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The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants

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Abstract

Although amino acids are critical for all forms of life, only proteogenic amino acids that humans and animals cannot synthesize de novo and therefore must acquire in their diets are classified as essential. Nine amino acids—lysine, methionine, threonine, phenylalanine, tryptophan, valine, isoleucine, leucine, and histidine—fit this definition. Despite their nutritional importance, several of these amino acids are present in limiting quantities in many of the world's major crops. In recent years, a combination of reverse genetic and biochemical approaches has been used to define the genes encoding the enzymes responsible for synthesizing, degrading, and regulating these amino acids. In this review, we describe recent advances in our understanding of the metabolism of the essential amino acids, discuss approaches for enhancing their levels in plants, and appraise efforts toward their biofortification in crop plants.

Contents

INTRODUCTION

Animals, including humans and monogastric livestock that serve as human food, cannot synthesize all of the 20 amino acids that are required for the formation of proteins. Therefore, they must obtain the amino acids that they cannot synthesize (termed essential amino acids) from external sources, which are based on plants. These nine essential amino acids are lysine (Lys), methionine (Met), and threonine (Thr) of the aspartate (Asp) family pathway; phenylalanine (Phe) and tryptophan (Trp) of the aromatic amino acids; valine (Val), isoleucine (Ile), and leucine (Leu) of the branched-chain amino acids (BCAAs); and histidine (His). The levels of four of these amino acids—Lys, Met, Thr, and Trp—are considered to limit the nutritional quality of plants, because their contents in plants are very low compared with the levels required for optimum growth of human and animals.

The major factors limiting these essential amino acids in crop plants are (*a*) regulatory factors that control the synthesis of the essential amino acids by feedback inhibition loops, in which the accumulating amino acids suppress the activity of enzymes in their biosynthesis pathways, and (*b*) the efficient catabolism of these amino acids. Indeed, amino acids also serve as precursors of a wide variety of plant natural products that play crucial roles in plant growth and development, including responses to biotic and abiotic stresses (205). Amino acids are also efficiently catabolized into the tricarboxylic acid (TCA) cycle to generate the cellular energy required for plant growth, particularly in response to stresses that create energy deprivation (105, 120).

METABOLISM OF THE AROMATIC AMINO ACIDS

Among the aromatic amino acids, Phe and Trp are considered essential, whereas tyrosine (Tyr) is regarded as nonessential (58). The synthesis of these three amino acids begins with the conversion of phospho*enol*pyruvate and erythrose 4-phosphate into chorismate via the shikimate pathway; this chorismate is subsequently converted into Phe and Trp via the aromatic amino acid biosynthetic pathways (18, 65, 190, 199) (**Figure 1**). Given that the shikimate and aromatic amino acid biosynthesis pathways have been described in considerable detail in recent reviews (125, 188, 189), we merely provide a brief overview of these pathways here.

The biosynthesis of the aromatic amino acid Phe from chorismate principally uses two different metabolic routes: one through phenylpyruvate as a metabolic intermediate, and one through arogenate (**Figure 1**). Chorismate mutase catalyzes the first committed step in Phe biosynthesis. Most plant species have a plastidial and a cytosolic isoform of the enzyme, with the former inhibited by Phe and Tyr and activated by Trp. Prephenate aminotransferase, which was only recently

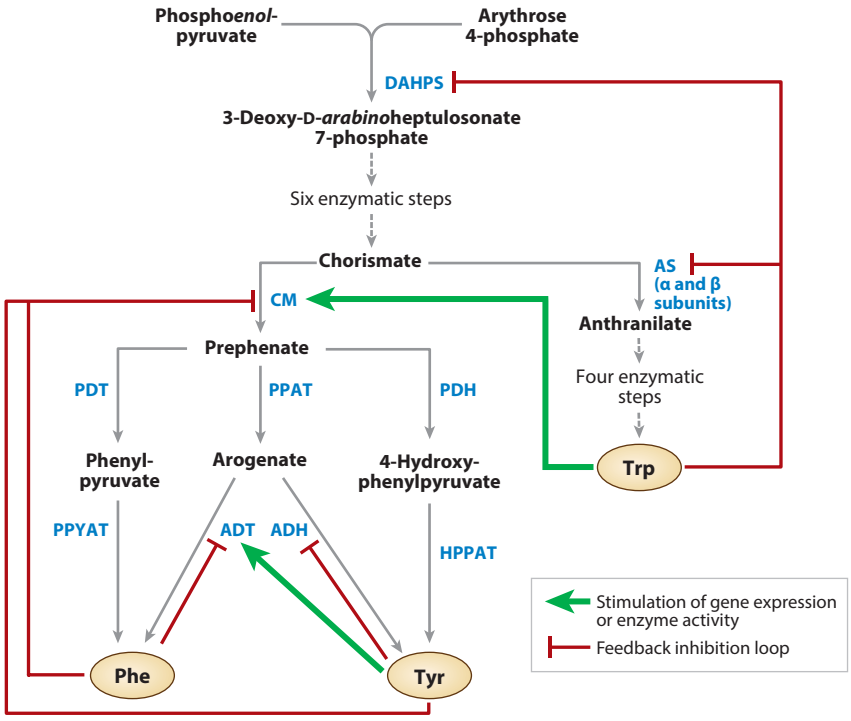

Figure 1

Aromatic amino acid biosynthesis leading to Trp, Tyr, and Phe in plants. Enzymes are indicated in blue text. Abbreviations: ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; AS, anthranilate synthase; CM, chorismate mutase; DAHPS, 3-deoxy-D-*arabino*heptulosonate 7-phosphate synthase; HPPAT, 4-hydroxyphenylpyruvate aminotransferase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PPAT, prephenate aminotransferase; PPYAT, phenylpyruvate aminotransferase.

identified at the molecular level (37, 72, 127), catalyzes the reversible transamination between prephenate and arogenate. This route to Phe is completed by the enzyme arogenate dehydratase. Studies of flowers of petunia plants, meanwhile, have revealed that expression of the arogenate dehydratase 1 isozyme was incredibly high in petals and that this high level was positively correlated with the biosynthesis of the endogenous Phe in the flowers (126).

It is important to note that arogenate dehydratase isolated from several species additionally possesses prephenate dehydratase activities (126, 201). Aiming to stimulate the production of Phe in plants, the Galili laboratory expressed a recombinant construct encoding a bacterial bifunctional PheA enzyme, containing chorismate mutase and prephenate dehydratase, in *Arabidopsis* (191). The PheA-expressing plants exhibited increased Phe content, indicating that plants, like bacteria, can convert prephenate into Phe, exposing an additional level of complexity in the biosynthesis of the aromatic amino acids in plants. Until very recently, this enzyme had only been speculated to be an aromatic amino acid transferase (190). However, this was subsequently determined at the molecular level when Maeda and coworkers (203) revealed that this conversion is mediated by a cytosol-localized Tyr:phenylpyruvate aminotransferase. This enzyme thereby links Phe production to a coordinated catabolism of Tyr in addition to linking the plastidial biosynthetic pathways to the downstream metabolic pathways of the aromatic amino acids. Phenylpyruvate also serves as precursor for several secondary metabolites, including phenylacetaldehyde, 2-phenylethanol, and

2-phenylethyl β-D-glucopyranoside (99, 186, 197). In addition, Phe itself is the precursor for a wide range of intermediary and secondary metabolites of considerable importance for both plant structure and defense (44).

The synthesis of Trp from chorismate requires the enzymes (*a*) anthranilate synthase, (*b*) phosphoribosylanthranilate transferase, (*c*) phosphoribosylanthranilate isomerase, (*d*) indole-3-glycerol phosphate synthase, and (*e*) α and β Trp synthase. In plants, anthranilate synthase is a heterotetramer consisting of two α and two β subunits and is feedback inhibited via the binding of Trp to the β subunit (146). Because anthranalite fluoresces a distinctive blue under UV light, it can be used as a phenotypic marker for identifying mutants in this step (88, 122); these mutants were additionally characterized as being feedback insensitive and thus accumulated Trp to three times the wild-type levels. The second enzyme in Trp biosynthesis converts anthranilate and phosphoribosylpyrophosphate into phosphoribosylanthranilate and inorganic pyrophosphate, and the expression of the gene encoding this enzyme is controlled by regulatory elements located inside the first two introns (165). The third enzyme is responsible for the conversion of phosphoribosylanthranilate into 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (**Figure 1**). *Arabidopsis* has three genes that are differentially regulated in response to UV irradiation and the elicitor silver nitrate in a tissue- and cell-specific manner (83). The subsequent enzyme, indole-3-glycerol phosphate synthase, catalyzes the formation of indole-3-glycerol phosphate from 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate and is the only enzyme known that catalyzes the formation of the indole ring (195). This reaction is therefore important in the production of indolic secondary metabolites, including auxin (indole-3-acetic acid), camalexin, indole glucosinolates, and indole alkaloids. The final step in Trp biosynthesis is carried out in two parts by the α and β subunits of Trp synthase: First, indole-3-glycerol phosphate is cleaved to indole and glycerol-3-phosphate by the α subunit, and then the indole is transferred to the β subunit, which catalyzes its condensation with serine (Ser) to form Trp (137).

Recent studies have shown that Phe and Trp levels are commonly upregulated during such diverse environmental conditions as light, water, and cold stress as well as during dark-induced senescence. Furthermore, it appears that plants are able to convert Phe and Trp into 2-oxoglutarate in a pathway that includes either isovaleryl coenzyme A (CoA) dehydrogenase or D-2-hydroxyglutarate dehydrogenase (9) in an as yet uncharacterized manner. The pattern of accumulation of Phe and Trp levels across the broad range of stress conditions mentioned above hints to potential in planta roles for either the free amino acids themselves or the metabolites thereof when plants are under stress. However, unequivocal elucidation of the functional importance of the aromatic amino acids under such conditions is presently lacking.

Because of the essential nature of aromatic amino acids, boosting their levels in plants was attempted by transforming *Arabidopsis* with a recombinant bacterial *AroG* gene encoding 3-deoxy-D-*arabino*heptulosonate 7-phosphate (DAHP), the product of which was insensitive to feedback inhibition by the aromatic amino acids (192). Expression of this gene yielded a major effect on the levels of intermediate primary metabolites, such as shikimate, Phe, and Trp, and a broad range of secondary metabolites derived from these amino acids, including phenylpropanoids, glucosinolates, and various hormone conjugates (192). Expression of this gene in tomato fruit and also in petunia resulted in enhanced levels of the aromatic amino acids as well as enhanced levels of volatile and nonvolatile phenylpropanoids (151, 193). Interestingly, the Phe levels did not correlate with those of phenylpropanoids in a tomato introgression line population (2); thus, these studies suggest that modifying aromatic amino acid metabolism can be an effective metabolic engineering strategy only when the feedback regulation mechanisms are circumvented.

HISTIDINE METABOLISM

The genetics of His biosynthesis has been regarded as a paradigm for metabolic regulation in fungi and bacteria. It was instrumental in both the discovery of operon structure and the regulation of amino acid biosynthesis by attenuation (3, 166), although its study in plants lagged greatly behind (159). As such, relatively little is known about His biosynthesis in plants. That said, several enzymes of this pathway (**Supplemental Figure 1**; follow the **Supplemental Materials link** from the Annual Reviews home page at **http://www.annualreviews.org**) were identified via the development of selective herbicides that block the pathway (141, 196). Consistent with an essential role of His biosynthesis, *Arabidopsis* mutants independently defective in multiple enzymatic steps of the pathway are present in surveys of embryo-defective genes (135, 143).

All nine enzymes of His biosynthesis have been identified in *Arabidopsis* (90), but it was only in 2010 that the gene encoding the final enzyme of the pathway, *myoinositol monophosphatase–like 2*, was demonstrated to encode a functional histidinol-phosphate phosphatase (155). Prior to this, an almost comprehensive study detailing the consequences of individually overexpressing the other nine enzymes revealed that the majority of the control of free His content in plants resides in the reaction catalyzed by overexpression of the first enzyme of the pathway, ATP-phosphoribosyltransferase (159). Interestingly, as also observed in other amino acid biosynthetic pathways (119), both ATP-phosphoribosyltransferase isoforms are feedback regulated by their product (150).

Another common feature shared with the other plant amino acid biosynthetic pathway is our relative lack of understanding of the transcriptional regulation of His biosynthesis. Indeed, current knowledge is limited to the fact that the genes in *Arabidopsis* are constitutively expressed, albeit only weakly in pollen. It is unclear whether the expression of His biosynthetic genes is strongly coordinated with other amino acid biosynthetic genes in plants, and given that His is often not detected in standard gas chromatography–mass spectrometry metabolite profiling (123), less is known about the coordination of its levels than is known for the other amino acids. That said, it has been reported that His levels correlate with those of eight other amino acids in potato shoots, although this correlation was not apparent in wheat (147) or *Arabidopsis* (159).

Having described the establishment and control of His biosynthesis, we now detail its role in plant cells. Uniquely among the proteinogenic amino acids, the side group of His has a pK_a of approximately 6, which enables it to alternate between protonated and nonprotonated forms under physiological conditions; this allows His to participate in general acid-base catalysis and, as such, renders it an important constituent of the active sites of many plant enzymes (90). In addition, it plays a role in the coordination of metal ions in a range of metallo-proteins such as zinc fingers (81). These features likely explain the above-described embryo-lethal phenotypes of *Arabidopsis* mutants of His biosynthesis.

Free His also plays an important role in nickel tolerance in many nickel-hyperaccumulating plant species, acting as a nickel-binding ligand (110). His biosynthesis is, moreover, intimately linked with nucleotide biosynthesis, sharing 5′-phosphoribosyl 1-pyrophosphate as a precursor with the de novo and salvage pathways of purines, pyrimidines, and the pyrimidine cofactors NAD^+ and $NADP^+$ (212). His also commonly acts as a precursor for Trp, arguably the amino acid that His is most closely linked to, although it is important to note that glutamic acid (Glu) and glutamine (Gln) both act as nitrogen donors in His biosynthesis (90).

Beyond the aromatic amino acids, His is the most energetically expensive amino acid to synthesize, costing between 31 and 41 ATP molecules (90), which probably explains its relatively low abundance outside of the active sites of proteins. In contrast to the other essential amino acids, however, practically nothing is known regarding His catabolism. Given that transcriptional

regulation of amino acid catabolism is a recurring theme in response to environmental stress (119), elucidation of the His catabolic pathway represents an important research priority to enable a comprehensive understanding of the network regulation of amino acid metabolism.

LYSINE METABOLISM

Lys biosynthesis is well characterized in plants, as comprehensively reviewed elsewhere (16, 59, 93). Our understanding of Lys synthesis dates to the 1950s, when the biosynthetic enzymes were studied on the basis of knowledge accrued from bacteria. In 1984, the first Lys-accumulating mutant was identified as a feedback-insensitive form of dihydrodipicolinate synthase (DHDPS) (144), and the 1990s saw the generation of a battery of transgenic plants in which the feedback-insensitive bacterial form of this enzyme was overexpressed in either a constitutive or seed-specific fashion, leading to significantly higher levels of Lys (19, 25, 53, 101, 154, 172, 174). Taken together with research documenting the expression of the genes encoding Lys biosynthesis (194, 209, 210), these studies led to a relatively comprehensive understanding of the regulatory networks underpinning Lys synthesis, revealing that, as in bacteria, Lys is synthesized through a branch of the Asp family pathway (**Figure 2**). The first enzyme of the pathway, Asp kinase (AK), consists of several isoforms that are feedback inhibited by Lys or Thr; Lys also allosterically inhibits DHDPS, the first unique enzyme of Lys synthesis. The other enzymes that participate in the biosynthesis of Lys are dihydrodipicolinate reductase, tetrahydrodipicolinate *N*-acetyltransferase, acyldiaminopimelate aminotransferase, acyldiaminopimelate deacylase, diaminopimelate epimerase, and diaminopimelate decarboxylase, which sequentially catalyze the subsequent conversion of 2,3-dihydrodipicolinate to Lys. However, these six enzymes do not appear to play important regulatory roles in Lys biosynthesis. Studies at the level of gene expression have revealed that the expression of both AK and DHDPS genes is light regulated (194, 209, 210) and that the expression of AK is additionally regulated by photosynthesis-related metabolites, sucrose, and inorganic phosphate (209), with broader analysis at the pathway level (and beyond) indicating that genes for Lys biosynthesis are generally downregulated under abiotic stress (118).

The process of Lys degradation is not fully understood; however, a range of important studies in both plants and mammals have begun to elucidate this important pathway (7, 9, 62, 139, 180, 211). In plants, the presence of a Lys catabolism pathway was confirmed using the [^{14}C]-labeled Lys to reveal that Lys was converted into Glu and α-amino adipic semialdehyde by the bifunctional Lys-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) enzyme (178). In addition, the degradative pathway has been targeted to boost Lys content in plants. In *Arabidopsis*, Zhu & Galili (207, 208) expressed bacterial DHDPS under the control of a seed-specific promoter in a background knockout mutant lacking the LKR/SDH enzyme; the transgenic seeds exhibited a nearly 64-fold increase in the level of free Lys. Additionally, a series of transgenic maize plants overaccumulating Lys have been produced using distinct strategies, including an endosperm-specific RNA interference (RNAi) suppression of the bifunctional LKR/SDH enzyme (57, 86, 163). These combined studies led to the accepted pathway of Lys degradation in plants (**Supplemental Figure 2**). However, results from a recent study of *ivdh-1* and *d2hgdh1-2* mutants of *Arabidopsis* that link protein degradation to the mitochondrial electron chain led to the postulation of a second independent pathway of degradation that is responsible for carrying a considerable proportion of the Lys degradative flux (9). The results of this study were consistent with Lys degradation occurring via a branched pathway partially similar to that described for the bacteria *Rhodospirillum rubrum* (48) (see **Supplemental Figure 2**). However, further genetic studies are still needed to fully elucidate the structure and importance of this alternative pathway in plants.

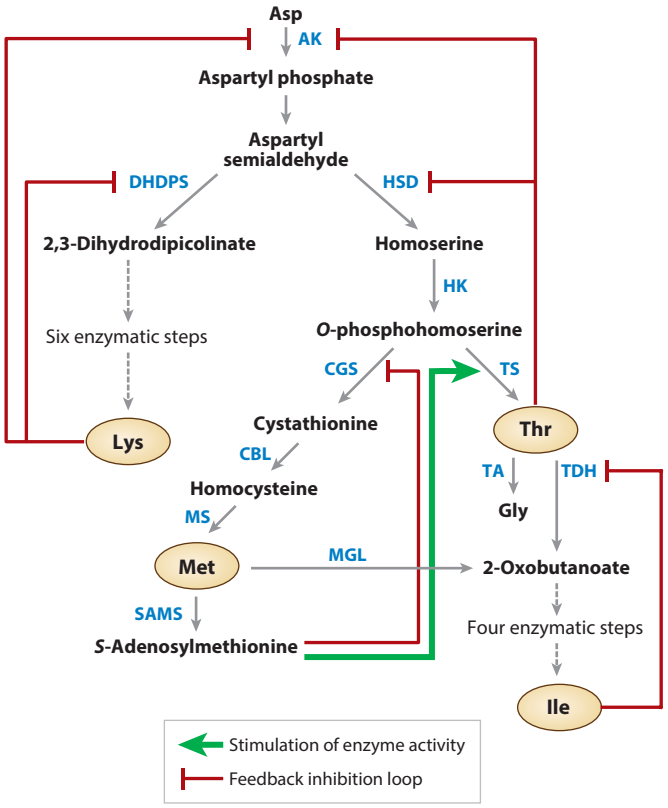

Figure 2

The Asp family of amino acids and the biosynthesis pathways leading to Lys, Thr, Met, and Ile in plants. Enzymes are indicated in blue text. Abbreviations: AK, Asp kinase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; DHDPS, dihydrodipicolinate synthase; HK, homoserine kinase; HSD, homoserine dehydrogenase; MGL, Met γ-synthase; MS, Met synthase; SAMS, *S*-adenosylmethionine synthase; TA, Thr aldolase; TS, Thr synthase; TDH, Thr dehydratase.

The above discussion highlights the interaction of Lys with other metabolic pathways and, as such, describes its important role as an alternative respiratory substrate when cellular carbohydrate supplies are depleted. In keeping with this, Lys levels increase during a wide range of abiotic stresses (148). Furthermore, Lys is important during seed germination, where its catabolism appears to be an important source of carbon to feed the TCA cycle and where levels of free Lys potentially affect protein stability (6). Moreover, as discussed in detail below, Lys, along with several of the other essential amino acids, represents an important energy source during periods of carbon starvation. However, further research is likely needed to establish the mechanisms by which Lys exerts its biological functions, and the recent finding that Lys metabolism also interacts with pathways of alkaloid biosynthesis (26), alongside our incomplete knowledge of Lys degradation, suggests that our understanding of this enigmatic metabolite remains far from comprehensive.

THREONINE METABOLISM

In plants, AK and homoserine dehydrogenase, the first and third enzymes in the Asp pathway, respectively, occur as either mono- or bifunctional proteins and are feedback regulated by Thr

(59), with the reaction catalyzed by Asp semialdehyde dehydrogenase sandwiched between them (**Figure 2**). Previous studies that analyzed mutants possessing a feedback-insensitive isozyme of AK (56) and transgenic tobacco plants overexpressing the bacterial feedback-insensitive form of AK (100) revealed significant accumulation of Thr. These studies proposed that AK activity limits and regulates Thr levels (59). In addition to AK, Thr synthase (TS), the last enzyme in Thr biosynthesis, might also play a regulatory role. Crystallization of TS in the presence (185) or absence (131) of *S*-adenosylmethionine (SAM), the main catabolic product of Met, revealed that its activity is allosterically regulated by SAM. The enzymes isolated from different species appear to exhibit remarkably similar properties, displaying no feedback inhibition by Thr, Met, or Ile with the exception of the enzymes isolated from duckweed and barley, which are repressed by excess Met (16). Given that TS is positioned at the branch point of Thr and Met biosynthesis, several researchers hypothesized that the regulation by SAM influences metabolite partitioning between the two pathways (reviewed in 5, 16, 61). The importance of TS was further confirmed in antisense TS potato and *Arabidopsis* plants, which displayed significant growth retardation (13, 204).

In addition to the regulatory roles of enzymes in the Thr biosynthesis pathway, the level of Thr is regulated by enzymes responsible for its catabolism (34). Two competing catabolic pathways exist in plants: one through Thr deaminase/dehydratase, which produces 2-oxobutanoate, an intermediate required for Ile synthesis (**Figures 2** and **3**), and one through Thr aldolase, which converts Thr to glycine (Gly) and acetaldehyde (94). Two *Arabidopsis* Thr aldolase–encoding genes (*THA1* and *THA2*) were able to complement the previously isolated *Saccharomyces cerevisiae* Gly auxotroph *gly1 shm1 shm2* mutant (95, 98). *THA1* mutations result in greatly enhanced Thr content (95), but *THA2* mutations are embryo lethal (98). Intriguingly, this embryo lethality could be complemented by the expression of a Thr deaminase, indicating that it was likely caused by the overaccumulation of Thr rather than a lack of Gly production.

Thr levels are also regulated by environmental stresses, increasing considerably in response to drought stress, high light, and other light stresses (148). The molecular mechanisms underlying these changes and their physiological significance remain unclear; however, several attempts have been made to understand the coordinate control of transcriptional regulation (97, 118, 119, 156), revealing the relation to Ile biosynthesis.

METHIONINE METABOLISM

The synthesis of Met initiates from an intermediate of the Thr pathway, *O*-phosphohomoserine (**Figure 2**). The first committed enzyme of Met biosynthesis, cystathionine γ-synthase (CGS), combines a carbon/amino skeleton derived from *O*-phosphohomoserine with a sulfur moiety donated by cysteine (Cys) (4, 61, 158). Overexpression of the *Arabidopsis* CGS (AtCGS) protein in tobacco, *Arabidopsis*, potato, and alfalfa plants leads to significant elevations in Met content, revealing the regulatory role of this enzyme in Met accumulation (14, 41, 76, 77, 103). Detailed analysis of AtCGS in *Arabidopsis* mutants with higher levels of Met has identified mutations in a conserved domain named MTO1, which is located in the N-terminal region of this enzyme. The mutations affect the feedback inhibition properties of this enzyme (152). Analysis of these mutants revealed that in the wild-type plants, accumulated levels of SAM induce a temporal arrest of the translation elongation process in the MTO1 domain, leading to mRNA degradation (153, 202). These studies proposed a unique feedback inhibition mechanism on AtCGS and on the Met content. Notwithstanding this regulation, no inverse correlation between high Met levels and low AtCGS mRNA levels was evident in transgenic plants constitutively expressing the endogenous AtCGS (41, 74, 77, 103). Therefore, it is likely that either other *trans* or *cis* elements outside

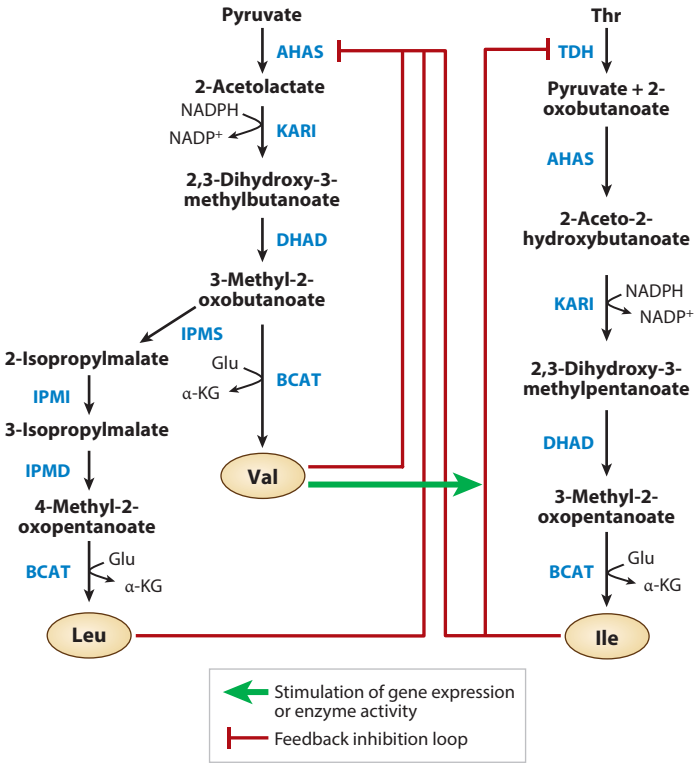

Figure 3

Branched-chain amino acid biosynthesis leading to Leu, Val, and Ile in plants. Enzymes are indicated in blue text. Abbreviations: α-KG, α-ketoglutarate; AHAS, acetohydroxyacid synthase (also known as acetolactate synthase); BCAT, branched-chain aminotransferase; CoA, coenzyme A; DHAD, dihydroxyacid dehydratase; IPMD, isopropylmalate dehydrogenase; IPMI, isopropylmalate isomerase; IPMS, isopropylmalate synthase; KARI, ketol acid reductoisomerase; TDH, Thr deaminase.

the MTO1 region or additional *trans*-acting elements are involved in regulating CGS transcript accumulation. At least one additional regulatory domain was indeed found within the N-terminal region of AtCGS very close to the MTO1 domain (77). Transgenic tobacco plants that overexpress an AtCGS lacking this domain (AtD-CGS), and thus are not subject to feedback regulation by high levels of Met, had Met levels that were nearly ten times those of wild-type plants (77).

Met is a fundamental metabolite in plant cells because, after its conversion to SAM, it controls the levels of several essential metabolites, including ethylene, polyamines, biotin, and phytosiderophores. SAM, as a primary methyl group donor, also regulates important processes such as the formation of chlorophyll, the formation of the cell wall, and the synthesis of many secondary metabolites (164). As expected from the major role that Met plays in plant metabolism, the level of AtCGS is tightly regulated. Its levels are downregulated by SAM (32) but upregulated by sucrose (75), Thr (14), and ethylene (102) in addition to being regulated by environmental stresses (120) and light (75).

Several studies using sense and antisense constructs in potato have demonstrated that cystathionine β-lyase and Met synthase, the two other enzymes of Met biosynthesis, do not play a regulatory role in determining Met content (128, 145). By contrast, several studies have suggested that TS significantly affects Met accumulation (for reviews, see 5, 61). Lower activity of

this enzyme in *Arabidopsis* mutants and in antisense *Arabidopsis* and potato plants led to increases in Met content (of 22-, 49-, and 239-fold in the experiments described in References 13, 17, and 206, respectively). Moreover, overexpression of an *Escherichia coli* TS resulted in retarded seedling growth that could be chemically complemented by the exogenous application of Met (115). Given that TS is positioned at the branch point of Thr and Met biosynthesis, it has been hypothesized that regulation by SAM is influential in metabolite partitioning (5, 61), because SAM negatively regulates the expression level of CGS (153) and positively regulates TS activity by an allosteric mechanism that increases the affinity of TS to *O*-phosphohomoserine (36).

To further study the nature of the competition between TS and CGS for *O*-phosphohomoserine, Lee et al. (115) fed homoserine, which exists upstream of *O*-phosphohomoserine, to *Arabidopsis* plants overexpressing AtCGS. The Met content in these plants increased 180-fold, whereas the Thr content was not significantly altered. These results suggest that the level of *O*-phosphohomoserine might limit Met synthesis (115). To test this assumption further, Hacham et al. (76) crossed tobacco plants overexpressing AtCGS with those expressing bacterial feedback-insensitive AK and exhibiting higher levels of Thr (173). Plants coexpressing these two heterologous genes had significantly higher Met and Thr levels, but the Met level did not increase beyond that found in plants overexpressing only AtCGS, likely owing to the function of the feedback inhibition regulation mediated by SAM on the transcript level of AtCGS (76). However, in plants expressing bacterial AK and AtD-CGS (a Met/SAM feedback-insensitive form), the Met level increased up to 173-fold compared with the level in wild-type plants, whereas the Thr levels increased 3-fold (76). These results suggest that, in the absence of feedback regulation, provision of the carbon/amino skeleton limits Met synthesis.

The AtD-CGS construct was also used to study the role of this enzyme in Met synthesis in seeds, as previous studies had suggested that Met in seeds is mostly transferred from the leaves in the form of *S*-methylmethionine (23, 55, 63, 116). However, seed-specific expression of AtD-CGS in tobacco, *Arabidopsis*, soybean, and kazoki bean led to significantly higher levels of Met, demonstrating the role of CGS in the de novo synthesis of Met in seeds (33, 132, 179). Unexpectedly, in these transgenic seeds, the levels of most other amino acids also significantly increased, leading to higher levels of the total proteins. Furthermore, metabolic and transcriptomic profiles showed that in the late stages of developing *Arabidopsis* seeds, a stimulation of metabolic and transcriptomic responses is associated with desiccation and drought stresses and an altered mitochondrial energy metabolism (33). In these seeds, the levels of glutathione—synthesis of which competes with that of Met for Cys as a precursor—are also significantly reduced. Probably as a result, the germination rate of these seeds is significantly reduced during oxidative stress. However, their germination rate increases under osmotic and salt stresses (33). One possible explanation is that these seeds have higher levels of stress-related metabolites such as sugars, BCAAs, and polyamines and different stress-related transcripts that assist the seeds during the germination process.

The level of Met is also regulated by its main catabolic enzymes, SAM synthase and Met γ-lyase (MGL). Studies that used radioactive Met and in silico modeling to examine the metabolic fates of Met indicated that in *Arabidopsis* leaves, 50% of Met is converted to SAM and its associated metabolites, and the rest is incorporated into proteins (157). As would be anticipated, a reduction in the expression level of SAM synthase leads to significantly higher levels of Met (up to a 443-fold increase) in plants (22, 70, 103, 175). However, owing to the central function of SAM in plant metabolism, the reduction in its content concomitantly leads to severe abnormal phenotypes. Similarly, lower levels of MGL led to a higher level of Met in *Arabidopsis* flowers and seeds (71, 96) and in potato tubers (87). This enzyme converts Met to Ile, and its expression level is upregulated in response to a simultaneous water deficit and nematode infection of roots (11). Indeed, plants overexpressing the *AtMGL* gene were more tolerant of nematode infection. MGL is also involved

in the synthesis of unique aroma volatiles (68, 69). In addition, Met is catabolized to produce glucosinolates, a group of plant secondary metabolites that can accumulate nearly 30% of the total sulfur content of plant organs and exhibit repellent activity against herbivore insects and pathogens (85).

METABOLISM OF THE BRANCHED-CHAIN AMINO ACIDS

Studies of the biosynthesis pathway of the BCAAs revealed that four enzymes operate in Val and Ile biosynthesis: acetohydroxyacid synthase, ketol acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), and branched-chain aminotransferase (BCAT) (20) (**Figure 3**). The biosynthesis of Ile is initiated by the Thr deaminase reaction, whereas the pathway toward Val starts from pyruvate, and Leu biosynthesis is initiated by the conversion of 3-methyl-2-oxobutanoate to 2-isopropylmalate (**Figure 3**). A combination of bioinformatic, cell biological, and proteomic data has revealed that these biosynthetic reactions are confined to the plastid (20). The first enzyme of Ile synthesis, Thr deaminase, cleaves Thr into ammonia and 2-oxobutanoate. It was first identified in a screen for L-*O*-methylthreonine-resistant mutants, which accumulate Ile levels that are 20 times those of the wild type (142) owing to abolition of the normal Ile-mediated feedback inhibition of the enzyme (176). Alternative routes from Thr to Ile also exist, as indicated by the inhibition of TS in potato (detailed above) and more recently by the finding that MGL overexpression can compensate for the loss of Thr deaminase (96). However, it is important to note that these alternatives are relatively minor routes under normal growth conditions. In addition, Thr deaminase is one of three enzymes of BCAA synthesis under allosteric control (**Figure 3**), with inhibition of Thr aldolase by Ile resulting from an increase in $K_{m(Thr)}$ in the presence of Ile, an effect that is antagonized by the presence of Val (80).

The next enzyme, acetohydroxyacid synthase, also known as acetolactate synthase, is the best-studied enzyme of the pathway, being the target for multiple classes of herbicide (134, 182). The enzyme requires thiamine diphosphate as an essential cofactor, which is anchored to the active site by a divalent metal ion such as Mg^{2+} and FAD^{+} as a third cofactor (46). It is a heterodimer consisting of a regulatory and catalytic subunit, with the regulatory subunit mediating feedback regulation by the BCAA, and has a similar structure to Thr deaminase, hinting at a common evolutionary origin (117).

Comparatively little is known about KARI and DHAD in plants. KARI from spinach and rice has been crystallized, revealing that it consistently forms a homodimer (21, 121). KARI is additionally distinguished by displaying an extremely high affinity toward Mg^{2+} (47); however, the biological relevance of this feature remains to be established. More recently, Kochevenko & Fernie (109) found that it colocalizes to quantitative trait loci (QTLs) for BCAA content in tomato. Although antisense inhibition of the tomato enzyme resulted in decreased levels of all BCAAs in leaves and fruits, it had few other metabolic consequences (109). Studies of DHAD are even sparser, although one study showed that it is essential for BCAA biosynthesis (176). Similar to the case for KARI, reverse genetics of *DHAD* in tomato confirmed that it could be a causal QTL for BCAA content and revealed alterations in the levels of several other amino acids and sugars (109).

After the DHAD reaction, ketoisovalerate is utilized either via BCAT, to yield Val, or by isopropylmalate synthase—the first committed step on the path to Leu synthesis (**Figure 3**). Four genes encode isopropylmalate synthase in *Arabidopsis*, and this enzyme has been the subject of many studies because of the importance of two of its isoforms, methylthioalkylmalate synthases 1 and 3 (MAM1 and MAM3, respectively), in controlling the levels of Met-derived glucosinolates (112, 184). The two other family members, MAM2 and MAM4, encode genuine isopropylmalate

synthases and have nearly identical substrate specificities (40). Furthermore, the lack of change in Leu content in MAM2 and MAM4 mutants suggests that the isoforms are able to compensate for one another (40, 54). Isopropylmalate synthase is the third enzyme of BCAA biosynthesis that is subject to feedback regulation; the enzyme acts as a branch point for Leu biosynthesis and competes with BCATs for the common intermediate 3-methyl-2-oxobutanoate. Feedback inhibition by Leu is widely documented (40, 78, 176), but the strength of the inhibition appears to vary substantially across species (35).

The isopropylmalate synthase reaction is followed by a reaction catalyzed by isopropylmalate dehydratase, which is a heterodimer. The functions of the encoding genes have been studied in various mutants; some of these display striking differences in glucosinolate composition, some have clear effects on Val levels, and some show no effect on either glucosinolate or amino acids (82, 106, 168). Intriguingly, a mutant impaired in the expression of large subunit 1 exhibited a severe developmental phenotype, but this phenotype could not be reversed by the application of Leu (67), suggesting that it did not result from a deficiency of this amino acid. More recently, Zhang et al. (206) demonstrated that the enzyme is important for proper gametophyte development and salinity tolerance in *Arabidopsis*. By contrast, even though this enzyme colocates to a QTL for BCAAs, downregulating it in tomato had little effect on the levels of Leu or, indeed, of any other amino acids (109).

The final steps in the biosynthesis of all three BCAAs are carried out by BCAT, which catalyzes the transamination of the respective 2-oxo acids into their corresponding amino acids—i.e., 4-methyl-2-oxopentanoate to Leu, 3-methyl-2-oxobutanoate to Val, and 3-methyl-2-oxopentanoate to Ile (169, 176). BCATs, which can also be involved in the degradation of these amino acids, have been characterized from diverse species, including *Arabidopsis*, spinach, potato, barley, *Nicotiana sylvestris*, melon, and tomato (20, 29, 64, 68, 79, 129, 130). In *Arabidopsis*, seven *BCAT* genes have been identified, of which six are expressed and five are capable of fully complementing the yeast *αbat1 βbat2* double mutant; the exception, *BCAT4*, was only able to restore Leu auxotropy (42). Of these genes, *BCAT2*, *BCAT3*, and *BCAT4* appear to be unequivocally located in the plastid, consistent with a predominantly synthetic role. BCAT2 is dramatically upregulated at the transcriptional level following a range of stresses and hormone treatments (133). BCAT3, by contrast, appears to be involved in both BCAA and glucosinolate biosynthesis (107). In *Arabidopsis*, BCAT4 exhibits unusual substrate specificity, and knockout mutants of this isoform show only a slight reduction in Leu content (170). The location of *Arabidopsis* BCAT5 is controversial: Green fluorescent protein (GFP) studies suggest a chloroplastic location, but proteomics studies imply that it resides in the mitochondria (183). Lächler et al. (114) recently demonstrated that BCAT6 is cytosolic and that mutants in this isoform alone resulted in altered BCAA levels, and quantitative genetics has been used to evaluate the control of amino acid content, with many amino acid QTLs found in recombinant inbred line populations (124, 167). Moreover, genome-wide association mapping has identified BCAT2 as causal for BCAAs in *Arabidopsis* seeds (8). In tomato, BCAT3 and BCAT4 are plastidial and function in BCAA biosynthesis, and overexpression of BCAT4 results in enhanced BCAA levels, confirming its colocation with a QTL on chromosome 3 (130). Interestingly, in some species [such as melon (68)] but not in others [such as tomato (108)], BCAA accumulation limits the production of volatiles derived from the BCAAs, such as 2-methyl-butanol, butylacetate, and ethyl 2-methylbutanoate.

Although the above discussion demonstrates that the biosynthesis of BCAAs is relatively well characterized (as is the intertwined biosynthesis of glucosinolates in the Brassicaceae), our understanding of the catabolic pathway remains far from complete (108) (**Supplemental Figure 3**). Catabolism is initiated in mitochondria, where the branched-chain keto acid dehydrogenase is located (183) and where some isoforms of the BCATs also reside. That the latter operate in the

degradative direction seems reasonable, given that BCAAs can act as precursors of the TCA cycle intermediates succinyl CoA and acetyl CoA as well as direct electron donors of the mitochondrial electron transport chain (9, 10, 91, 92). The various isoforms of the enzymes thus play a critical role in regulating steady-state levels of the BCAAs in plant cells (130). However, the second step in the degradation, which is catalyzed by the high-molecular-weight branched-chain keto dehydrogenase complex, is better characterized; it is similar to pyruvate dehydrogenase and the mitochondrial 2-oxoglutarate dehydrogenase (140, 187) and comprises three subunits, E1 (α-keto acid dehydrogenase), E2 (dihydrolipoyl acyltransferase), and E3 (dihydrolipoyl dehydrogenase). Indeed, the E3 subunit is shared by these complexes, and *Arabidopsis* plants deficient in its expression are characterized by altered levels of Leu and Ile (187) as well as a decreased response to arsenate (30). Moreover, in a heterotrophic cell suspension culture of *Arabidopsis*, Taylor et al. (183) detected branched-chain keto acid dehydrogenase activity with the 2-oxo acids of all three BCAAs, albeit with slight preference for 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate. Furthermore, when they provided BCAAs to isolated mitochondria, they observed high rates of oxygen consumption for Val and Leu but virtually none for Ile, suggesting that Val and Leu are degraded in the mitochondria. The importance of this complex in the formation of acyl sugars, which play important roles in defense against insects, has additionally been demonstrated in *Solanum pennellii* and *Nicotiana benthamiana* (177).

In the next step, the different acyl coesters are oxidized by an acyl CoA dehydrogenase. The isovaleryl CoA dehydrogenase has been relatively well characterized, with proteins identified with differing substrate specificities in *Arabidopsis*, potato, and pea (38, 39, 52, 160). Screening for *Arabidopsis* mutants defective in free amino acid accumulation identified a line in which the levels of 12 amino acids were altered, including at least 16-fold increases of all three BCAAs as well as changes in glucosinolates. These chemotypes were subsequently attributed to an inactivated isovaleryl CoA dehydrogenase, confirming the links between BCAAs, Met, and glucosinolate metabolism (73). Detailed evaluation of the *Arabidopsis* isovaleryl CoA dehydrogenase further revealed that it plays an important role not only in BCAA degradation but also in the breakdown of Lys (10, 50).

The catabolism of amino acids feeds into the electron transfer flavoprotein pathway via reactions catalyzed by isovaleryl CoA dehydrogenase and 2-D-hydroxyglutarate dehydrogenase (10, 50). Moreover, and most importantly, the results observed under a range of different growth conditions suggest a role for the electron transfer flavoprotein pathway not only during the severe stress imposed by extended darkness but also under conditions experienced by most plants at some stage during their life cycles (9, 28). Although 2-hydroxyglutarate, the substrate of 2-D-hydroxyglutarate dehydrogenase, is a well-known metabolic intermediate in bacteria, yeast, and mammals, little or nothing is known about the biological function of this metabolite in plant tissues (50). Based on feeding experiments, Araujo et al. (9) suggested that in plants, Lys can be degraded via 2-hydroxyglutarate or α-ketovalerate, and in *Arabidopsis*, the flux loads carried by these alternative pathways are approximately equivalent. In plants, the electron donation to the electron transfer flavoprotein system occurs via the catabolism of BCAAs, aromatic amino acids, and Lys in the case of isovaleryl CoA dehydrogenase, and by the catabolism of aromatic amino acids and (particularly) Lys in the case of 2-D-hydroxyglutarate dehydrogenase. In addition, metabolite analysis of the mitochondrial protein complex composition following oxidative stress suggested that enhancement of amino acid catabolism into the TCA cycle might compensate for a reduced electron supply from the TCA cycle (149). The fact that degradation of BCAAs, aromatic amino acids, and Lys provides electrons to the mitochondrial electron transport chain by both direct and indirect routes represents a strong connection between amino acid metabolism and the TCA cycle (105).

After the oxidation reaction of isovaleryl dehydrogenase and the concomitant electron transfer reaction documented above, Leu degradation proceeds via a carboxylation reaction catalyzed by methylcrotonyl CoA carboxylase, which was the first described enzyme of Leu degradation (1) (**Supplemental Figure 2**). *Arabidopsis* knockouts of methylcrotonyl CoA carboxylase revealed that inhibition of the expression of either subunit blocks mitochondrial Leu catabolism and results in an impaired reproductive growth phenotype, including aberrant flower and silique development and decreased seed germination (43). This step is followed by the action of an enoyl CoA hydratase; two genes encoding this enzyme have been predicted to be imported into the mitochondria, and proteomic evidence has substantiated these predictions in one instance (138). Surprisingly, however, these genes are not coexpressed with other genes of BCAA catabolism. The reactions catalyzed by enoyl CoA hydratase result in the formation of a range of hydroxyl acyl CoAs, which are subsequently catabolized by a range of as yet poorly characterized enzymes located in the mitochondria or peroxisome (51, 66, 162). It thus appears that BCAA metabolism is a good example of a mosaic pathway occurring across several metabolic compartments (181). Moreover, coexpression analysis links BCAA degradation to that of Lys as well as that of the ubiquitination and autophagic pathways of protein degradation (10). Interestingly, a recent study documented that all of the essential amino acids measured were significantly reduced in autophagy mutants following carbon starvation (12), further demonstrating the links between these processes. Despite these findings, however, Val and Leu levels correlated neither with the levels of other primary and secondary metabolites nor with yield-associated traits in the above-mentioned *S. pennellii* introgression line population (2). By contrast, Ile levels correlated with those of homoserine, Phe, proline (Pro), ribose, uracil, fructose, and galactonic acid and with fresh weight and total yield.

The above-mentioned gas chromatography–mass spectrometry survey of plant metabolic responses to abiotic stress revealed that Ile levels and Thr levels react similarly to stress: Both are considerably elevated in response to drought stress, high light and other light stresses (148), and heat stress. Val and Leu generally also react similarly, but Val is unique in accumulating considerably in response to UVB stress (113). Our present understanding of the roles of the BCAAs under such conditions remains poor; however, it is worth noting that studies in several species, including tomato, wheat, and barley, have indicated that BCAAs play a role in plant responses to drought (24, 171, 200). Thus, although these studies provide first hints of the in planta role of free BCAAs, it is clear that considerable further research effort is required in order to fully understand their roles beyond feeding into other biosynthetic pathways or being catabolized to provide energy.

BIOFORTIFICATION OF LYSINE, METHIONINE, THREONINE, AND TRYPTOPHAN

Among the nine essential amino acids discussed here, the levels of four—Met, Lys, Thr, and Trp—are considered to limit the nutritional quality of plants because their contents in plants are very low compared with the levels required for optimum growth of humans and animals. Additionally, at least two other amino acids, Cys and Tyr, which are classified as conditionally essential amino acids, are synthesized in mammals only from the essential amino acids Met and Phe, respectively, and thus their levels also limit nutritional quality (198). When one amino acid is present at a low level, the others are catabolized and used as an energy source, which hinders protein synthesis and thus lowers protein content below that required for normal body function. In cultures with a primarily vegetarian diet and in developing countries in which plant-derived foods are predominant, this

can lead to nonspecific protein deficiencies that can affect mammalian organs, including kidney function; the brain function of infants and young children; gut mucosal function and permeability; and the immune system. The physical signs of protein deficiency include decreased blood proteins, retarded mental and physical development in young children, poor musculature, and dull skin (84). This syndrome is referred to as protein–energy malnutrition, and the World Health Organization (198) estimates that approximately 30% of people in the developing world suffer from it. In many cultures, cereals (deficient in Lys, Thr, Trp, and Tyr) are combined with legumes (deficient in Met and Cys) to overcome this limitation, but the levels of these amino acids still limit the nutritional value of plant-based diets. Hence, in many Western countries, synthetic or fermented amino acids are added to animal-based foods in order to gain a more balanced diet (49).

Classical breeding and mutant screening have mostly failed to increase the levels of these amino acids, and even when the content per se has been increased, it has normally come at the cost of a significantly reduced crop yield (89, 161). Genetic engineering research appears to be more promising, particularly given that heterologous gene choice can be informed by considerable prior research. Moreover, the use of tissue-specific promoters is able to circumvent some abnormal phenotypes.

Four different approaches (and combinations of them) have largely been used in attempts to increase the levels of these four amino acids in the vegetative tissues and seeds of transgenic crop plants (**Supplemental Table 1**). The first approach is to increase the synthesis of these amino acids, mainly by expressing feedback-insensitive regulatory enzymes in their biosynthesis pathways. The second is to reduce their catabolism by suppressing the expression of the main catabolic enzymes through approaches such as RNAi or antisense techniques. This method has succeeded mainly in elevating the levels of Lys and Met (60) and has been less successful for Thr and Trp. The reasons for this are unknown but might be related to the roles of Thr and Trp catabolic products in plant metabolism: Thr is used for the synthesis of the three BCAAs, Gly, and acetaldehyde (94), and Trp serves as a precursor for indole-3-acetic acid, tryptamine, serotonin, melatonin, phytoalexins, vitamin B_3, indole glucosinolates, and other indole alkaloids that play important roles in plant development, plant-insect interactions, and defense against pathogens (e.g., 27, 45). The third approach is to express storage protein genes that are rich in codons for one or more of these four amino acids. This technique is usually used for seed storage proteins, which are sometimes fortified with additional codons for these essential amino acids or have synthetic peptides enriched with these amino acids (reviewed in 4, 16, 111). The fourth approach is to suppress a gene encoding a seed storage protein that is poor in the content of one or more of these amino acids. This sometimes leads to the accumulation of other storage proteins enriched in the desired amino acids, as observed in mutants such as *opaque 2*, which has a low level of α-zein but high levels of other Lys-rich storage proteins (15, 136).

Although these approaches have made considerable progress, efforts are still needed to combine them in order to further elevate the levels of these amino acids. Additionally, it will be worthwhile to test plants under different growth conditions in order to define the best growth conditions for high essential amino acid production. Such studies should also investigate the effects of different nitrogen and sulfate levels in the soil, because these nutrients are known to affect the protein profile (31, 104, 111). It will be also valuable to elucidate the effects of the manipulations on the phenotype and on genome-wide gene expression programs and primary metabolic profiles. Because all four of these amino acids plays important metabolic roles in plants, such manipulations could also shed light on how plants tolerate the metabolic perturbations that occur in their central core pathways.

SUMMARY POINTS

1. Evaluation of breeding populations and natural variants indicates that, as with many metabolites, the genetic architecture underlying the control of essential amino acids tends to be highly complex.
2. The biosynthesis pathways of all nine essential amino acids are feedback regulated by their products in inhibitory loops.
3. The pathways of amino acid degradation remain relatively poorly understood, and in many cases our understanding of the reactions that constitute the catabolism of these metabolites is incomplete.
4. Transcriptional regulation of essential amino acid biosynthesis has been investigated under a wide range of stresses, suggesting that the abundance of the amino acids is upregulated not only by protein degradation but also by de novo synthesis under these conditions.
5. There is considerable evidence showing that essential amino acids serve as alternative respiratory substrates and as substrates for secondary metabolite production.
6. Reverse genetics strategies have had considerable success in elevating of the contents of Lys, Met, Thr, and Trp in crop species.

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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8. Describes the first genome-wide association study of plant amino acid levels.

10. Summarizes data showing that in plants, amino acids are not only used for protein synthesis but also serve as energy donors via their degradation in the TCA cycle.

12. Shows that autophagy serves as an essential process, helping organisms mobilize proteins as an energy source and thus survive when they are starved of various important metabolites (particularly carbohydrates).

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