The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants

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Abstract

L-Tryptophan, L-phenylalanine, and L-tyrosine are aromatic amino acids (AAAs) that are used for the synthesis of proteins and that in plants also serve as precursors of numerous natural products, such as pigments, alkaloids, hormones, and cell wall components. All three AAAs are derived from the shikimate pathway, to which ≥30% of photosynthetically fixed carbon is directed in vascular plants. Because their biosynthetic pathways have been lost in animal lineages, the AAAs are essential components of the diets of humans, and the enzymes required for their synthesis have been targeted for the development of herbicides. This review highlights recent molecular identification of enzymes of the pathway and summarizes the pathway organization and the transcriptional/posttranscriptional regulation of the AAA biosynthetic network. It also identifies the current limited knowledge of the subcellular compartmentalization and the metabolite transport involved in the plant AAA pathways and discusses metabolic engineering efforts aimed at improving production of the AAA-derived plant natural products.

Contents

INTRODUCTION

L-Tryptophan (Trp), L-phenylalanine (Phe), and L-tyrosine (Tyr) are aromatic amino acids (AAAs) required for protein biosynthesis in all living cells. In plants, these AAAs also serve as precursors of a wide variety of plant natural products that play crucial roles in plant growth, development, reproduction, defense, and environmental responses (**Figure 1**). Trp is a precursor of alkaloids, phytoalexins, and indole glucosinolates as well as the plant hormone auxin, whereas Tyr is a precursor of isoquinoline alkaloids, pigment betalains, and quinones (tocochromanols and plastoquinone) (**Figure 1**) (105, 146). Phe is a common precursor of numerous phenolic compounds, which include flavonoids, condensed tannins, lignans, lignin, and phenylpropanoid/benzenoid volatiles (196). Of the three AAAs, the highest carbon flux is often directed to Phe, as Phe-derived compounds can constitute up to 30% of organic matter in some plant species (142, 148, 201). All three AAAs are produced from the final product of the shikimate pathway, chorismate, which is also a precursor for vitamins K_1 and B_9 and the plant defense hormone salicylic acid (**Figure 1**).

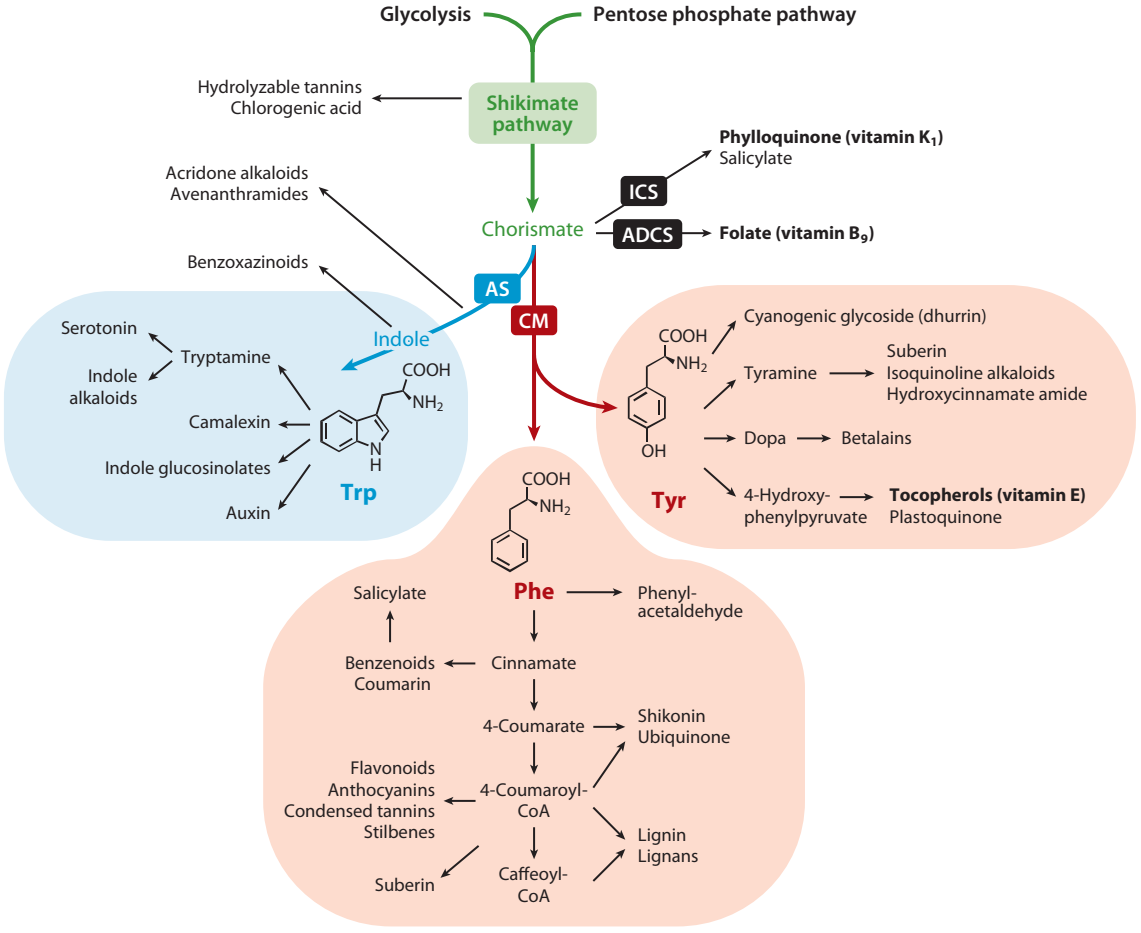


Figure 1

The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in *green*) produces chorismate, a common precursor for the tryptophan (Trp) pathway (*blue*), the phenylalanine/tyrosine (Phe/Tyr) pathways (*red*), and the pathways leading to folate, phylloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diverse array of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (*bold*). Other abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoA, coenzyme A; ICS, isochorismate synthase.

The AAA pathways consist of the shikimate pathway (the prechorismate pathway) and individual postchorismate pathways leading to Trp, Phe, and Tyr (the Trp, Phe, and Tyr pathways, respectively; **Figure 1**). These pathways are found in bacteria, fungi, plants, and some protists but are absent in animals. Therefore, AAAs and some of their derivatives (vitamins) are essential nutrients in the human diet, although in animals Tyr can be synthesized from Phe by Phe hydroxylase (53). In animals, Trp and Tyr are also precursors of the serotonin and catecholamine neurotransmitters, respectively (50, 53). Some of the aforementioned AAA-derived plant natural products have pharmacological or biological activities and thus are widely used in human medicine and nutrition (105, 112). The absence of the AAA pathways in animals also makes these pathways attractive targets for antimicrobial agents and herbicides (6).

Despite the importance of AAAs and their derivatives for both agriculture and human health, our knowledge of the AAA pathways has often been inferred from microbial studies, and as a result, the regulation of AAA biosynthesis is poorly understood in plants. This fundamental knowledge gap also creates a bottleneck in effective plant breeding and metabolic engineering for the improved production of AAA-derived target compounds. In this review, we focus on recent molecular identification and genetic analysis of enzymes involved in plant AAA biosynthesis. By integrating previous biochemical and recent genetic data, we present an overview of the transcriptional and posttranscriptional regulations of the AAA pathways. We also highlight knowledge gaps in the transport of AAAs and pathway intermediates across plastid membranes. Finally, we discuss metabolic engineering efforts and perspectives for improved production of AAAs and their downstream metabolites in plants.

THE SHIKIMATE PATHWAY

The seven enzymatic reactions of the shikimate pathway connect central carbon metabolism and the AAA network by converting phospho*enol*pyruvate (PEP) and D-erythrose 4-phosphate (E4P)—intermediates in glycolysis and the pentose phosphate pathways, respectively—to chorismate, the universal precursor for all AAAs and numerous metabolites derived from them (**Figures 1** and **2**) (12, 80, 81, 164). The pathway was named after the first identified intermediate, shikimate, which was isolated from the fruit of *Illicium religiosum* (commonly known as the Japanese star anise, *shikimi*) (19, 46). All enzymes involved in the shikimate pathway have been biochemically characterized, and the corresponding genes have been identified from both microbes and plants (**Table 1**). A full set of the shikimate pathway enzymes exists in the plastids, based on experimental evidence and predictions of their subcellular localization (**Table 1**). Whereas microbial enzymes have been subjected to extensive genetic analysis that has provided a comprehensive understanding of shikimate pathway regulation, only limited genetic studies have been performed with plant enzymes, and in plants this regulation remains poorly understood.

3-Deoxy-D-*Arabino*-Heptulosonate 7-Phosphate (DAHP) Synthase

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase catalyzes the first committed step of the shikimate pathway, an aldol condensation of PEP and E4P to produce DAHP (**Figure 2**). DAHP synthases are metalloenzymes containing a divalent metal cation (e.g., Mn^{2+} or Co^{2+}) (88, 161). All DAHP

Figure 2

The aromatic amino acid pathways in plants. One D-erythrose 4-phosphate (E4P) and two phospho*enol*pyruvate (PEP) molecules are used for biosynthesis of chorismate via seven enzymatic reactions of the shikimate pathway (shown in *green*). Tryptophan (Trp) is produced from chorismate via six enzymatic reactions of the Trp pathway (*blue*), whereas phenylalanine (Phe) and tyrosine (Tyr) are produced via three reactions in the arogenate or phenylpyruvate/4-hydroxyphenylpyruvate routes (*red*). DHD and SDH form a bifunctional DHD-SDH enzyme in plants, whereas ASα and ASβ as well as TSα and TSβ form noncovalent AS and TS enzyme complexes, respectively. The intermediates of the DHD-SDH and TS enzyme-catalyzed reactions (3-dehydroshikimate and indole, respectively) are shown in brackets. Other abbreviations: CdRP, 1-(*o*-carboxyphenylamino)-1-deoxy-ribulose 5-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; EPSP, 5-*enol*pyruvylshikimate 3-phosphate; G3P, glyceraldehyde 3-phosphate; Gln, glutamine; Glu, glutamate; α-KG, α-ketoglutarate; P_i, inorganic phosphate; PP_i, inorganic diphosphate; Ser, serine. Enzyme abbreviations: ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; ASα, anthranilate synthase α subunit; ASβ, anthranilate synthase β subunit; CM, chorismate mutase; CS, chorismate synthase; DHD, 3-dehydroquinate dehydratase; DHQS, 3-dehydroquinate synthase; HPP-AT, 4-hydroxyphenylpyruvate aminotransferase; IGPS, indole-3-glycerol phosphate synthase; PAI, phosphoribosylanthranilate isomerase; PAT, phosphoribosylanthranilate transferase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PPA-AT, prephenate aminotransferase; PPY-AT, phenylpyruvate aminotransferase; SDH, shikimate dehydrogenase; SK, shikimate kinase; TSα, tryptophan synthase α subunit; TSβ, tryptophan synthase β subunit.

synthases have a core $(\beta/\alpha)_8$ barrel monomer structure and display large variability in their peripheral small domains responsible for AAA-mediated allosteric regulation. Based on sequence similarity, DAHP synthases are classified into two types, which share <10% amino acid sequence identity. Examples of type I enzymes include DAHP synthases from *Escherichia coli* (AroF, AroG, and AroH) and *Saccharomyces cerevisiae* (Aro3 and Aro4) containing an N-terminal Phe/Tyr-binding domain (12), bifunctional chorismate mutase (CM)–DAHP synthase enzymes (e.g., from *Bacillus subtilis*) (205), and unregulated DAHP synthase possessing only the core barrel structure (e.g., from *Pyrococcus furiosus*) (167). Type II DAHP synthases are found in plants and some microbes (e.g., *Mycobacterium tuberculosis*) and

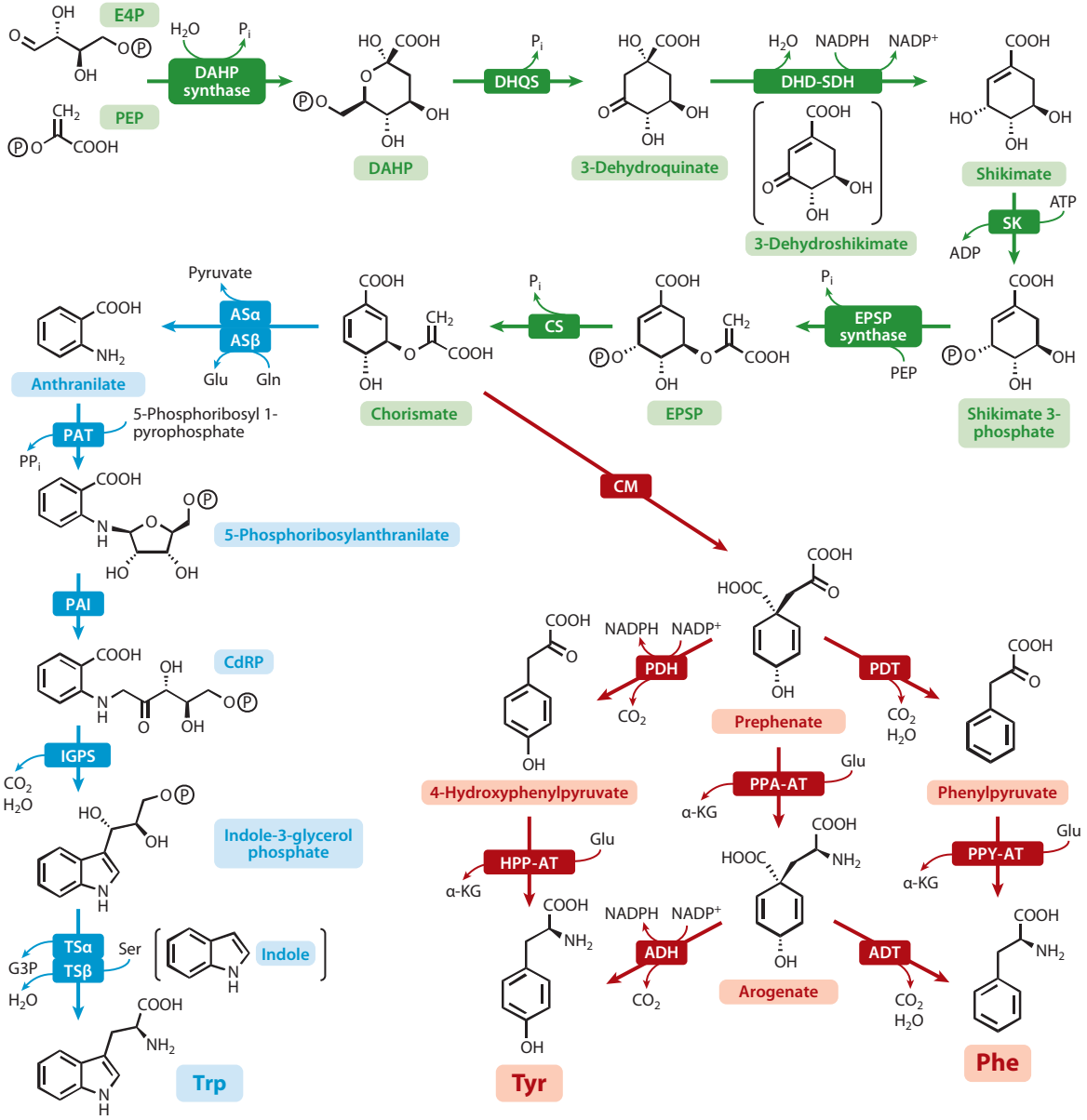


Table 1 Enzymes and genes involved in the aromatic amino acid biosynthetic pathways in plants

Enzyme name	Abbreviation	EC number	*Escherichia coli* genes	Number of isolated genes (plant species)	Subcellular localization (experimental methods)[a]	Cofactor(s)	Regulation of plant enzymes	Reference(s)
Shikimate pathway								
3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase	DAHP synthase	4.1.2.15	*AroF* *AroG* *AroH*	2 (*Solanum tuberosum*) 2 (*Arabidopsis thaliana*) 2 (*Lycopersicon esculentum*)	Plastids (F, I) (cytosol, Co^{2+}-dependent putative DAHP synthase)	Mn^{2+} (or Co^{2+})	Redox control Activation by Trp Inhibition by arogenate	44, 47, 76, 95, 127, 161, 185, 214
3-Dehydroquinate synthase	DHQS	4.6.1.3	*AroB*	1 (*Lycopersicon esculentum*)	Plastids (F)	NAD^+,[b] Co^{2+}	—	15, 127
3-Dehydroquinate dehydratase	DHD[c]	4.2.1.10	*AroD*	1 (*Lycopersicon esculentum*) 1 (*Arabidopsis thaliana*)	Plastids (F, G) (cytosol, tobacco DHD/SDH2, G)	—	—	16, 38, 127, 175
Shikimate dehydrogenase	SDH[c]	1.1.1.25	*AroE*	2 (*Nicotiana tabacum*)		NADPH	—	
Shikimate kinase	SK	2.7.1.71	*AroL* *AroK*	1 (*Lycopersicon esculentum*) 2 (*Arabidopsis thaliana*) 3 (*Oryza sativa*)	Plastids (F, I)	ATP, Mg^{2+} (or Mn^{2+})	Energy charge control	60, 61, 93, 127, 165, 166
5-*Enol*pyruvylshikimate-3-phosphate synthase	EPSP synthase	2.5.1.19	*AroA*	1 (*Arabidopsis thaliana*) 1 (*Solanum lycopersicum*) 2 (*Petunia hybrida*)	Plastids (F, I)	—	—	35, 67, 96, 127, 169
Chorismate synthase	CS	4.6.1.4	*AroC*	1 (*Corydalis sempervirens*) 2 (*Solanum lycopersicum*)	Plastids (F)	Reduced flavin mononucleotide (FMN)	Redox control	74, 128, 162
Tryptophan (Trp) pathway								
Anthranilate synthase α subunit	ASα	4.1.3.27	*TrpE*	2 (*Arabidopsis thaliana*) 2 (*Ruta graveolens*)	Plastids (F, I)	—	Inhibition by Trp	17, 18, 130, 212
Anthranilate synthase β subunit	ASβ		*TrpG*	1 (*Arabidopsis thaliana*)	Plastids (predicted)	—	—	131
Phosphoribosylanthranilate transferase	PAT	2.4.2.18	*TrpD*	1 (*Arabidopsis thaliana*)	Plastids (F)	—	—	157, 212
Phosphoribosylanthranilate isomerase	PAI	2.4.2.18	*TrpF*	3 (*Arabidopsis thaliana*)[d]	Plastids (F, I)	—	—	115, 212
Indole-3-glycerol phosphate synthase	IGPS	4.1.1.48	*TrpC*	1 (*Arabidopsis thaliana*)	Plastids (predicted)	—	—	114

Tryptophan synthase α subunit	TSα	4.2.1.20	*TrpA*	1 (*Arabidopsis thaliana*) 1 (*Zea mays*)	Plastids (F, I)	—	—	101, 145, 147, 212
Tryptophan synthase β subunit	TSβ		*TrpB*	2 (*Arabidopsis thaliana*) 2 (*Zea mays*)	Plastids (F, G)	Pyridoxal 5-phosphate (PLP)	—	106, 204, 212
Phenylalanine/tyrosine (Phe/Tyr) pathways								
Chorismate mutase	CM	5.4.99.5	*AroQ* *PheA*[e] *TyrA*[e]	2 (*Petunia hybrida*) 3 (*Arabidopsis thaliana*)	Plastids (F, G) (cytosol, *Arabidopsis* CM2)	—	Inhibition by Phe and Tyr Activation by Trp Inhibition by caffeic acid (CM2)	10, 29, 45, 70, 104, 124
Prephenate aminotransferase	PPA-AT	2.6.1.79	Absent	1 (*Arabidopsis thaliana*) 1 (*Petunia hybrida*) 1 (*Lycopersicon esculentum*)	Plastids (F, G)	PLP	—	21, 32, 75, 120, 172, 173
Arogenate/prephenate dehydratase	ADT/PDT	4.2.1.91/ 4.2.1.51	*PheA*[e]	6 (*Arabidopsis thaliana*) 3 (*Petunia hybrida*) 1 (*Oryza sativa*)	Plastids (G)	—	Inhibition by Phe Activation by Tyr	26, 92, 119, 153, 171, 206
Arogenate/prephenate dehydrogenase	ADH/PDH	1.3.1.78/ 1.3.1.12	*TyrA*[e]	2 (*Arabidopsis thaliana*) 4 (*Zea mays*)	Plastids (G)	$NADP^+$ (NAD^+ in maize)	Inhibition by Tyr	23, 30, 84, 152, 153
Phenylpyruvate/ 4-hydroxyphenylpyruvate aminotransferase	PPY-AT/ HPP-AT	2.6.1.5	*TyrB* *AspC* *IlvE*	1 (*Cucumis melo*) 1 (*Papaver somniferum*) 1 (*Arabidopsis thaliana*)	Cytosol (predicted)	PLP	—	72, 109, 141
Phenylalanine hydroxylase	Phe hydroxylase[f]	1.14.16.1	Absent	1 (*Pinus taeda*) 1 (*Physcomitrella patents*) 1 (*Chlamydomonas reinhardtii*)	Plastids (G)	10-formyltetra-hydrofolate	—	142

[a]F, subcellular fractionation; G, GFP (green fluorescent protein) localization study; I, chloroplast import assay.
[b]The reaction is redox neutral and NAD^+ is not consumed (9, 181).
[c]DHD and SDH form a bifunctional enzyme and are encoded in a single *DHD-SDH* gene in plants.
[d]Four genes were found in several *Arabidopsis* ecotypes (e.g., Wassilewskija) (7).
[e]CM is fused with PDT or PDH to form a bifunctional CM-PDT (PheA) or CM-PDH (TyrA) enzyme in *E. coli*.
[f]Phe hydroxylase is not found in angiosperms, including *Arabidopsis* (142).

have unique AAA-binding elements as additions to the core barrel structure (199).

Despite the fact that plant DAHP synthases are distantly related to *E. coli* and yeast enzymes, two copies of DAHP synthase genes have been isolated from several plant species through complementation of *E. coli* or yeast mutants deficient in the corresponding activity (44, 95). The two DAHP synthase genes (*DAHPS1* and *DAHPS2*) display differential expression in *Arabidopsis thaliana* (*Arabidopsis*), *Solanum lycopersicum* (tomato), and *Solanum tuberosum* (potato). *DAHPS2* (*shkB* in potato) is constitutively expressed, whereas *DAHPS1* (*shkA* in potato) is strongly induced in response to wounding and pathogen infection (73, 95). *Arabidopsis* DAHP synthase 1 is subject to redox regulation by a ferredoxin-thioredoxin system that links carbon flow into the shikimate pathway with photosynthetic electron flow (47). Although plant DAHP synthases 1 and 2 both use Mn^{2+} (88, 137), an additional Co^{2+}-dependent DAHP synthase activity has been detected in the cytosol of several plant tissues (66, 161). However, the corresponding genes have not been identified, and their physiological functions are currently unknown.

3-Dehydroquinate Synthase (DHQS)

3-Dehydroquinate synthase (DHQS) converts DAHP to 3-dehydroquinate using a divalent cation (e.g., Co^{2+}) and NAD^+ cofactors via five consecutive chemical reactions: alcohol oxidation, β-elimination of inorganic phosphate, carbonyl reduction, ring opening, and intramolecular aldol condensation (9, 181). Based on crystal structure studies, DHQS performs these reactions in one active site without forming by-products (24).

A plant *DHQS* gene has been isolated from tomato through complementation of an *E. coli* mutant (15). This single-copy gene is highly expressed in tomato roots, and its expression is induced by elicitor treatment in suspension cell culture (15). DHQSs from *E. coli* (AroB) and plants are monofunctional enzymes, whereas those from fungi are part of a pentafunctional enzyme (AROM complex) that catalyzes five consecutive reactions converting DAHP to 5-*enol*pyruvylshikimate 3-phosphate (EPSP) in the shikimate pathway (42).

3-Dehydroquinate Dehydratase (DHD)–Shikimate Dehydrogenase (SDH)

The third and fourth enzymatic reactions in the shikimate pathway include (*a*) the dehydration of 3-dehydroquinate to 3-dehydroshikimate to introduce the first double bond in the ring and (*b*) the reversible reduction of 3-dehydroshikimate into shikimate using NADPH (**Figure 2**). 3-Dehydroquinate dehydratase (DHD) and shikimate dehydrogenase (SDH; also referred to as shikimate:$NADP^+$ oxidoreductase) catalyze the respective reactions and have markedly different enzyme organizations in three kingdoms. These enzymes are part of the AROM complex in fungi (42) and are monofunctional enzymes in *E. coli* (AroD and AroE, respectively) (1, 41), whereas in plants they are fused to form a bifunctional DHD-SDH enzyme (16, 51, 138). The crystal structure of the *Arabidopsis* enzyme shows that the active sites of DHD and SDH are localized in close proximity and face each other, which facilitates an optimal, local 3-dehydroshikimate concentration for effective SDH catalysis (175). In plant tissues, roughly 10 times higher activity of SDH than that of DHD (51, 127) further ensures that the 3-dehydroshikimate intermediate will be efficiently converted to shikimate, thus increasing metabolic flux through the shikimate pathway.

A single gene encoding DHD-SDH, which carries a putative plastid transit peptide, has been identified in several plant species, including *Arabidopsis* (**Table 1**) (16, 38, 175). An exception is the genome of *Nicotiana tabacum* (tobacco), which contains two genes encoding both plastidic and cytosolic DHD-SDHs (38). RNA interference (RNAi) suppression of the plastidic *DHD-SDH* in tobacco leaves resulted in accumulation of both 3-dehydroquinate and shikimate—the substrate and product of the

enzyme, respectively—along with decreased levels of Phe, Tyr, lignin, and chlorogenic acid (38). However, the underlying mechanisms for the increased shikimate level in the RNAi lines as well as the role(s) of the cytosolic DHD-SDH in tobacco plants remain to be investigated.

Shikimate Kinase (SK)

Shikimate kinase (SK), the fifth enzyme of the shikimate pathway, catalyzes the phosphorylation of the C3 hydroxyl group of shikimate using ATP as a cosubstrate to yield shikimate 3-phosphate (**Figure 2**). SK requires a divalent cation (e.g., Mg^{2+} or Mn^{2+}) for its activity (100) and consists of core, lid, shikimate-binding, and nucleotide-binding domains, which undergo substantial conformational changes upon binding of shikimate and ATP (61, 65). Whereas *E. coli* has two isozymes, AroL and AroK (33), plants have different numbers of isozymes depending on species: one in tomato, two in *Arabidopsis*, and three in *Oryza sativa* (rice) (**Table 1**) (60, 61, 93, 165). The purified *Spinacia oleracea* (spinach) SK is modulated by the status of the energy charge (the relative concentrations of ATP, ADP, and AMP) (166), similar to *B. subtilis* SK and other ATP-utilizing enzymes (86, 134). Thus, SK may provide a regulatory link between the energy-requiring shikimate pathway and cellular energy balance in plants.

5-*Enol*pyruvylshikimate 3-Phosphate (EPSP) Synthase

EPSP synthase (also referred to as 3-phosphoshikimate 1-carboxyvinyltranferase) catalyzes the penultimate step of the shikimate pathway, the formation of EPSP, by transferring the enolpyruvyl moiety of PEP to the 5-hydroxyl position of shikimate 3-phosphate (**Figure 2**). This C3 enolpyruvyl unit eventually becomes the side chain of Phe and Tyr and is removed during the biosynthesis of Trp. Crystal structure studies have shown that the binding of the first substrate, shikimate 3-phosphate, triggers a global conformational change to form the active site in the interdomain cleft of EPSP synthase (168). EPSP synthase is the primary target of the nonselective, broad-spectrum herbicide glyphosate [*N*-(phosphonomethyl)glycine, originally sold under the name Roundup®]. Glyphosate competitively inhibits EPSP synthase with respect to the second substrate, PEP, by occupying the PEP binding site of the enzyme-shikimate 3-phosphate complex (168). EPSP synthases from different organisms have been divided into two classes based on glyphosate sensitivity: All plants and most bacteria, including *E. coli*, have glyphosate-sensitive class I EPSP synthases, whereas some bacteria, such as *Agrobacterium* sp. strain CP4, have class II EPSP synthases that are relatively resistant to glyphosate and therefore have been used to generate glyphosate-resistant crops (62).

Genes encoding EPSP synthases have been isolated from a number of plant species (**Table 1**) (67, 96, 169). Consistent with the purification of two isozymes with very similar kinetic properties in *Zea mays* (maize) (56), the *Arabidopsis* genome contains two genes encoding one functional (96) and one putative (2) EPSP synthase. The EPSP synthase gene is in general constitutively expressed at low levels but exhibits tissue-specific and developmentally regulated expression in *Petunia hybrida* (petunia) flowers (11, 67), likely for the production of Phe-derived volatiles.

Chorismate Synthase (CS)

Chorismate synthase (CS) catalyzes the final reaction of the shikimate pathway, the 1,4-*anti*-elimination of the 3-phosphate and C6-*pro*-R hydrogen from EPSP, introducing the second double bond in the ring to produce chorismate (117). Although the reaction is redox neutral, CS requires reduced flavin mononucleotide (FMN) as a cofactor that transiently donates one electron to the EPSP substrate to facilitate the phosphate cleavage, and it is involved in the C6 hydrogen abstraction (117, 118). Although CSs from different kingdoms are highly homologous and have similar

structural folds, cofactor specificity, and kinetic properties (118, 143, 163), two classes have been distinguished based on their ability to reduce the oxidized FMN. CSs from fungi are associated with NADPH-dependent flavin reductase as a part of a bifunctional enzyme (163), whereas most bacteria and plants have monofunctional enzymes that depend on an external source of reduced FMN. In plants, the reduced FMN may be provided by blue-light-mediated FMN photoreduction or by flavin reductase activity not physically associated with the enzyme (128, 162). Further investigation into the sources of reduced FMN in the plastids will allow us to understand the role of redox potential in regulating CS activity in plants. To date, plant genes encoding CSs have been isolated from *Corydalis sempervirens* and tomato, the latter having two differentially expressed genes (74, 162).

THE TRYPTOPHAN PATHWAY

In plants, chorismate is a common precursor for at least four branches of metabolic pathways leading to the formation of Trp, Phe/Tyr, salicylate/phylloquinone, and folate (**Figure 1**). Four enzymes—CM, anthranilate synthase (AS), isochorismate synthase (ICS), and aminodeoxychorismate synthase (ADCS)—catalyze the committed step of the respective pathways and compete for chorismate (**Figure 1**). The Trp pathway converts chorismate to Trp via six enzymatic reactions (**Figure 2**) (146, 170). All enzymes involved in the Trp biosynthetic pathway are shown or predicted to localize in the plastids (**Table 1**) (17, 101, 212). In contrast to multifunctional enzymes often found in the microbial Trp biosynthetic pathway (31, 180), all plant enzymes are monofunctional (146), although the first and last reactions are catalyzed by noncovalent enzyme complexes. Isolation and analysis of Trp biosynthetic mutants have provided genetic evidence for the involvement of these enzymes in Trp biosynthesis and its regulation *in planta*, and have advanced our understanding of the formation of other indole compounds.

Anthranilate Synthase (AS)

AS is an amino-accepting chorismate-pyruvate lyase that catalyzes the first step in Trp biosynthesis, the formation of anthranilate. AS consists of large α and small β subunits (ASα and ASβ, respectively), which form an α/β heterodimer or an α_2/β_2 tetramer (155). ASα binds to chorismate and catalyzes the amination and pyruvate elimination reactions, whereas ASβ hydrolyses glutamine and provides ammonia to ASα (99). The binding of chorismate to ASα triggers a conformational change to an active state and creates an intermolecular channel for ammonia transfer from ASβ to ASα (126). The AS enzyme is allosterically inhibited by Trp, which binds to ASα and restricts its conformational change (126), suggesting that ASα (but not ASβ) is responsible for feedback inhibition by Trp. Most ASα subunits in plants are feedback-sensitive to Trp, with the known exceptions of feedback-insensitive tobacco and *Ruta graveolens* ASα (18, 177). Higher plants examined to date contain at least two genes encoding ASα and one gene encoding ASβ (**Table 1**) (17, 130, 131, 177). One of the *ASα* genes is constitutively expressed, whereas the other is regulated developmentally and induced in response to wounding and pathogens (17, 130, 188), suggesting its involvement in the production of Trp pathway–derived natural products as a part of plant defense.

Phosphoribosylanthranilate Transferase (PAT)

Phosphoribosylanthranilate transferase (PAT) transfers the phosphoribosyl moiety from phosphoribosylpyrophosphate to anthranilate and produces 5-phosphoribosylanthranilate. The first isolated *Arabidopsis* mutant impaired in Trp biosynthesis, *trp1* (107), carries a mutation in a single-copy gene encoding PAT (157). The *PAT* gene is constitutively expressed in *Arabidopsis*, and the first two introns were shown to enhance the abundance of *PAT* messenger RNA (mRNA) posttranscriptionally (158). The *trp1* mutant exhibits a series of

auxin-deficient phenotypes (e.g., reduced size and apical dominance) as well as a blue fluorescence phenotype owing to the accumulation of anthranilate glucosides (144, 159). Feeding of Trp did not restore the auxin-deficient phenotype of *trp1* (107, 159), highlighting the importance of the Trp-independent pathway of auxin biosynthesis.

Phosphoribosylanthranilate Isomerase (PAI)

Phosphoribosylanthranilate isomerase (PAI) catalyzes the irreversible rearrangement of 5-phosphoribosylanthranilate to 1-(*o*-carboxyphenylamino)-1-deoxy-ribulose 5-phosphate (CdRP), a reaction that can also occur nonenzymatically. *Arabidopsis* possesses three or four highly similar *PAI* genes depending on ecotype (7, 115). *PAI1* and *PAI2* encode functional PAI enzymes (122), and their mRNAs represent major transcripts differentially expressed in response to environmental stresses and in a cell type–specific manner (78, 122). Similar to the *PAT* mutant (*trp1*), reduction in PAI activity in *PAI* antisense plants or deficiency in the tandem *PAI1* and *PAI4* genes in the *trp6* mutant resulted in the blue fluorescence phenotype (78, 115). The relative contribution of each isogene to Trp biosynthesis and other indole compounds has not been fully resolved owing to their high sequence similarities (115) and coordinated epigenetic control of the *PAI* gene family in some *Arabidopsis* ecotypes (7, 122).

Indole-3-Glycerol Phosphate Synthase (IGPS)

Indole-3-glycerol phosphate synthase (IGPS) catalyzes the irreversible conversion of CdRP to indole-3-glycerol phosphate. One of two *Arabidopsis* genes encoding IGPSs (*IGPS1*) has been isolated through complementation in an *E. coli trpC* mutant defective in IGPS (114). Antisense suppression of *IGPS1* reduces the levels of both Trp and auxin, whereas the *trp2* and *trp3* mutants defective in Trp synthase (see below) have less Trp but accumulate more auxin, suggesting that indole-3-glycerol phosphate serves as a key branch-point intermediate in Trp-independent auxin biosynthesis (133).

BX1: BENZOXAZINELESS 1 (see Reference 57)

Tryptophan Synthase (TS)

The final two reactions of the Trp pathway are catalyzed by the Trp synthase α subunit (TSα) and β subunit (TSβ), respectively. TSα catalyzes the reversible retro-aldol cleavage of indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate (G3P), and TSβ subsequently condenses indole and serine to produce Trp using pyridoxal 5-phosphate (PLP) as a cofactor (4). TSα and TSβ form an $\alpha_2\beta_2$ heterocomplex, and indole is transferred from the active site of TSα to that of TSβ through a 25-Å-long intermolecular tunnel (4). Whereas fungi possess a single gene encoding a bifunctional TSα-TSβ enzyme, bacteria such as *E. coli* have two separate genes encoding TSα and TSβ (31). The *Arabidopsis* genome contains at least two and three putative genes encoding TSα and TSβ, respectively (2, 106, 147). The indispensable roles of *Arabidopsis* TSα1 and TSβ1 in Trp biosynthesis have been genetically proven based on the Trp auxotrophic phenotypes of the *trp3* and *trp2* mutants, respectively (106, 145), whereas the functions of the other genes remain to be investigated. In contrast to *Arabidopsis*, maize contains two highly identical and redundant TSβ genes, and only simultaneous loss of both functions leads to Trp auxotrophy (204).

Plants possess other enzymes in addition to TSα that convert indole-3-glycerol phosphate to indole, a common precursor of not only Trp but also other plant natural products (**Figure 1**). In maize, indole-3-glycerol phosphate lyase (IGL) produces volatile indole under herbivore attack (59), whereas BX1 (BENZOXAZINELESS 1) catalyzes the first step in the biosynthesis of the natural pesticides benzoxazinoids (57, 58). In contrast to maize

IGL and BX1, which possess a predicted plastid transit peptide (57, 59), the *Arabidopsis* genome also contains an indole-producing TSα-like enzyme that localizes in cytosol (209). Although all three genes are TSα paralogs, the corresponding enzymes catalyze indole formation independent of a TSβ-like subunit (57, 59, 209). Because the production of indole or benzoxazinoids sometimes exceeds that of Trp (58), the heterocomplex formation of TSα and TSβ prevents the release of the indole intermediate and ensures a basal level of Trp production for protein and auxin biosynthesis.

THE PHENYLALANINE AND TYROSINE PATHWAYS

In contrast to the Trp pathway, our knowledge of the plant Phe and Tyr pathways is still in its infancy, and genes encoding the pathway enzymes have only recently been identified. Phe and Tyr are derived from chorismate, the final product of the shikimate pathway. Chorismate is converted by CM to prephenate, whose subsequent conversion to Phe and Tyr may occur via two alternative pathways (**Figure 2**) (170). In one route (the arogenate pathway), prephenate is first transaminated to L-arogenate (previously known as pretyrosine) followed by dehydration/decarboxylation to Phe or dehydrogenation/decarboxylation to Tyr catalyzed by arogenate dehydratase (ADT) or arogenate dehydrogenase (ADH), respectively. In the other route (the phenylpyruvate or 4-hydroxyphenylpyruvate pathway), these reactions occur in reverse order: Prephenate is first subjected to dehydration/decarboxylation by prephenate dehydratase (PDT) or to dehydrogenation/decarboxylation by prephenate dehydrogenase (PDH), followed by transamination of the corresponding products, phenylpyruvate and 4-hydroxyphenylpyruvate, to Phe or Tyr, respectively (**Figure 2**). Most microorganisms studied to date use the phenylpyruvate pathway primarily for Phe and Tyr biosynthesis, with a few exceptions (207). Recent genetic evidence indicates that the arogenate pathway is the predominant route for Phe biosynthesis in plants (119), whereas the major pathway for Tyr biosynthesis remains to be determined. Although the two possible pathways differ only in the sequence of aromatization and transamination, determination of the actual biosynthetic route(s) will allow understanding of carbon flux partitioning and its regulation within the Phe and Tyr pathways *in planta*.

Chorismate Mutase (CM)

CM catalyzes the first committed step in Phe and Tyr biosynthesis, the conversion of chorismate to prephenate via a pericyclic Claisen rearrangement (156). Functionally and structurally, CMs exist in multiple forms in different AAA-producing organisms. In addition to monofunctional enzymes, which are more prevalent in nature, there are several bifunctional enzymes, such as CM-PDT and CM-PDH (both found in *E. coli*) as well as CM-DAHP synthase (from *B. subtilis*) (156). On the basis of protein folds, two classes of CMs with α-helical or α/β-barrel structures (AroQ or AroH types, respectively) have been identified (27, 108). Whereas the AroH class is found in only a few cases (e.g., monofunctional CM in *B. subtilis*), most bacteria, fungi, and plant CMs are of the AroQ type.

Plants contain at least two isozymes, CM1 and CM2 (174). The plastidic CM1 is generally inhibited by Phe and Tyr and activated by Trp, whereas CM2 lacks a putative plastid transit peptide and is usually insensitive to allosteric regulation by AAAs (10, 45, 70, 104). Although the physiological function of CM2 remains unclear, it always has roughly 10-fold higher affinity toward chorismate than does CM1 (10, 124), which is consistent with presumably lower chorismate concentration in the cytosol than in the plastids. *Arabidopsis* has an additional gene encoding CM3 (124). Like CM1, CM3 contains a putative plastid transit peptide and is subject to allosteric regulation, but its affinity toward chorismate is closer to that of CM2 (124). All three *Arabidopsis* genes display differential

expression, with *CM1* and, to a lesser extent, *CM3* being inducible in response to elicitors and pathogen treatments (45, 124), suggesting their distinct physiological functions under different developmental and environmental conditions. Recent RNAi suppression of petunia *CM1* showed its involvement in the formation of Phe-derived volatile phenylpropanoid/benzenoid compounds in flower petals (29).

Prephenate Aminotransferase (PPA-AT)

Prephenate aminotransferase (PPA-AT) catalyzes the initial step of the arogenate pathway in Phe and Tyr biosynthesis, a reversible transamination between prephenate and arogenate using PLP as a cofactor (**Figure 2**). PPA-AT activities have been detected in some bacteria (49, 182) as well as in many plant species (21, 160, 173). PPA-AT enzymes use L-glutamate or L-aspartate as amino donors and exhibit the highest affinity toward prephenate among three keto acid intermediates in Phe and Tyr biosynthesis (i.e., prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate) (21, 120, 173). Despite extensive biochemical characterization of PPA-ATs, the corresponding genes had not been identified in any organisms until recently. Identification of *PPA-AT* genes from *Arabidopsis*, petunia, and tomato (32, 75, 120) revealed that PPA-ATs belong to the class Ib aspartate aminotransferases, which contain a lysine residue at position 109 important for the recognition of the dicarboxylic acid substrates (e.g., glutamate, aspartate, prephenate) (34, 132). Kinetic analysis of plant PPA-AT enzymes showed that they have roughly 10-fold higher affinity toward prephenate than toward arogenate (120, 172), suggesting that PPA-ATs predominantly catalyze the forward reaction and direct the carbon flux from prephenate to arogenate. Similarly to most aminotransferases, which often exhibit broad substrate specificity (203), plant PPA-ATs that are purified from plant tissues or recombinantly expressed display aspartate aminotransferase activity as well (21, 75, 120). However, RNAi suppression of *PPA-AT* in petunia petals significantly reduced only total PPA-AT activity without affecting aspartate aminotransferase activity, suggesting that the *PPA-AT* gene product plays a major role in the conversion of prephenate to arogenate but has only a minor contribution toward total aspartate aminotransferase activity *in planta* (120).

Aspartate aminotransferase: an enzyme that converts α-ketoglutarate or oxaloacetate into glutamate or aspartate, respectively

ACT domain: aspartokinase, chorismate mutase, and TyrA domain

Prephenate and Arogenate Dehydratases (PDT and ADT)

PDT catalyzes the decarboxylation and dehydration of prephenate to phenylpyruvate, the initial step in the phenylpyruvate route of Phe biosynthesis, whereas ADT converts arogenate to Phe, the final step in the arogenate route (**Figure 2**). Prephenate and arogenate can be spontaneously converted to phenylpyruvate and Phe, respectively, under acidic conditions in vitro (40, 208). However, crystal-structure and site-directed-mutagenesis studies of PDTs showed that the acidic residues of the enzyme are not directly involved in the catalysis (210). Instead, the geometric distortion of prephenate at the PDT catalytic site seems to promote decarboxylation and dehydration in a concerted manner (193, 210). PDTs and ADTs consist of two domains: a catalytic domain and a C-terminal ACT (aspartokinase, chorismate mutase, and TyrA) regulatory domain that is involved in the allosteric regulation by Phe (97). Whereas fungi and most bacteria have monofunctional PDTs or ADTs, some eubacteria such as *E. coli* have PDTs that are fused at the N-terminal with CM, thus forming a bifunctional CM-PDT enzyme (PheA) (97).

ADT activity has been detected in a variety of plant tissues and species (92, 170, 171), whereas PDT activity has been shown only in etiolated *Arabidopsis* seedlings (198). Genes homologous to bacterial PDTs have recently been identified in *Arabidopsis*, rice, and petunia (six, four, and three genes, respectively, though additional genes might exist in the rice and petunia genomes) (26, 119, 206). These plant genes encode monofunctional dehydratases that, on the basis of localization studies, are

targeted to the plastids (119, 153). All of these recombinant plant enzymes display strict or preferred substrate specificities toward arogenate over prephenate (26, 119, 206) and thus were designated as ADTs. RNAi suppression of *ADT* with strict substrate specificity toward arogenate has been shown to reduce Phe levels by ~80% in petunia flowers, providing genetic evidence that Phe is predominantly produced via the arogenate route in this plant organ (119). Moreover, comparative analysis of PPA-AT and ADT activities in petunia petals revealed that PPA-AT activity is at least three orders of magnitude higher than ADT activity (119, 120), suggesting that ADT, rather than PPA-AT, catalyzes a rate-limiting step within the arogenate pathway of Phe biosynthesis.

In general, PDT activity and phenylpyruvate have rarely been detectable in plant tissues (119, 198). However, when carbon flux through the shikimate pathway was increased by feeding with exogenous shikimate, phenylpyruvate became readily detectable in petunia petals and the reduced Phe levels in *ADT*-RNAi transgenic petals were recovered, suggesting that the PDT activity of plant ADT enzymes can contribute to Phe biosynthesis via a functional phenylpyruvate route under this condition (119). The role of the phenylpyruvate route in plant Phe biosynthesis under physiological conditions remains to be determined.

Given that both the arogenate and phenylpyruvate pathways can potentially be active in plants (119, 190), an open question is why the major flux goes via the arogenate route in plant Phe biosynthesis. Recent characterization of PPA-AT and ADT enzymes has provided a biochemical answer to this long-standing question (120). ADTs that can use prephenate in addition to arogenate compete with PPA-ATs for the prephenate substrate (**Figure 2**). Although both enzymes have similar K_m values for prephenate, the catalytic efficiencies of PPA-ATs are 50- to 250-fold higher than those of ADTs (26, 119, 120), suggesting that the high catalytic efficiency of PPA-AT directs carbon flux from prephenate into the arogenate pathway.

Arogenate and Prephenate Dehydrogenases (ADH and PDH)

ADH and PDH catalyze the oxidative decarboxylation of arogenate and prephenate to Tyr and 4-hydroxyphenylpyruvate, respectively, using an NAD^+ or $NADP^+$ cofactor. Whereas most fungi and bacteria possess PDHs, some cyanobacteria and brevibacteria have ADHs (49, 182). Similar to PDT enzymes, PDHs exist as either monofunctional or bifunctional enzymes in which the PDH domain is fused with other enzymes such as CM (AroQ), EPSP synthase (PDH-AroF), or AAA aminotransferase (HisH) (178). PDHs/ADHs are active as tetramers, and each monomer consists of an N-terminal nucleotide-binding domain and a C-terminal substrate-binding domain (111). Unlike PDTs/ADTs that contain a Phe-binding regulatory domain, PDHs/ADHs lack an independent Tyr-binding domain and are subject to inhibition by Tyr competitively with respect to the arogenate substrate (23, 30, 111, 152).

ADH activities have been detected in a variety of plant species (23, 30, 160), whereas PDH activities have been found only in legumes (64, 160). Plant ADHs specifically use $NADP^+$ as a cofactor (30, 152), with the exception of the NAD^+-dependent ADH in maize (23). Plant genes encoding ADHs have been isolated only from *Arabidopsis* and maize (two and four, respectively) (84, 152), and both *Arabidopsis* ADHs were shown to be targeted to the plastids (153). Biochemical characterization of *Arabidopsis* enzymes revealed that ADH1 exhibits strict substrate specificity toward arogenate, whereas ADH2 can also accept prephenate but at three orders of magnitude lower catalytic efficiency than arogenate (152). In vivo functions of *Arabidopsis* ADHs have not been investigated, but a transposon insertion in the *ADH1* gene in maize resulted in an opaque endosperm phenotype with decreased levels of zein storage proteins in kernels. However, only a minor reduction in free Tyr levels was detected in this mutant (84), likely owing to genetic redundancy of *ADH* genes or the presence of redundant Tyr pathways in maize.

Phenylpyruvate and 4-Hydroxyphenylpyruvate Aminotransferases (PPY-AT and HPP-AT)

Phenylpyruvate aminotransferase (PPY-AT) catalyzes a reversible transamination between phenylpyruvate and Phe using a PLP cofactor (**Figure 2**). Similarly, 4-hydroxyphenylpyruvate aminotransferase (HPP-AT) interconverts 4-hydroxyphenylpyruvate and Tyr. The forward reactions, phenylpyruvate and 4-hydroxyphenylpyruvate transaminations, represent the final steps of the phenylpyruvate and 4-hydroxyphenylpyruvate pathways in Phe and Tyr biosynthesis, respectively (**Figure 2**). The reverse reactions, Phe and Tyr transaminations, can be the initial steps of Phe and Tyr catabolism. In *E. coli*, two distinct aminotransferases [an AAA aminotransferase (AroAT/TyrB) and one aspartate aminotransferase (AspC)] catalyze the phenylpyruvate/4-hydroxyphenylpyruvate transamination to Phe/Tyr. However, an additional enzyme [a branched-chain amino acid aminotransferase (IlvE)] can also convert phenylpyruvate to Phe (68).

Similarly to those of microbes, AAA aminotransferase activities detected in a number of plant species exhibit broad substrate specificity with respect to amino donors and keto acid acceptors (63, 72, 203). Seven genes encoding putative Tyr aminotransferases exist in the *Arabidopsis* genome (2), and two of these enzymes have been biochemically characterized (91, 116, 141). Recently, an AAA aminotransferase gene was identified from *Cucumis melo* (melon) (72) and *Papaver somniferum* (opium poppy) (109), and the involvement of the latter enzyme in benzylisoquinoline alkaloid biosynthesis was shown by a virus-induced gene silencing (109). However, the *in planta* role of these aminotransferases in Phe and Tyr biosynthesis has not been demonstrated.

Phenylalanine Hydroxylase

In animals, Phe is an essential amino acid and can be converted to Tyr by Phe hydroxylase (53). Protists and some bacteria also contain iron-dependent monooxygenases, which hydroxylate the aromatic ring of Phe to Tyr. Recently, Phe-specific AAA hydroxylases have been identified in nonflowering plants, including gymnosperms, mosses, and algae, but not in angiosperms (142). These plant Phe hydroxylases are targeted to the plastids and use 10-formyltetrahydrofolate as a cofactor, providing a plant-specific novel link between folate and AAA metabolism.

REGULATION OF AROMATIC AMINO ACID BIOSYNTHESIS

Both microorganisms and plants regulate carbon flux toward AAA biosynthesis at the transcriptional and posttranscriptional levels (12). Besides basal levels of AAA production for protein biosynthesis, plants have to maintain their production for the biosynthesis of downstream natural products, including the major cell wall component lignin and defense compounds, the levels of which often drastically change under specific developmental and environmental conditions. Thus, the regulation of AAA biosynthesis in plants should be coordinated with the activities of downstream metabolic pathways and different from those of microorganisms.

Transcriptional Regulation

In microbes, the expression of the first gene in the shikimate pathway (*DAHP synthase*) is regulated in response to the cellular levels of AAAs, playing a key role in controlling the carbon flux into the pathway. In *E. coli*, the transcriptional repressors (TyrR and TrpR) directly bind AAAs and modulate expression of DAHP synthase genes (12, 22). In fungi, the expression of DAHP synthase genes (Aro3p and Aro4p) is regulated through a master transcriptional activator (Gcn4p), which is induced under general amino acid starvation (129). In plants, there is limited information about the effect of AAA levels on the expression of the shikimate pathway genes. Reduction of AAA biosynthesis

through the glyphosate-mediated inhibition of EPSP synthase induces DAHP synthase protein level and activity in potato cells either transcriptionally or translationally (136). In addition, reduced Phe levels in petunia flowers of *ADT1*-RNAi lines increase the expression of the shikimate pathway genes (119). Although the underlying molecular mechanisms are currently unknown, these results suggest that reduced levels of AAAs or their downstream products may act as a signal to induce the expression of the shikimate pathway genes and restore the carbon flux through the pathway in plants.

The expression of many plant genes encoding enzymes in the AAA pathways is regulated developmentally (11, 119) and in response to various environmental stimuli, such as wounding (43, 95), ozone (90), and pathogen infection or elicitors (73, 95, 213). In *Arabidopsis*, methyl jasmonate treatment induces the expression of genes encoding DAHP synthase and Trp pathway enzymes via a COI1 (CORONATINE INSENSITIVE 1)–dependent signaling pathway (37). In addition, salicylate is involved in the transcriptional activation of the Trp pathway, as demonstrated by the fact that salicylate-deficient *Arabidopsis* NahG plants show a reduction in pathogen-induced expression of the Trp pathway genes (213). The *Arabidopsis ups1* (*underinducer after pathogen and stress 1*) mutant exhibits reduced *PAT* expression and a compromised salicylate-, jasmonate-, and reactive oxygen species–mediated induction of defense gene expressions (36), implying that UPS1 is an upstream component involved in transcriptional activation of the Trp pathway and plant defense (**Figure 3*a***).

Many transcription factors that regulate biosynthesis of AAA-derived natural products have been isolated, and some have been shown to coregulate expression of genes in the AAA pathway as well (**Figure 3*a***). In *Arabidopsis*, MYB transcription factors regulating indole glucosinolate biosynthesis (HIG1/MYB51 and ATR1/MYB34) also activate genes encoding DAHP synthase 1 and ASα1, respectively (8, 69). In addition, *ASα1* expression is under control of the ORCA3 transcription factor, which regulates the expression of several biosynthetic genes involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (191). RNAi suppression of *MYB8* in *Nicotiana attenuata* significantly reduces the expression of all seven shikimate pathway genes, resulting in complete elimination of phenylpropanoid-polyamine conjugates (94). Expression of genes encoding 6 out of 10 enzymes required for Phe biosynthesis was reduced in an *Arabidopsis* double

COI1 (CORONATINE INSENSITIVE 1): an F-box protein, part of the E3 ubiquitin ligase complex, involved in the perception of the plant defense hormone jasmonic acid

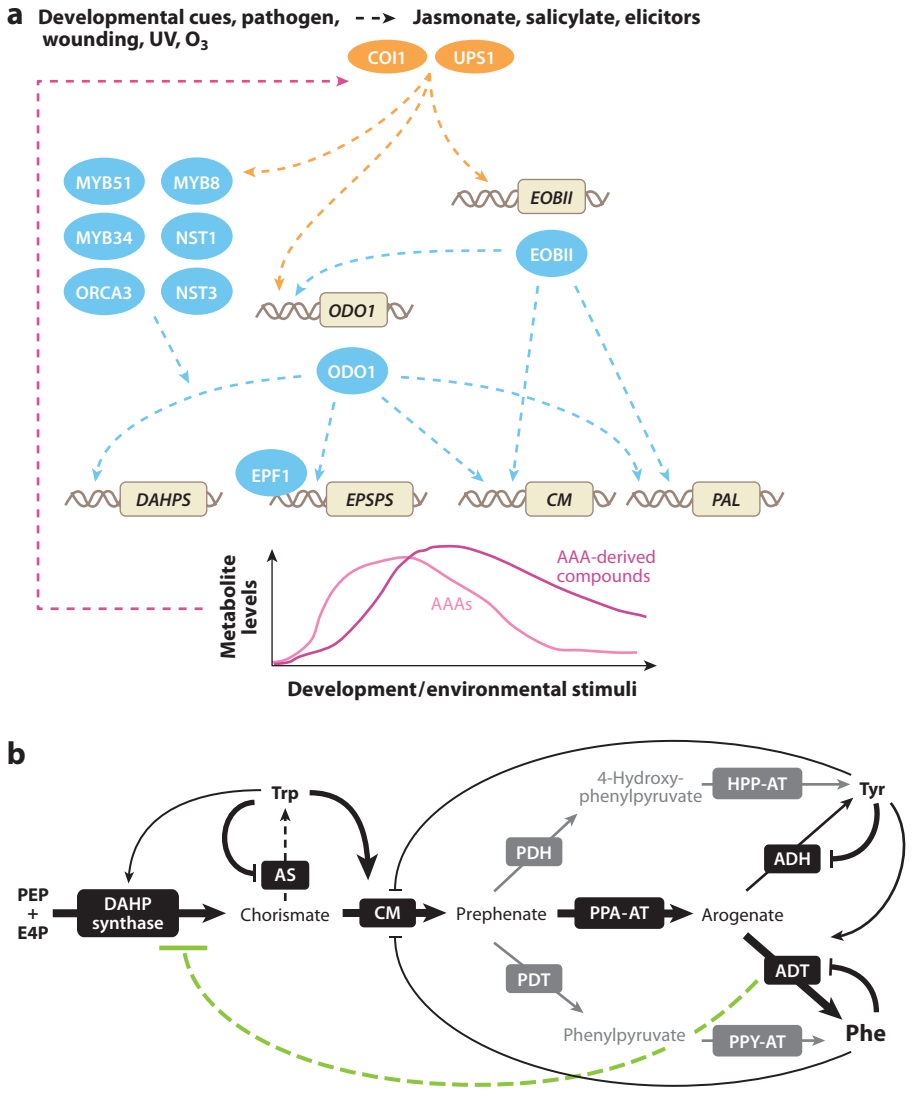
***ups1* (*underinducer after pathogen and stress 1*) mutant:** an *Arabidopsis* mutant that was isolated on the basis of reduced expression of *PAT* and is thus defective in Trp biosynthetic pathway regulation

Figure 3

Transcriptional and posttranscriptional regulations of plant aromatic amino acid (AAA) biosynthesis. (*a*) Various developmental cues and environmental stimuli induce the signaling pathways (shown in *orange*), leading to transcriptional and/or posttranscriptional activations of transcription factors (*blue*). These factors either directly or indirectly activate genes encoding enzymes in the AAA pathways. Resulting changes in the levels of AAAs and/or AAA-derived metabolites act as signals to further fine-tune the transcriptional network (*pink dotted line*). (*b*) Tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr) allosterically inhibit enzymes catalyzing the committed steps in each pathway [anthranilate synthase (AS), arogenate dehydratase (ADT), and arogenate dehydrogenase (ADH), respectively]. In addition, Trp and Tyr activate chorismate mutase (CM) and ADT, respectively, to ensure that the major carbon flux is directed toward Phe biosynthesis in plants. Phe and Tyr inhibit CM, and in some cases, Trp activates 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase in vitro, although the in vivo roles of these regulations remain to be investigated. The early part of the shikimate pathway is negatively regulated via one or more unknown posttranscriptional mechanisms (*green dotted line*). The contributions of the phenylpyruvate and 4-hydroxyphenylpyruvate pathways (shown in *gray*) for Phe and Tyr biosynthesis remain to be determined in plants. The thickness of the lines indicates the relative strength of flux and allosteric regulation. Other abbreviations: E4P, D-erythrose 4-phosphate; HPP-AT, 4-hydroxyphenylpyruvate aminotransferase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PEP, phospho*enol*pyruvate; PPA-AT, prephenate aminotransferase; PPY-AT, phenylpyruvate aminotransferase.

mutant defective in NST1 and NST3, NAC transcription factors regulating secondary wall formation (123). These results indicate that in plants the expression of genes encoding enzymes in the AAAs and their downstream pathways are coordinately regulated, often by the same transcription factor, to achieve the required production of AAA-derived natural products. However, it remains to be determined whether these transcription factors activate the target promoters directly or indirectly and whether additional factors are involved.

Petunia flowers produce high levels of Phe-derived phenylpropanoid/benzenoid volatiles in a developmentally and diurnally regulated fashion. Three transcription factors controlling shikimate pathway genes—a C2H2-type zinc finger DNA-binding protein, EPF1, and two R2R3-type MYB transcription factors, ODORANT1 and EOBII—have been isolated in petunia flowers and provided new insights into this transcriptional regulatory network (**Figure 3*a***) (179, 186, 195). EPF1 directly binds to the *EPSP synthase* promoter and



controls its spatial and developmental expression (186). ODORANT1 and EOBII appear to have only partial overlap in their targets: RNAi suppression of both *ODORANT1* and *EOBII* leads to reduced expression of *CM* and *PAL*, whereas *DAHP synthase* and *EPSP synthase* expression is affected only by *ODORANT1* but not *EOBII* suppression (**Figure 3*a***) (179, 195). Recent studies have shown that EOBII binds to the *ODORANT1* promoter via a putative MYB binding site (192) and that *EOBII* suppression results in partial *ODORANT1* downregulation (179). Because the overexpression of *EOBII* did not significantly alter the *ODORANT1* and *CM* expressions (179), one or more additional factors are likely involved in the EOBII-mediated activation of these promoters. Future investigations of the regulation of activities of transcription factors themselves, their redundancy, and epistatic interactions will provide a comprehensive understanding of the transcriptional regulatory networks of AAA biosynthesis in plants.

Posttranscriptional Regulation

In addition to the transcriptional regulation, AAA biosynthesis is subject to complex posttranscriptional regulations, which control carbon flux into the shikimate pathway as well as the carbon allocation toward individual AAAs. Within the pathway, the partitioning of carbon flux between the Trp and Phe/Tyr pathways is controlled at the level of two enzymes, AS and CM, both of which compete for chorismate as a substrate (**Figure 3*b***). AS and CM are feedback inhibited by the final product(s) of the corresponding pathways (i.e., Trp and Phe/Tyr, respectively) in both microbes and plants (18, 99, 156). In addition, Trp activates CM to redirect flux from Trp to Phe/Tyr biosynthesis (10, 103). Likewise, the enzymes localized at the branch points of Phe and Tyr biosyntheses, ADT and ADH (or PDT and PDH, depending on the organism), are feedback inhibited by Phe and Tyr, respectively (30, 152, 171, 206). In some cases, Tyr activates ADT to redirect flux from Tyr to Phe biosynthesis (**Figure 3*b***) (92, 171).

In plants, the biochemical characterization of CMs revealed that their inhibition constants (K_i) with Phe and Tyr are relatively high (0.3–1.1 mM) (10, 103) compared with those of ADTs/ADHs (8–61.5 μM) (30, 152, 171, 206). Thus, the feedback regulation of ADT/ADH, rather than CM, likely plays a key role in the regulation of Phe/Tyr biosynthesis via the arogenate pathway *in planta*. Indeed, a single mutation within the ACT regulatory domain of ADT makes the enzyme less sensitive to regulation by Phe and results in elevated Phe levels in *Arabidopsis* and rice mutants (87, 206), suggesting that CM inhibition by elevated Phe levels is unable to prevent Phe overproduction. These mutants also accumulate high levels of Trp (87, 206), which can contribute to Phe overaccumulation because Trp effectively activates CM with activation constants (K_a) of 1.2–2.4 μM and can reverse Phe/Tyr-mediated CM inhibition (10, 103). The tight regulation of Trp and Tyr biosyntheses in plants ensures that the major carbon flux is directed toward Phe biosynthesis for the production of abundant phenolic compounds (e.g., lignin) (**Figure 3*b***).

How the carbon flow into the shikimate pathway is regulated in plants remains ambiguous. In contrast to microbial enzymes (12, 205), plant DAHP synthases are not inhibited by AAAs (80, 88). The exceptions include DAHP synthases from maize shoots (76) and *Pisum sativa* (pea) leaves (150), which are feedback inhibited by Trp and Tyr, respectively. In addition, activation of DAHP synthases by Trp was found in carrot and potato (137, 185). To our knowledge, a Phe-sensitive DAHP synthase has not been identified in plants. Although arogenate has been shown to inhibit spinach and mung bean DAHP synthases in vitro (39, 161), such arogenate feedback regulation has not been demonstrated in vivo.

In petunia flowers, suppressing ADT expression via an RNAi strategy reduced the flux through the shikimate pathway, resulting in decreased levels of shikimate and Trp (119). Considering that expression of genes encoding the shikimate and Phe pathway enzymes (i.e., DAHP synthase, EPSP synthase, and CM) were

upregulated and feeding of exogenous shikimate restored the reduced Trp and Phe levels in these transgenics, these results suggest that one or more biosynthetic steps upstream of shikimate are negatively regulated via unknown posttranscriptional mechanism(s) (**Figure 3*b***) (119). Given that plant DAHP synthases belong to the type II class and contain AAA-binding elements, it is likely that the correct conditions and/or combinations of AAAs that affect plant DAHP synthase activity have not been found (200).

E4P and PEP Precursor Supply to the Shikimate Pathway

The supply of the DAHP synthase substrates, E4P and PEP, can also play an important role in the regulation of the carbon flux into the shikimate pathway. In photosynthetic tissues, transketolase (TK) in the Calvin cycle converts G3P and fructose 6-phosphate (F6P) to xylose 5-phosphate (X5P) and E4P (**Figure 4**). A slight decrease in TK activity in transgenic tobacco leaves leads to a substantial reduction in the levels of AAAs and their downstream metabolites (e.g., phenylpropanoids, tocopherols) (79), suggesting that the E4P supply via TK can be a limiting factor for AAA biosynthesis. In nonphotosynthetic tissues, transaldolase (TA) and TK in the oxidative pentose phosphate pathway (OPPP) likely play key roles in E4P supply to the shikimate pathway (**Figure 4**) (102). In bacteria, overexpression of TK rather than TA was found to be more effective in directing the carbon flux into the AAA pathways (20); however, the relative contributions of these enzymes to E4P supply for the shikimate pathway in plants have not been investigated. The broad substrate specificity of TK and TA and the presence of additional intermediates (e.g., octulose 8-phosphate) potentially involved in the OPPP (13, 54, 194) suggest that the OPPP and its regulation may be much more dynamic and complex than currently thought to meet the high demand of E4P for biosynthesis of AAAs, especially in plants.

Plastidic PEP can be derived from (*a*) plastidic glycolysis via phosphoglyceromutase (PGyM) and enolase (ENO1) (140), (*b*) import from the cytosol via the PEP/phosphate translocator (PPT) (52, 184), and/or (*c*) phosphorylation of pyruvate catalyzed by plastidic pyruvate orthophosphate dikinase (PPDK) (**Figure 4**) (55). Although analysis of *ENO1*, *PGyM*s, *PPT*s, and *PPDK* expression revealed that the relative contributions of different pathways are tissue specific (98, 135, 140), mutant analysis showed that multiple pathways can simultaneously contribute to the plastidic PEP internal pool in plants. The *Arabidopsis ppt1* (*cue1*) knockout mutant displays a mesophyll-specific defect in chloroplast development (113) that can be rescued by the constitutive overexpression of *PPDK* in the plastids (197), suggesting that sufficient levels of pyruvate exist in the chloroplasts to compensate for the lack of PEP transport from the cytosol (197). The gametophyte-lethal phenotype of the *ppt1eno1* double-knockout mutant indicates that PEP generation through both the plastidic glycolysis and its import from the cytosol are essential during early *Arabidopsis* development (139). However, considering that plastidic PEP is also used for the biosyntheses of fatty acids, branch-chained amino acids, and isoprenoids (via the MEP pathway) (**Figure 4**), the mutant phenotypes described above cannot be attributed to a single metabolic pathway (e.g., the shikimate pathway) and the major route for PEP supply for the shikimate pathway remains to be determined.

SUBCELLULAR LOCALIZATION OF AROMATIC AMINO ACID BIOSYNTHESIS IN PLANTS

Several lines of evidence conclusively indicate that the plastids contain a full set of biosynthetic enzymes for the production of all AAAs from PEP and E4P. (*a*) Feeding of radiolabeled precursors (e.g., PEP) to isolated chloroplasts led to the production of labeled Trp, Phe, and Tyr (3, 14, 85). (*b*) Activities of almost all enzymes involved in AAA biosynthesis have been

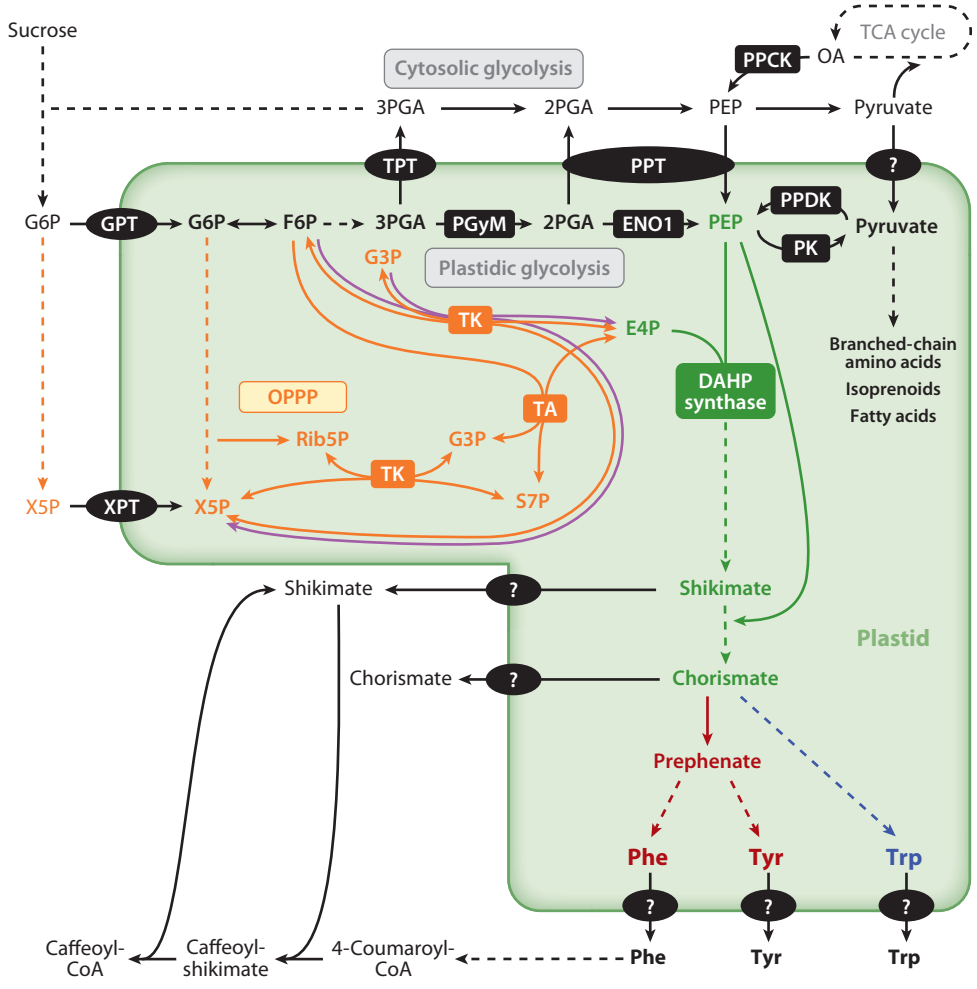


Figure 4

Supply of the shikimate pathway precursors and transport of aromatic amino acids (AAAs), precursors, and pathway intermediates across the plastid membranes. D-Erythrose 4-phosphate (E4P) can be synthesized by transketolase (TK) as part of the Calvin cycle (*purple lines*) or by either transaldolase (TA) or TK through the oxidative pentose phosphate pathway (OPPP; *orange*). Plastidic phospho*enol*pyruvate (PEP) can be generated from three different routes: via plastidic enolase (ENO1), via PEP/phosphate translocator (PPT), and/or via pyruvate orthophosphate dikinase (PPDK). Within the plastids (*light green shape*), PEP is used not only for the shikimate pathway (*green*) but also for the synthesis of branched-chain amino acids, isoprenoids, and fatty acids after being converted to pyruvate by pyruvate kinase (PK). PEP as well as precursors for PEP and E4P biosyntheses are imported into the plastids through membrane-localized transporters (*filled circles*). The intermediates and products of the AAA pathways are exported from the plastids via unknown transporters (*filled circles with question marks*). Dotted arrows indicate that multiple reactions are involved. Other abbreviations: CoA, coenzyme A; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GPT, G6P/phosphate translocator; OA, oxaloacetate; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; PGyM, phosphoglyceromutase; Phe, phenylalanine; PPCK, PEP carboxykinase; Rib5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; TCA, tricarboxylic acid; TPT, triosephosphate translocator; Trp, tryptophan; Tyr, tyrosine; X5P, xylose 5-phosphate; XPT, X5P/phosphate translocator.

detected in the plastid fractions of plant tissue extracts (10, 92, 127, 128, 173, 212). (*c*) The amino acid sequence of at least one isozyme responsible for each biochemical step in the plant AAA pathway contains a plastid transit peptide. And (*d*) plastidic localization for most pathway enzymes has been confirmed using GFP (green fluorescent protein) fusion proteins or protein import assays (17, 29, 32, 35, 38, 93, 142, 153, 165, 214), with the exception of CS, ASβ, IGPS, and PPY-AT/HPP-AT (**Table 1**).

An open question is whether some of the biochemical steps are also present outside of the plastids. Subcellular fractionation and localization studies showed that some enzymes of the AAA pathway (i.e., DAHP synthase, CM, DHD/SDH) exist in the cytosol (164). Genes encoding cytosolic tobacco DHD/SDH2 (38) and *Arabidopsis* and petunia CM2 (29, 45) have been isolated, and the extraplastidic localization of the corresponding proteins was shown using a GFP fusion protein and a protein import assay, respectively (29, 38). A gene encoding cytosolic DAHP synthase has not been identified; however, a PPY-AT recently isolated from melon lacks a plastid transit peptide and is likely localized in the cytosol (72), raising the possibility that the last step of the phenylpyruvate pathway of Phe biosynthesis might take place outside of the plastids. Further investigation of molecular identity, subcellular localization, and physiological functions of potential cytosolic enzymes will clarify whether the AAA pathways exist in more than one cellular compartment.

TRANSPORT OF AROMATIC AMINO ACIDS AND PATHWAY INTERMEDIATES ACROSS PLASTID MEMBRANES

Although the AAA pathways are predominantly localized in the plastids, all three AAAs and some of the pathway intermediates (e.g., shikimate) are exported to the cytosol for the biosynthesis of proteins and other AAA pathway–derived compounds (e.g., indole compounds, phenylpropanoids, alkaloids) (**Figure 4**). However, in contrast to the large number of AAA transporters identified in microbes (28, 149), little is known about the biochemical and molecular mechanisms of metabolite transport across plastid membranes in plants. Although multiple members of amino acid transporters have been shown to transport AAAs in *Arabidopsis* (151), none of the transporters have been shown to localize in the plastid membranes (77, 82, 110). Shikimate is conjugated with *p*-coumaroyl-coenzyme A (CoA) by hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase in the cytosol, forming *p*-coumaroyl shikimate, an intermediate in the monolignol biosynthesis (83). Thus, shikimate has to be exported from the plastids. Most plant enzymes utilizing chorismate are localized in the plastids (**Figure 1**) (5, 17, 29, 183); however, CM2 resides in the cytosol (29) and is inhibited in tobacco by caffeic acid, which is synthesized in the cytosol (71). Furthermore, the overexpression of *E. coli* chorismate pyruvate lyase in tobacco cytosol results in the production of 4-hydroxybenzoate (176), indicating that a substantial pool of chorismate is present in the cytosol as well. Future identifications of plastid membrane transporters will allow us to understand the roles of shikimate and chorismate in the crosstalk between the plastidic AAA pathway and cytosolic metabolic pathways.

METABOLIC ENGINEERING OF AROMATIC AMINO ACID BIOSYNTHESIS

Although numerous attempts have been made in plants to modify the yield of substances derived from AAAs (112, 187, 202), metabolic engineering of flux toward AAAs themselves and examination of subsequent effects on downstream products have been conducted in only a few cases. To increase Phe production and elucidate the effect of high Phe levels on the AAA metabolic network, a bacterial bifunctional CM/PDT that converts chorismate into phenylpyruvate via prephenate was recently overexpressed in *Arabidopsis* plastids (190). The C-terminal allosteric domain was removed from the introduced CM/PDT to prevent its

feedback inhibition by Phe. Up to a 100-fold increase in Phe levels was observed in transgenics relative to control plants, suggesting that *Arabidopsis* plants possess an endogenous aminotransferase activity that converts the phenylpyruvate produced by the introduced bacterial enzyme into Phe. Interestingly, these transgenic lines contain increased levels of Phe- and Tyr-derived metabolites (e.g., phenylpropanoids, glucosinolates, and vitamin E), whereas the levels of Trp-derived secondary metabolites were reduced (190), suggesting that increased utilization of chorismate by feedback-insensitive CM/PDT for Phe overproduction limits the substrate availability for Trp biosynthesis.

The overexpression of tobacco feedback-insensitive ASα in tobacco and *Astragalus sinicus* resulted in up to a 10-fold increase in the levels of free Trp (25, 211). Much greater increases in the internal pools of free Trp (up to 431-fold) were achieved when rice feedback-insensitive ASα was overexpressed in rice, potato, and *Arabidopsis* (89, 121, 188). These examples show that the overexpression of feedback-insensitive ASα is generally effective in enhancing Trp accumulation, but the degree of enhancement depends on the biological system. One may expect that the activation of Trp biosynthesis increases chorismate consumption, which in turn might limit the production of Phe and Tyr as well as compounds derived from them. However, *Arabidopsis* Trp-overproducing lines show high levels of Phe and Tyr (89), which may be attributed to an activation of CM by elevated levels of Trp in transgenics (10, 103). Thus, in contrast to upregulation of Phe biosynthesis, an increase in carbon flux toward Trp does not compromise biosynthesis of other AAAs and their derived compounds.

Trp is a precursor of auxin as well as a diverse range of secondary metabolites, including indole alkaloids, glucosinolates, and phytoalexins (**Figure 1**). In rice calli and potato shoots overexpressing feedback-insensitive ASα, the levels of indole-3-acetic acid were increased by up to 57- and 39-fold, respectively (121, 125). In contrast, a 200-fold increase in Trp in the *Arabidopsis* transgenic lines led to only a 2-fold increase in the levels of indole-3-ylmethyl glucosinolate and unaltered levels of the indole phytoalexin camalexin after pathogenic fungus inoculation (89). These results show that the level of Trp is not a major limiting factor for indole glucosinolates and phytoalexin biosyntheses, as had been previously suggested (213). Thus, simultaneous upregulation of Trp biosynthesis and downstream pathways may be required to significantly increase the yield of target Trp-derived compounds.

In plants, Tyr and Phe biosynthesis diverge at the level of arogenate, with the majority of carbon flux being directed to Phe for phenylpropanoid production. Much less carbon is generally incorporated into Tyr, which is further converted to 4-hydroxyphenylpyruvate and homogentisate, the aromatic precursors of tocochromanols collectively known as vitamin E. To increase flux toward 4-hydroxyphenylpyruvate and production of vitamin E, prephenate was directly converted into 4-hydroxyphenylpyruvate by the expression of Tyr-insensitive *S. cerevisiae* PDH in the plastids of tobacco plants (154). Although tobacco plants expressing only the yeast PDH transgene accumulate trace amounts of tocotrienols, the coexpression of the yeast PDH with an *Arabidopsis* 4-hydroxyphenylpyruvate dioxygenase that converts 4-hydroxyphenylpyruvate to homogentisate resulted in a massive accumulation of tocotrienols (154). Because an overexpression of *Arabidopsis* 4-hydroxyphenylpyruvate dioxygenase alone had only a limited effect on tocopherol production (48, 189), both supply of 4-hydroxyphenylpyruvate and its subsequent conversion to homogentisate represent bottlenecks in vitamin E accumulation in plants. Taken together, these results show that the elimination of posttranscriptional feedback regulatory mechanisms by introducing feedback-insensitive enzymes can lead to an increase in AAA production; however, upregulation of additional downstream biochemical steps is likely required for effective production of target AAA-derived metabolites.

SUMMARY POINTS

1. Discovery of genes encoding enzymes required for AAA biosynthesis appears to have been completed in plants.
2. In contrast to most microbes, Phe biosynthesis in plants occurs predominantly via the arogenate pathway.
3. Transcription factors controlling AAA-derived natural product formation often coregulate expression of upstream AAA pathway genes to increase carbon flux toward one or more target pathways.
4. Posttranscriptional feedback regulation in the AAA network exerts significant control over flux distribution between three AAAs. In plants, Trp and Tyr biosyntheses are tightly regulated to ensure that the major flux is directed toward Phe, the level of which is under control of ADT allosteric inhibition by Phe.
5. AAA biosyntheses occur in the plastids, whereas AAAs and pathway intermediates are transported across the plastid membrane for their further utilization.
6. Metabolic engineering efforts revealed that elimination of allosteric regulation by overexpressing feedback-insensitive branch-point enzymes in AAA biosynthesis leads to increased production of corresponding AAAs and sometimes metabolites derived from them.

FUTURE ISSUES

The following points remain to be determined for further understanding of AAA biosynthesis in plants:

1. The contribution of the phenylpyruvate and 4-hydroxyphenylpyruvate routes for Phe and Tyr biosyntheses (respectively) in plants, and the molecular identity of plant aminotransferases involved in these alternative pathways.
2. The major biosynthetic route for Tyr biosynthesis in plants.
3. Additional transcription factors, their genetic/physical interactions with previously isolated regulators, and their roles in the regulatory network of AAA biosynthesis.
4. Regulatory mechanisms controlling the carbon flux into the shikimate pathway.
5. The major pathways providing precursors (E4P and PEP) for the shikimate pathway, and how the levels of these precursors derived from different pathways are coordinately regulated and contribute to the overall flux through the shikimate pathway.
6. The crucial transporters that translocate AAAs and pathway intermediates across the plastid membranes, and their role in the control of the relative AAA levels in different cellular compartments.
7. How to efficiently direct increased levels of AAAs, obtained via elimination of negative feedback regulation, to target metabolites by increasing the activity of specific downstream biochemical steps.

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.

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Annual Review of Plant Biology
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Contents