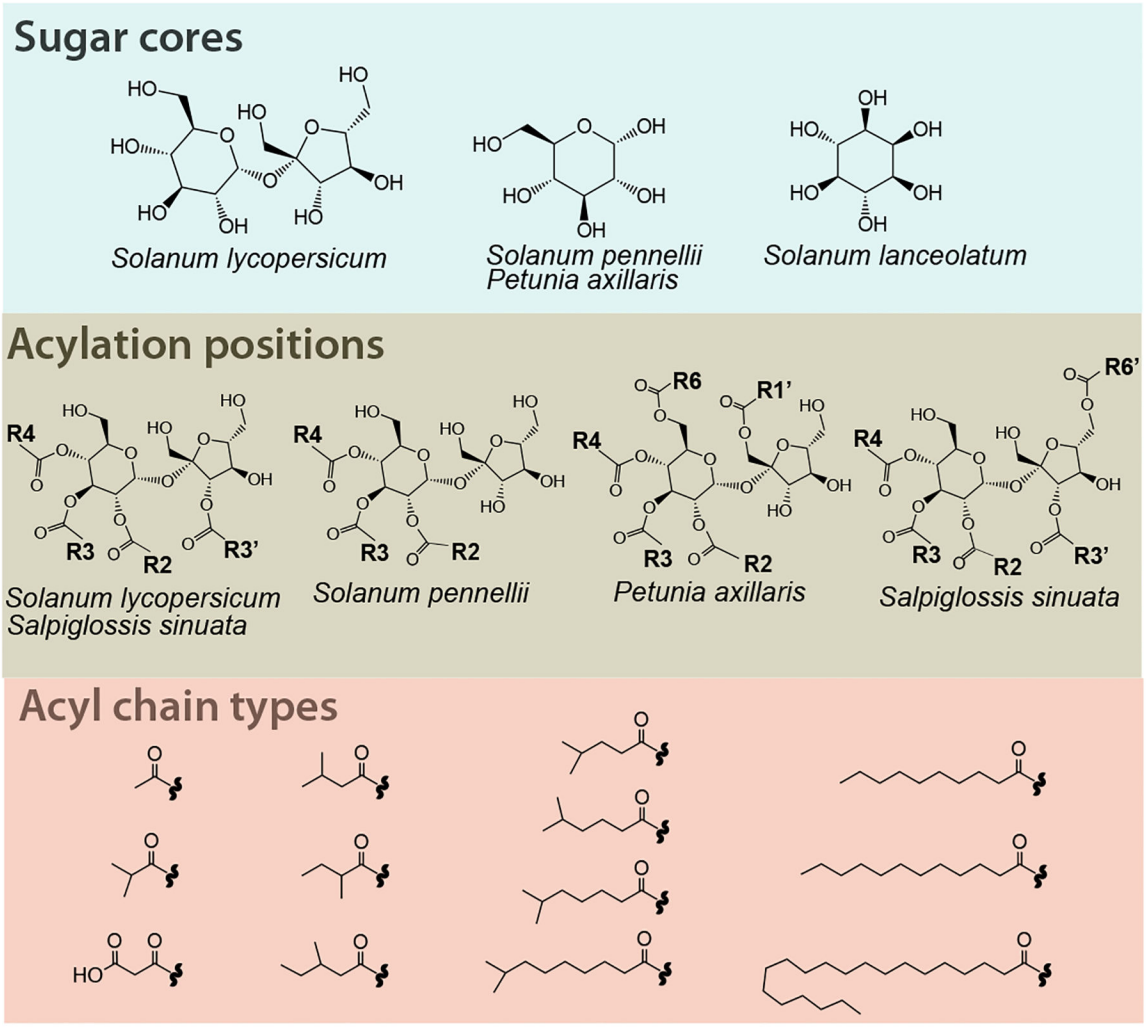

Figure 1.

Acylsugar structural diversity is built on simple design principles. More than one hundred known acylsugar structures are based on three varied components: sugar cores (top panel: sucrose, glucose and inositol are shown from left to right), acylation positions (middle panel shown for sucrose esters), and acyl chain types (bottom panel). The species are used as representative examples.

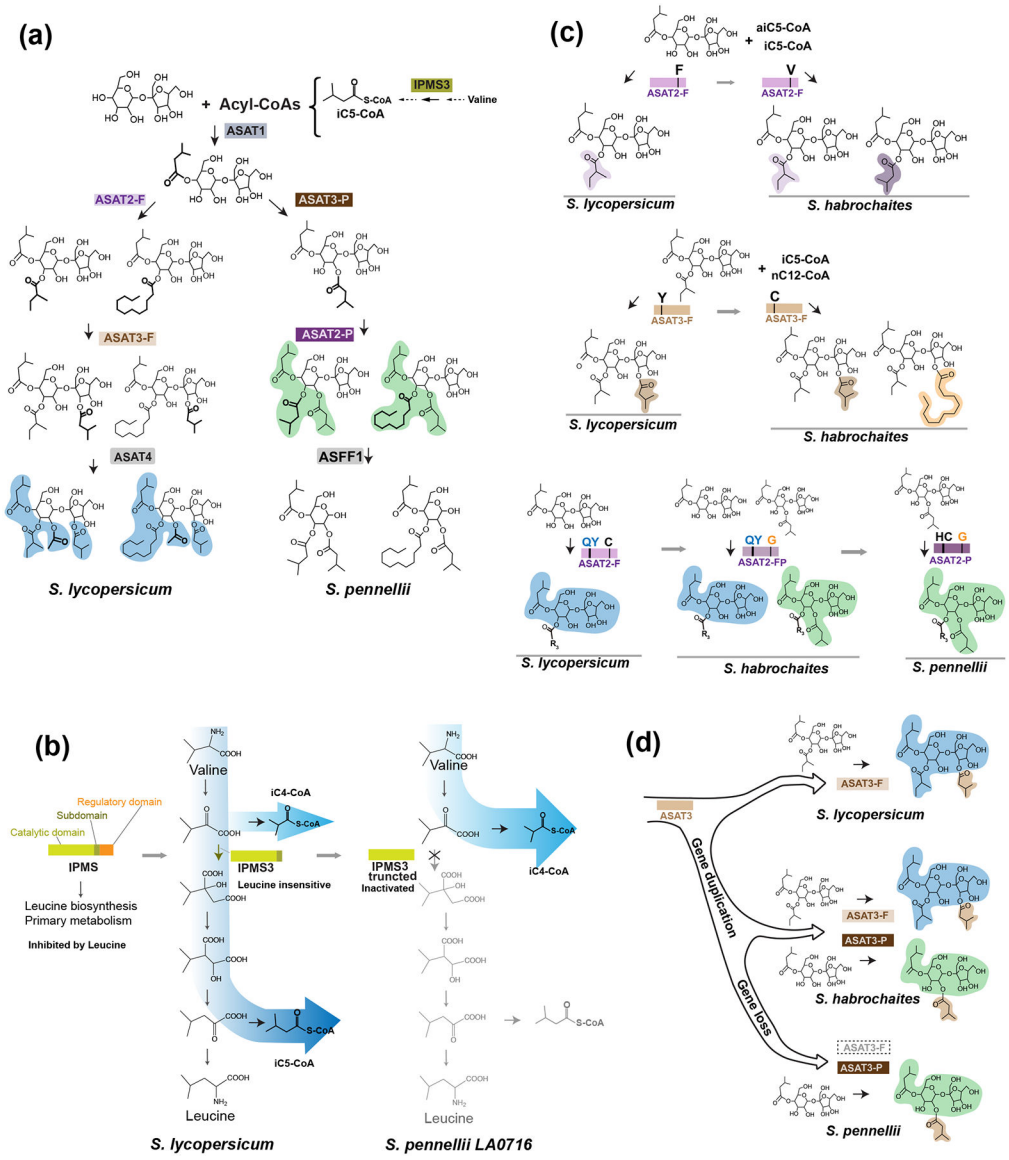

Figure 2.

Acylsugar biosynthetic pathway diversity in tomato species. **(a)** The acylsugar biosynthetic pathways characterized from the cultivated tomato *S. lycopersicum* and wild relative *S. pennellii*. IPMS3, isopropylmalate synthase-like 3; ASAT, acylsucrose acyltransferase; ASFF1, acylsucrose fructofuranosidase 1. **(b)** Left, recruitment of the feedback insensitive and trichome gland cell expressed SlIPMS3 in cultivated tomato *S. lycopersicum* for biosynthesis of acylsugar isoC5-CoA acyl chain precursor. Right, the *IPMS3* allele is further truncated at the C-terminus and inactivated in *S. pennellii* LA0716, diverting the acyl CoA precursor pathway to produce more isoC4-CoA derived acylsugars. **(c)** Examples of how small numbers of amino acid differences influence ASAT2 and ASAT3 enzyme acyl CoA donor and sugar core substrate preference and promiscuity. The tomato species from which the ASAT alleles are derived are labeled beneath the product(s). **(d)** Functional

diversification of ASAT3 through gene duplication, neofunctionalization, and loss in the tomato clade.

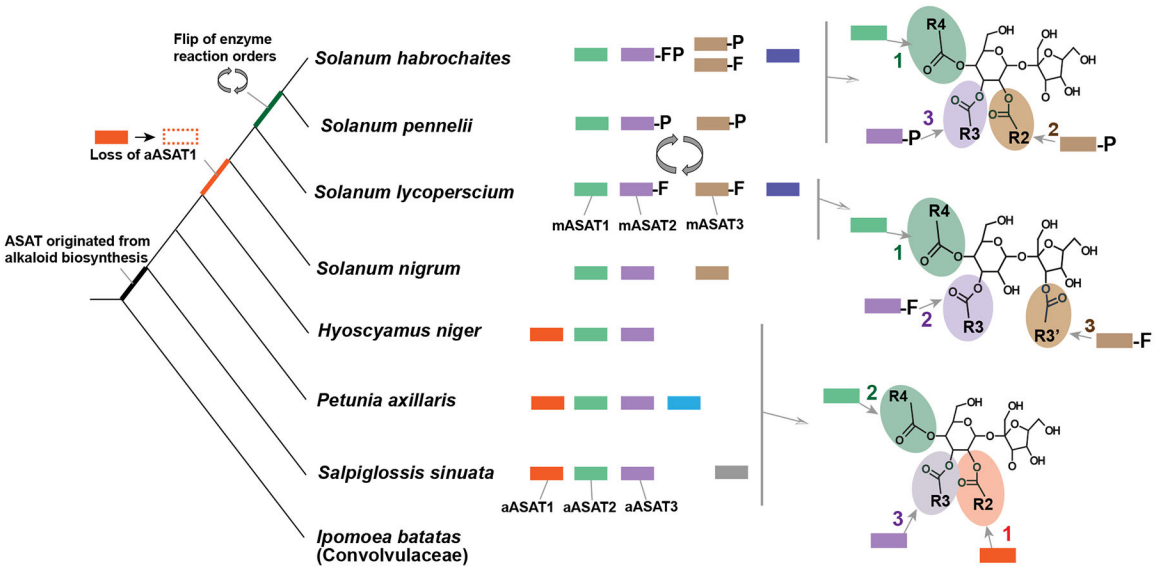

Figure 3.

Variation in acylsucrose acyltransferases in a phylogenetic context. A combination of enzymology, metabolite profiling, genomics and transcriptomics approaches led to a model for evolution of the core acylsucrose biosynthetic network over the tens of millions of years since divergence from the last common ancestor with Convolvulaceae [8]. The rectangular boxes represent ASAT homologs found in the corresponding species. The same color represents the closest ASAT homologs across species. All ASATs shown were biochemically characterized except the ones in *Solanum nigrum* and *Hyoscyamus niger*. The triacylated sucroses with all acyl chains on the pyranose ring (P-type acylsucroses) produced by *S. pennellii* have the same acylation pattern as those produced in *Petunia* and *Salpiglossis*. However, the enzymes, and the order of acylation to produce these P-type acylsucroses, differ as depicted. In fact, 'flipped pathway' leading to synthesis of the *S. pennellii* P-type acylsucroses appears to be a metabolic innovation that originated after the last common ancestor of the *Solanum* tomato clade and before the divergence from the last common ancestor of *S. pennellii* and *S. habrochaites*. This model is consistent with P-type acylsucroses evolving multiple times in the Solanaceae.

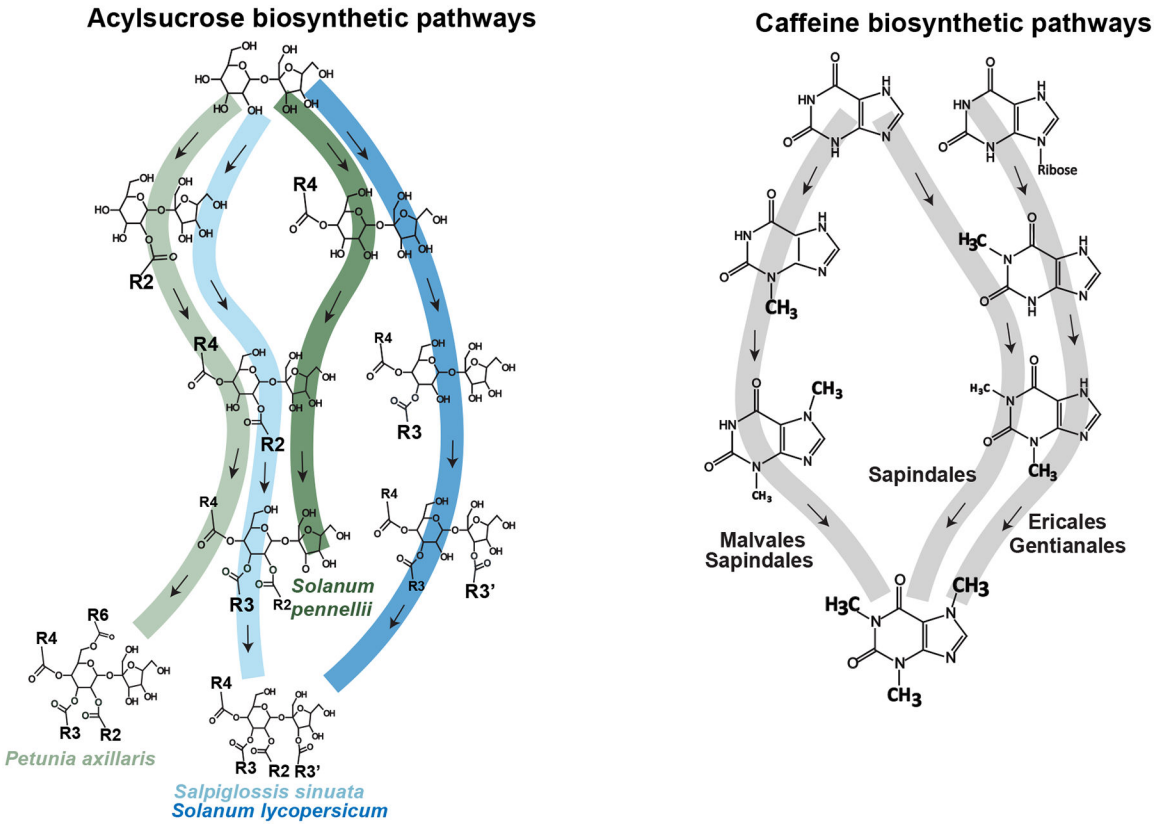

Figure 4.

Comparison of acylsucrose and caffeine biosynthesis: different biosynthetic routes generate similar products. Different orders of sucrose acylation in *S. pennellii* and *P. axillaris* (left panel, indicated by green ribbon) produce similarly structured acylsucroses with all acyl chains on the pyranose ring (P-type acylsucroses). In *S. lycopersicum* and *S. sinuata*, distinct biosynthetic routes (left panel, indicated by blue ribbons) generate acylsucroses with acyl chains on both pyranose and furanose ring (F-type acylsucroses). This theme of multiple biosynthetic routes to similar acylsucroses is reminiscent of caffeine biosynthesis, which convergently evolved in different plant orders (right panel). Both acylsugar and caffeine biosyntheses require sequential chemical modifications on the backbone structures that were 'hijacked' from primary metabolism. Each arrow on the left or right panels represents a biochemical reaction catalyzed by an acylsucrose acyltransferase for acylsugars or xanthine methyltransferase for caffeine.