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Promiscuity, impersonation and accommodation: evolution of plant specialized metabolism

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Abstract

Specialized metabolic enzymes and metabolite diversity evolve through a variety of mechanisms including promiscuity, changes in substrate specificity, modifications of gene expression and gene duplication. For example, gene duplication and substrate binding site changes led to the evolution of the glucosinolate biosynthetic enzyme, AtIPMDH1, from a Leu biosynthetic enzyme. BAHD acyltransferases illustrate how enzymatic promiscuity leads to metabolite diversity. The examples 4-coumarate:CoA ligase and aromatic acid methyltransferases illustrate how promiscuity can potentiate the evolution of these specialized metabolic enzymes.

Introduction

Plant specialized metabolites are lineage-specific compounds, many of which are involved in ecological interactions, such as herbivore defense or pollinator attraction [1,2]. The number of specialized metabolites produced across all plant species is estimated to be in the hundreds of thousands [3]. Specialized metabolic enzymes tend to have lower catalytic efficiency [4] and greater substrate promiscuity [5] than primary metabolic enzymes. This review explores factors involved in enzyme evolution and discusses how these result in metabolite diversity.

We focus on mechanisms that play roles in “potentiation” (Figure 1A); metabolic examples of what was more generally described by Blount et al. [6] as factors that allow for the realization of a new trait. In recent years, enzymatic promiscuity – the ability of an enzyme to catalyze reaction(s) in addition to its primary reaction – has been documented to “potentiate” the evolution of new specialized metabolic activities (Figure 1A) [6]. Substrate promiscuity is documented to play a central role in the evolution of specialized metabolic

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enzymes (Figure 1B) [5]. Changes in substrate specificity also can result in emergence of novel activities and chemical diversity. Such a shift in substrate specificity can change the primary substrate of an enzyme from an intermediate in an existing biosynthetic pathway to a new substrate, which – in turn – can potentiate novel enzymatic reactions (Figure 1C). Gene duplication and divergence in gene expression patterns or enzyme activities also potentiate the evolution of specialized metabolic enzymes (Figure 1D) [7]. We highlight examples from the past five years in which promiscuity, changes in substrate specificity, gene duplication, and changes in gene expression were shown to play prominent roles in evolution of specialized metabolic enzymes and generation of chemical diversity. These examples illustrate the power of structural analysis – especially in a comparative evolutionary context – to reveal constraints and opportunities to facilitate the modification or engineering of these enzymes.

Gene duplication and changes in substrate specificity in the evolution of a glucosinolate biosynthetic enzyme

Glucosinolates are a group of structurally diverse, amino-acid derived plant specialized metabolites that mediate interactions between crucifers and insects or pathogens [8]. The biosynthesis of methionine-derived glucosinolates involves a repeated three step elongation process similar to Leu biosynthesis: condensation with acetyl-CoA, isomerization, and oxidative decarboxylation to successively add one carbon units to the aliphatic side chain [9]. The glucosinolate oxidative decarboxylation step is catalyzed by the *A. thaliana* isopropylmalate dehydrogenase (AtIPMDH1), while two other *A. thaliana* IPMDH enzymes catalyze the same reaction in Leu biosynthesis; all three enzymes have between 85–93% amino acid identity with each other [10,11]. The Leu biosynthetic substrate (3-isopropylmalate) and glucosinolate substrate (3-(2'-methylthio)-ethylmalate), have the same carboxyl and hydroxyl group configuration but differ in side chain length and composition, suggesting similarities in the binding dynamics between the enzymes and substrates in the two pathways (Figure 2A, side chains are in color).

The AtIPMDH2 Leu biosynthetic enzyme crystal structure with 3-isopropylmalate (3-IPM) revealed several binding interactions with the polar portion of the substrate [12]. The structure revealed that residues interacting with the polar groups of 3-IPM are conserved between all IPMDH enzymes (Figure 2B) [13]. This, combined with the similarity of the polar groups of 3-IPM and (3-(2'-methylthio)-ethylmalate, suggested that recognition of the side chain is responsible for substrate discrimination. There are no specific substrate-enzyme interactions between the 3-IPM aliphatic isopropyl side chain and the residues in the largely hydrophobic pocket in the active site (Figure 2B) [12,13]. Thus, the differences between the glucosinolate biosynthetic enzyme AtIPMDH1 and Leu IPMDH enzymes presumably are responsible for the difference in substrate specificity.

Sequence alignments of Leu IPMDH enzymes with AtIPMDH1 revealed a key feature that affects the ability of the enzyme to discriminate between Leu and glucosinolate substrates. AtIPMDH1 carries a position 133 Leu:Phe change at a site in the hydrophobic pocket at a site that is invariant in the Leu biosynthetic enzymes from bacteria to plants [10,13].

Reciprocal substitutions of residues at this site led to a decrease in the *in vitro* catalytic efficiency with the native substrate – e.g. 3-IPM for AtIPMDH2 and AtIPMDH3 and 3-(2'-methylthio)-ethylmalate for AtIPMDH1 – and an increase with the non-native substrate [13]. Because the chemical structures of 3-(2'-methylthio)-ethylmalate and 3-IPM differ only by the length and structure of the side chain, this result demonstrates that the substitution of Leu by Phe is sufficient to facilitate the accommodation of the 3-(2'-methylthio)-ethylmalate side chain in the enzyme [12].

This example illustrates many themes found throughout the evolution of specialized metabolic enzymes [7]. A model for the emergence of the glucosinolate IPMDH can be constructed [13], starting with a simple gene duplication that led to the opportunity for 'recruitment' of the highly conserved Leu biosynthetic enzyme, IPMDH. This was followed by the L137F change in the amino acid sequence, leading to alteration of substrate specificity. In this model, gene duplication (Figure 1D) was critical for the divergence in structure and function of AtIPMDH1, allowing maintenance of the Leu pathway, while facilitating the incorporation of IPMDH activity into the glucosinolate pathway. The residue change (Figure 1C) facilitated the alteration of substrate specificity resulting in incorporation of the IPMDH activity into the glucosinolate pathway and increased chemical diversity through the production of elongated glucosinolates.

Acyltransferase promiscuity modulates metabolite diversity

Acylsucrose acyltransferases (ASATs) are BAHD (BEAT, AHCT, HCT, and DAT) [14] acyltransferases involved in biosynthesis of protective glandular trichome acylsugars in cultivated tomato and wild relatives [15–17]. The large BAHD family includes enzymes that perform *O*- and *N*-acylation of structurally diverse acceptor substrates such as alkaloids, phenylpropanoids, terpenoids and acylsugars [6]. Acylsucroses are produced through consecutive ASAT-mediated conjugation of acyl-CoAs, starting with sucrose to form mono-, di-, tri- and tetra-acylated sucroses [18–20]. These enzymes contribute to intraspecific and interspecific diversity in the profiles of insecticidal acylsugars of tomato and its wild relatives.

The second enzyme of acylsugar biosynthesis in cultivated and wild tomato species (ASAT2) exhibits an interesting pattern of substrate preference for iso- (3-methylbutyrate) and anteiso branched (2-methylbutyrate) C5 as well as nC12 acyl chains [18]. ASAT2 in cultivated tomato shows higher specificity, using aiC5-CoA but not the structurally similar iC5-CoA as a donor substrate. In contrast, the ASAT2 orthologs in other wild tomatoes are promiscuous, using both aiC5-CoA and iC5-CoA. [18]. A combination of primary sequence comparisons and homology modeling of the isozymes using the crystal structure of a distantly related BAHD enzyme led to identification of the amino acid responsible for the difference in promiscuity [18,21]. Specifically, Phe408 restricts and Val408 enables iso-branched C5 acyl chain utilization. This simple change contributes to accumulation of more diverse acylsugars in the wild tomato, *Solanum habrochaites* LA1777, compared to the cultivated species [22]. This example illustrates that enzymatic promiscuity can arise due to simple changes and facilitate the generation of product diversity in specialized metabolism (Figure 1B).

Hydroxycinnamoyltransferases illustrate how the interface of enzyme and substrate structure shape promiscuity

In addition to sequence changes, structural similarities of the substrates and how they interact with structural elements of the enzymes can also influence enzyme promiscuity. This theme is exemplified by the hydroxycinnamoyltransferases (HCTs), which play a role in phenylpropanoid biosynthesis [23–26]. HCTs are involved in the conjugation of *p*-coumaroyl CoA to shikimate to form *p*-coumaroyl shikimate, and can be highly promiscuous [23–26]. For example, the *A. thaliana* HCT (AtHCT) uses nine substrates besides the native substrate shikimate – some better than shikimate (Figure 2C) – to produce a diversity of products *in vitro*. [24].

Crystallography and molecular dynamics analysis of AtHCT highlighted two mechanisms by which HCT maintains its *in vivo* function while being such a promiscuous enzyme [26]. First, comparison of AtHCT apoenzyme:substrate crystal structure and molecular dynamics revealed that this enzyme undergoes a conformational change upon *p*-coumaroyl-CoA or *p*-coumaroylshikimate binding: this reduces the volume of the active site and induces changes in the position of residues involved in substrate binding and catalysis [26]. Second, a conserved Arg ‘handle’ in the active site forms electrostatic interactions with the shikimate carboxyl group, orienting the 5-hydroxyl towards the catalytic center (Figure 2D) [26]. An HCT structure from *Coleus blumei* (CbHCT) was used to corroborate both features [26]. CbHCT was crystallized as a ternary complex with *p*-coumaroyl-CoA and 3-hydroxyacetophenone (Figure 2C), an uncharged non-native substrate [26]. The CbHCT structure revealed that the active site does not shrink when bound to 3-hydroxyacetophenone [26]. Furthermore, mutation of the Arg to Ala, Asp, or Glu caused a drastic decrease in activity with shikimate [26]. Conversely, those mutations increased activity with positively-charged non-native substrates such as 3,4-dihydroxybenzylamine and dopamine (Figure 2C), demonstrating that the Arg helps discriminate against some non-native substrates [26].

These two structural features play roles in substrate specificity with 3-hydroxyacetophenone or positively-charged substrates, but may not discriminate against substrates that are more structurally similar to shikimate. The Arg residue was shown to form electrostatic interactions with the shikimate carboxyl, discriminating against substrates lacking the carboxyl group [26]. Perhaps more interesting are the promiscuous activities of AtHCT [24]. AtHCT has activity with the non-native substrates gentisate and 3-hydroxyanthranilate, which share the negatively charged carboxyl group with shikimate (Fig 2). We hypothesize that the Arg handle – in combination with the carboxyl moiety in the promiscuous substrates – may facilitate activity with non-native substrates [24]. This is an example of how multiple structural elements of an enzyme – in this case the Arg handle and a dynamic conformation change – help maintain the primary activity despite numerous alternative reactions. These studies also highlight how the interplay of structural similarities of substrates and enzymatic features facilitate promiscuity. Discovery of more mechanisms that enable enzymes to maintain a primary metabolic activity despite being promiscuous will lead to general principles that can guide efforts to engineer specialized metabolic enzymes.

How promiscuity allows 4-coumarate:CoA ligase to catalyze multiple steps in phenylpropanoid biosynthesis

4-coumarate:CoA ligase (4CL) catalyzes multiple steps in the biosynthesis of phenylpropanoids including structural lignins and defensive anti-microbial compounds (Figure 3D). Promiscuity plays a central role in the ability of the enzyme to conjugate at least three different hydroxylated and methylated forms of hydroxycinnamic acids to coenzyme A in multiple plant species (Figure 3A) [27–29]. Two 4CL crystal structures inform our understanding of substrate promiscuity [30,31]. The *Populus tomentosa* 4CL1 structure revealed a largely hydrophobic hydroxycinnamate binding pocket [30]. Studies of *Nicotiana tabacum* 4CL2 complexed with feruloyl-adenylate confirmed the existence of this hydrophobic binding pocket, and revealed how it accommodates hydroxycinnamic acids of varying structure [31]. This binding flexibility is mediated by interaction with a tyrosine residue responsible for ring stacking with the substrate phenyl ring (Figure 3B). The enzyme binding pocket accommodating the hydroxycinnamoyl moiety is spacious at one of the *meta* positions on the phenyl ring and constrained at the other, which accommodates the hydroxy and methoxy groups in caffeic and ferulic acid, respectively (Figure 3C, red arrow shows constrained region and blue arrow identifies the spacious area). A serine residue in the active site hydrogen bonds with the *para* hydroxyl present in many monolignols (Figure 3B). These combined structural features accommodate diverse modified substrates allowing 4CL to catalyze multiple steps in phenylpropanoid metabolism.

Four isoforms of 4CL in *A. thaliana* use multiple hydroxycinnamate substrates *in vitro* and have distinct – but overlapping – roles [27,32,33]. Recent work suggests that one or more of the At4CL isoforms – in addition to ligating 4-coumaric acid to CoA – catalyzes the formation of caffeoyl-CoA, and this appears to be relevant *in vivo*, representing a later biosynthetic step towards guaiacyl and syringyl lignin (Figure 3D) [32,34]. The action of 4CL in multiple biosynthetic steps is conferred by the catalytic flexibility provided by promiscuous activities. In addition, At4CL3 is involved in flavonoid biosynthesis, which shares the common intermediate – 4-coumaroyl-CoA – with the lignin pathway, but uses different enzymes to synthesize their downstream products (Figure 3D). Flavonoids and lignins accumulate in different tissues, and differential expression of At4CL3 – along with the downstream flavonoid enzyme genes – diverts metabolites away from the lignin pathway, towards flavonoid biosynthesis [32,35]. 4CL illustrates how a combination of promiscuity, gene duplication, and divergence in gene expression potentiate the diverse roles that 4CL plays in phenylpropanoid biosynthesis.

Promiscuity in Salicylic and Benzoic acid methyltransferase evolution

Salicylic Acid (SA) and Benzoic Acid (BA) methyltransferases provide another example where promiscuity plays a role in enzyme function and evolution. These enzymes are in the SABATH (SAMT, BAMT, and Theobromine synthase) methyltransferase family, involved in the methylation of structurally diverse specialized metabolites [36]. BA and SA differ by the presence of a 2-hydroxyl group on the aromatic ring (Figure 4A), and the enzymes catalyzing methylation of BA and SA are promiscuous [37,38]. The volatile methylated

benzenoids are produced in differing ratios across characterized plant groups [39–41], and have ecological roles including insect pollinator attraction [1]. The enzyme responsible for SA methylation in *Clarkia breweri* is promiscuous, possessing activities with BA, 3-hydroxybenzoic acid, and cinnamic acid substrates (69%, 2%, and 2% of SA activity, respectively) [42].

The structural determinants that facilitate SA carboxyl methyltransferase promiscuity — including those involved in the binding of *C. breweri* SAMT (CbSAMT) to SA — were explored by X-ray crystallographic analysis of CbSAMT [43]. Methionine residues at positions 150 and 308 form a clamp on both faces of the SA benzyl ring, and the side chain nitrogens of Gln-25 and Trp-151 form hydrogen bonds with the SA carboxylate moiety (Figure 4B) [43]. The SA 2-hydroxyl moiety is not close enough to any active site residues or water molecules to form hydrogen bonds; thus there are no obvious enzyme structural elements that would discriminate between BA and SA [43]. However, the 2-hydroxyl forms an intramolecular hydrogen bond with the carboxylate group of SA, which restricts its movement, potentially influencing SA and BA activities [43]. Finally, the remaining active site residues sterically restrain the SA substrate, allowing for methylation of the carboxylate to occur (Figure 4C) [43]. This example illustrates how similarity of the favored substrate and structural variants can influence promiscuity. These binding site structural elements can accommodate both SA and BA, and this promiscuity appears to have influenced the evolutionary history of a clade of SABATH methyltransferases [44].

A combination of phylogenetic analysis, ancestral sequence reconstruction, and enzyme assays was used to infer that the major activity of the enzyme switched between BA and SA methylation two times during evolution of the clade that includes SAMTs and BA/SA carboxyl methyltransferases from plants in the Apocynaceae and Solanaceae families [44]. A unique feature of this integrative study is that ancestral sequence reconstruction was used to predict and test the enzymatic activity of extinct specialized metabolic enzymes at different nodes in the evolutionary history of this methyltransferase clade [44]. All predicted ancestral enzymes tested possessed some level of activity with both BA and SA [44]. These results led to the hypothesis that two shifts in substrate preference occurred over time, with minor promiscuous activities of ancestral enzymes emerging as the primary activity of descendants [44]. In this example, the promiscuity of these different ancestral enzymes potentiated the shifts in enzymatic activity. This work elegantly demonstrates evolution acting on minor activities of promiscuous enzymes, leading to new major enzyme activities [44].

Conclusion

The examples in this review illustrate multiple ways in which plant specialized metabolic enzymes generate product diversity and metabolic flexibility. Substrate promiscuity, changes in enzyme activity, gene duplication as well as changes in gene expression all provide potentiating environments [6] for the evolution of metabolic novelty. In addition to its importance in understanding evolution of protein form and function, a deeper appreciation of the structural basis of simple changes in substrate specificity (Figure 1C) and how

promiscuous enzymes maintain a primary activity despite the ability to utilize one or more alternate substrates should inform protein engineering and synthetic biology approaches.

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Highlights

- Promiscuity plays a role in the evolution of specialized metabolic enzymes and metabolic product diversity
- Changes in substrate specificity can result in the evolution of novel enzyme activities and pathways
- Gene duplications potentiate specialized metabolic enzyme evolution
- Substrate structural similarity affects enzyme promiscuity

Figure 1. Mechanisms leading to evolution of chemical diversity and enzymatic novelty

A) Potentiation – The different shapes and colors represent factors involved in the evolution of a novel function or enzymatic activity. These factors enable the realization of novel functions or enzymatic activities. In this example, the green circle represents suitable localization of gene expression, the purple square represents substrate availability in the tissue of interest, and the blue hexagon represents the ability of the enzyme to utilize the substrate [6]. B) Substrate promiscuity – primary metabolic enzymes typically catalyze a specific reaction, while specialized metabolic enzymes tend to be promiscuous and catalyze reactions using multiple substrates. C) Substrate specificity – specific amino acid changes result in alteration of enzyme substrate specificity, resulting in a new enzymatic activity or function. D) Gene duplication and neofunctionalization – a primary metabolic gene is duplicated, facilitating diversification of one isoform into a specialized metabolic function.

Figure 2. Structural analysis of AtIPMDH2 and AtHCT

A) Structure of substrates for IPMDH enzyme variants in Arabidopsis — 3-IPM is the leucine biosynthetic intermediate, and 3-(2'-methylthio)-ethylmalate participates in aliphatic glucosinolate biosynthesis. B) Interactions of AtIPMDH2 active site residues with 3-IPM demonstrate extensive interactions with the polar moieties, while not specifically interacting with the 3-IPM hydrophobic side chain. 3-IPM structural backbone is green. Active site residues are cyan. Blue represents nitrogen atoms, and red represents oxygen atoms. Green and red crosses are magnesium and water respectively [PDB ID: [5J32](#)]. The Leu 133 residue responsible for the change in substrate specificity is labeled. C) Substrates used for AtHCT analysis: Shikimic acid is the native substrate; 3-hydroxyacetophenone is a neutral non-native substrate of AtHCT; 3,4-dihydroxybenzylamine and dopamine are substrates that are positively charged under physiological pH. AtHCT has greater *in vitro* activity with gentisate, a non-native substrate, than with shikimate. D) Three-dimensional representation of Arg-356 interaction with the carboxyl group of *p*-coumaroyl-shikimic acid substrate in the active site of AtHCT. *p*-coumaroyl shikimic acid is colored green. Active site residues are colored cyan and red crosses represent water [PDB ID: [5KJU](#)].

Figure 3. 4-coumarate ligase characteristics that impact product diversity

A) At4CL utilizes substrates with hydrogen or hydroxyl and *O*-methyl substitution at the *meta* position of the aromatic ring. B) Three-dimensional representation of the active site residue interactions with feruloyl-adenylate from the *N. tabacum* 4CL2 co-crystal structure. Feruloyl-adenylate backbone is in green, while residues that interact with the feruloyl moiety are cyan. The red color represents oxygen atoms, the blue color represents nitrogen atoms, and the orange represents phosphorus atoms [PDB ID: 5BSV]. C) Mesh model of active site around feruloyl-adenylate. Presences or absence of steric hindrance in the active site allows substitution at one of the *meta* positions on the ring, but not the other. Red arrow indicates the *meta* position that is blocked by steric hindrance, while the blue arrow points to position at which substitutions are permitted by the enzyme [PDB ID: 5BSV]. D) Representation of the phenylpropanoid biosynthetic pathway impacted by 4CL activity. 4CL is involved in biosynthetic steps colored green [32].

Figure 4. Promiscuity in SAMTs and BSMTs is facilitated by substrate structural similarity
A) SA and BA have structures that differ by a single hydroxyl at the 2 position. B) Three-dimensional representation of *C. breweri* SAMT active site interactions with SA. Salicylic acid is colored green, while the interacting active site residues are colored cyan. The red color represents oxygen atoms, the blue represent nitrogen atoms, and the yellow represent sulfur atoms [PDB ID: [1M6E](#)]. C) Mesh representation of the *C. breweri* SAMT active site demonstrating the steric environment around SA [PDB ID: [1M6E](#)].