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The aspartic acid metabolic pathway, an exciting and essential pathway in plants

Review Article

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Summary. Aspartate is the common precursor of the essential amino acids lysine, threonine, methionine and isoleucine in higher plants. In addition, aspartate may also be converted to asparagine, in a potentially competing reaction. The latest information on the properties of the enzymes involved in the pathways and the genes that encode them is described. An understanding of the overall regulatory control of the flux through the pathways is undisputedly of great interest, since the nutritive value of all cereal and legume crops is reduced due to low concentrations of at least one of the aspartate-derived amino acids. We have reviewed the recent literature and discussed in this paper possible methods by which the concentrations of the limiting amino acids may be increased in the seeds.

Keywords: Aspartic acid – Asparagine – Isoleucine – Lysine – Methionine – Threonine

Introduction

Amino acids in the soluble form are key participants in the general metabolism of plants, but their major role is as a constituent of proteins (Medici et al., 2004a, b). In addition, over 300 non-protein amino acids have been isolated from plants (Lea and Azevedo, 2003), having important roles in medicine, nutrition and agriculture. They also act as metabolic pathway intermediates and nitrogen storage molecules (Ferreira et al., 2005a), but in some cases are toxic to humans (Camargos et al., 2004). Among the protein amino acids, nine are termed as ''essential'', since they cannot be synthesized by humans and monogastric animals, and must be obtained through the diet (Ferreira et al., 2005b). Lysine, threonine, methionine and isoleucine are essential amino acids which share a common precursor, aspartate, in a branched and complex regulated pathway (Fig. 1) (Azevedo and Lea, 2001).

Although the classic role of agriculture in producing food crops to feed an increasing population is still valid, there has been for many years a strong and growing demand for better and improved nutritional quality (Azevedo, 2002). For instance, cereal crops represent approximately 50% of the plant protein consumed worldwide; however, cereal seeds are nutritionally deficient in important amino acids such as lysine, threonine and tryptophan (Helm et al., 2004), a situation that has presented researchers with the major task of improving the nutritive value of cereal seeds.

In 1997, Azevedo et al. published a review article in which each step of the aspartic acid metabolic pathway was reported and all the properties of the enzymes, and regulatory mechanisms were discussed in detail. Taking into account the information available at the time, the authors reassessed the five main conclusions originally proposed by Giovanelli et al. (1989) and also questioned whether transgenic crop plants containing elevated concentrations of aspartate-derived amino acids in the seed would be available to farmers within a five-year period. Has such a goal been achieved?

It can be seen from Fig. 1 that the synthesis of asparagine could compete for aspartate, with metabolism to lysine, threonine and methionine. The literature has rarely examined in an integrative manner the interaction between these two pathways. However, we think it is important to discuss in this review the route of asparagine

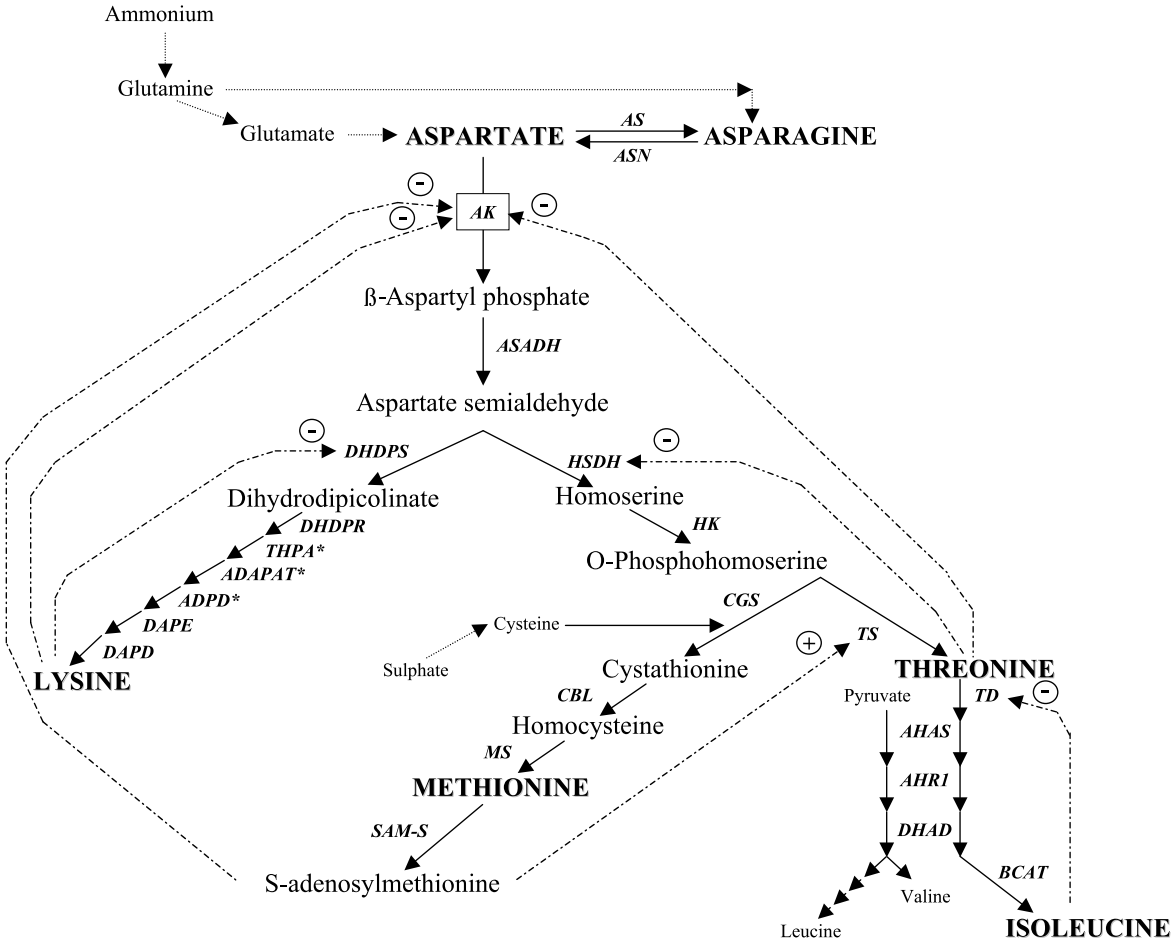

Fig. 1. The aspartate metabolic pathway of higher plants. *AS*, asparagine synthetase (E.C. 6.3.5.4); *ASN*, asparaginase (E.C. 3.5.1.1); *AK*, aspartate kinase (E.C. 2.7.2.4); *ASADH*, aspartate semialdehyde dehydrogenase (E.C. 1.2.1.11); *HSDH*, homoserine dehydrogenase (E.C. 1.1.1.3); *HK*, homoserine kinase (E.C. 2.7.1.39); *CGS*, cystathionine γ-synthase (E.C. 4.9.99.9); *TS*, threonine synthase (E.C. 4.2.99.2); *CBL*, cystathionine β-lyase (E.C. 4.4.1.8); *MS*, methionine synthase (E.C. 2.1.1.13); *SAM-S*, *S*-adenosylmethionine synthetase (E.C. 2.5.1.6); *TD*, threonine deaminase (E.C. 4.2.1.16); *AHAS*, acetohydroxyacid synthase (E.C. 4.1.3.18); *AHRI*, acetohydroxy acid isomeroreductase (E.C. 1.1.1.86); *DHAD*, dihydroxy acid dehydratase (E.C. 4.2.1.9); *BCAT*, branched-chain amino acid aminotransferase (E.C. 2.6.1.42); *DHDPS*, dihydrodipicolinate synthase (E.C. 4.2.1.52). *DHDPR*, dihydrodipicolinate reductase (E.C. 1.3.1.26); *THPA**, tetrahydrodipicolinate acylase (E.C. 2.3.1.117); *ADAPAT**, *N*-acyl-L,L-diaminopimelate aminotransferase (E.C. 2.6.1.17); *ADPD**, *N*-α-acyl-L,L-diaminopimelate deacylase, (E.C. 3.5.1.18); *DAPE*, diaminopimelate epimerase (E.C. 5.1.1.7); *DAPD*, diaminopimelate decarboxylase (E.C. 4.1.1.20). $\ominus$, negative feeback. $\oplus$, positive feedback. An asterisk indicates an enzyme for which Hudson et al. (2005) were unable to obtain evidence that they were involved in lysine biosynthesis in higher plants

synthesis and present some elements that could help put it into perspective with the aspartate pathway. We are hopeful that the recent advances in metabolic profiling (Fernie et al., 2005) will offer a possibility to examine the dynamic of the aspartate metabolic pathway and understand better its regulation in the context of wider metabolite fluxes such as asparagine.

The aspartate metabolic pathway

Aspartate is the initial starting compound, which is formed by the transamination of oxaloacetate, and may be derived from the Krebs tricarboxylic acid cycle in the mitochondria, or through the operation of phospho*enol*-pyruvate carboxylase or phospho*enol*pyruvate carboxykinase in the cytoplasm (Lea et al., 2001). Aspartate is a precursor for two main pathways, the first one leading to the synthesis of asparagine, a key compound used for the transport and storage of nitrogen in plants, which is synthesized by the transfer of the nitrogen from the amide group of glutamine to aspartate, catalyzed by asparagine synthetase. The enzyme asparaginase carries out the catabolism of asparagine forming ammonia, which is reassimilated through the glutamate synthase cycle (Andrews et al., 2004). Aspartate is also the precursor of the aspartate-derived amino acids, lysine, threonine, methionine

and isoleucine (Azevedo et al., 1997) (Fig. 1). The enzymes of asparagine metabolism are located in the cytosol, whilst gene sequencing studies have recently confirmed that the enzymes involved in the synthesis of the remainder of the aspartate-derived amino acids are located in the chloroplasts of the leaves, or in the plastids of non-photosynthetic organs, such as seeds and roots (Lea and Azevedo, 2003). Due to the low lysine and threonine concentrations in cereal seeds and their importance as essential amino acids, studies have been carried out in order to obtain a better understanding of the regulation of the pathway, with the ultimate aim of constructing plants by genetic manipulation that can overproduce and accumulate higher amounts of lysine and threonine in the seeds (Hesse and Hoefgen, 2003; Zhu and Galili, 2003; Hesse et al., 2004a; Tang and Galili, 2004).

Aspartate kinase and homoserine dehydrogenase

Aspartate kinase (AK, EC 2.7.2.4) catalyzes the first enzymatic reaction of the pathway which involves the phosphorylation of aspartate to produce β-aspartyl phosphate (ASA). AK was initially studied in bacteria and later characterized in detail in a wide range of plant species (Ferreira et al., 2005b). At least two distinct AK isoenzymes can occur in higher plants, a monofunctional lysine-sensitive isoenzyme, which is involved in the overall regulation of the pathway and can be synergistically inhibited by *S*-adenosylmethionine (SAM) (Rognes et al., 1980), normally accounting for the majority of AK activity, with rare exceptions (Lugli et al., 2002). The other isoenzyme is a bifunctional threonine-sensitive AK and homoserine dehydrogenase (HSDH, EC 1.1.1.3) protein, first proposed in plants by Aarnes and Rognes (1974), as previously reported for some but not all bacterial enzymes. This finding was later confirmed by Wilson et al. (1991) and Azevedo et al. (1992a) using molecular and biochemical approaches, respectively. HSDH is the first enzyme committed to the synthesis of threonine and methionine and also exists as separate isoenzymes, the cytoplasmic threonine-resistant HSDH isoenzyme, whose physiological function is still unknown, and the bifunctional threonine-sensitive AK-HSDH as described above (Azevedo et al., 1992a, b).

Following over-expression in *Escherichia coli* (Paris et al., 2002a, 2003), the protein structure of *Arabidopsis thaliana* AK-HSDH was shown to comprise a 66-amino-acid N-terminal region, a 276-amino-acid AK region, a 246-amino-acid intermediary threonine regulatory domain and a 328-amino-acid C-terminal HSDH region. The intermediary regulatory domain of threonine-sensitive AK-HSDH contains two similar subdomains, each with a common loop–α-helix–loop–β-strand–loop–β-strand motif in a manner analogous to that of threonine deaminase (see below). Paris et al. (2003) carried out a series of mutations of key glutamine residues which had been proposed to be in the threonine binding region. Steady-state kinetic experiments on wild-type and mutant enzymes demonstrated that each regulatory domain of the monomers of AK-HSDH contained two nonequivalent threonine binding sites, constituted in part by Gln443 and Gln524. The results also demonstrated that interaction of threonine with Gln443 led to inhibition of AK activity and facilitated the binding of a second threonine on Gln524. Interaction of this second threonine with Gln524 led to inhibition of HSDH activity.

A new AK-HSDH cDNA of *A. thaliana* designated *akthr2* has been cloned by functional complementation of a *Saccharomyces cerevisiae* strain mutated in the HSDH gene (*hom6*) (Rognes et al., 2003). The expression pattern of the *akthr2* gene was studied in transgenic *A. thaliana* in comparison with the *akthr1* gene of tobacco and shown to be expressed in meristematic cells, leaves and stamens. The main differences between the two genes concerned the time-restricted or absent expression of the *akthr2* gene in the stem and the gynoecium and during seed formation, while *akthr1* was less expressed in roots (Rognes et al., 2003).

Two different cDNAs were initially isolated from *A. thaliana* that encode a monofunctional lysine-sensitive AK (Frankard et al., 1997; Tang et al., 1997). Yoshioka et al. (2001) screened a gene trap library of *A. thaliana* and isolated a third gene encoding a lysine-sensitive AK, designated as *AK-lys3*, which was highly expressed in the xylem of leaves and hypocotyls and stele of roots. Significant expression of this gene was also observed in trichomes after bolting. Slight expression of *AK-lys3* was detected in vascular bundles and mesophyll cells of cauline leaves, inflorescence stems, sepals, petals, and stigmas. These results indicated that the *AK-lys3* gene is not expressed uniformly but in a spatially specific manner.

A transgenic forage plant species (*Medicago sativa*) over-expressing the *E. coli* feedback-insensitive AK exhibited enhanced levels of both free and protein-bound threonine, which was in some of the transgenic lines accompanied by significant reduction in aspartate and glutamate. This data suggests that in alfalfa, AK might not be the only limiting factor for threonine biosynthesis, and that the concentration of soluble threonine may limit

its incorporation into protein (Galili et al., 2000). Some limited success has been obtained in increasing the concentration of methionine in legume seeds by simultaneous expression of a feedback-insensitive AK and Brazil nut 2S albumin (Demidov et al., 2003). A desensitized AK gene has been developed as a non-antibiotic selection marker for use in the production of transgenic chickpea, which showed that the level of AK activity detected in lysine- plus threonine-selected plants was higher than that detected in the non-transformed control plants (Tewari-Singh et al., 2004).

Aspartate semialdehyde dehydrogenase

The β-aspartyl phosphate produced in the reaction catalyzed by AK, is then converted to β-aspartyl semialdehyde (ASA) in a reaction catalyzed by the enzyme aspartate semialdehyde dehydrogenase (ASADH, EC 1.2.1.11) in an NADPH-dependent reaction. Following this, the pathway is divided into two branches, one leading to lysine formation, whereas the other branch is subsequently divided to form two subbranches, with one leading to the synthesis of threonine and the other methionine (Fig. 1). It has been suggested that inhibitors of ASADH may serve as useful antibacterial, fungicidal, or herbicidal agents (Hadfield et al., 2001) and the crystal structure and regulation of gene expression of bacterial ASADH have been determined (Hadfield et al., 2001; Tunca et al., 2004). However, ASADH has still not been well characterized in plants and perhaps the first and only detailed characterization of a plant ASADH is the one reported by Paris et al. (2002b), who isolated a cDNA clone encoding ASADH with a plastid transport sequence from *A. thaliana*. The enzyme was purified following expression in *E. coli* and shown to be homodimeric with subunits of 36 kDa, which exhibited a cooperative behavior for aspartyl phosphate. There was no evidence of channeling of substrates between the AK-HSDH and the ASADH enzyme proteins (Paris et al., 2002b).

Homoserine kinase

The homoserine produced as a result of the HSDH reaction is phosphorylated to *O*-phosphohomoserine, with the production of ADP, by the action of the enzyme homoserine kinase (HK, EC 2.7.1.39) in the presence of ATP. HK is the fourth enzyme of the aspartate pathway and belongs to a large yet unique class of small metabolite kinases termed the GHMP kinase superfamily (Zhou et al., 2000). In a manner similar to that of ASADH, this enzyme has been characterized in bacteria and the structure determined by X-ray crystallography, which has revealed the presence of a highly specific homoserine and a novel nucleotide binding site (Krishna et al., 2001). In plants, the enzyme is a dimer of approximately 75 kDa and requires potassium ions for activity. A HK cDNA clone isolated from *A. thaliana* was shown to be composed of an 1113 bp continuous open reading frame, encoding a 38 kDa protein, with an amino acid terminal sequence exhibiting features of a plastid transit peptide. An active enzyme protein was expressed in *E. coli* which was not subject to regulation by end-product amino acids (Lee and Leustek, 1999). When HK was overexpressed in *A. thaliana*, there was no effect on concentration of soluble *O*-phosphohomoserine, methionine and threonine. However, the addition of homoserine stimulated the accumulation of all three amino acids, indicating that the rate of synthesis of homoserine and not *O*-phosphohomoserine, is a major limiting factor (Lee et al., 2005a).

Threonine synthase and cystathionine γ-synthase

The *O*-phosphohomoserine produced by the action of the enzyme HK is an important intermediate of the aspartate pathway, since it is the branch-point where threonine and methionine synthesis diverge (Fig. 1). *O*-Phosphohomoserine can be converted to threonine by the allosteric enzyme threonine synthase (TS, EC 4.2.99.2), the fifth enzyme involved in the synthesis of threonine from aspartic acid. *O*-Phosphohomoserine can also form cystathionine by the action of the two-substrate enzyme cystathionine γ-synthase (CGS, EC 4.2.99.9), the first enzyme committed to methionione biosynthesis (Hesse et al., 2004b). Furthermore, modelling analysis revealed that TS and CGS display similar kinetic efficiencies in the metabolic context considered and are first-order for the *O*-phosphohomoserine substrate (Curien et al., 2003). It is interesting to note, however, that in vitro activity measurements indicate that in plants TS has a 250- to 500-fold higher affinity for *O*-phosphohomoserine than the competing enzyme of the methionine pathway, CGS. This would suggest that most of the carbon from aspartate is channelled to threonine rather than methionine.

TS and CGS have been very well characterized in microorganisms and plants, particularly in recent years. Both enzymes have significant impact on threonine and methionine biosynthesis, which play important roles in cellular metabolism. Furthermore, methionine is not only an important amino acid for protein synthesis but is a

precursor of several metabolites (Nikiforova et al., 2002), and it is the immediate precursor of SAM, which is a major methyl group donor in transmethylation reactions and is also an intermediate in polyamines biosynthesis and of the phytohormone ethylene (Ravanel et al., 1998). Interestingly, CGS and TS, do not appear to exist as isoenzymes (Azevedo et al., 1997).

The crystal structure of TS has been reported for microorganisms (Garrido-Franco et al., 2002; Omi et al., 2003) and plants (Laber et al., 1999; Thomazeau et al., 2001). Perhaps the main feature of plant TS, in contrast to its bacterial counterpart, is the allosteric regulation by SAM, which is able to stimulate dramatically TS activity (Hesse et al., 2004b), although TS can also be inhibited by AMP (Curien et al., 2003). The potato *StTS* gene encodes a TS protein with a molecular mass of 57 kDa. The amino-terminal sequence contains a number of serine and threonine residues, which are common features of transit peptides. Gene expression was differentially regulated in a tissue-specific manner, with the *StTS* transcripts being most abundant in source leaves and flowers. Less expression was detected in sink leaves, stems, stolons and roots, and the lowest level of expression was found in tubers. Photosynthesis-related metabolites such as sucrose, oxaloacetate and phosphate, nitrogenous compounds, or intermediates/products such as *O*-phosphohomoserine, threonine, homoserine, asparagine, and glutamine did not influence gene expression (Casazza et al., 2000).

The crystal structure of the CGS from *Nicotiana tabacum* has been characterized (Steegborn et al., 2001). In contrast to other regulatory enzymes in the aspartate pathway, the CGS enzyme is not an allosteric enzyme and is not subject to feedback inhibition by methionine or any of its metabolites. Following ground breaking experiments by Giovanelli and colleagues with *Lemna paucicostata* (Giovanelli et al., 1989), it is now established that CGS is regulated at the level of transcription and also post-transcription in some but not all plants. CGS mRNA and enzyme activity are reduced in the presence of methionine in *A. thaliana*, following destabilisation of mRNA by the MTO1 region of the nascent polypeptide chain (Chiba et al., 1999, 2003; Lambein et al., 2003). Somewhat surprisingly, in potato there is no evidence of any change in CGS mRNA or enzyme activity, in the presence of increased methionine (Kreft et al., 2003).

Efficient plant transformation techniques and the isolation of the genes encoding the key enzymes in the regulation of the aspartate pathway has allowed the engineering of plants with altered enzyme regulation and activity (Nikiforova et al., 2002). TS and CGS transgenic plants have been produced and a considerable amount of new information has been obtained. Cultured cells of transgenic tobacco expressing an *E. coli* SAM-insensitive TS exhibited a 5-fold increase in soluble threonine (Muhitch, 1997). In the leaves of an *A. thaliana* mutant (*mto2*) with reduced TS activity, a 16-fold reduction in soluble threonine and 22-fold increase in methionine was recorded (Bartlem et al., 2000). A series of antisense lines of transgenic potato with gradually reduced TS mRNA and enzyme activity were constructed by Zeh et al. (2001). A maximum of a 239-fold increase in methionine was determined in the leaves with a small decrease in threonine. However, in the tubers there was a 20-fold higher methionine concentration and also an increase in isoleucine, without a reduction in threonine.

Transgenic *A. thaliana* plants overexpressing CGS exhibited l8- to 20-fold increases in soluble methionine and *S*-methylmethionine (SMM) in the leaf and root of seedlings and the flowers, and roots of mature plants. However, analysis of the CGS protein indicated that it was present mainly in the leaf tissue and thus methionine must be transported rapidly away from the point of synthesis to the flowers and roots (Kim et al., 2002). Initial attempts to increase methionine production in potato by the overexpression of a potato CGS gene were not successful, despite the presence of small increases in enzyme activity (Kreft et al., 2003). However, when an *A. thaliana* CGS gene was introduced into potato, there was evidence of increased concentrations of soluble methionine and methional in the leaves and tubers. Methional is the compound responsible for the aroma of potatoes after cooking and baked potatoes obtained from field-grown transgenic plants were still shown to contain elevated concentrations (Di et al., 2003). Transgenic alfalfa overexpressing *A. thaliana* CGS in chloroplasts contained greatly increased concentrations of soluble cysteine, glutathione, methionine and SMM and over twofold increases in both protein-bound methionine and cysteine in the leaves (Avraham et al., 2005). The increase in cysteine and glutathione demonstrated by Avraham et al. (2005) was somewhat surprising, but very encouraging for the production of animal feed containing high concentrations of sulfur-containing amino acids. A series of methionine overaccumulating *A. thaliana* mutants (mto) have been obtained by specific mutations in the CGS (*mto1-1*; Inaba et al., 1994), TS (*mto2-1*; Bartlem et al., 2000) and *S*-adenosylmethionine synthetase (*mto3-1*; Shen et al., 2002; *mto3-2*; Goto et al., 2002) genes.

On the other hand, antisense expression of CGS RNA in transgenic *A. thaliana* led to reduced (90%) CGS

activity, severe stunting of growth, morphological abnormalities and inability to flower, with little change in soluble methionine and SMM contents (Kim and Leustek, 2000). Similar stunted morphology was detected in lines of *A. thaliana* that exhibited gene silencing of CGS, although growth could be restored to a certain extent following the addition of methionine (Kim et al., 2002). However, when antisense constructs were inserted into potato, there was a 93% reduction in CGS activity and a 80% decrease in the soluble leaf methionine concentration. Conversely, there was no reduction in protein methionine and no morphological phenotypic changes were observed in the potato plants with low CGS activity (Kreft et al. 2003).

Although CGS plays a key role in methionine biosynthesis, recent reports have clearly confirmed a much broader importance for CGS, since the enzyme is thought to catalyze the synthesis of Se-cystathionine from Se-cysteine, the first step in the conversion of Se-cysteine to volatile dimethylselenide (Van Huysen et al., 2003). Indian mustard transgenic lines overexpressing *A. thaliana* CGS exhibited 2- to 3-fold higher Se volatilization rates when compared to the wild type, suggesting the overexpression of CGS in plants could provide a promising phytoremediation approach to remove Se from contaminated sites (Van Huysen et al., 2003; Gratão et al., 2005a, b).

Cystathionine β-lyase, methionine synthase, *S*-adenosylmethionine synthetase

Methionine and SAM biosyntheses can be considered together due to their key role in the regulation of methionine cycling. Cystathionine β-lyase (CBL, EC 4.4.1.8), methionine synthase (MS, EC 2.1.1.14) and *S*-adenosylmethionine synthetase (SAM-S, EC 2.5.1.6) are the three enzymes involved (Hesse et al., 2004b).

CBL is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the penultimate step in the synthesis of methionine in microorganisms and plants by the β-cleavage of cystathionine to yield homocysteine, pyruvate and ammonia. Such as for CGS and TS, plant CBL has been extensively studied in the last few years. The enzyme has been purified to homogeneity from a range of plant sources and shown to be a tetrameric quaternary structure with a 45.7 kDa subunit. The three-dimensional structure of CBL from *A. thaliana* has been determined and a catalytic mechanism postulated. The active site of the plant enzyme is larger than that of *E. coli*, due to the absence of an arginine residue and allows the *A. thaliana* enzyme to use djenkolate as a substrate as well as cystathionine (Breitinger et al., 2001). The role of CBL in methionine synthesis has been recently investigated in more detail by different strategies involving the use of transgenic plants. An antisense construct of potato CBL was used to produce potato transgenic plants with reduced CBL levels, that expressed a severe stunted phenotype. As would be expected there was a reduction in methionine content and increases in cystathionine, homoserine, cysteine but also somewhat unexpectedly an increase in homocysteine was observed. The results demonstrated a central role of CBL in methionine synthesis, plant growth and development (Maimann et al., 2000). On the other hand, when potato CBL was overexpressed in potato, resulting in accumulation of both CBL transcript and protein and a 2.5-fold increase in enzyme activity, there was no change in the growth habit of the plants. Analysis of the amino acid concentrations both upstream and downstream of CBL in the leaves did not demonstrate any significant changes in the metabolites (Maimann et al., 2001). When the *A. thaliana* CBL gene was overexpressed in the chloroplast of *A. thaliana*, despite a 4-fold increase in CBL activity and protein in the transgenic plants, there was no change in the soluble methionine, SMM, cysteine or threonine contents (Gakière et al., 2002). The two sets of data taken together suggest that CBL does not play a major role in regulating the flux through the methionine synthetic pathway.

The conversion of homocysteine to methionine is catalyzed by MS, using N5-methyltetrahydrofolate as the methyl donor. The enzyme has been purified from plant species and the crystal structure of an *A. thaliana* MS complexed with zinc, homocysteine and methyltetrahydrofolate has recently been reported (Ferrer et al., 2004). The MS gene was shown to be differentially expressed in potato, being highest in the flowers and lowest in stems and tubers. The expression of MS in the leaves was induced by light, sucrose and sucrose-derived products (Zeh et al., 2002). In barley, MS has been shown to be expressed extensively throughout the pericarp and to a lesser extent in the transfer cell layer of the barley endosperm (Sreenivasulu et al., 2002) and to be induced by salt stress (Narita et al., 2004).

The majority of the earlier enzyme localisation and organelle feeding studies have indicated that the final step in methionine synthesis takes place in the cytoplasm and that homocysteine was transported out of the chloroplast. However, recent studies by Ravanel et al. (2004) in which they provided compelling evidence for the existence of a separate chloroplastic form of MS have revolutionized this hypothesis. In *A. thaliana*, three genes encoding

vitamin B12-independent MS isoenzymes were identified, two of which were directed towards the cytoplasm (*atms1*, *atms2*) and one to the chloroplast (*atms3*). The three different mRNAs were detected in all analyzed organs (roots, stems, leaves, flowers, siliques, and seeds), demonstrating that the corresponding genes were transcriptionally active. However, the *atms1* mRNA coding the first cytosolic MS isoform was the most abundant transcript in all organs, with the highest level in flowers and the lowest level in roots. The *atms2* mRNA coding the second cytosolic MS isoform largely followed the *atms1* mRNA expression pattern and was generally 4- to 10-fold less abundant than *atms1*, with the exception of flowers. The *atms3* gene displayed a fairly constant and very low expression level in stems, leaves, flowers, and siliques. In these organs the mRNA coding the plastidial MS isoform was 65- to 430-fold less abundant than the mRNAs coding the cytosolic isoforms. This low level of expression of the *atms3* gene may well explain the earlier inability to detect the enzyme in leaf chloroplasts. Ravanel et al. (2004) proposed that the plastid isoenzyme is most likely required for the methylation of homocysteine that is synthesized de novo, suggesting that plastids are the only site of methionine synthesis in plant cells. The other MS isoenzymes present in the cytosol are most probably involved in the regeneration of methionine from homocysteine, produced in SAM-dependent methylation reactions.

The conversion of methionine to SAM is catalyzed by SAM-S in an ATP-dependent reaction. As indicated above there is now new evidence that the last step of methionine synthesis can occur in chloroplasts, nevertheless all the evidence still indicates that SAM is synthesized solely in the cytosol. There is however a considerable demand for SAM inside the chloroplast for a range of methylation reactions including the synthesis of chlorophyll, tocopherols, and plastoquinones and several proteins of the thylakoid membranes and the stroma. Thus SAM is transported into chloroplasts by a carrier-mediated facilitated diffusion process, which may also transport *S*-adenosylhomocysteine (Ravanel et al., 2004).

In *A. thaliana*, four *SAMS* genes have been identified by analysis of the sequenced genome. Two *SAMS* genes, *SAMS1* and *SAMS2*, have been previously characterized, and they are 97% identical at the amino-acid level (Shen et al., 2002). Genomic and cDNA clones of SAM-S were isolated from mustard (*Brassica juncea*) (Lim et al., 2002). Expression analysis revealed that the amount of SAM-S transcripts varied, showing strong accumulation in the roots, flower buds, seed pods and leaves of flowering plants, but were less abundant in the stems, petioles and leaves of vegetative plants. Exogenous application of several compounds, including polyamines and NaCl, upregulated SAM-S transcripts, whereas polyamines inhibited ethylene production, indicating that ethylene production in mustard may be in part regulated by SAM-S transcript level in a gene-specific manner (Lim et al., 2002). Up-regulation of SAM-S transcript and increase in enzyme activity have also been observed in *Suaeda salsa* plants subjected to NaCl stress (Ma et al., 2003). It appears that SAM-S responds to several stress conditions. For instance, recent proteomic analysis of two barley lines, abiotic-stress-tolerant and -sensitive, subjected to heat stress resulted in differential expression of SAM-S between the two cultivars (Sule et al., 2004). Proteomic analysis of *A. thaliana* seeds also revealed differential accumulation of MS and two distinct forms of SAM-S during germination, indicating that both enzymes may be involved in controlling metabolism during the transition from the quiescent to highly active state during seed germination (Gallardo et al., 2002).

Papaver somniferum tissue culture cells transformed with the SAM-S gene (*sam-1*) from *A. thaliana* exhibited an increase in SAM-S activity when compared to the control whereas polyamine levels varied (Nabha et al., 1999). The different expression of the *sam-1* gene of *A. thaliana* in *N. tabacum* callus resulted in increased SAM-S activity and SAM accumulation, but also reflected in the pattern of secondary products present in the cell lines (Belbahri et al., 2000). The consequences on methylation of lignin precursors and pectins have also been investigated in *Linum usitatissimum* transgenic cell lines overexpressing the *A. thaliana sam-1* gene, which exhibited an increase of pectin methylesterification but no effect on other compounds (Lamblin et al., 2001). Lignin is a major metabolic sink for the methyl groups of SAM and was shown to decrease by 21.75% in the *mto-3* methionine-overaccumulating *A. thaliana* mutant, probably due to the reduced SAM supply and total SAM-S activity observed in this mutant (Shen et al., 2002).

Enzymes involved in isoleucine synthesis

Threonine deaminase

Threonine deaminase (TD, EC 4.2.1.16) is the first and only unique enzyme in the isoleucine biosynthetic pathway, catalyzing the deamination and dehydration of threonine to produce 2-ketobutyrate and ammonia. One isoenzyme which is predominantly present in younger developing tissues is inhibited by isoleucine and has been

termed ''biosynthetic''. A second ''biodegradative'' isoenzyme that is insensitive to inhibition by isoleucine has been demonstrated in older senescing tissues (Singh, 1999). The *A. thaliana* biosynthetic TD is a tetramer composed of identical 59.6 kDa subunits. The inhibitory binding of isoleucine to TD induces dimerization of the enzyme, whereas tetramerization is restored by addition of high concentrations of valine (Halgand et al., 2002). Kinetic and binding experiments demonstrated that each regulatory domain of the monomers of *A. thaliana* TD possesses two different effector-binding sites constituted in part by Tyr-449 and Tyr-543. It has been demonstrated that Tyr-449 belongs to a high-affinity binding site whose interaction with a first isoleucine molecule induces conformational modifications yielding a protein with an enhanced ability to bind a second isoleucine at a lower-affinity binding site containing Tyr-543. Isoleucine interaction with this latter binding site is responsible for conformational modifications leading to final inhibition of the enzyme. Tyr-449 interacts with both regulators, isoleucine and valine, however, the interaction of valine with the high-affinity binding site induces different conformational modifications leading to the reversal of isoleucine binding and reversal of inhibition (Wessel et al., 2000).

The transformation of tobacco with both wild-type and isoleucine-insensitive forms of *E. coli* TD (*ilvA*) gave rise to plants with elevated concentrations of isoleucine, most of which displayed a stunted growth habit (Ebmeier et al., 2004). Site-directed mutagenesis in the two regulatory regions of *A. thaliana* TD allowed the construction of isoleucine-insensitive forms of the enzymes. *A. thaliana* plants transformed with TD genes mutated in one site exhibited increases in isoleucine concentration of between 2- and 15-fold. However a 106-fold increase in isoleucine concentration was demonstrated in a double mutant that was affected in both sites, which could be used as a novel selectable marker for plant transformation (Garcia and Mourad, 2004).

The biosynthetic form of TD has been identified as a major soluble protein in tomato flowers and the gene encoding a 55 kDa peptide has been isolated and characterized (Samach et al., 1991). A 51-amino-acid chloroplast transit peptide rich in serine and threonine was identified from the cDNA sequence. The expression of the TD gene was shown to be 250- to 500-fold higher in floral organs as compared to leaves, roots or seeds. Immunolocalization studies indicated that the enzyme protein was quite specifically located in the parenchyma cells of petals stamens and sepals. Later studies showed that dramatic increases in the expression of TD could be induced by wounding and methyl jasmonate in the parenchyma of leaves and flowers (Samach et al., 1995). In experiments to determine the effects of the insect *Manduca sexta* on gene expression in the leaves of its natural host *Nicotiana attenuata*, the expression of biosynthetic TD gene showed the strongest and most specific response to insect attack. mRNA transcripts encoding TD accumulated to high concentrations after herbivory and the application of methyl jasmonate to leaves (Hermsmeier et al., 2001). The strong herbivore-induced expression of TD may reflect the high demand for enzyme activity for the synthesis of defence compound precursors derived from 2-ketobutyrate.

Acetohydroxyacid synthase

AHAS (EC 4.1.3.18), also known as acetolactate synthase (ALS), carries out a series of parallel reactions required for the biosynthesis of leucine, valine and isoleucine. In the pathway that produces isoleucine, the enzyme catalyzes the condensation of pyruvate with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate. In the reaction that produces valine and leucine, AHAS catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate. The enzyme requires thiamine pyrophosphate, flavine adenine dinucleotide and a divalent cation for both condensation reactions (Azevedo et al., 1997). AHAS is the target of a range of potent low-dose herbicides and for this reason the enzyme has been the subject of intense study, particularly with the respect of changes to the amino acid sequence of the protein that confer herbicide resistance (Kim et al., 2004; Pang et al., 2004; McCourt et al., 2005).

An unusual feature of plant AHAS is that it is inhibited by all three of the branched-chain amino acids, unlike most bacterial and fungal enzymes that are sensitive to valine only. Moreover, the plant enzyme is also subject to synergistic inhibition by the combination of leucine plus valine. Initially there was some confusion as to the localization of the binding site(s) of the feedback inhibitor end product amino acids, until it became clear that the native plant AHAS enzyme contained not only a catalytic subunit but also a smaller regulatory subunit (Lee and Duggleby, 2001). A molecular model of the *A. thaliana* AHAS regulatory subunit has been proposed in which the amino acid sequence contains two domains created by an internal duplication, with one binding valine or isoleucine and the other leucine. The evidence for a leucine-specific site is that reconstitution of the catalytic subunit with the one domain of the regulatory subunit yields

an enzyme that is inhibited by leucine but not by valine or isoleucine. The second domain is unable to bind leucine because saturation with leucine would abolish the effects of valine (or isoleucine), whereas it is observed that saturation with leucine enhances the affinity for valine (Lee and Duggleby, 2002). More recently, the crystal structure of the catalytic subunit of yeast AHAS has been resolved at 0.28 nm (Kim et al., 2004), whereas crystallization and preliminary X-ray diffraction analysis of the *A. thaliana* catalytic subunit in complex with the sulfonylurea herbicide chlorimuron ethyl has been reported (Pang et al., 2004).

Higher plants have a variable number of AHAS genes, mainly depending on the level of ploidy. Diploid *A. thaliana* has one constitutive copy (Mazur et al., 1987), allotetraploid *N. tabacum* has two unlinked AHAS loci, whose genes are expressed in a coordinated manner in all tobacco organs; *Zea mays*, which has been defined a cryptic allopolyploid, has two very similar AHAS genes; *Brassica napus*, an amphidiploid of *B. campestris* and *B. oleracea*, has five AHAS genes, with the different isoforms being developmentally regulated in a tissue-specific manner (Singh, 1999). No introns have yet been found in AHAS genes, whilst putative chloroplast transit peptides have been identified in all cDNA clones so far sequenced, confirming the localization of AHAS activity (Miflin, 1974). Unlike for the gene for TD as discussed earlier, there is no evidence that AHAS is overexpressed in flowers. Most studies showed an overall pattern of constitutive expression with higher levels being detected in young meristematic tissue (Singh, 1999). A wide range of AHAS genes conferring tolerance to herbicides have been discovered in plants through mutagenesis and selection, the majority of this work is beyond the scope of this review (Kochevenko and Willmitzer, 2003; Jung et al., 2004; Tan et al., 2004).

Acetohydroxy acid isomeroreductase

Acetohydroxy acid isomeroreductase (AHRI, EC 1.1.1.86), also known as ketolacid reductoisomerase (KARI), isomerises and then reduces the two acetohydroxyacids to produce dihydroxyacids. The enzyme is also of considerable interest in agrochemical research because it is absent from animals, making it a potential target for specific herbicides and fungicides (Dumas et al., 2001; Lee et al., 2005b). The enzyme has been purified from spinach and barley and studies on the spinach enzyme overexpressed in *E. coli* indicate that the enzyme probably exists as a dimer with a subunit of 59 kDa (Wessel et al., 1998). The enzyme has similar high affinity for the two substrates, which appear to compete for the same binding site. However the V_{max} for the two plant enzymes is 6–11 times higher for 2-aceto-2-hydroxybutyrate than for 2-acetolactate (Dumas et al., 1992; Durner et al., 1993). H–D exchange experiments indicated that Mg^{2+} ions and NADPH binding to AHRI lead to an initial conformational change at the interface of the two domains of each monomer, with the binding of the two cofactors altering the structure of the active site to promote either substrate or inhibitor binding (Halgand et al., 1999). The crystal structure of plant AHRI complexed with its substrate, NADPH and Mn^{2+} ions has been solved to a resolution of 0.16 nm (Thomazeau et al., 2000) and also with an inhibitor (Biou et al., 1997).

A gene encoding AHRI has been isolated from spinach and *A. thaliana*, and only one copy could be detected per haploid genome. The *A. thaliana* gene, which contained nine introns, encoded a protein precursor of 591 residues including a putative chloroplast transit peptide of 67 amino acids (Dumas et al., 1993).

Dihydroxy acid dehydratase

Dihydroxy acid dehydratase (DHAD, EC 4.2.1.9) again carries out two reactions in which 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate is dehydrated to form 2-oxoisovalerate or 2-oxo-3-methylvalerate, respectively. A range of auxotrophic mutants requiring isoleucine, leucine and valine for growth have been isolated (Singh, 1999), indicating that it is likely that only one copy of the gene is present (Singh, 1999). The enzyme has been purified to homogeneity from spinach and shown to be a dimer with a subunit of about 62–63 kDa. The enzyme was shown to contain a [2Fe–2S] cluster, a novel finding for an enzyme of the hydrolyase class (Flint and Emptage, 1988).

Aminotransferase

The final metabolic step in the production of the amino acids leucine, isoleucine and valine is a transamination reaction catalyzed by the branched-chain amino acid aminotransferases (BCAT, EC 2.6.1.42). The crystal structure of the *E. coli* enzyme and the mechanism of the binding of the substrates have been identified by Goto et al. (2003). In plants, Hagelstein et al. (1997) isolated two different forms of aminotransferases from spinach chloroplasts, one which was able to use

2-oxoisovalerate as a substrate and thus form valine and another which could use either 2-oxo-3-methylvalerate or 2-oxoisocaproate as substrates to form isoleucine and leucine respectively.

Two different cDNAs have been isolated from potato encoding for BCAT (*BCAT1* and *BCAT2*). Southern analysis indicated that *BCAT1* and *BCAT2* were not in identical regions of the genome and that *BCAT1* may exist in multiple copies. The deduced amino acid sequences for both cDNAs contained putative transit peptides for plastid or mitochondrial localization. Analysis of gene expression, indicated an induction of *BCAT1*, relative to *BCAT2*, in leaf callus cultures exposed to treatment with naphthalene acid and 6-benzylaminopurine (Campbell et al., 2001). More recently, seven putative *BCAT* genes have been identified in *A. thaliana* and six of the cDNA sequences cloned (Diebold et al., 2002). The deduced amino acid sequences exhibited between 47.5% and 84.1% identity to each other and 30% to the homologous enzyme from yeast. Analysis of the subcellular localization of BCATs in *A. thaliana* using GFP fusion proteins, indicated that three of the genes encoded plastid forms and one gene encoded a mitochondrial form of the enzyme. The localization of the enzymes encoded by the two remaining genes was not clear. Diebold et al. (2002) proposed that the chloroplast aminotransferases are involved in the biosynthetic reactions and that a separate pathway of branched-chain amino acid degradation takes place in the mitochondria.

Enzymes involved in lysine metabolism

In an article in this issue (Stepansky et al., 2006), the metabolism of lysine, particularly the molecular genetics and roles of the enzymes involved in lysine degradation, will be discussed in much greater detail. We present here a general view of the lysine branch of the pathway and a more general view of the main enzymes studied in plants.

Dihydrodipicolinate synthase

The first enzyme unique to lysine synthesis, dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) catalyzes the condensation of pyruvate and aspartate semialdehyde to form 4-hydroxy-2,3,4,5-tetrahydrodipicolinate. DHDPS has been purified and characterized from a range of plant species and shown to be very sensitive to inhibition by low concentrations of lysine (Azevedo et al., 1997; Lee et al., 2001). The crystal structure of *Nicotiana sylvestris* DHDPS with and without inhibitory lysine bound to the enzyme has been solved to a resolution of 0.28 nm. The molecule is a homotetramer composed of two tightly bound dimers. Comparison with the structure of *E. coli* DHDPS (which is a 100-fold less sensitive to lysine) showed that following the binding of lysine there is a profound rearrangement of the dimers forming the tetramer (Blickling et al., 1997). Further work on the crystal structure of *E. coli* DHDPS and three mutant forms confirmed that a catalytic triad of Tyr133, Thr44 and Tyr107 played a major role in the enzyme reaction (Dobson et al., 2004).

Genes encoding DHDPS have been isolated from several plant species and a range of mutagenized forms of maize DHDPS were isolated that contained single amino acid changes that reduced inhibition by lysine (Shaver et al., 1996). Substitution of arginine for tryptophan at position 53, or asparagine for isoleucine at position 80, in mutant forms of *A. thaliana* and tobacco DHDPS were shown to prevent the inhibition of the enzyme by lysine. The possible structure of the lysine binding allosteric site of plant DHDPS has been discussed in more detail by Vauterin et al. (2000).

Initially only one gene encoding DHDPS was isolated from *A. thaliana*, and the expression of promoter-GUS constructs in transformed *A. thaliana* and tobacco was found to be cell type-specific and mainly in the fast growing tissues, where protein synthesis rates were high (Vauterin et al., 1999). However, more recently a second gene (*dhdps-2*) has been characterized by two independent laboratories. It exhibits 84% identity at the nucleotide level with the previously cloned *dhdp-1* cDNA (Craciun et al., 2000; Sarrobert et al., 2000). Both groups confirmed that the *dhdps-1* gene is located on chromosome III, whilst the *dhdps-2* gene is on chromosome II. The *dhdps-2* gene was expressed in *E. coli* to form a functional enzyme that was strongly inhibited by lysine, with a 50% loss of activity at 30 μM lysine, which is slightly less sensitive compared to DHDPS-1, for which 50% inhibition was at a lysine concentration of 10 μM.

Craciun et al. (2000) observed that expression of the *dhdps-2* gene in *A. thaliana* was strikingly similar to that observed for the *dhdps-1* gene. In vegetative parts of the plants, GUS expression driven by the *dhdps-2* promoter was detected in meristems and vasculature. Roots showed expression in the root tips, mainly in the elongation zone and in the meristems of emerging lateral roots. Expression of the *dhdps-2* gene was strongly detected in the vasculature of stems and leaves but only slightly in the mesophyll cells of the leaves. In the reproductive organs, strong *dhdps-2* expression was observed in anthers, developing

pollen, carpels and developing seeds. Sarrobert et al. (2000) observed some differences in the expression of *dhdps-2*, which they showed to be predominant in the elongation zone of the root tips and pollen grains. No *dhdps-2* promoter driven GUS activity was detected in the leaves or stems. The key question is whether the two enzymes encoded by the two genes play a different role in the growth of the plant. Knockout mutants of *A. thaliana* with insertions in the *dhdps-2* gene exhibit close to normal growth but have a reduced lysine and greatly increased threonine content (Craciun et al., 2000; Sarrobert et al., 2000).

The final steps of lysine synthesis

Following the formation of dihydrodipicolinate, a further six enzymes are required for the synthesis of lysine. Some basic information is available concerning 2,3-dihydrodipicolinate reductase (DHDPR, EC 1.3.1.26) (Tyagi et al., 1983), diaminopimelate epimerase (DAPE, EC 5.1.1.7) (Tyagi et al., 1982) and *meso*-2,6-diaminopimelate decarboxylase (DAPD, EC 4.1.1.20) (Kelland et al., 1985), which catalyze the pyridine nucleotide-linked reduction of dihydrodipicolinic acid to tetrahydrodipicolinic acid and the pyridoxal phosphate-dependent decarboxylation of *meso*-diaminopimelic acid to lysine, respectively. However, no information is available concerning the other enzymes in the pathway of lysine synthesis in plants as shown in Fig. 1, which is based on the synthetic route demonstrated in bacteria, which can use either *N*-acetyl or *N*-succinyl derivatives.

Very recently Hudson et al. (2005) set out with the positive intention of identifying the missing steps in the biosynthesis of lysine in higher plants. They were unable to measure significant activity of *N*-α-succinyl-α-amino-ε-ketopimelate-glutamate aminotransferase, *N*-α-succinyl-L,L-diaminopimelate deacylase or *N*-α-acetyl-L,L-diaminopimelate deacylase in extracts of three higher plant species and one algal species, despite the fact that lysine synthesis was determined. Hudson et al. (2005) then carried out a search of the *A. thaliana* complete gene sequence database for orthologues of genes encoding putative lysine biosynthetic enzymes. Two genes encoding dihydrodipicolinate synthase, three encoding dihydrodipicolinate reductase, four encoding *N*-α-acyl-L,L-diaminopimelate deacylase, one encoding diaminopimelate epimerase and two encoding diaminopimelate decarboxylase were detected and shown to be expressed. In contrast, genes encoding tetrahydrodipicolinate acylase and *N*-acyl-L,L-diaminopimelate aminotransferase were not identified in the *A. thaliana* database. Hudson et al. (2005) cast doubt on the role of *N*-α-acyl-L,L-diaminopimelate deacylase in lysine biosynthesis on the grounds that a substrate would not exist and that the enzyme protein could not be transported into the chloroplast. Thus it would appear likely that the pathway from tetrahydrodipicolinate to diaminopimelate in higher plants shown in Fig. 1 is incorrect, but at the present time the precise route is not known.

Lysine breakdown

The lysine catabolic pathway in plants was barely investigated in the 70s and 80s, but due to the necessity of producing cereal crop seeds with a balanced amino acid distribution, which directly involve increasing lysine content, and to the relatively little success in producing high-lysine seeds by only altering enzymes involved in lysine biosynthesis, much more attention was given to the control of lysine breakdown in the 90s.

Initial studies of lysine catabolism in plants were carried out using ^{14}C-labelled lysine (Sodek and Wilson, 1970) and showed that lysine is catabolized via saccharopine in a two consecutive enzymes reaction, involving lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8, also known as lysine α-ketoglutarate reductase [LKR]), which catalyzes the formation of saccharopine, and saccharopine dehydrogenase (SDH, EC 1.5.1.9), which hydrolyzes saccharopine to glutamate and α-aminoadipic acid (Arruda et al., 2000).

Most of the LOR-SDH activities are present in a single bifunctional polypeptide (Gaziola et al., 1997; Galili, 2002; Lima et al., 2003a, b), although monofunctional forms of both enzymes have also been detected (Zhu et al., 2000; Tang et al., 2002). Analysis of recombinant *A. thaliana* LOR-SDH and monofunctional LOR enzymes expressed in yeast cells revealed that the K_m of the monofunctional LOR for lysine was nearly 10-fold lower than that of the LOR-SDH bifunctional enzyme (Tang et al., 2002). Biochemical properties such as optimum pH, K_m for saccharopine and NAD were similar for monofunctional and bifunctional *A. thaliana* SDH, although SDH activity may be limiting in vivo because of its apparent non-physiological pH optimum of activity (Zhu et al., 2000). LOR activity has been shown to be stimulated by lysine via a complex cascade of calcium and protein phosphorylation/dephosphorylation (Miron et al., 1997). Similar results have also been suggested for the enzyme from other plant species (Kemper et al., 1998; Gaziola et al., 2000).

After some success in producing high-lysine biochemical mutants and transgenic plants expressing altered regulation of lysine biosynthetic enzymes (Azevedo et al., 1990; Shaul and Galili, 1992a, b; 1993; Heremans and Jacobs, 1997; Lee et al., 2001), it became apparent that the rate of lysine breakdown may regulate the amount of lysine that can accumulate in the seed (Azevedo, 2002). Studies with the opaque-2 maize mutant were the most significant ones concerning lysine catabolism, since this mutant is characterized by a high-lysine concentration when compared to wild-type maize seeds, which is due to an altered storage protein distribution and higher accumulation of lysine in the soluble form (Gaziola et al., 1999). However, it was also confirmed that LOR and SDH activities were drastically reduced in the opaque-2 maize mutant endosperm (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996). Biochemical and molecular analyses revealed that the *o2* gene, which encodes a transcriptional activator factor, regulates LOR activity in the opaque-2 maize mutant endosperm, where LOR enzymatic activity, protein content and mRNAs have been shown to be reduced in accordance with expression pattern alterations during the development of the grain (Kemper et al., 1999). More recently, higher lysine concentrations were observed in the mature seeds of a LOR-SDH *A. thaliana* knockout mutant (Zhu et al., 2001). In order to obtain a better understanding of the role of lysine synthesis and degradation in determining lysine concentration in the seed, a bacterial lysine feedback-insensitive DHDPS was expressed in both wild-type and LOR-SDH knockout *A. thaliana* plants (Zhu and Galili, 2003). Although DHDPS overexpressing and LOR-SDH knockout plants exhibited severalfold increases in soluble lysine, a tremendous synergistic 80-fold increase in lysine was observed in plants in which both traits were combined, in addition there was a dramatic increase in methionine and, to a lesser extent, glutamine and asparagine (Zhu and Galili, 2003).

Today, it is generally accepted that for the production of plants with high lysine concentrations, several strategies may be employed. These include the identification and selection of naturally occurring high-lysine mutants such as opaque-2 and floury maize, which are excellent examples of the success of a simple and traditional, but effective approach (Azevedo, 2002). Unfortunately, the high-lysine mutants exhibited undesirable agronomic characteristics and only several years after the discovery of the opaque-2 mutant has such an agronomic setback been partially overcome with the development of Quality Protein Maize (QPM) (Lopes et al., 1995; Gaziola et al., 1999). Other techniques such as the production of biochemical mutants and in particular transgenic plants are quite efficient as long as the main regulatory steps are properly targeted.

It may also be interesting to investigate in more detail other natural high-lysine mutants, in addition to the opaque-2 maize mutant. For instance, several other recessive and semidominant high-lysine maize mutations have been available for some time, but only recently have they been investigated concerning lysine metabolism and storage proteins (Hunter et al., 2002). An analysis of AK, HSDH, LOR and SDH in opaque and floury high-lysine maize mutants revealed major changes in activity in the developing endosperm, which were dependent on the genotype (Azevedo et al., 2003, 2004a, b). These results suggest that the final total lysine concentration in the maize seed is dependent not only on the storage protein synthesis but also on the rate of lysine biosynthesis and degradation, which are differently affected by the opaque and floury mutations (Azevedo et al., 2003, 2004a, b). Other plant high-lysine mutants such as those already obtained for sorghum and rice may be of considerable interest to study further lysine, threonine and methionine metabolism in cereal seeds (Fornazier et al., 2005).

Asparagine synthetase

Enzyme

The major biosynthetic pathway for asparagine in plants is via the glutamine-dependent asparagine synthetase (AS, EC 6.3.5.4). This enzyme catalyzes the transfer of amide group from glutamine to aspartate in an ATP-dependent reaction. Due to the many difficulties encountered in assaying AS activity from plants, most of the data regarding this enzyme has been obtained via molecular analyses. The reasons suggested have been the rapid turnover of the enzyme, the presence of endogenous inhibitors and asparaginase activity (Joy, 1988; Lam et al., 1996). In 2000, Romagni and Dayan described an extraction procedure and assay conditions for a reliable and direct assay of AS activity in crude plant extracts in several leguminous species. In peas, aminooxyacetic acid (AOA, 1 mM), which prevents the assimilation of aspartate into the TCA cycle, was necessary to measure AS activity, but not in lupin or soybean.

AS appears to be encoded by a small gene family (one to three genes) in most plant species (Nakano et al., 2000). AS cDNAs have been characterized from many plant species including pea (2), asparagus (1),

arabidopsis (3), maize (1), rice (1), soybean (2), alfalfa (1), sunflower (3), barley (2) and common bean (2) (Moeller et al., 2003). The full-length clones isolated indicate that plant AS proteins are composed of 579- to 591-amino-acid residues of a molecular mass of approximately 65 kDa. The identity between all the AS sequences is very high (approximately 70–80%) at the N terminus, whilst major divergences are observed in the last 30–40 amino acids of the C terminus. AS enzymes in plants are believed to be glutamine dependent, as they have four amino acids (Met-Cys-Gly-Ile) binding glutamine that have been characterized in their human counterpart (van-Heeke and Schuster, 1989).

Asparagine metabolism

Asparagine is an important form of nitrogen in plants, as it is used for both storage and transport. It is involved in nitrogen mobilization during grain filling, germination and natural senescence (Joy, 1988; Lea et al., 1990) and accumulates in response to sugar starvation (Brouquisse et al., 1992), dark/light transitions (Schultz, 1998) and to various stresses such as mineral deficiencies, salt or drought (Stewart and Larher, 1980; Pinheiro et al., 2004). In tomato roots, the asparagine concentration is increased in response to cadmium treatment (Chaffei et al., 2004). Asparagine also increases during sulfur deprivation in tobacco (Migge et al., 2000), suggesting a redirection of the nitrogen surplus and carbon that cannot be used in methionine synthesis towards asparagine via aspartate. However, a metabolic study of the sulfur network by Nikiforova et al. (2004) showed that in *A. thaliana* seedlings subjected to sulfur deprivation, although the concentrations of cysteine and glutathione and somewhat unexpectedly lysine decreased, those of the remainder of the aspartate family, including methionine, remained unaffected.

During sugar starvation in tomato roots, protein degradation led to the release of ammonium and storage of nitrogen mainly as asparagine and glutamine (Brouquisse et al., 1992; Devau et al., 2003). The accumulation of asparagine during carbon starvation is probably triggered by an increase in AS gene transcription (Chevalier et al., 1996; Baldet et al., 2002), associated with a strong induction of the enzymatic activity (Brouquisse et al., 1992). Asparagine, however, does not seem to be an unequivocal marker of C starvation, as Gary et al. (2003) showed that in prolonged darkness, asparagine accumulated in the leaves and flowers of tomato, whilst it remained constant in the root and fruit, despite the exhaustion of carbon. N starvation conditions caused by prevention of nitrogen fixation in nodulated soybean, led to diminished asparagine and elevated aspartate concentrations in the xylem sap, which correlated with a reduction in AS enzyme activity (Lima and Sodek, 2003).

Asparagine is also involved in the response of plants to pathogen attack (Yang et al., 2005). An increase in asparagine and a strong accumulation of AS transcripts was observed in tomato leaves infected by *Pseudomonas syringae* (Olea et al., 2004). Following infection, immunolocalization studies on AS demonstrated that the enzyme protein accumulates in the phloem cells of the main vascular bundles and in secondary bundles of the leaf blade. This localization data indicates a possible role of cytosolic glutamine synthetase and AS in the synthesis of asparagine for long-distance transport via the phloem (Olea et al., 2004).

So far, little attempt has been made to correlate changes in asparagine metabolism with those in the aspartate pathway. In a study by Zhu and Galili (2003) on a *A. thaliana* LKR/SDH knockout mutant transformed with a bacterial DHDPS expressed in the seed, the authors observed a considerable increase in lysine, a small increase in soluble asparagine and glutamine along with a decrease in aspartate and glutamate. Zhu and Galili (2003) proposed that, although these steady-state level measurements do not necessarily reflect metabolic processes, glutamine and asparagine could operate as pools for lysine synthesis, via glutamate and aspartate.

AS gene regulation

The specific regulation of the AS isogenes and their particular function are poorly understood, but it is known that AS expression is mainly regulated by the carbon/nitrogen status of plant tissues (Lam et al., 1998).

Regulation by nitrogen

The *ASN1* and *ASN2* genes are induced by nitrate in *A. thaliana* (Wang et al., 2001, 2003). The non-protein amino acid γ-aminobutyrate induces AS transcription in *A. thaliana*, in correlation with senescence and ethylene-induced genes (M. Lancien, unpublished data). After dark treatment of *A. thaliana* to induce senescence, microarray expression analysis in the senescing leaves showed that *AS* genes and the pathway for asparagine synthesis involving pyruvate orthophosphate dikinase (PPDK) were up-regulated (Lin and Wu, 2004). Both maize and rice AS have been reported to display enhanced

ammonia-dependent activity and the *A. thaliana ASN2* is induced by increased ammonium concentrations (Lam et al., 1998; Kawachi et al., 2002). Wong et al. (2004) showed that stress such as exogenous ammonium supply and cold treatment increased the abundance of detectable *ASN2* mRNA transcripts and the internal plant ammonium concentration. Transgenic lines of *A. thaliana* overexpressing *ASN2* accumulated less ammonium than the wild type under stress condition, suggesting that asparagine metabolism may play a role in ammonium detoxification, although the mechanism is not known, due to the inability to determine AS enzyme activity. In plants underexpressing *ASN2*, ammonium increased in response to high light stress, suggesting a role of *ASN2* in an alternative route to provide nitrogen for the photorespiratory pathway. In lines of transgenic *A. thaliana* overexpressing 35S-*ASN1*, seeds exhibited an enhanced nitrogen status, which included an elevated protein concentration and a greater capacity to germinate and grow on nitrogen-limited media In addition there was evidence of increased accumulation of asparagine in the leaves and flowers of the overexpressing plants (Lam et al., 2003).

Regulation by light and sugar

In plants, AS gene expression is repressed by light and induced in the dark in both leaves and roots. The light/dark repression of AS genes has been reported for a number of species such as soybean, common bean, alfalfa, maize, Arabidopsis and sunflower (Lam et al., 1998; Moeller et al., 2003; Herrera-Rodriguez et al., 2004). In other plants species such as *Lotus japonicus*, AS expression is unaffected by light (Waterhouse et al., 1996). Light regulation occurs either through phytochrome or by increasing the photosynthetic production of carbon metabolites. In *A. thaliana*, the aspartate pool required for asparagine synthesis in siliques and etiolated seedlings has been proposed to be mainly formed via the cytosolic aspartate aminotransferase AAT2, as mutants deficient in this enzyme show a decrease in asparagine synthesis (Miesak and Coruzzi, 2002).

Repression by exogenous sucrose was demonstrated in etiolated and light-grown seedlings of *A. thaliana*, in roots of maize and common bean and in asparagus cell cultures. Under a high C/N ratio (light, sucrose addition) the expression of AS is reduced. In contrast, when the C/N ratio is low (darkness, roots, nitrogen solute addition), the plant activates genes for asparagine biosynthesis and assimilated nitrogen is transported as asparagine, confirming earlier enzymological data (Joy, 1988). A combinatorial design analysis on *ASN1* and *ASN2* gene expression in *A. thaliana* seedlings showed the existence of a complex interaction between light quality, metabolites and plant development for the expression of these genes (Thum et al., 2003). In brief, for *ASN1*: (a) light overrides the regulation by carbon in etiolated plants; (b) carbon affects the far-red light repression in etiolated seedlings; (c) carbon affects the far-red light repression in light-grown plants. For *ASN2* it was concluded: (a) carbon overrides light regulation in light-grown plants; (b) blue light high fluence (BHF) and carbon have a repressive effect in etiolated seedlings; (c) carbon attenuates light induction in light-grown plants; (d) carbon potentiates far-red light induction in light-grown plants.

ShAS, a cDNA encoding an asparagine synthetase, has recently been isolated from the parasitic weed *Striga hermonthica*. In a manner similar to that of *ASN2*, *ShAS* was shown to be upregulated by light in mature leaves, light-grown calli and senescing calli growing in continuous light. The increase in *ShAS* transcription correlated with an accumulation of asparagine, suggesting that this isoform is involved in asparagine synthesis in both the active nitrate-metabolising and protein-degrading organs (Simier et al., 2005).

Asparaginase

L-Asparaginase (EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia. Asparaginases have been classified into two main families, bacterial type and plant type. The most studied enzymes in the plant type class are from legume plants and are involved in metabolic pathways connected with the assimilation of atmospheric nitrogen and amide transport (Joy, 1988; Lea et al., 1990). There are two groups of proteins in plants, designated potassium-dependent and potassium-independent asparaginases. cDNAs encoding potassium-independent enzymes have been characterized first from lupin seed and then from other legumes and plants (Lough et al., 1992; Casado et al., 1995). All plant type enzymes characterized so far have a conserved catalytic threonine residue also found in bacterial enzymes (Borek and Jaskólski, 2001). Recombinant asparaginases are capable of deaminating asparagine and isoaspartyl dipeptides (Hejazi et al., 2002; Borek et al., 2004).

The *E. coli* enzyme (EcAIII), which is homologous to the lupin potassium-independent asparaginase (LIA) and exhibiting both isoaspartyl dipeptidase and L-asparaginase activities, has recently been crystallised (Prahl et al., 2004).

Existing biochemical data and sequence homology indicate that lupin asparaginase belongs to the family of N-terminal nucleophile (Ntn) amidohydrolases (Borek et al., 2004). Recombinant plant type asparaginases from cyanobacteria, *A. thaliana* and lupin expressed in *E. coli* form heterotetramers of 70 kDa, following the autocatalytic cleavage of the protein into two peptides at a Gly-Thr bond (Hejazi et al., 2002), confirming molecular mass data obtained previously (Sodek and Lea, 1993; Chagas and Sodek, 2001). Plant potassium-independent asparaginases exhibit about 60% amino acid sequence identities with aspartylglucosamidases found in humans, which are involved in the degradation of glycosylated proteins and can also act as an asparaginase.

Very little has been described regarding asparaginase gene expression in plants. In the mature root nodule of *Lupinus angustifolius*, the transcription of L-asparaginase is repressed (Vincze et al., 1994). The repressor protein (termed rep2037) has been isolated from *Mesorhizobium loti* and shown to be sarcosine oxidase. Jones et al. (2004) proposed that one possible role of sarcosine oxidase is to switch off the asparaginase gene of *L. corniculatus* by binding to and perhaps methylating the CTAAAAT of the promoter region. Such repression, which occurs upon establishment of an effective symbiosis in the *L. angustifolius* nodule, greatly reduces asparaginase activity and prevents asparagine breakdown, thus allowing subsequent transport of this compound to aerial parts of the plant for use in growth and development.

Epilogue

It is clear that for some of the minor enzymes of the lysine, methionine and threonine branches of the aspartate pathway, little new research has been carried out in order to characterize and understand their regulation. However, for the key regulatory enzymes or those that are targets of herbicide action, major advances have been made. Single and often multiple genes have been cloned and the expression of these in *E. coli* has ensured that several enzymes have been crystallized and their reaction mechanisms studied. Transgenic cereal and legume crop species containing both increased and reduced amounts of enzyme activity have demonstrated that the regulation of the pathway can be dramatically altered, results confirmed by recent developments in metabolic profiling. However, the goal of producing viable and commercial lines with enhanced protein quality and increased essential amino acid content in the seeds has still to be achieved.

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