ANNUAL REVIEWS

Annual Review of Plant Biology

BAHD Company: The Ever-Expanding Roles of the BAHD Acyltransferase Gene Family in Plants

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Annu. Rev. Plant Biol. 2023. 74:165–94

First published as a Review in Advance on November 30, 2022

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

https://doi.org/10.1146/annurev-arplant-062922-050122

Keywords

hydroxycinnamoyltransferase, HCT, phenylpropanoid, acylsugars, ester, amide, land plant evolution

Abstract

Plants' ability to chemically modify core structures of specialized metabolites is the main reason why the plant kingdom contains such a wide and rich array of diverse compounds. One of the most important types of chemical modifications of small molecules is the addition of an acyl moiety to produce esters and amides. Large-scale phylogenomics analyses have shown that the enzymes that perform acyl transfer reactions on the myriad small molecules synthesized by plants belong to only a few gene families. This review is focused on describing the biochemistry, evolutionary origins, and chemical ecology implications of one of these families—the BAHD acyltransferases.

The growth of advanced metabolomic studies coupled with next-generation sequencing of diverse plant species has confirmed that the BAHD family plays critical roles in modifying nearly all known classes of specialized metabolites. The current and future outlook for research on BAHDs includes expanding their roles in synthetic biology and metabolic engineering.

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Serine carboxypeptidase-like acyltransferases (SCPLs): a family of acyltransferases related to the serine carboxypeptidase-like proteins

BAHD acyltransferases (BAHDs): acyltransferase family named according to the first four biochemically characterized enzymes of this family (BEAT, AHCT, HCBT, DAT)

INTRODUCTION

Within the realm of plant specialized metabolism, the majority of acylated compounds are the products of two distinct gene families, the serine carboxypeptidase-like acyltransferases (SCPLs) and the BAHD acyltransferases (BAHDs) (115). The latter are typically among the top 15 largest metabolic protein domain families in land plant genomes (**Supplemental Table 1**), suggesting their important in planta functional roles. The name BAHD is derived from the names of the first four biochemically characterized enzymes discovered (144), and family membership is typically defined from protein sequence using the Protein family (Pfam) domain PF02458. Considerable evidence in support of the essentiality of acylation modifications in plant growth, reproduction, and response to biotic and abiotic stress is increasingly available. Esters and amides are also critical biomolecule modifications for both industrial (e.g., biofuels) and pharmaceutical plant-derived products (e.g., morphine), and thus more and more resources are being dedicated to studying BAHDs for their roles in synthetic biology and metabolic engineering.

BAHD family members were critical during the transition from aquatic to terrestrial environments, and their diversification accompanied the radiation of spermatophytes during the colonization of land. Extensive gene gains and losses, in conjunction with the ecological roles these genes play in the interaction of plants with their surrounding biotic and abiotic environments,

have affected the number of genes and their distribution in plant genomes. Understanding the functional diversity of BAHDs and their evolutionary dynamics not only is necessary to ascertain how this family contributed to the explosion of plant metabolic diversity but can also be instrumental in guiding future biotechnological and metabolic engineering approaches.

More than 2,500 articles regarding BAHDs have been published since the first major BAHD review in 2006 (21). Reflecting the increasing pace of BAHD discovery, more than 1,500 publications have been released in the last five years. This review provides a comprehensive look at the BAHD family within the context of the evolution of structure–function relationships and with respect to the relevance BAHD products have in plant biology.

Protein family (Pfam): a protein family database (**https://www.ebi.ac.uk/interpro/entry/pfam/**)

Acyltransferase: enzyme using activated acids (as CoA-thioester or sugar ester) to transfer to hydroxyl or amine moieties forming esters or amides

EVOLUTIONARY HISTORY OF THE BAHD FAMILY

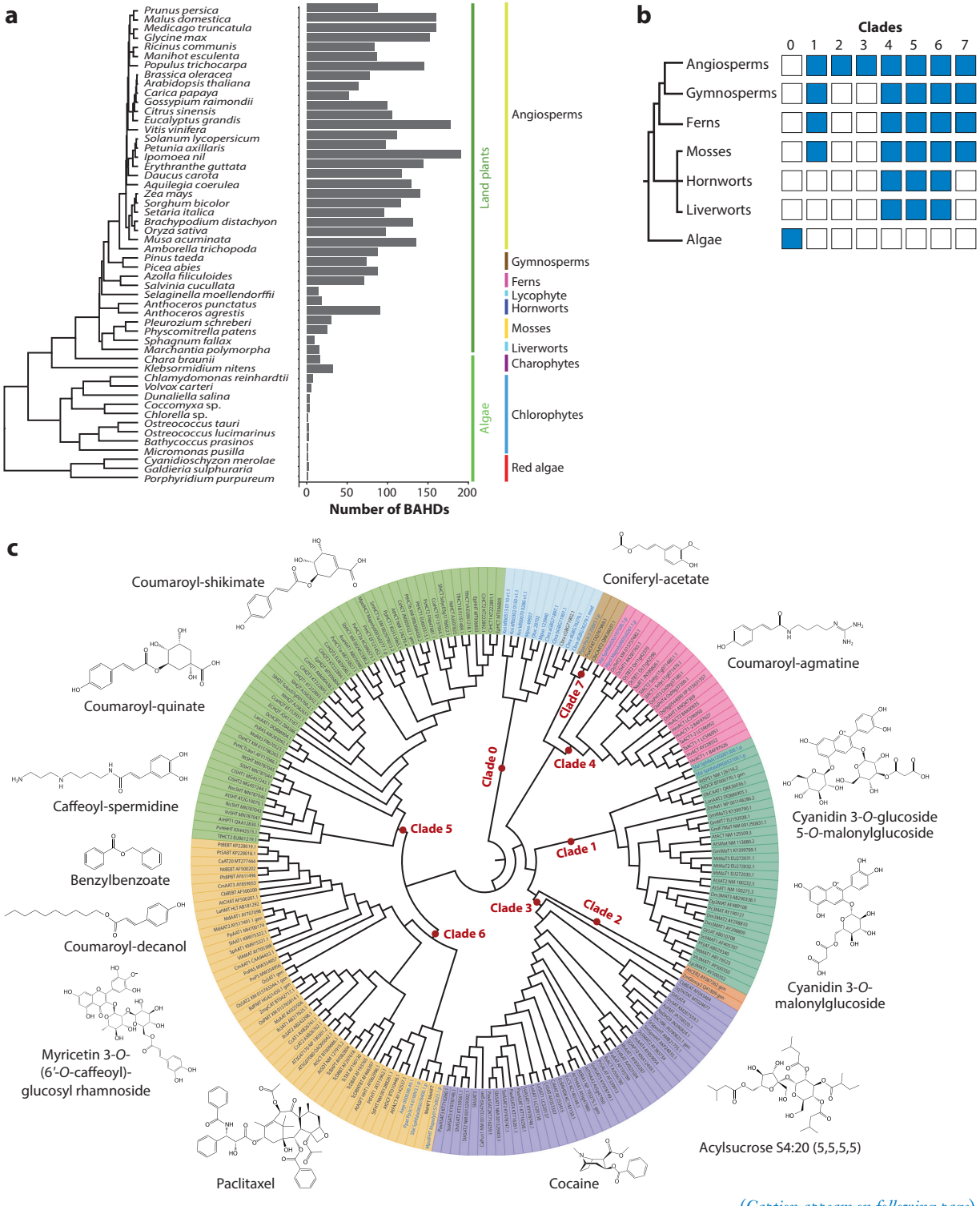
Origins of BAHDs Beyond the Plant Kingdom

The BAHD acyltransferase protein fold is found in plants and fungi, indicating that the BAHD fold itself emerged beyond the plant kingdom and is older than plants. Fungi typically contain only a handful of copies of BAHD-encoding genes per genome, whereas flowering plants contain dozens to hundreds (70, 152). Fungal BAHD domain–containing proteins also contain additional domains, such as those involved in adenosine monophosphate binding (PF00501), dityrosine biosynthesis (PF05141), and enoyl-coenzyme A (CoA) hydratase activity (PF00378), whereas in plants, most BAHDs contain only the BAHD domain. At least two BAHD activities from yeasts are characterized: trichothecene 3-*O*-acetyltransferase [AYT1 (open reading frame: *YLL063c*, *Saccharomyces cerevisiae*)] and 15-*O*-trichothecene acetyltransferase (TRI3, in *Fusarium* and *Trichoderma* species). These enzymes are associated with the metabolism of trichothecenes—mycotoxins produced by many fungi (3). While TRI3 performs an essential acetylation of 15-OH of 15-decalonectrin in trichothecene biosynthesis (87), AYT1 acetylates the 3-OH position, resulting in partial inactivation of the mycotoxin (3). AYT1 homologs are found in many fungal lineages and may be a defense mechanism against the mycotoxin (150).

Several potential origins for BAHDs from nonplant protein families were suggested based on the presence of motifs similar to their catalytic HXXXD motif (144), such as the nonribosomal peptide synthetases (NRPSs; containing HHUUXDG), glycerolipid acyltransferases (NHXSXUD), choline/carnitine acyltransferases (CATs; EHSXXDG), chloramphenicol acetyltransferases (HH[A,S]VCDG), and dihydrolipoamide acyltransferases (DHRUUDG). Of these, the CATs (PF00755) are found in mostly nonplant eukaryotes. They catalyze the transfer of various acyl chains, such as acetyl, octanoyl, and palmitoyl, to the hydroxyl group of substrates in the biosynthesis of amino acids and their derivatives. In recent years, genome sequences have also revealed another domain—fungal alcohol acyltransferases (fAATs; PF07247)—that contains the signature HXXXD (137), as well as surrounding residues similar to BAHDs. Also similar to some BAHDs, fAATs such as ATF1 and ATF2 from *S. cerevisiae* (40, 101) are involved in the production of volatile esters such as ethyl, isoamyl, and phenylethyl acetates, producing fruity aromas (19). These observations suggest that BAHDs are ancestrally related to fAATs and/or NRPSs. The BAHD domain was also suggested to be a fusion of NRPS and CAT proteins (144). Similarly, fusions between fAATs/CATs or fAATs/NRPSs are additional possibilities.

Expansion of BAHDs in Plants

The BAHD family in plants expanded significantly during plant evolution. While algal species have 1–5 BAHD copies in their genome, angiosperm genomes typically have 50–200 (70) (**Figure 1*a***). There are significant structural differences among BAHDs in algae versus land plants.



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

(*a*) BAHD counts in the genomes of plant species. The number of BAHDs increased significantly in land plants (50–200 genes) in comparison to the few genes in algae species (0–5 genes). (*b*) The heatmap illustrates the presence/absence of a specific BAHD clade in plant lineages, based on presence/absence of characterized activities and homologs in that given lineage. (*c*) Phylogenetic tree of characterized BAHDs (in vitro and in vivo). Clade numbers are based on Reference 21, except for clade V, which is split into clades 5, 6, and 7, as well as a newly defined clade 0 (algal clade). Clade 7 comprises unclassified members and is functionally and taxonomically loosely defined. Red circles on branches indicate nodes with bootstrap values >70 used to define clades 1–6. The phylogenetic tree was inferred using IQ-TREE with 1,000 nonparametric bootstrap replicates and illustrated in Interactive Tree of Life (iTOL) (80). Exemplary structures for characteristic enzyme products of each clade are provided. Sequences written in blue are uncharacterized BAHDs from early diverging plant lineages that were included to illustrate the spread of certain clades. Not all sequences mentioned in the text are included in this tree. See **Supplemental Figure 2** for a larger version of panel *c*. Panel *a* and *c* adapted from Reference 70.

The algal BAHDs, despite consisting of a single BAHD domain, are larger and have more than one intron. By contrast, BAHDs from land plants typically are intronless. Due to the paucity of characterized algal BAHD activities, the roles they play in algae are unclear.

Supplemental Material >

The dramatic expansion of BAHDs in land plants is associated with plants' conquest of new land environments, suggesting an important role for BAHDs in the adaptation to terrestrial life. To facilitate analyses, multiple studies have grouped BAHDs into different clades (**Figure 1*b*,*c***). Kruse et al. (70) grouped BAHDs into eight clades, building on the classification scheme of D'Auria (21). The published catalytic activities of BAHDs (**Supplemental Table 2**) were classified into 10 different substrate classes, namely aromatic alcohols, aliphatic alcohols, aromatic amines, aliphatic amines, terpenoids, flavonoids, anthocyanins, phenolic glucosides, sugar derivatives, and alkaloids. Cataloged functions were then mapped onto the different clades, which were defined based on phylogenetic analyses.

Clade 0 (**Figure 1*c***) comprises exclusively algal enzymes. One enzyme from this clade, a BAHD from the charophytic alga *Chara braunii*, was tested in vitro for coumaroyltransferase activity against multiple acceptor substrates representative of the above classes. Only quinate was acylated; therefore, it was labeled as *Chara braunii* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (CbHQT)-like. Charophytic algae do not possess (true) lignin (118, 121); however, compounds that can be considered prelignin occur in charophytic algae (60, 121, 123). Charophytic algae generally also possess the necessary genetic machinery for the biosynthesis of phenylpropanoids and their derivatives (121). Based on the CbHQT-like activity as well as other BAHD activities from mosses and liverworts, it is possible that the ancestral BAHDs of land plants had the ability to catalyze acylation reactions with substrates like shikimate and quinate.

Two core BAHD activities—hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (HST) and/or hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (HQT)—participate in multiple steps of the general phenylpropanoid pathway in plants. All HST and HQT enzymes are clustered within clade 5 (**Figure 1*c***), which contains members from every land plant lineage, including liverworts, mosses, hornworts, and ferns (**Figure 1*b***). Similarly broadly conserved, and thus likely essential, is a subclade of clade 6 (**Figure 1*b*,*c***), whose members *Marchantia emarginata* ω-hydroxyacid/fatty alcohol hydroxycinnamoyltransferase (MeHFT) and *Marchantia polymorpha* feruloyltransferase (MpFHT) are involved in the biosynthesis of cutin monomers (161). In gymnosperms, a specialized subclade includes members from *Taxus* spp. involved in acylation of paclitaxel pathway intermediates (106). Paclitaxel, which is typically isolated from *Taxus brevifolia*, is an important natural anticancer drug (89). Several other clade 6 members such as *Arabidopsis thaliana* acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (AtCHAT) and *Solanum lycopersicum* alcohol acyltransferase 1 (SlAAT1), which are able to acylate aliphatic and aromatic alcohols using both aliphatic and aromatic CoA-activated donors, have also been characterized in multiple

Lignin: apoplastic polymer in plant secondary cell walls formed by a complex network of aromatic subunits (monolignols) that can be acylated

Hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (HST): hydroxycinnamoyltransferase acting on shikimate as an acceptor molecule

Hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (HQT): hydroxycinnamoyltransferase acting on quinate as an acceptor molecule

angiosperm species. These BAHDs produce volatile alcohol esters that play important roles in pollination, insect defense, and production of fruit aromas (47).

Acylsugar acyltransferase (ASAT): acyltransferase acting on a sugar core, which is esterified with one or more fatty- and amino-acid-derived acyl groups

Clade 1 (**Figure 1*c***) comprises mostly BAHDs involved in anthocyanin/flavonoid/phenolic glucoside acylation. These enzymes cluster closely with enzymes involved in salicylic acid metabolism and aliphatic alcohol, for example, eugenol acylation in *Ocimum basilicum* (sweet basil) and *Lavandula angustifolia* (lavender). Interestingly, *Vitis vinifera* anthocyanin acyltransferase 1 (Vv3AT1; clade 3), *Salvia splendens* anthocyanin 5-*O*-glucoside-4‴-*O*-malonyltransferase (Ss5MAT; clade 3), and *Iris* × *hollandica* anthocyanin-3-(4-coumaroyl)transferases 1 and 2 (Ih3AT1,AT2; clade 6), which are distantly related to clade 1 enzymes, have also been characterized, indicating that there are multiple routes to emergence and/or fixation of the anthocyanin acyltransferase activity.

Clade 2 (**Figure 1*c***) contains only three characterized members—*A. thaliana* Eceriferum (AtCER2), *Zea mays* Glossy2 (ZmGlossy2), and ZmGlossy2-like—which have all been implicated in the biosynthesis of epicuticular waxes (105) through complementation and other genetic assays. Both ZmGlossy2 and ZmGlossy2-like are able to complement loss of function of AtCER2 in *A. thaliana* (2), indicating mostly conserved function. Some differences in very long chain fatty acyl lipids were observed, suggesting functional diversification between the two maize enzymes. Functionally, this clade may appear analogous to clade 6 enzymes involved in cutin monomer biosynthesis; however, while clade 6 enzymes are conserved across plants, orthologs of the characterized members of clade 2 are found only in angiosperm genomes.

Clade 3 (**Figure 1*c***) contains several diverse activities, including the well-studied acylsugar acyltransferases (ASATs) that are restricted to the Solanaceae family. ASATs are produced in the type I/IV trichome tip cells on leaf and stem surfaces (90). Oligosaccharide sugar acylation is found in multiple plant families, for example, in Convolvulaceae resin glycosides (69); however, the acylating enzymes have not yet been identified. Clade 3 also contains enzymes involved in aliphatic alcohol, terpene, anthocyanin glycoside, aliphatic amine, and alkaloid acylation characterized across different angiosperm species.

Clade 4 (**Figure 1*c***) comprises multiple characterized enzymes from monocot and dicot clades involved in aliphatic amine acylation. This includes agmatine coumaroyltransferases in *Hordeum vulgare* (HvACT), *Oryza sativa* (OsAHT1), and *Triticum aestivum* (TaACT1) as well as putrescine hydroxycinnamoyltransferases in *Nicotiana attenuata* (NaAT1), *O. sativa* (OsPHT1–4), and *S. lycopersicum* (SlACT1). Despite their annotations, multiple enzymes from both types use diverse acceptors including agmatine, putrescine, spermine, and spermidine, and donors such as 4-coumaroyl-, caffeoyl-, feruloyl-, and cinnamoyl-CoA (70). Although homologs of these enzymes are found even in liverworts and mosses (**Figure 1*b***), only the angiosperm enzymes have been functionally characterized. The HvACT enzyme is involved in the biosynthesis of antifungal hordatines in barley seedlings (12), while NaAT1 plays a role in phenolamide biosynthesis in response to multiple biotic and abiotic stresses (110).

Clade 7 (**Figure 1*c***)—a loosely defined clade—consists of coniferyl alcohol acetyltransferases from *Petunia* × *hybrida* (PhCFAT1) and *O. basilicum* (ObCAAT2), both of which are involved in acetylation of aromatic alcohols such as benzyl, coniferyl, and cinnamyl alcohol as well as aliphatic alcohols/phenylpropenes/terpenes such as octanol, eugenol, and geraniol. While PhCFAT1 is responsible for producing floral volatiles, ObCAAT2 produces these volatile esters in the glandular trichomes of sweet basil leaves.

While the characterized enzymes can be classified into eight clades, additional undefined clades likely exist, especially in nonseed plants as a result of lineage-specific duplications and because of insufficient studies of BAHDs in those clades. Such studies may reveal novel chemistries and products that presently do not feature in the BAHD chemical substrate space.

MECHANISTIC BASIS FOR BAHD FUNCTIONAL DIVERSITY

Alcohol Acyltransferases

Alcohol acyltransferase (AAT): an acyltransferase acting on various alcohols as acceptor molecules

Substrate promiscuity: the capacity of an enzyme to catalyze alternative reactions using different substrates; nonnative reactions commonly occur at lower efficiencies

BAHD family members have been implicated in volatile ester production since the earliest reports of the biochemical characterization of such enzymes (1, 22, 30). The alcohol acyltransferases (AATs) often have the ability to use various acceptor substrates and can produce a wide range of products. As a result, substrate promiscuity is considered a hallmark for this subset of BAHD members.

A common theme underpinning many structure–function studies in the BAHD family is understanding how the substrate channel in the enzymes' tertiary structure modulates the catalytic mechanism. AAT1 from *Vasconcellea pubescens* fruit contains a solvent channel that is critical for orientation of the CoA-thioesters. Several Asp residues were identified via molecular dynamics simulations to be intricately involved in influencing substrate binding (96). A later study used similar techniques to examine the contribution of the conserved DFGWG motif. Three mutants (Y52F, D381A, and D381E) were tested and found to contain unfavorable free energies for the interaction with the substrates, although no large changes were witnessed in the overall structure of the enzyme (95). Van der Waals forces were the main contributing factor in the energetics of the electrostatic potential on the solvent channel surface. These results were also reconfirmed with studies on an AAT from *Fragaria* × *ananassa* (103).

Mono- and sesquiterpene alcohols are often esterified to produce a wide range of volatile products. In roses, the floral headspace is dominated by geranyl and citronellyl acetate, both produced by the enzyme RhAAT1 (135). The isolation and biochemical characterization of a geraniol acetyltransferase from *Cymbopogon martinii* revealed an atypical DFGWG motif, suggesting increased plasticity in substrate recognition (136). Eight AATs from *Wurfbainia villosa* produce bornyl acetate (WvBAT); however, while all eight can use (−)-borneol as a substrate, only five of the WvBATs are capable of using (+)-borneol (83). This suggests that stereoselectivity can be achieved with a few amino acid changes. While not volatile, phytosterols are another class of terpenes known to be acylated. In an attempt to isolate the enzyme responsible for producing the cardenolide 21-*O*-malonyl-5β-pregnane-3β,14β-diol-20-one, a hybrid homology-based approach using two *Arabidopsis* BAHD enzymes was used in docking experiments with various 21-hydroxypregnanes using a known malonyltransferase from tobacco as the model. Once they identified the critical residues putatively involved in substrate binding, candidate genes were isolated from *Digitalis lanata*. The resulting recombinant proteins were shown to be 21-hydroxypregnane 21-*O*-malonyltransferases (151).

C6 volatile esters are common in plants since they are related to oxylipin biosynthesis and are the result of tissue damage and wounding. The so-called green leaf volatiles are produced rapidly upon tissue damage, and the production of the esters follows the original appearance of the more toxic C6 aldehydes produced by lipoxygenases (23). In addition to its presence in wounded leaves, the enzyme PaAAT1 from *Prunus armeniaca* was found to be a major contributor to the aroma of ripe apricots (176). C6 volatile esters are also important for the flavor of several apple varieties (177). MpAAT1 has a substrate preference for aliphatic C6 compounds, but production of volatiles seems at least partly controlled by the kinetic properties of the enzyme (55, 142). During the development of the fruit, certain substrates are available in higher concentrations and therefore dominate the aroma profile at that particular time point (141). Lastly, the orthologous enzyme MdAAT1 in *Malus* × *domestica* not only is involved in aliphatic ester biosynthesis but was also identified via quantitative trait locus (QTL) mapping to be involved in phenylpropanoid biosynthesis (173). The enzyme produces 4-hydroxycinnamoyl acetates, which serve as substrates

for enzymes that produce chavicol and eugenol. This activity may also be important for other agriculturally important fruit-bearing plants, such as strawberry and tomato.

Hydroxycinnamoyltransferase (HCT): an enzyme transferring a hydroxycinnamate moiety from an activating cofactor to an acceptor molecule

Hydroxycinnamoyltransferases

BAHD hydroxycinnamoyltransferases (HCTs) form esters or amides with hydroxycinnamic acids. The transfer of a hydroxycinnamoyl moiety from an activated precursor can be mediated by different enzyme families (115). BAHD-HCTs use CoA-activated hydroxycinnamates or, rarely, hydroxycinnamate esters such as chlorogenate as hydroxycinnamoyl donors (73, 91, 92). In some cases, benzoyl-CoA is also accepted. Hydroxycinnamates derive from the phenylpropanoid pathway starting with L-phenylalanine and L-tyrosine, which ultimately yields 4-coumaroyl-CoA. The substitution pattern of caffeoyl-CoA is established via shikimate esters or direct hydroxylation of 4-coumarate/4-coumaroyl-CoA (116). A depiction of coenzyme A–activated donor substrates used by HCTs alongside a wide variety of acceptor substrates involved in hydroxycinnamoyl transfer is available in **Supplemental Figure 1**.

Supplemental Material >

Hydroxycinnamoyl-CoA:shikimate and/or Quinate Hydroxycinnamoyltransferases

Chlorogenate (caffeoylquinate) is a widespread phenolic ester in plants that can accumulate to high levels. By contrast, caffeoylshikimate is rarely found in high concentrations. In 2001, the importance of 4-coumaroylshikimate as a substrate for the introduction of the meta-hydroxyl group by cytochrome P450 (CYP98A family) to form caffeoylshikimate and other caffeate derivatives was discovered (132). This was followed by the description of the first gene sequence encoding a hydroxycinnamoyl-CoA:shikimate HCT (HST, NtHCT in clade 5) (**Figure 1*c***) in tobacco. This enzyme also accepts quinate as a substrate, together with differently substituted hydroxycinnamoyl-CoAs (54). Since then, multiple genes encoding enzymes with HCT activities toward shikimate (HST), quinate (HQT), or both have been reported. Some enzymes accept other nonrelated substrates, including 3-hydroxyanthranilate, 5-hydroxyanthranilate, 3-hydroxybenzoate, 2,3-dihydroxybenzoate, 3-aminobenzoate, gentisate, catechol, and protocatechuate (31, 127). Interestingly, some enzymes only accept shikimate or quinate, while others do not strictly discriminate between these two substrates. **Supplemental Table 3** compiles characterized HST and/or HQTs and their biochemical characteristics. A number of HCTs catalyze the reverse reaction, producing hydroxycinnamoyl-CoA and the previous acceptor substrate. Cleavage of hydroxycinnamoyl-CoA to free hydroxycinnamic acid and CoA is also often observed.

HQTs additionally form dicaffeoylquinic acids, although to a lower extent (73, 91, 92). Chlorogenate is the substrate for 3,5-dicaffeoylquinate production at low coenzyme A concentrations (73, 91, 92). Chlorogenate:chlorogenate HCT activity takes place at low pH values, but studies with green fluorescent protein (GFP)/HCT fusion proteins excluded a vacuolar localization (91).

The crystal structure of coffee HST [Protein Data Base identification (PDB ID) 4G0B, CcaHCT in clade 5] (**Figure 1*c***) was the first from this clade to be identified (72). Refined experiments used a more stable protein with two Lys residues mutated to Ala in the loop between the two domains (PDB ID 4G2M, 4G22) (73). Structural and biochemical properties were similar to those of the native protein. The protein is composed of two large chloramphenicol acyltransferase–like domains connected by a loop stretching from Pro206 to Thr224. In three of the four obtained structures, the catalytic His153 residue is oriented toward the solvent channel, which is supposedly the active form of the enzyme. Docking studies placed the CoA residue into the solvent channel with the SH group positioned into the channel. Binding is mediated mainly by residues of the C-terminal domain. Three substrate binding pockets were identified (SBP-1–SBP-3). Quinate

and shikimate are bound into the hydrophobic pocket SBP-1, but H bonds can be formed between polar amino acids and polar substrate substituents. SBP-2 harbors the hydroxycinnamoyl moiety by π stacking of the aromatic ring with Trp372 and formation of H bonds between the aromatic OH group and Ser38 and Tyr40. The bulkiness of amino acids at the entrance of this binding pocket may be involved in substrate discrimination between smaller (e.g., malonyl-CoA) and larger CoA-activated acids (e.g., hydroxycinnamoyl-CoA). When 3,5-dicaffeoylquinate was docked into the protein, C3 of quinate was placed in SBP-2 while C5 was in SBP-3. Mutation of His153 to Ala abolished enzyme activity, while mutation of His53 to Ala reduced it. This residue may be important for orienting His153 properly for catalysis. A number of HCTs have two neighboring His residues (His153 and His154). Interestingly, mutation of His154 resulted in considerably higher 3,5-dicaffeoylquinate formation. Leu400 and Phe402 are involved in substrate discrimination between shikimate and quinate. The amino acid exchanges Leu400Thr and Phe402Tyr and the corresponding double mutation shifted the acceptance from shikimate to quinate (73).

The structure of *Sorghum bicolor* HST (SbHCT) was solved in the apo-form (PDB ID 4KE4; resolution 2.0 Å) (**Figure 1*c***) and as a ternary complex with 4-coumaroyl-CoA and shikimate (PDB ID 4KEC; resolution 2.4 Å) (157). Substrate binding resulted in a closure of the substrate binding pockets. The ternary complex showed the substrate binding tunnel blocked by the product. This tunnel was lined predominantly with hydrophobic amino acids. The two substrate molecules probably enter this tunnel from opposite sides. CoA was only weakly coordinated by the protein, and its adenine ring was located at the surface. On the other hand, strong protein–product interactions were visible. The aromatic ring of 4-coumaroylshikimate interacted with Tyr40 and Ser38 via an ordered water molecule. The carbonyl oxygen forms an H bond with Trp386. The shikimate moiety interacts with Arg371 and Thr384. Amino acids important for substrate binding and catalysis were mutated to alanine, namely Thr36Ala, Ser38Ala, Tyr40Ala, His162Ala, Arg371Ala, and Thr384Ala. No activity was shown for His162Ala and Arg371Ala, while the other mutants displayed residual activities between 6% and 62%. The catalytic His162 is located approximately in the middle of the substrate binding tunnel. It has significantly different positions in the apo-form and the complex form, where it interacts with Pro32, Phe34, and Thr36, and thus shows high flexibility. Catalysis starts with the abstraction of a proton from C3-OH of shikimate by the $N_{\varepsilon 2}$ atom of His162 supported by Thr36. This enables a nucleophilic attack of the formed oxyanion on the γ-C of 4-coumaroyl-CoA. After protonation, CoA is released. Calorimetric data indicated that 4-coumaroyl-CoA binds first, then the acceptor substrate. Substrate discrimination against quinate was mediated by a steric clash with Pro32. Similar results were obtained for HST from *Panicum virgatum* (PvHCT2a) (PDB ID 5FAL, 5FAN) (31) (**Figure 1*c***).

The structure of HST from *A. thaliana* (AtHCT) was solved in the native form (PDB ID 5KJS) (**Figure 1*c***) and complexed with 4-coumaroyl-CoA (PDB ID 5KJT) and 4-coumaroylshikimate (PDB ID 5KJU) (81). This enabled comparisons between the apo-form and forms with bound donor substrate and product. The catalytic His153 showed a switch-like conformational shift, while Arg356 shifted more inside upon substrate/product binding. The volume of the active site decreased by 28.2% and 52.5% after binding of 4-coumaroyl-CoA and 4-coumaroylshikimate, respectively. The acceptor substrate specificity and fixation were related to the so-called arginine handle (Arg356) and the surrounding amino acids 354–362, Thr369, and Trp371. Changes in this region alter substrate acceptance. Mutation of Arg356 completely abolished enzyme activity. Similar observations were made for HST from *Selaginella moellendorffii* (SmHCT) (17) (**Figure 1*c***).

Ester-forming hydroxycinnamoyl- and benzoyltransferases. Less abundant hydroxycinnamic acid esters and the enzymes involved in their formation are summarized in

Supplemental Material >

Supplemental Table 4. Benzoyl-CoA:benzyl alcohol benzoyltransferase from *Clarkia breweri* forms volatile esters but also accepts acetyl-CoA and cinnamoyl-CoA as donors (22). Rosmarinic acid synthase (RAS; hydroxycinnamoyl-CoA:hydroxyphenyllactate HCT) from *Coleus blumei* (syn. *Plectranthus scutellarioides*, Lamiaceae; CbRAS) (**Figure 1*c***) transfers various hydroxycinnamoyl units to the aliphatic OH groups of phenyllactate derivatives (11, 127). RAS has been isolated and characterized from other Lamiaceae species (29, 75, 124, 126, 145, 161), *Phacelia campanularia* (Boraginaceae) (82), and *Sarcandra glabra* (Chloranthaceae; P. Bömeke and M. Petersen, unpublished results). In contrast to Lamiaceae RAS, RAS from Boraginaceae displayed higher sequence identity to spermidine HCTs and lower catalytic efficiencies (82), indicating that different RAS genes have undergone repeated evolution. RAS exhibits increased substrate promiscuity and catalyzes ester as well as amide formation (75, 127). CbRAS was crystallized in complex with 4-coumaroyl-4′-hydroxyphenyllactate (PDB ID 6MK2), showing the typical two-domain structure of BAHDs with the substrate/product binding site in the solvent channel; PcRAS from *Phacelia* was modeled accordingly (82). Although overall sequence similarity between CbRAS and PcRAS was low due to the independent evolutionary origin, amino acid replacements in positions relevant for substrate binding were conservative exchanges, yielding similar functionality. The Arg handle conserved in HST was replaced by Lys in both RAS sequences, revealing that this Lys has the same function for acceptor binding.

BAHD-catalyzed ester formation with other substrates, for example, malate, tartrate, glucarate, galactarate, or piscidate, is summarized in **Supplemental Table 4**.

Amide-forming hydroxycinnamoyltransferases. *N*-HCTs (amide-forming) are found in all BAHD acyltransferase clades except clades 2 and 7 (**Figure 1*c***). Other *N*-acyltransferases are ascribed to a different superfamily: the GNAT superfamily containing tyramine HCT–like enzymes (123). Clade 4 HCTs use agmatine, tyramine, tryptamine, serotonin, and putrescine as acceptors (**Supplemental Table 5**). They are mainly found in members of monocotyledonous plants. Agmatine is also the acceptor for the only known clade 1 HCT in *A. thaliana* (AtACT) (**Figure 1*c***) induced by fungal infection (99). The members of other clades mainly use spermidine and in some cases acylated spermidine, spermine, or (5-hydroxy)anthranilate (**Supplemental Table 5**). Spermine and spermidine can be substituted with one to three (spermidine) or four (spermine) hydroxycinnamoyl units. Clade 3 and 6 HCTs form mono- or dihydroxycinnamoylated products as they can use only primary amine groups for their reaction. Clade 5 HCTs acylate primary and secondary amine groups and thus catalyze multiple hydroxycinnamoyl transfers, resulting in spermidine/spermine derivatives carrying three or four hydroxycinnamoyl residues (123). In addition to hydroxycinnamoyl-CoA, some enzymes also transfer (4-hydroxy)benzoyl moieties (57, 113, 172).

Recently, the first *N*-acyltransferases, agmatine coumaroyltransferase from *H. vulgare* (HvACT) and *T. aestivum* (TaACT) (**Figure 1*c***), have been crystallized and structurally elucidated (170, 171). HvACT was solved as the apo-form (PDB ID 7CYS; resolution 1.81 Å) and strongly resembled the structures of *O*-HCTs. The catalytic His152 of the HXXXDG motif faces the wall of the solvent channel. Mutagenesis of this His to Ala resulted in severe loss of activity (170). TaACT was crystallized and structurally elucidated as the apo-form (PDB ID 7DTP; resolution 2.3 Å). Some structural differences from HvACT were observed, mainly the orientation of Phe39 involved in the hydroxycinnamoyl binding pocket (171).

Flavonoid and Anthocyanin Acyltransferases

Acylation is a commonly occurring decoration of flavonoids and is distributed among a wide range of species as well as in different tissues and developmental stages (7). Acylation is catalyzed by

various acyltransferases, primarily BAHDs or SCPLs (5, 20), which transfer the acyl group to the sugar moieties of the flavonoids.

Flavonoid-acylating BAHDs are members of clade 1 (14, 21) (**Figure 1*c***). Acylation plays key roles in the alteration of flavonoids and anthocyanins, affecting their solubility, stability, and interaction with other molecules (155). It also confers protection from adverse ultraviolet (UV) radiation (148), acts as a signal for transport and/or storage, and confers specific hues to tissues (10).

BAHDs catalyze the transfer of acyl groups as either CoA-activated aromatic acids (including caffeate, 4-coumarate, ferulate, gallate, 4-hydroxybenzoate, or sinapate) and/or aliphatic acids (such as acetate, malate, malonate, oxalate, or succinate) (5). Given this wide range of donors and acceptors, the diversity of potential products is considerable. That said, phenylacylated flavonoids are by far the best characterized of these products, exhibiting increased solubility and higher stability under acidic pH.

Enzymes catalyzing the (phenyl)acylation of anthocyanins were among the first biochemically characterized BAHDs, namely the *Gentiana triflora* anthocyanin-5-Glc-6″-*O*-phenylacyltransferase (Gt5AT) (41) (**Figure 1*c***). Two genes encoding anthocyanin-3-*O*-glucoside 4-coumaroyltransferases have been characterized in *Arabidopsis*, the major anthocyanins of which are acylated with 4-coumaroyl, sinapoyl, and malonyl moieties (24, 84, 125) (see At5MAT and At3AT1/AtAT2 in **Figure 1*c***). Acylated anthocyanins are also of great interest for the food industry given the effect this modification has on color and stability under processing protocols (5, 125).

The enzymology of the acylation reactions has long been studied, and several comprehensive reviews cover this in detail (see, for example, 102, 160). In brief, the following are critical features of the enzymes: (*a*) Do they specifically or generally use an acyl donor such as malonyl-CoA or acetyl-CoA; (*b*) are the acceptor types specific to subsets of anthocyanins such as pelargonidin or flavonols such as astragalin or are they rather general (155); (*c*) are there sequence signatures in the case of specificity? Recently, these features have been studied in gentian, tomato, and grape (98, 122, 149). In parallel, metabolite or protein structural biological approaches have yielded several important insights. First, Unno et al. (153) described crystallographic and mutational analyses of *Chrysanthemum* petal acyl-CoA-dependent anthocyanin acyltransferases, revealing the residues responsible for acyl acceptor specificities as well as providing the first structure of the enzyme complexes with acyl-CoA (see Dm3MAT3 in **Figure 1*c***). Second, the structural analysis of a *G. triflora* anthocyanin 5,3′-aromatic acyltransferase also uncovered residues for substrate specificity as well as suggested that acyl transfer selectivity is most likely determined by the C-terminal lobe of the protein (98). Montbretia BAHDs (CcAT1 and CcAT2 in clade 6) (**Figure 1*c***) acylate myricetin-rhamnosyl-glucoside using caffeoyl-CoA to form mini-montbretin A (mini-MbA), the third step of the MbA assembly pathway (56). MbA is a highly potent inhibitor of human pancreatic α-amylase (HPA) (164), suggesting that this unusual activity may possess additional ecological roles that remain to be investigated.

Acylsugar Acyltransferases

Acylsugars constitute specialized metabolites found in many unrelated plant families such as Solanaceae, Convolvulaceae, Caryophyllaceae, and Gentianaceae (69); however, a unique class of acylsugars in the Solanaceae family has been the most extensively studied from a biochemical standpoint (32, 90). These Solanaceae-type acylsugars are produced primarily in type I/IV trichomes and are implicated in insect defense and possibly desiccation tolerance (37). They comprise a sucrose, glucose, or, rarely, inositol core esterified with 2–12 carbon long aliphatic acyl

chains (32, 79). While fatty acid and amino acid biosynthetic enzymes are involved in generating the components (36, 84, 108), the core pathway consists of ASATs that are expressed in the type I/IV trichome tip cells (32). There are variable numbers of ASATs in different species; while cultivated tomato (*S. lycopersicum*) has four ASATs (ASAT1–ASAT4), another species (*Salpiglossis sinuata*) is predicted to have at least five. Starting from sucrose, each ASAT typically adds acyl chains to a specific hydroxyl on the sugar molecule. However, building from this simple template, the rapidly changing substrate specificity of the ASATs has resulted in a wide range of acylsugar structural differences within and between species. In *S. sinuata*, for example, over 400 acylsugar-like features can be detected from the surface of a single leaf. In other species such as *Solanum pennellii*, the products of these ASATs are further modified by enzymes such as acylhydrolases and invertases, further expanding acylsugar diversity.

Mechanistic studies of ASATs have revealed that their acceptor substrate preferences change rapidly (34, 35). Even within species of the Lycopersicon section of the *Solanum* genus, the orders of ASATs are switched in some species, producing flipped pathways and generating acylsugar structural diversity (reviewed in 32). In *S. lycopersicum*, SlASAT2 uses monoacylated sucrose and catalyzes the second acylation step in the pathway. In *Solanum habrochaites*, orthologs of the same enzyme can use both mono- and diacylsucroses. However, in *S. pennellii* (whose geographical species range lies in Peru), the activity retained in northern Peru accessions acylates both mono- and diacylsucroses, while the one retained in southern Peru acylates only diacylsucroses. These activity differences were traced to three amino acids in the ASAT2 enzyme, whose combinations resulted in promiscuity and activity shifts (33).

A similar dynamic is seen for ASAT3 between these species. While SlASAT3 uses diacylsucrose and adds an acyl chain to the furanose ring of the sugar, its ortholog experienced duplication in the ancestor of *S. pennellii* and *S. habrochaites*, producing two variants ASAT3-P and ASAT3-F (129). One of the duplicate copies (ASAT3-F) was lost in *S. pennellii*, but both were retained in *S. habrochaites*. *S. habrochaites* thus has two ASAT3 activities, the ancestral activity acylating diacylsucrose on the furanose ring (ShASAT3-F) and a novel activity acylating monoacylsucrose on the pyranose ring (ShASAT3-P). *S. pennellii* only has the novel activity (SpASAT3-P) (129). Elegant phylogeny-guided experiments identified specific amino acid changes in the active site that resulted in ASAT2 and ASAT3 activity differences (33). This approach revealed that the patterns of acylation in *S. habrochaites* and *S. pennellii* are also population dependent (65, 82, 108). These differences were linked to specific amino acid changes in the ASAT enzymes between different populations, which could be associated with population migration and expansion along the native Andean mountain range of these species (74). Comparative studies of ASAT evolution across the broader Solanaceae family, that is, in *Petunia axillaris* (100), *S. sinuata*, and *Hyoscyamus niger* (90), further revealed evolutionary dynamics resulting in the reorganization of the entire acylsugar biosynthetic pathway across the family.

The acceptor substrate diversity of ASATs is evidenced by other enzymes such as ASAT4 and ASAT5 (which acylate sucrose-core acylsugars), inositol-acylating acyltransferases in *Solanum quitoense* (78, 79), and glucose-acylating acyltransferases (84) appearing in phylogenetic subclades that are unrelated to the ASAT1/ASAT2/ASAT3 subclade. Unlike most other BAHD clades, clade 3, to which ASATs belong, is composed of a mixture of acceptor substrate types, including alkaloids, anthocyanins, and alcohols. The donor substrate preference of ASATs is also enzyme-dependent. While some ASATs such as SsASAT1 can use a number of acyl-CoAs from 2 to 12 carbons long (90), others such as ASAT4 (using acetyl CoA) are much more specific. Despite a multitude of products that ASATs can theoretically make, the eventual acylsugar profile in a species is dictated by substrate preferences of subsequent ASATs, effectively

narrowing the realized acylsugar structural space. Together, these findings highlight the functional malleability of ASATs and how changes at the amino acid level influence ASAT substrate preferences and promiscuity, leading to biochemical variation in individuals, populations, genera, and families.

Alkaloid Acyltransferases

Heterocyclic nitrogen-containing compounds produced by plants have a wide and varied origin for their precursor substrates. This gives rise to the vast diversity in plant-derived alkaloids. It is not surprising, therefore, that the acyltransferases involved in modifying alkaloids exhibit a scattered distribution in their relatedness within the BAHD superfamily. Furthermore, the acyl transfer reactions for alkaloid-related BAHD members depend on their modification of hydroxyl or amine moieties within the alkaloids.

Several of the first BAHD family members to be biochemically characterized are alkaloid acyltransferases. These include enzymes involved in the production of vindoline from *Catharanthus roseus* (71). Interestingly, the first reported crystal structure of a BAHD enzyme was that of vinorine synthase (*Rauvolfia serpentina*, RsACT) (**Figure 1*c***), and it confirmed several key properties shared by canonical BAHD members, including their monomeric active forms that consist of two domains and almost equal numbers of β-strands and α-helices (86).

In the biosynthesis of another well-known alkaloid, capsaicin, the terminal step of the pathway involves *N*-acylation of vanillylamine with a fatty acyl-CoA donor of variable length, which is in turn derived from the elongation of a branched-chain amino acid. The amine substrate of this reaction (vanillylamine) and the presence of an acyl donor, although aliphatic, make this reaction similar to those catalyzed by other *N*-acylating BAHD transferases, most of which are part of clade 4 and involved in the synthesis of other aromatic amine conjugates (113). Given that capsaicin synthase, the BAHD encoded by AT3 (a.k.a. *Pun1* in clade 3) (**Figure 1*c***), is highly insoluble, its purported biochemical activity has never been reported. Proof of the role of AT3 in the accumulation of capsaicinoids remains entirely based on genetic studies, although, in the absence of biochemical data from AT3, the possibility of other non-BAHD acyltransferase genes showing capsaicin synthase activity cannot be entirely discarded.

The pharmaceutically important anticancer drug paclitaxel is a complex diterpenoid-alkaloid that requires five acyl modifications (158) resulting in both ester and amide formation (106). Obtaining soluble protein in heterologous expression systems is a common problem with BAHD enzymes and is observed for several members of the paclitaxel-related BAHD members. In order to increase solubility, two point mutations (Q19P and N23K) were introduced into the enzyme 2-*O*-benzoyltranserase (104). More than a fivefold increase in solubility was obtained, while also achieving slightly higher turnover rates and nearly tenfold higher catalytic efficiencies. In addition, the regiospecificity and regioselectivity of several of the acetyltransferases can be attenuated, depending on the order of deacetylated substrates provided (109). This could provide a way in which the semisynthetic production of novel taxane structures could be produced for future pharmaceutical development.

The reaction mechanisms of alkaloid BAHDs have also been studied through substrate docking and molecular modeling studies based on their crystal structure. The catalytic mechanism of the BAHD responsible for producing the neurotoxic compound β-*N*-oxalyl-L-α,β-diaminopropionate (β-ODAP) was studied by running over 385 different docking simulations using a multitude of acyl substrates. The study used the solved structure of β-ODAP synthase to predict that both substrates interact along a V-shaped active site tunnel and that a proton shuttle mechanism via conserved residues Asp166 and His162 is likely to be critical for catalysis (44).

Glucoarabinoxylan and Lignin Acyltransferases

Commelinids: a clade of monocotyledonous plants (orders Commelinales, Arecales, Poales, and Zingiberales) distinguished by the presence of ferulic acid in their cell walls

Glucoarabinoxylan (GAX): hemicellulose type (mostly formed from xylose and arabinofuranose) that is particularly abundant in primary cell walls of grasses and other commelinids

Acylation is also a common chemical modification found in different polymers of the cell wall of monocots. In the commelinid clade (which includes grasses, palms, bromeliads, and Zingiberales), up to 40% of the polysaccharide matrix is made up of glucoarabinoxylan (GAX), whose arabinofuranosyl moieties are frequently esterified with ferulic acid (FA). Oxidation of these FA residues, giving rise to diferulate, allows the formation of links between adjacent GAX strands in hemicellulose. The presence of FA residues on GAX also allows cross-linking with lignin monomers and oligomers during lignification. The extent of GAX feruloylation is therefore related to digestibility of the biomass, with higher amounts of FA/diferulate conferring an increased recalcitrance in the conversion of biomass to ethanol. In cell walls from grasses, lignin and, more rarely, GAX, can also be esterified with another hydroxycinnamate, 4-coumarate, which, although it does not form dimers, can be readily oxidized to its radical, and can thus cross-link monolignols and be incorporated into the lignin polymer (25).

Given the interest that acylation of GAX can have in the industrial processing of biomasses, several efforts have been made in the study of (di)ferulate distribution in the cell walls of monocots and in the search for the potential genes able to acylate GAX and lignin. Initially, several putative hydroxycinnamoyltransferases, classified within PF02458 (transferases), were identified from screening collections of expressed sequence tags (ESTs) of various species. The rationale of this approach was based on the assumption that potential feruloyltransferase and 4-coumaroyl transferase, acting on GAX, were overrepresented in EST data sets from monocots and, in particular, from Gramineae (rice, barley, and wheat) with respect to their orthologs in dicots, whose primary cell walls generally have a low amount of GAX and lack ferulate esters (128). This initial survey of ESTs across monocots and dicots led to the identification of a new group of putative BAHDs, which has been found to be almost exclusive of commelinid monocots (175). Since then, several in-depth studies have focused on the characterization of some of these BAHD members from grasses.

Very little is known about the location where the GAX acylation reactions might occur in the cell. GAX and in general all xylan backbones are assembled in the Golgi apparatus. The initial hypothesis of a transport of feruloyl-CoA and 4-coumaroyl-CoA across the Golgi membrane, with the subsequent acylation of GAX taking place in this compartment, seemed intuitive. However, given that BAHDs are cytosolic, it seems more probable that the acylation reaction may occur in the cytosol, with the BAHD using uracil-diphosphate (UDP)-arabinofuranose as a substrate and converting it to a UDP-acylated-arabinofuranose. This product can then be transported through the Golgi membrane and finally incorporated into the growing GAX backbone by the action of glycosyltransferases. This latter model seems more consistent with the majority of BAHDs acting in the cytosol and with the Golgi localization of various glycosyltransferases involved in the synthesis of xylan (6, 18).

In addition to their role as modifiers of hemicelluloses, some BAHD enzymes are also implicated in lignin modification. Lignin is a major component of secondary cell walls; depending on the species, tissue, and specific developmental stage, a significant fraction of lignin may be acylated with acetate, 4-coumarate, 4-hydroxybenzoate, and ferulate (61). The current model is consistent with acylation occurring in the cytosol on monolignol monomers (4-coumaroyl, coniferyl, and sinapyl alcohol), which are then transported to the apoplast and incorporated, as acyl conjugates, in lignin polymers. The transport of acylated monolignols to the apoplast is not well understood, nor are the biological implications of such chemical modifications of the lignin (27). Lignin acylation can provide increased defense against plant stresses or modify the rate of its polymerization. The knowledge accumulated so far about the BAHD genes responsible for the acylation of monolignols

may be helpful in designing strategies to optimize lignin composition and amount in biorefinery processes.

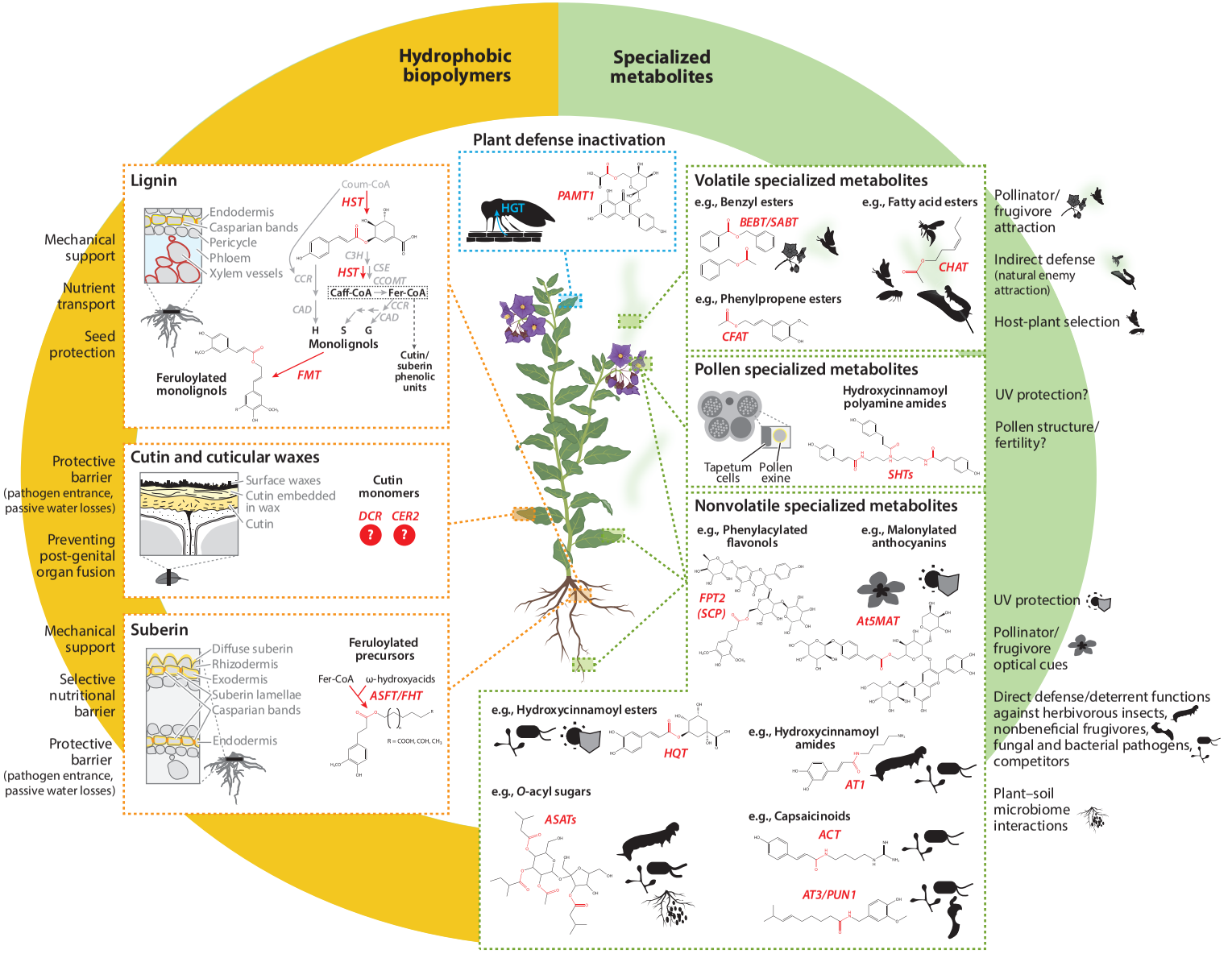
Perhaps the best of these examples comes from the discovery of a feruloyl-CoA monolignol transferase (FMT) from the Chinese angelica (*Angelica sinensis*), a dicotyledonous medicinal plant widely grown in China. AsFMT, part of clade 3 (**Figure 1*c***), is rather specific in accepting only feruloyl-CoA as the acyl donor but is pliable in accommodating all the three canonical monolignols as acyl acceptors (163). When *AsFMT* was used to stably transform poplar, the acylated monolignols were shown to be incorporated, through the formation of labile ester bonds, into the growing lignin polymers. In both cases, upon mild alkaline treatment, lines with increased levels of feruloylated conjugates showed an increase of saccharification yield (163). Most of the monolignol transferases characterized to date are from monocots and are strictly related to those GAX-specific acyltransferases that are also part of clade 6 (**Figure 1*c***), which specifically expanded in commelinid monocots (62, 138).

FUNCTIONAL IMPORTANCE OF BAHDS

Role of BAHDs in the Conquest of Land and Resilience to Abiotic Stress

BAHDs play a role in both primary and specialized metabolism, and thus their activities are critical across growth, reproduction, and stress responses. The BAHD enzyme family expansion coincides with the emergence of land plants (70), raising the possibility that BAHDs played a role in the adaptation to land life. There are four main polymers that are needed for land plants in order to provide stability and waterproof their cell walls—lignin, sporopollenin, suberin, and cutin—and BAHDs play a role in the biosynthesis of all of them (107). From a phylogenetic perspective, BAHDs involved in lignin biosynthesis and those important for the synthesis of cutin and suberin are the most broadly conserved, with orthologs being found in all land plant lineages (70, 121) (**Figure 1*b*,*c***). The widespread occurrence of these BAHD clades indicates that these enzymes and their associated activities likely emerged in the last common ancestor of all land plants and may have assisted in the conquest of land.

Lignin and plant architecture. Because of its importance to humans, for example, in the food, paper, construction, and biofuel industries, lignin biosynthesis has been studied in detail. Lignin is synthesized mainly from monolignols (coumaroyl, coniferyl, and sinapyl alcohols). By polymerization of the different monolignols, the varied units of lignin are generated: H (4-hydroxyphenyl) units from coumaroyl alcohol, and G (guaiacyl) and S (syringyl) units from coniferyl and sinapyl alcohol, respectively (154) (**Figure 2**). A key enzyme in this pathway is HST (see the section titled Hydroxycinnamoyltransferases), which synthesizes central intermediates in the monolignol and phenylpropanoid biosynthesis and is conserved across land plants. HSTs from nonseed plant lineages such as *M. polymorpha*, *Physcomitrium patens*, and *S. moellendorffii* are able to complement *A. thaliana* HST T-DNA insertion lines, indicating conserved functions (17, 68). In planta evidence from *P. patens* loss-of-function mutants suggests that PpHST is also important for the biosynthesis of caffeoylthreonate esters that are involved in the formation of an intact cuticle (17). Loss-of-function assays in vascular plants support the central role of HST in plant growth and development. For example, RNA silencing of HST in *Arabidopsis* and *Nicotiana benthamiana* resulted in a dwarf phenotype as well as drastic changes in the lignin composition. Specifically, larger ratios of H to S units were observed, consistent with the role of HST in the synthesis of G and S units (53). Altered lignin phenotypes upon HST silencing—a 31% increase of H units in comparison to wild-type plants and a 42% decrease in lignin content in tracheary elements—were also observed in gymnosperms (*Pinus radiata*) (156).



(Caption appears on following page)

Figure 2 (*Figure appears on preceding page*)

Roles of BAHD enzymes in plants' adaptation to the terrestrial environment and engagement in ecological interactions. (*Left*) Current knowledge of the role of BAHDs in the biosynthesis and modification of monomers for the production of apoplastic biopolymers acting as hydrophobic protective barriers. (*Right*) The cornerstone role of BAHDs in propelling specialized metabolism diversity and providing a chemical playground for plant–environment interactions. Focus is placed on BAHD-dependent metabolites with demonstrated functions in the context of plants' biotic interactions. Abbreviations: ACT, agmatine coumaroyltransferase; ASAT, acylsugar acyltransferase; ASFT, aliphatic suberin feruloyl transferase; AT1, acyltransferase1 (hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyl transferase); At5MAT, *Arabidopsis thaliana* anthocyanin 5-*O*-glucoside-4‴-*O*-malonyltransferase; BEBT, benzoyl-CoA:benzyl alcohol benzoyltransferase; Caff-CoA, caffeoyl-coenzyme A; CER2, ECERIFERUM2; CFAT, coniferyl alcohol acetyltransferase; CHAT, acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase; CoA, coenzyme A; Coum-CoA, Coumaroyl-CoA; DCR, Defective of cuticular ridges; Fer-CoA, feruloyl-CoA; FHT, feruloyltransferase; FMT, feruloyl-CoA monolignol transferase; FPT2, flavonol-phenylacyltransferase 2; HGT, horizontal gene transfer; HQT, hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase; HST, hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase; PAMT1, plant-derived phenolic glucoside malonyltransferase1; PUN1, Pungency locus1; SABT, benzoyl-CoA:salicyl alcohol *O*-benzoyltransferase; SHT, hydroxycinnamoyl-CoA:spermidine hydroxycinnamoyltransferase; UV, ultraviolet.

HST activity results in the production of intermediates that can lead to multiple metabolite classes relevant for growth and adaptation, such as phenylpropenes, hydroxycinnamates, catechins, flavonoids, and anthocyanins. Furthermore, duplications of HSTs coupled with functional associations with other enzymes have also given rise to or altered other metabolic phenotypes important for adaptation, for example, chlorogenates (140), rosmarinate biosynthesis in Lamiaceae (115), and possibly hydroxycinnamoyl amides in Brassicaceae and other eudicots (117).

Sporopollenin and the stress resilience of spores. BAHDs also play functionally important roles in pollen wall generation and are potentially involved in the biosynthesis of sporopollenin, characterized as one of the strongest biopolymers on earth (49). Sporopollenin was proposed to have emerged prior to the conquest of land (121). Loss-of-function mutant phenotypes of *Arabidopsis* spermidine hydroxycinnamoyltransferase (AtSHT, clade 5) (**Figure 1*c***) suggested that cinnamoyl spermidine derivatives are involved in the formation of the pollen wall (48). The rice *defective pollen wall 2* (*dpw2*) mutant of a BAHD acyltransferase, which shows complete male sterility and abnormal anther cuticle, transfers hydroxycinnamoyl groups to ω-hydroxy fatty acids in the tapetum of the anther, the site of sporopollenin biosynthesis (159, 168). The incorporation of coumaroyl moieties esterified with ω-hydroxy fatty acids suggests the involvement of BAHDs. The fixation of the BAHD activities in pollen wall formation may have assisted in producing more stress-resilient spores adapted to dry land climates.

Cuticular waxes and drought. Cutin, which is part of the cuticle, is another critical biopolymer for the conquest of land. Cutin, cutan, suberin, and other extracellular waxes such as polyesters found in the stigma and in sporopollenin provide a protective layer on the surface of plants to withstand water loss and uncontrolled water absorption and to provide protection against pathogens, and thus their evolution was important in conquest of land (118). Cutin is typically found at the surface of aboveground tissues and in the root cap and lateral roots, while suberin is primarily present in the root endodermis and periderm (38, 39). Cutin typically consists of C16 and C18 ω-hydroxy fatty acids, while suberin is predominantly built from longer-chain fatty acids (>C20), incorporating phenylpropanoids (e.g., ferulate), fatty alcohols, glycerol, and a high level of α,ω-dicarboxylic acids (118, 119). Several BAHDs involved in the biosynthesis of these polymers are distributed across three clades: Defective of cuticular ridges (DCR)-like BAHDs in clade 1, ECERIFERUM2 (CER2)-like BAHDs in clade 2, and several others in clade 6 (45, 93, 111) (**Figures 1*c*** and **2**).

Of these three groups, clade 6 BAHDs are the most widely conserved across land plants. Two of the characterized enzymes in this clade are *M. emarginata* feruloyl transferases (MeHFT) and

A. thaliana aliphatic suberin feruloyl transferase (AtASFT), which are involved in suberin formation. AtASFT loss-of-function mutants contain severely reduced levels of ferulate monomers in root and seed coat suberin, and biochemical assays confirmed that ASFT is responsible for the transfer of the ferulate moiety to ω-hydroxy fatty acids (46, 94). The findings for AtASFT were corroborated by studies of homologs in other species such as potato and poplar (16, 134). Although more studies are needed to define the true role of ferulate esters in the suberin macromolecule (133), they are believed to act as a linker by forming C-C and ether bonds, enabling the linkage of suberin to the cell wall (39). The DCR-like BAHDs found in clade 1 have only been studied using knockout or knockdown assays in multiple plant species; however, the major evidence for their activities stems from *Arabidopsis*. DCR-like mutants were not able to properly incorporate 9(10),16-dihydroxy-hexadecanoic acid into the cutin polymer and showed severe phenotypes after exposure to stresses such as water deprivation, high salt, and osmotic stress (76, 111). Several possible activities for DCR-like BAHDs have been proposed, but it is likely that this enzyme catalyzes an acyl exchange to provide a more suited substrate to the cytochrome P450 enzyme CYP77A6 in the suberin assembly pathway (93).

CER2-like BAHDs (clade 2) are required for cuticular wax production in *Arabidopsis* and maize via very long chain fatty acid elongation [>C26 (108) or C28 (88)]. Very long chain fatty acids are required in all plant cells for the production of sphingolipids and in specific cell types for the synthesis of other very long chain fatty acid derivatives such as cuticular waxes, pollen coat, and suberin (59). *Arabidopsis* CER2 mutants show strong wax-deficient phenotypes and a lack of >C28 cuticular wax components, giving the plants a glossy appearance (66). The *glossy2* mutant in maize also shows a similar phenotype, suggesting functional conservation in angiosperms (51, 52, 113). Male sterility was also observed in CER2 mutants under drought conditions, indicating the importance of CER2-like BAHDs for proper pollen coat formation and the adaptation to dry conditions (120). Several mutant phenotypes that cause small changes in carbon chain length of cuticular waxes have strong effects on the physiological properties of the cuticle, affecting water repellency, particle adhesion, light reflection, and herbivore resistance (50, 51, 112).

Flavonoids, hydroxycinnamoyl amides, and ultraviolet protection. Acylation of flavonoids is among the most-characterized of BAHD-dependent functional modulations. More specifically, phenylacylation, which is a commonly described final step in anthocyanin biosynthesis, affects thermostability and the transport of flavonoids. This modification appears to confer enhanced UV-B absorption, particularly in the 310–360-nm range of the UV-B spectrum (43). This functionality is likely provided by the formation of a so-called bridge-piled structure between the A ring of the flavonoid backbone and the phenolic ring within the acyl group, resulting in an intramolecular copigmentation-like effect (58, 148).

Another broad class of BAHD-dependent specialized metabolites that likely functions as a chemical shield against UV is the hydroxycinnamoyl amides. These metabolites accumulate at high levels in the floral parts of many plant families and likely contribute to UV protective functions (123). This functional prediction is reinforced by the chemical identification in members of the Asteraceae of tetra-substituted spermine amides characterized by broad UV absorbance spectra (10). However, functional evidence for a role in UV adaptation remains unclear.

The Chemical Ecology of BAHD-Derived Metabolites

In parallel to their roles in biopolymer synthesis, BAHDs also contribute markedly to plant specialized metabolite diversity. In concert with other large gene families of decorating enzymes, for example, cytochrome P450s or UDP-glycosyltransferases, BAHDs carry out chemical modifications of small molecule scaffolds, thereby propelling specialized metabolite structural diversity and

providing a chemical playground for plant–environment interactions. Important progress has been made within specific metabolic classes in connecting BAHD-dependent biochemistry to specific functional modulations that are likely important for plant adaptation to environment (**Figure 2**).

Indirect defense: inducible trait via the production of chemicals advertising to predators the presence of actively feeding insect herbivores

Phytoanticipins: preformed specialized compounds in plants that act in defense against pathogens and herbivores and can be released after attack

Optimal defense theory: predicts higher defense allocation to tissues and developmental stages with the highest contribution to fitness and/or probability of herbivory

Flavonoids, anthocyanins, and pollinator attraction. An important ecological dimension to BAHD functions in flavonoid metabolism is readily inferable from the variation and patterning of flower petal hues—traits for which anthocyanins frequently act as core pigments and which are part of the (optical) strategies used by plants to attract and guide pollinators. Acylation increases anthocyanin stability and increases their vacuolar uptake and sequestration. A study on the evolution of the spatial pigmentation of *Mimulus* flowers in regard to bumblebee versus hummingbird pollination detected an important MYB transcription factor controlling the flux between anthocyanin versus flavonol conditions and subsequently on dependent BAHDs (174). In sexually deceptive orchids, visual mimicry has, in several examples, been linked to the complex and tissue-specific coordination of higher levels of specific acylated anthocyanins (165).

Floral volatiles and pollinator attraction. More direct functional links exist for the widespread effects of BAHDs on flower–pollinator interactions via the biosynthesis of floral volatiles. A long list of volatiles dominating floral scents have their biosynthesis anchored on BAHD-dependent acylation reactions, with emblematic examples of aliphatic and aromatic alcohol esters such as esters of (*Z*)-3-hexen-1-ol in many angiosperms (23); isoeugenol, produced from coniferyl acetate as intermediate in *Petunia* (26); and esters of benzoate, whose biosynthesis was initially characterized in *Clarkia* and *Petunia* (13). Of central importance is the multifunctionality of these floral bouquets sculpted by the balancing selection pressures exerted by pollinators and florivores (63). For example, in *Petunia* isoeugenol and benzoate esters also act as florivore deterrents in addition to their predicted pollinator attraction functions (63). This differential chemical ecology of BAHDs also extends to other volatile-emitting plant parts such as fruits and leaves (47). For example, C6 esters such as those of (*Z*)-3-hexen-1-ol are ubiquitous components of foliar herbivory-induced volatiles, acting as indirect defenses via recruitment of natural enemies (4) but also in host-plant selection for oviposition and herbivory (167) (**Figure 2**).

BAHD products in insect and pathogen defense. Structurally diverse BAHD-dependent specialized metabolites accumulate in tissues/organs that are decisive to a plant's fitness. This accumulation occurs either constitutively, with many of these phytoanticipins being distributed according to the optimal defense theory, or specifically, upon interactions with pathogens and insect herbivores, for example, hydroxycinnamoyl amides in Solanaceae (42). Beyond these correlations, rigorous demonstrations of the true fitness benefits of BAHD products during these interactions are, however, often lacking. Previously mentioned BAHD-dependent hydroxycinnamoyl amides and *O*-acyl sugars are classes for which firm ecological demonstrations of defense-related modes of action have been achieved (**Figure 2**). In the case of hydroxycinnamoyl amides, a large body of work indicates their role as phytoalexins against necrophytic pathogens, such as for apoplast-exported hydroxycinnamoyl putrescine and agmatine amides in *Arabidopsis* and potato leaves infected by *Phytophthora infestans* (28), and as antiherbivore defenses (8). Acylated putrescines are structurally reminiscent of neurotoxins present in spider and wasp venoms and could exert defensive functions against insects as poisons of neuromuscular junctions. *O*-acyl sugars produced by glandular trichomes and covering aerial surfaces of Solanaceae plants act as defense molecules against native fungal pathogens (85) and as chemical glue entrapping small insects, while those exuded from roots influence rhizobiome recruitment (67). Most strikingly, field experiments have shown that *O*-acyl sugars from wild tobaccos, when ingested by larvae of the lepidopteran insect *Manduca sexta*, have their ester bonds—the direct translation of ASAT BAHD

activities—hydrolyzed in the alkaline larval midgut, thereby releasing volatile short acyl chains that tag these larvae and their frass with a specific odor, betraying their presence to predatory ants (162). Such postingestive odor-tagging indirect defense mechanisms critically depend on the activity of the plant's diversity of ASAT BAHDs, and biogeographical variations in acyl chain diversity within *O*-acyl sugar chemotypes of wild tomatoes could reflect on BAHD-dependent adaptations for diverse ecological interactions (129). Interestingly, the critical role of BAHDs in also shifting plant metabolite properties postingestively has been hijacked by opportunistic herbivores, as in the case of whiteflies that exploit a horizontally transmitted plant-derived phenolic glucoside malonyltransferase gene BtPMaT1 to neutralize phenolic-based defenses (166) (**Figure 2**).

Being restricted to the fruits, and acting as potent antimicrobials, capsaicinoids offer an ideal system to test their value as deterrents in wild populations of *Capsicum* (**Figure 2**). Field experiments showed mammals to be clearly deterred by pungent fruits, with the seeds they incidentally ingested not capable of germinating anymore when excreted. Capsaicin showed instead no deterrence to birds like thrashers, which fed on *Capsicum* fruits despite their pungency, and dispersed viable seeds after ingestion (146). Pungency manifests itself as a polymorphic trait in the wild, with populations of *Capsicum chacoense* showing a gradient of pungency in response to environmental heterogeneity. Polymorphism in pungency is constrained by the existence of a cline in moisture determining the differential pressure of microbial pathogens, which enter fruits through scars formed by foraging insects (147). On one hand, in a high-moisture environment, where the pressure of the pathogens is high, pungent plants predominate as capsaicin is toxic to microbes; on the other hand, in low-moisture environments, fungal infection is rare, and the proportion of pungent plants decreases.

SYNTHETIC BIOLOGY AND METABOLIC ENGINEERING USING BAHD MEMBERS

Many modern research programs that seek to understand and manipulate biological processes in plants are employing the tools of synthetic biology for such purposes. The use of BAHDs as part of the toolbox for the modulation of plant specialized metabolism has already begun and will become more prominent in the future. Pioneering metabolic engineering studies began with the introduction of single genes into different plants and microorganisms to achieve the creation of a small number of novel products. More recent reports incorporating BAHDs into pathway reconstruction experiments have been reviewed (160).

The manipulation of lignin and cell wall polymers can result in plants that are useful in the generation of biofuels. In one early example, the overexpression of rice OsAt10 drastically changed the cell wall ferulate and 4-coumarate content of glucuronoarabinoxylan (9). The reduction of ferulate substitutions would greatly aid in the digestibility of grass cell walls. In a related case, a gene encoding a feruloyl-CoA monolignol transferase was isolated from *A. sinensis* and when introduced into poplar under a 35S promoter was able to change the cell wall monolignol configuration (163). Additional manipulations were performed in poplar by introducing a rice BAHD that increased the 4-coumaroyl monolignols without negatively affecting overall plant performance (139). Similar results were achieved in a more recent study which used RNA interference to silence a hydroxycinnamoyltransferase from the grass *Setaria viridis.* Biomass saccharification increased 32% following acid pretreatment without changing total lignin amounts (97). Altering the cell wall can result in other phenotypes that are also of interest to modern agriculture. In one case, the ectopic expression of PtFHT1 from poplar in *Arabidopsis* increased the ferulate modifications in suberin as well as leaf cutin (16). The resulting root polyester changes increased salt stress tolerance in the transgenic plants.

Hydroxycinnamoyl-containing compounds are often the focus of metabolic engineering studies. Many of these metabolites have value in the nutraceutical market and are found in the so-called superfoods. In one instance, tobacco HST and HQT were introduced into *Escherichia coli* along with several other up- and downstream pathway components to ultimately produce 235 mg/L and 450 mg/L of 4-coumaroylshikimate and chlorogenate, respectively (64). A set of BAHDs capable of using both hydroxycinnamoyl- and benzoyl-CoA were introduced in a *S. cerevisiae* system alongside several other pathway-relevant genes to produce more than 30 compounds, including rosmarinate. This study was also the first to combine cocaine synthase from *Erythroxylum coca* with 4-coumarate:CoA ligase (4CL), which, when fed the precursors benzoate and 3β-tropine, produce tropacocaine and cinnamoyl 3β-tropine (31). Cocaine synthase (EcCS) (**Figure 1*c***) was later used again in a yeast-based system in which the full solanaceous tropane biosynthetic pathway was introduced (15 genes). In that case, the strain synthesized α-tropine as a substrate, and the resulting product obtained was cinnamoyl 3α-tropine (143). Interestingly, the enzyme is incapable of using α-hydroxy tropine in vitro, suggesting that stereoselectivity may be augmented based on the chosen heterologous host (130).

The exponential increase in the annotation of BAHD members due to genome sequencing projects does not extend to the determination of their biochemical functions. In an effort to utilize high-throughput screening of enzyme activities via microplate cultures, Lee et al. (77) designed a platform to study the activity of AATs. This system, which was tested using AATs from different domain families including some plant BAHDs, is capable of rapidly screening for novel wild-type chemotypes but is also amenable for use with engineered sequences for the testing of novel product formation (77). More studies that use synthetic biology tools for the purposes of pathway inference and gene discovery are being performed. The full pathway for the plant-produced salvianolic acid B is still incomplete. However, a reconstruction of the genes involved in rosmarinate biosynthesis, including the required BAHD SmRAS, was able to use endogenous *Saccharomyces* genes to substitute for the missing steps and allow for the de novo production of 30 μg/L salvianolic acid B (169).

Taking advantage of the promiscuity of certain BAHD members allows for the production of novel acylated products that might be useful for the development of pharmaceuticals. In one recent case, the use of spermidine *N*-hydroxycinnamoyltransferase in a yeast-based production system was able to produce eight novel trihydroxycinnamoyl spermidines (114). In addition, the same system was able to produce structures containing halogenated hydroxycinnamoyl groups, further enabling the possibilities for similar systems to be utilized in rational drug design. Another example of using BAHD substrate versatility is in the *S. cerevisiae* and *Lactococcus lactis* production system for the biosynthesis of clovamide. The BAHD family member HDT1 from red clover produces *N*-hydroxycinnamoyl-L-amino acids, compounds that are hard to synthesize and only present in small amounts in plants. However, when introduced with the requisite CoA-ligase gene, more than 20 novel compounds were obtained (15). Lastly, piperamide synthase from *Piper nigrum* is found in developing fruits and is coexpressed with piperine synthase, the enzyme responsible for the pungent taste of black pepper. Piperamide synthase is less restricted in its substrate specificity and can produce a wide variety of pharmaceutically relevant amides when placed in a microbial expression system (131).

CONCLUDING REMARKS

Our understanding of structure–function relationships within the BAHD family and of its evolutionary diversification during plant terrestrialization has dramatically increased within the last decade. However, our appreciation of the diversity of ecological functions fulfilled by

BAHD-dependent metabolites remains often predictive or at best partial. Hence, a more systematic exploration, guided by macro- and microevolutionary phylogenetics studies and bottom-up comparative metabolomics, of BAHD gene function in the context of abiotic and biotic stress adaptation and in ecologically relevant settings represents one of the research frontiers for this and other plant enzyme families.

SUMMARY POINTS

1. The expansion of BAHD acyltransferases (BAHDs) has given rise to at least eight clades, with enzymes involved in the biosynthesis of compounds important for growth, reproduction, and defense.
2. The BAHD family has dramatically expanded together with the radiation of land plants, and seed plant genomes typically encode dozens to hundreds of BAHDs.
3. Fresh insights into structure–function relationships within the BAHD family are being provided by a combination of crystallography, electron microscopy, and molecular dynamics.
4. Besides being important enzymes for the formation of numerous specialized metabolites involved in the adaptation of plants to their environment, hydroxycinnamoyltransferases are pivotal for the formation of monolignols and thus lignin biosynthesis.
5. BAHDs not only are used in synthetic biology systems to produce their normally catalyzed products in heterologous systems but are also being exploited to produce novel compounds useful in the pharmaceutical and nutraceutical marketplace.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Work presented in this review was supported by the Deutsche Forschungsgemeinschaft (DFG) award 411255989 (to L.H.K.). Some work presented here was conducted in connection with the Priority Program MAdLand—Molecular Adaptation to Land (SPP2237), financially supported as PE360/37-1 (to M.P.) by the DFG.

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162. This study on *O*-acylsugar multitrophic roles illustrates the importance of natural history-guided research for exploring ecological sophistication in the mode of action of BAHD-dependent metabolites.

163. A BAHD feruloyl-CoA monolignol transferase from *Angelica sinensis* was overexpressed in poplar to introduce ester labile linkages in the lignin polymers, increasing saccharification yield and demonstrating the possibility to modify lignin composition through biotechnological approaches.

166. Expands our understanding of plant BAHDs to the postingestive side of plant-insect interactions by describing the horizontal gene transfer of a plant-derived BAHD by whiteflies as a key determinant to plant toxin inactivation.