Amino Acid Metabolism

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Amino Acids

Amino acids are the building blocks of proteins. They are not only important for cell structure but direct and control the chemical reactions that constitute life processes. Proteins are composed of many copies of 20 amino acids linked in chains by covalent bonds. Thus a 100-unit protein has 20^{100} possible structures. This enormous variability means that cells and organisms can differ greatly in structure and function, even though they are made up of similar macromolecules produced by the same type of reaction.

The monomers that make up proteins are called amino acids because, with one exception, each contains a basic amino group (-NH_2) and an acidic carboxyl group (-COOH). The exception, proline, has an imino group (-NH-) instead of an amino group. All amino acids are constructed according to a general design: a central carbon atom, called the α-carbon (because it is adjacent to the carboxyl group), which is bonded to the amino (or imino) group, the carboxyl group, a hydrogen atom, and to one variable group, called a side chain or R group. This side chain gives the amino acids their individuality. The 20 common amino acids normally occurring in proteins are classified according to whether their side chains are basic, acidic, uncharged polar or non polar. These 20 amino acids are given either three-letter or one-letter abbreviations; thus alanine = Ala = A.

The structures of all amino acids except glycine are asymmetrically arranged around the α-carbon, because it is bonded to four different atoms or groups of atoms (- NH_2, -COOH,-H and-R). Thus all amino acids, except glycine, have at least two stereo-isomeric forms. By convention, these mirror-image structures are called the D and the L forms of amino acids. With very rare exceptions, only the L forms of amino acids are found in proteins.

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At typical pH values in cells, the amino and carboxyl groups are ionised as NH_3^+ and $-COO^-$. The side chains of some amino acids are also highly ionised and therefore charged at neutral pH. Amino acids are acidic because they have a carboxyl group that tends to dissociate to form the negatively charged $-COO^-$ carboxylate ion. The amino group is a base, because it can take up a hydrogen ion to form the positive - NH_3^+ amino ion. The degree to which a dissolved acid releases hydrogen ions or to which a base takes them up, depends partly on the pH of the solution. In neutral solutions, amino acids exist predominantly in the doubly ionised form. Such a dipolar ion is called a zwitterion. In solutions at low pH, carboxylate ions recombine with the abundant hydrogen ions, so that the predominant form of amino acid molecule is ^+H_3N-R-CH-COOH. At high pH, the scarcity of hydrogen ions decreases the chance that an amino group or a carboxylate ion will pick up a hydrogen ion, so that the predominant form of an amino acid molecule is H_2N-R-CH-COO^-.

At neutral pH, arginine and lysine are positively charged, histidine is also positively charged, but only weakly, and aspartic and glutamic acids are negatively charged. Serine and threonine, whose side chains have an -OH group, can interact strongly with water by forming hydrogen bonds. The side chains of asparagine and glutamine have polar amide groups with even more extensive hydrogen-bonding capacities. These nine amino acids constitute hydrophylic, or polar amino acids. The side chains of several other amino acids, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, and valine consist only of hydrocarbons, except for the sulphur atom in methionine and the nitrogen atom of tryptophan. These seven non polar amino acids are hydrophobic; their side chains are only slightly soluble in water. Tyrosine is also strongly hydrophobic because of the presence of a benzene ring, but the hydroxyl group allows it to interact with water, making its properties somewhat ambiguous.

Cysteine plays a special role in proteins, because the -SH group allows it to dimerise through an -S-S- bond to a second cysteine to give cystine. When the SH remains free, cysteine is quite hydrophobic. Two other special amino acids are glycine and proline. Glycine has a hydrogen atom as the R group, thus it is the smallest amino acid and has no special hydrophobic or hydrophylic character. Proline, as an imino acid, is very rigid and creates a fixed link in a polypeptide chain, proline is also quite hydrophobic.

The chemical bond that connects two amino acids in a polymer is the peptide bond. It is formed between the α-amino group of the amino acid and the α-carboxyl of another one. This reaction, called condensation, liberates a water molecule. Because the carboxyl carbon and oxygen atoms are connected by a double bond, the peptide bond between C and N exhibits a partial double-bond character. A single linear array of amino acids connected by peptide bonds is called a polypeptide. If the polypeptide is short (i.e. fewer than 30 amino acids long), it may be called an oligopeptide or peptide. Polypeptides in plant cells differ greatly in length. They generally contain between 40 to 1000 amino acids. Each polypeptide has a free amino group at one end (N terminus) and a free carboxyl group at the other end (C terminus). The polypeptides fold up to form fibrous or globular proteins. Proteins give tissues their rigidity or catalyse chemical reactions; these latter proteins are enzymes. The proteins can be modified further by the attachment of additional small molecules (i.e. enzyme cofactors, sugars, phosphate etc.). The amino acids represent the alphabet by which linear proteins are written .

However, a very large number of non-protein nitrogenous compounds are also found in plants. These range from universal nitrogen molecules such as γ-aminobutyric acid, β-alanine, ornithine, hydroxyproline, homocysteine, *O*-acetylhomoserine,

S-adenosylmethionine, *S*-adenosylhomocysteine and glutathione to some well-identified substances that occur widespread, but not universally (homoserine and pipecolic acid), or to those that occur infrequently in often highly specific but seemingly unpredictable situations (azetidine-2-carboxylic acid and canavanine). Still other substances are present infrequently and apparently at random (γ-methyleneglutamine). There are others that are still regarded as extremely rare in plants (hypoglycine A). Some non-protein nitrogen compounds are considered as intermediates of synthesis. This is the case for homoserine, ornithine, citrulline, diaminopimelate, *O*-acetylserine, cystathionine, β-cyanoalanine and Δ^1-pyrroline-5-carboxylic acid. Some others are products of breakdown of proteins and nucleic acids (γ-aminobutyric acid, β-alanine, β-aminoisobutyric acid). Homoserine is involved, for example, in nitrogen transport in peas; it is also the case in γ-methyleneglutamine, some amides and ureides. Indoleacetic acid, azetidine-2-carboxylic acid and N-formylmethionine are considered as metabolic or growth regulators. Some amino acids are toxic to animals and found in seeds (canavanine in legumes, for example); so they appear to serve both as a storage reserve and as a feeding deterrent to herbivores. Non-protein amino acids generally have a wide range of function in plants. They are involved in the transportation of nitrogen between roots, leaves and harvested organs (fruits, seeds, etc.), and are precursors in the synthesis of chlorophyll and enzyme cofactors (biotin, thiamine pyrophosphate, and coenzyme A). Amino acids are also involved in the production of some secondary natural products such as alkaloids, phenolic acids, etc. (Fowden 1981).

The 20 common amino acids are of central biological importance as they are the constituents of proteins. Considering this importance, it is surprising that mammals can synthesise only ten amino acids by de novo pathways; the remainder, called the essential amino acids, must be supplemented in their diet. Higher plants and many bacteria and fungi possess the ability to synthesise all of the protein amino acids.

By the 1970s, the development of laboratory techniques, particularly in the areas of biochemistry and enzymology, and in the use of labelled precursors (chromatography and all sophisticated and physical techniques of analysis) and instrumentation (automation and miniaturisation), had provided a good understanding of the amino acid biosynthetic pathways in bacteria. However, in plants, the pathways generally accepted for the synthesis of amino acids have been assumed to be the same as those in bacteria and it is only relatively recently that these have been confirmed, or novel pathways demonstrated in plants. However, the synthesis of some amino acids in plants still remains unclear, as is the case with histidine, lysine and many a non-protein amino acids. Moreover, elucidating the regulation of the amino acid biosynthetic mechanisms of plants has come up against some difficulties. While several enzymes involved in the pathways are catalytically similar between plants and bacteria, the mechanisms regulating gene expression and activity of these enzymes are quite distinct. In plants, a number of amino acids can be synthesised by several pathways, depending on the tissue concerned, stage of growth and development and environmental conditions. Consequently, in plants, the branched and the interwoven nature of the pathways for amino acid synthesis require strict and precise control. The carbon and nitrogen destined for one particular amino acid do not all end up in this single amino acid, but must be partitioned between different amino acids, in response to the needs of the plant organs at a particular time of development. Thus, the metabolic pathways of amino acids have to maintain a harmonious balance in the partitioning of C and N fluxes between each amino acid pool. This requires, in a defined manner, a

very strict regulation of enzyme activities. Enzymes are regulated to different degrees. For many amino acid biosynthetic pathways, for example, end-product inhibition appears to be the principal mechanism by which amino acids are regulated. There are, however, reported cases where the accumulation of amino acids is regulated at the level of gene expression or by a balance between biosynthesis and degradation.

Research on amino acid metabolism in plants has been complicated by the fact that some of the metabolic reactions are catalysed by multiple isoenzymes located in distinct subcellular compartments. With traditional biochemical approaches, it has been impossible to sort out the function of each isoenzyme. Traditional biochemical methods have involved tissue disruption, which artificially mixes isoenzymes that may not coexist in the same cell types *in vivo*. Moreover, the *in vitro* biochemical methods commonly used to define the controlling enzyme in a pathway in unicellular organisms may lead to erroneous interpretations when employed to study plant metabolic pathways. An alternative way to define the *in vivo* or *in planta* function of a particular isoenzyme is by mutant analysis. Plant mutants, defective in particular isoenzymes in biosynthetic amino acid pathways, have been identified and characterised in *Arabidopsis thaliana*, barley, maize, tobacco, pea, soybean etc., and have greatly added to our understanding of amino acid biosynthetic regulatory mechanisms in plants (Lea and Forde 1994). However, generating mutants is not straightforward for polyploid plants and for specific members of multigene families. The development of molecular genetics and efficient plant transformation techniques has provided transgenic plants containing altered levels of an individual amino acid biosynthetic enzyme in a cell-and tissue-specific manner. Consequently, the reductionist approach that has prevailed recently in molecular biology and has led to the great accumulation of factual minutiae about molecular and cellular mechanisms, now needs to be balanced by the holistic vision of integrated whole-plant biology.

Research in this field is still in its infancy and while we have not made great strides in crop improvement, analysis of the different transformants, with both sense and antisense constructs, has improved our understanding of the regulation of amino acid metabolism in plants. This understanding will also have an impact on genetic engineering strategies in applied research, such as increasing the potential yield, or improving nutritional protein quality.

Because amino acids are derived from a single "head" amino acid, they can be grouped together into different families. Conversely, because more than one amino acid may be involved in the synthesis of another, a single amino acid may be assigned to more than one family. This chapter will be confined to the 20 amino acids normally incorporated into proteins.

Amino acids are synthesised either in roots, leaves, seeds, or in fruits, depending on the sites of nitrate reduction and nitrogen remobilisation. For example, in legumes, much of the nitrate is reduced and converted to organic form (asparagine and glutamine) in the roots prior to transport in the xylem. In developing cereal leaves, nitrate transported in the xylem from the roots is reduced to ammonia and then incorporated into amino acids. Senescing leaves hydrolyse a lot of their proteins and degrade other nitrogenous molecules for transport to the developing fruits and seeds, which receive most of their nitrogen in the form of amino acids translocated in the phloem. Synthesis and metabolism of amino acids may require that carbon skeletons such as α-oxo acids be withdrawn from glycolysis, and that the citric acid and pentose phosphate pathways (reductive and oxidative) replenish the amino acids pools.

Ammonia Assimilation and Transamination

Ammonia assimilation

As described in Chapter 2.2, it appears that nearly all plants assimilate ammonia first into glutamine, owing to the high affinity of glutamine synthetase (EC.6.3.1.2) for ammonia with the concurrent hydrolysis of one molecule of ATP.

$$\text{L-Glutamate} + NH_3 + \text{ATP} \rightarrow \text{L-glutamine} + H_2O + \text{ADP} + P_i.$$

The ammonia assimilated into the amide group of glutamine is then transferred to the α-oxo position of α-oxoglutarate to form glutamate via the reaction of glutamate synthase NAD(P)H-dependent (EC 1.4.1.14) or ferredoxin dependent (EC 1.4.7.1). This amination reaction requires the oxidation of one molecule of NAD(P)H or equivalent.

$$\text{L-Glutamine} + \alpha\text{-oxoglutarate} + 2H \rightarrow 2\ \text{L-glutamate}.$$

Thus, glutamine synthetase and glutamate synthase are able to act in conjunction with the net production of one molecule of glutamate (the GS/GOGAT cycle) (Ireland and Lea 1999). A similar result is achieved by the reversible enzyme glutamate dehydrogenase (EC 1.4.1.2), which can also produce glutamate from α-oxoglutarate and ammonia, with the oxidation of only one molecule of NADH (Lea and Ireland 1999).

$$\text{L-Glutamate} + H_2O + NAD^+ \leftarrow \alpha\text{-oxoglutarate} + NH_3 + \text{NADH} + H^+.$$

Other pathways that have been implicated in ammonia assimilation, include alanine dehydrogenase (EC 1.4.1.1), aspartate dehydrogenase (EC 1.4.3.1), and aspartase (EC 4.3.1.1). Alanine dehydrogenase catalyses the amination of pyruvate to alanine in presence of NAD(P)H. In a similar reaction, aspartate dehydrogenase aminates oxaloacetate to aspartate. Aspartase is another ammonia-assimilating enzyme which catalyses the addition of ammonia to fumarate, yielding aspartate. These latter enzymes have been detected in a few plants, but only the GS/GOGAT is thought to make a significant contribution to ammonia assimilation in plant tissues (Lea and Ireland 1999).

Transamination reactions

These are central to all areas of amino acid metabolism. These reactions result in the redistribution of nitrogen from glutamate to other amino acids. Aminotransferases also known as transaminases, catalyse the transfer of an amino group from the α-carbon of an amino acid to the α-carbon of an α-oxo acid, producing a new amino acid and a new oxo acid (Givan 1980 ; Lea and Ireland 1999).The transamination reactions are considered as an oxidative deamination of the donor (primary amino acid or amine), linked with the reductive amination of the acceptor (α-oxo acid or an aldehyde). Although the aminotransferase reaction might be regarded as oxidoreduction with no net change, the unique and distinctive factor is the transfer of the amino group.The aminotransferases transfer nitrogen from one compound to another in a system described as a donor/acceptor group transferase. The aminotransferases have a tightly bound coenzyme: pyridoxal-5-phosphate. This coenzyme accepts an amino group from the amino acid substrate, becoming aminated to pyridoxamine phosphate. The oxo acid thus produced is released and the ami-

nated form of the coenzyme then undergoes a reversal of the process, giving up the newly acquired amino group to another oxo acid substrate to produce a new amino acid product.

The plant aminotransferases can catalyse the transamination of all of the common protein amino acids, except proline, which is an imino acid and thus has no primary amino group available for transamination. It appears that aminotransferases are not highly specific, and may react with a number of amino acids or oxo acids. In plants, many aminotransferases can use glutamate as amino donor in the synthesis of a wide range of amino acids. The extensive use of glutamate results in the production of large amounts of α-oxoglutarate, which can be reaminated by the GS/GOGAT cycle during ammonia assimilation. Aminotransferases do not exhibit any specific regulatory properties. Their activities are solely dependent on pH and substrate/product concentrations (Ireland and Lea 1999 ; Lea and Ireland 1999).

Aminotransferases serve to redistribute the nitrogen to a range of amino acids as glutamate, aspartate and alanine, which then provide nitrogen for the synthesis of the other amino acids. They contribute to the maintenance of relatively stable amino acid pools. In the plant, for example, aspartate : α-oxoglutarate aminotransferases (EC 2.6.1.1) can catalyse the amination of oxaloacetate to yield aspartate. Alanine : oxoglutarate aminotransferase (EC 2.6.1.2) is the enzyme catalysing the amination of pyruvate to alanine. In the peroxisome, a glutamate : glyoxylate aminotransferase (EC 2.6.1.4) ensures the transfer of nitrogen group from glutamate to glyoxylate to form glycine. Aminotransferases are also implicated in the transfer of many amino acids that are often present in millimolar concentration in the vacuole. These aminotransferases have functions that are unique in leaves, such as those involved in carbon assimilation and other processes requiring the shuttling of metabolites during C_4 photosynthesis and photorespiration (Ireland and Lea 1999 ; Lea and Ireland 1999).

At the molecular level, *A.thaliana* has been shown to contain five genes encoding the aspartate aminotransferase isoenzymes localised in distinct subcellular compartments, cytosol, chloroplasts, mitochondria, and peroxisomes (Schultz et al. 1998). A study of the evolutionary relationships of tyrosine, histidinol phosphate and aspartate aminotransferases was carried out by Metha et al. (1989). Among the transferases from several species, it was revealed the existence of 12 amino acid residues that were found to be invariant in all the 16 known sequences of α-aminotransferases.

Synthesis of Amino Acids Derived from Glutamate

Glutamate is the precursor of the amino acids glutamine (as previously described), arginine and proline.

Arginine Synthesis

Arginine is a major nitrogen storage compound in plants, where it may form 40% of the nitrogen in seed protein and 50 to 90% of the soluble nitrogen in trees, flower bulbs and tuberised roots of chicory (Ameziane et al. 1997).

The synthesis of arginine proceeds through ornithine, which is derived from glutamate in an acetylated pathway (Fig. 1). In the synthesis of arginine, glutamate

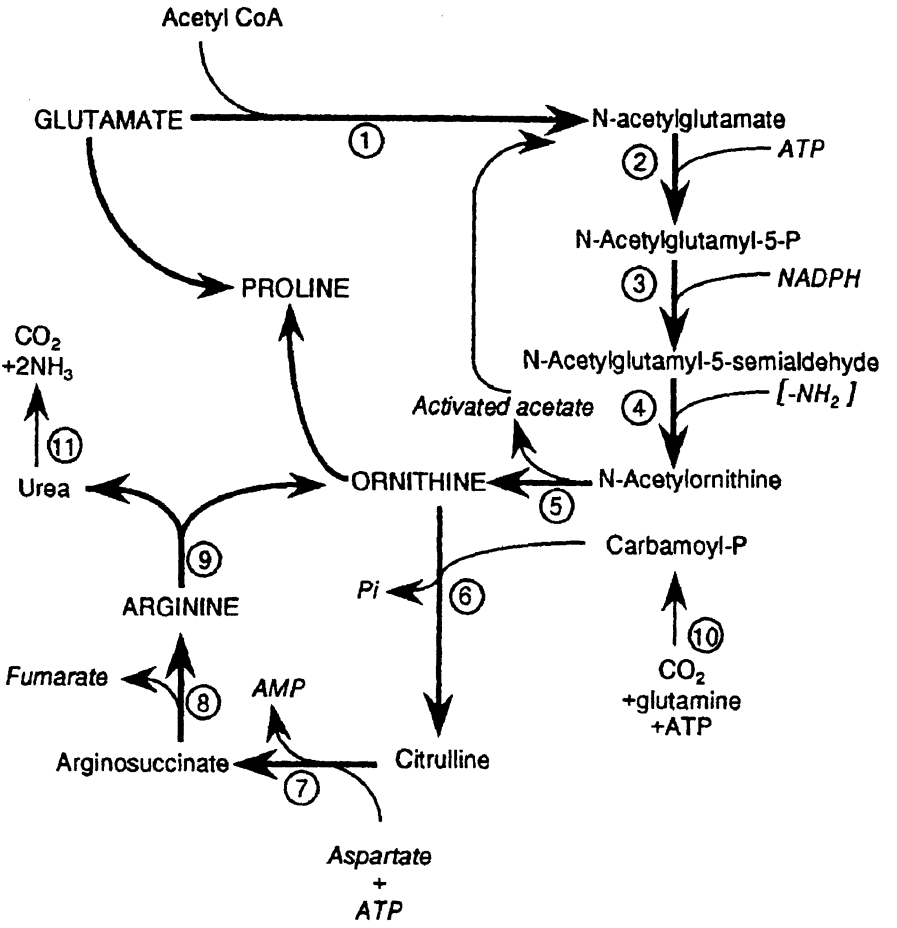

Fig. 1 : The biosynthetic pathway of arginine. Enzymes: *1.* N-acetyl transferase; *2.* N-acetylglutamate kinase; *3.* N-acetylglutamyl-5-phosphate reductase; *4.* N-acetylornithine aminotransferase; *5.* N-acetyl transferase; *6.* ornithine carbamoyl transferase; *7.* arginosuccinate synthetase; *8.* arginosuccinate lyase; *9.* arginase; *10.* carbamoyl phosphate synthetase, *11.* urease.

after acylation is metabolised via phosphorylation, reduction and transamination to the non-protein amino acid, ornithine (Verma and Zhang 1999). The acylation of glutamate to N-acetylglutamyl-5- phosphate, in the presence of acetyl-CoA and ATP, is catalysed by an N-acetyl transferase (acetyl-CoA :glutamate N-acetyl transferase) (EC 2.3.11.1) and N-acetylglutamate kinase (EC 2.7.2.8). Then, two enzymes, N-acetylglutamyl-5-phosphate reductase (EC 1.2.1.38) and N-acetylornithine aminotransferase (EC 2.6.1.11), transform the acetylated intermediate to N-acetylornithine which is converted to ornithine by a N-acetyl transferase (N^2-acetylornithine :glutamate N-acetyl transferase) (EC 2.3.1.35). The acetyl-protecting group can be reused to form N-acetylglutamyl-5-phosphate as described previously (Taylor and Stewart 1981; Micallef and Shelp 1989).

Ornithine reacts with carbamoyl phosphate and yields citrulline. This reaction is catalysed by ornithine carbamoyltransferase (EC 2.1.3.3). Carbamoyl phosphate is formed from glutamine, ATP, and CO_2, via the action of carbamoyl phosphate synthetase (EC 6.3.5.5). Citrulline, in the presence of ATP and aspartate, is converted into arginosuccinate. This reaction is catalysed by arginosuccinate synthetase (EC 4.3.2.1).The arginosuccinate so formed, is subsequently cleaved via the reaction of arginosuccinate lyase (EC 4.3.2.1) to give arginine and fumarate. Then, arginine is broken down by arginase (EC 3.5.3.1) to yield urea and ornithine. Arginase activity was observed to increase in *A. thaliana* seedlings 6 days after germination. The product of hydrolysis, urea, appears to have a function in recycling nitrogen into ammonia via urease (EC 3.5.1.5) (Zonia et al. 1995).

Although there have been a number of studies on the subcellular localisation of arginine-metabolising enzymes in plants, the results have been contradictory. Taylor and Stewart (1981), utilising pea leaf protoplasts, have shown that all the enzymes involved in the metabolism of acetylated intermediates of glutamate, that is glutamate acetyl transferase, carbamoyl phophate synthetase, and ornithine carbamoyl transferase, were localised in the chloroplast, whilst arginosuccinate lyase was present only in the cytoplasm, and arginase was mitochondrial. Arginine is able to regulate the rate of its own synthesis. It inhibits the synthesis of N-acetylglutamate from acetyl-CoA but not from N-acetylornithine. Arginine also appears to control the activity of N-acetylglutamate kinase, another example of an endproduct inhibiting an early reaction in its synthesis.

Glutamate can be decarboxylated into γ-aminobutyrate (GABA).

$$\text{L-Glutamate} + H^+ \rightarrow \text{L-}\gamma\text{-aminobutyrate} + CO_2.$$

This reaction is catalysed by glutamate decarboxylase (EC 4.1.2.15), which can be activated by calcium and calmodulin. GABA is metabolised through a reversible transamination to succinic semialdehyde. This reaction is catalysed by GABA transaminase (EC 2.6.1.19). The product of the reaction is then oxidised to succinate in an irreversible reaction catalysed by succinate semialdehyde dehydrogenase (EC 1.2.1.16). These three reactions constitute a pathway known as the GABA shunt. Evidence indicates that glutamate and GABA are produced during storage protein mobilisation, as a means of recycling arginine-derived N and C. Thus, the GABA shunt could be of considerable importance in the N economy of plants. There is also considerable literature demonstrating the rapid and large accumulation of GABA in response to many stimuli, e.g. cold shock, mechanical stimulation, hypoxia, cytosolic acidification, water stress and phytohormones. GABA accumulation and efflux may play a part in an intercellular signal transduction pathway, leading to the regulation of growth and development (Bown and Shelp 1997; Ireland and Lea 1999).

Proline Synthesis

The accumulation of proline is a striking metabolic response to osmotic stress. Proline has been suggested to function as an osmoticum, as an energy or a reducing power sink, a nitrogen storage compound, a hydroxy-radical scavenger, a compatible solute that protects enzymes, a means of reducing acidity, and a way to regulate cellular redox potentials (Taylor 1996 ; Hua et al. 1997, Hare et al. 1999).

In the first step of proline synthesis, glutamate in the presence of ATP and NAD(P)H is phosphorylated and then reduced to glutamyl-5-semialdehyde via a

bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) exhibiting both γ-glutamyl kinase (EC 2.7.2.11) and glutamate semialdehyde dehydrogenase activities (EC 1.2.1.41) (Fig. 2). The semialdehyde spontaneously cyclises to give Δ^1-pyrroline-5-carboxylate (P5C) which is then reduced in presence of the NAD(P)H to proline. This reaction is catalysed by the Δ^1-pyrroline-5-carboxylate reductase (P5CR) (EC 1.5.1.2) (Hu et al.1992). Note that the formation of the semialdehyde is similar to the initial stages of ornithine synthesis. However, during ornithine synthesis the presence of the N-acetyl group prevents the non-enzymic cyclisation reaction.

Proline is reported to inhibit its own synthesis though a feedback effect, as the P5CS enzyme is allosterically inhibited by proline (Hu et al. 1992). Proline synthesis probably occurs in the cytosol, and proline degradation in the mitochondria. This pathway of proline synthesis was first established in *Escherichia coli* and *Saccharomyces cerevisiae*, and the corresponding plant genes were identified through the complementation of yeast and bacterial mutants defective in the various steps of the pathway (Delauney and Verma 1993, Verma and Zhang 1999).

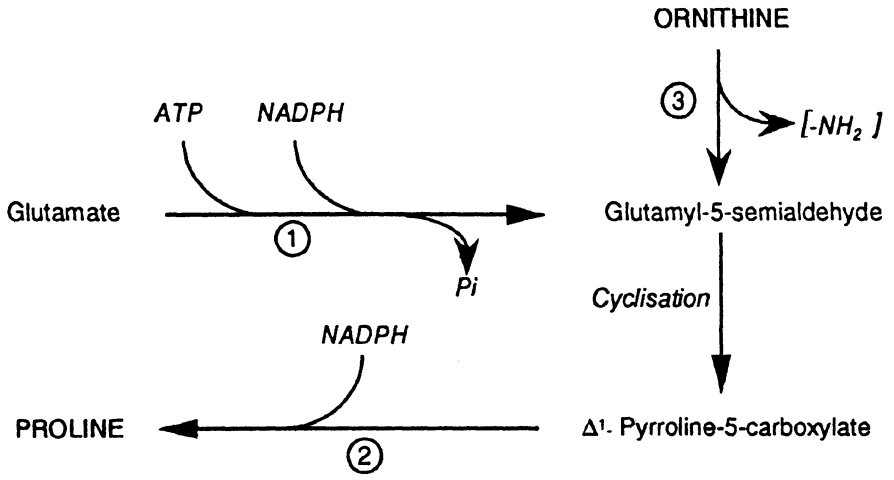

Fig. 2 : The biosynthetic pathway of proline. Enzymes : *1.* Δ^1-pyrroline-5-carboxylate synthetase; *2.* Δ^1-pyrroline-5-carboxylate reductase; *3.* ornithine δ-aminotransferase.

Ornithine can be converted to Δ^1-pyrroline-5-carboxylate via the loss of the δ-amino group by the action of the ornithine δ-aminotransferase (EC 2.6.1.13), as well as to Δ^1-pyrroline-2-carboxylate. There are thus two potential ways of converting ornithine to proline, depending on whether the 2-amino or 5-amino position is transaminated first. Only the route involving the transamination of ornithine into Δ^1-pyrroline-5-carboxylate was strongly indicated by the functional complementation of a defective *E. coli* mutant (Delauney et al.1993).

It was initially suggested that the biosynthesis of proline from glutamate would be enhanced under stress conditions, whereas the ornithine pathway would be inhibited (Delauney et al. 1993). However, Roosens et al. (1998) demonstrated that the ornithine pathway could serve an important role in very young *A. thaliana* plantlets. Under salt-stress conditions, the main task of this pathway is devoted to proline

production and contributes, together with the glutamate pathway, to proline accumulation. In later stages of differentiation, the ornithine pathway contributes to a lesser extent to proline biosynthesis. It has been suggested that under normal growth conditions, the role of the ornithine pathway would not be to produce proline, but would be related to the necessity for the plants to synthesise glutamyl semialdehyde via δ-ornithine aminotransferase. Glutamyl semialdehyde is then oxidised to glutamate via Δ^1-pyrroline-5 -dehydrogenase (EC 1.5.1.12). Glutamate can then be used for transamination or to generate new carbon constituents upon entry into the Krebs cycle as α-oxoglutarate.

Less is known about the genes controlling proline catabolism in plants. Proline is oxidised to Δ^1-pyrroline-5-carboxylate by the mitochondrial inner membrane enzyme, proline dehydrogenase (EC 1.5.99.8) (Hare et al. 1999). In plants this enzyme is linked to the respiratory electron transfer system and thus couples proline degradation to ATP formation (Elthon and Stewart 1981). In plants undergoing water deficit, proline degradation in the mitochondria is reduced, whereas proline synthesis is increased in the cytosol. Furthermore, an increase in proline concentration in the phloem of alfalfa under water deficit has been demonstrated, suggesting that increased proline transport may indeed contribute to the ability of the plants to withstand water deficit (Girousse et al. 1996). Recently, Verslues and Sharp (1999) have observed an increase in proline uptake in maize primary roots at low water potentials, which suggests increased proline transport. The molecular mechanisms controlling proline transport during water deficit and salt stress have also been studied in *A. thaliana* (Kiyosue et al. 1996).

Maize mutants, whose growth is limited by the availability of proline, have been isolated by Racchi et al. (1981). The rate of proline synthesis in the maize *Prol 1-1* mutant was not affected, but the rate of proline catabolism seemed to be increased (Dierks-Ventling and Tonelli 1982). The glutamate-synthase-deficient barley mutant which lacks the ability to accumulate proline showed premature symptoms of stress when subjected to a gradual increase in water deficit (Al-Sulaiti et al. 1990).

As proline acts as an osmotic protectant in plants that have been subjected to either drought or high salt-stress, many genetic manipulations have been made to modify the expression of the genes encoding the enzymes involved in proline pathway. Kishor et al. (1995) have shown that the overexpression of P5CS leads to an increase in proline concentration in transgenic tobacco. The higher proline levels correlated with enhanced productivity of the transgenic plants under water deficit conditions. The levels of both P5CS and P5CR are elevated in salt stressed plants (Hu et al. 1992). However, Szoke et al. (1992) have observed that tobacco plants containing a 50-fold enhancement of soybean P5CR, exhibited no significant increase in proline concentration. These results have confirmed the suggestion that P5CR contrary to P5CS, is not a flux-controlling enzyme.

It was observed that high salinity and dehydration resulted in increased accumulation of proline in *A. thaliana* and rice, and have been shown to be accompanied by an increase in the P5CS message level (Verbruggen et al. 1996; Savoure et al. 1997; Strizhov et al. 1997; Igarashi et al. 1997). The expression of the gene encoding P5CS is strongly induced under water deficit and following the addition of NaCl (Hu et al. 1992; Yoshiba et al. 1995). The regulation of expression and structures of two evolutionary divergent genes for Δ^1-pyrroline-5-carboxylate synthetase were studied in tomato by Fujita et al. (1998).

An understanding of the regulation of the proline biosynthesis, transport, and degradation is required for the successful engineering of drought and salinity tolerance in crop plants. It should provide insight into the mechanisms through which proline accumulation contributes to the capacity of plants to survive such stresses.

Synthesis of Amino Acids Derived from Aspartate

Asparagine

Asparagine is almost universally used by higher plants as a storage and transport compound, although glutamine and arginine are also used by some plants (Lea and Ireland 1999). Asparagine synthesis is particularly important in the root nodules of legumes (see Chap. 2.2 and 3.1). Much of the nitrogen fixed by the rhizobium is rapidly transferred to asparagine through the activities of both glutamine synthetase and asparagine synthetase. Thus, much of the nitrogen in legume plants is transported in the xylem, away from the nodule, in the form of asparagine.

The incorporation of ^{15}N into asparagine tends to be much slower than into glutamine, although rapid turnover has been demonstrated (Woo et al. 1982; Ta et al. 1988). Most evidence suggests that ammonia is first incorporated into the amide position of glutamine and is then transferred to aspartate to yield asparagine. Asparagine is synthesised by asparagine synthetase (EC 6.3.5.4) which catalyses the amidation of aspartate by glutamine in an ATP-dependent reaction (Brouquisse et al. 1992; Ireland and Lea 1999).

L-Glutamine + L-aspartate + ATP → L-asparagine + L-glutamate + AMP + PP_i.

Asparagine synthetase is present in many plants. The enzyme has been isolated from the cotyledons of germinating seeds, maize roots, and root nodules. Expression of asparagine synthetase is enhanced by low carbohydrate concentration and during senescence. In some cases, there is evidence that the enzyme is able to utilise ammonia. Stulen et al. (1979) have claimed that in maize roots the K_m for ammonia is low enough to allow direct incorporation under certain physiological conditions. In most cases, however, it appears that the *in vivo* substrate for this enzyme is actually glutamine, but not ammonia. Hence, asparagine synthetase does not usually constitute a route for ammonia assimilation in normal conditions (see Hirel and Lea. Chap. 2.2). Asparagine may also be synthesised from the hydrolysis of β-cyanoalanine by β-cyanoalanine hydrolase (EC 4.2.1.65) which is formed from hydrogen cyanide and cysteine, a condensation catalysed by β-cyanoalanine synthase (EC 4.4.1.9). This pathway has been demonstrated in lupin, sorghum, pea and asparagus. This pathway, however, is probably a detoxification mechanism rather than a major pathway of asparagine synthesis. Transamination of α-oxosuccinamate (the β-amide of oxaloacetate) with a suitable amino donor can lead to asparagine synthesis, but this reaction proceeds far more favourably in the reverse direction. Sieciechowicz et al. (1988) have suggested that this pathway is more significant in asparagine catabolism.

Asparagine may be metabolised by two pathways, one involving transamination, and the other immediate deamination by asparaginase (EC 3.5.1.1).

L-Asparagine + H_2O → L-aspartate + NH_3.

This reaction of deamination by asparaginase is very efficient in seeds. Studies have shown that most asparagine is metabolised in the seed coat, with the embryo receiving the products of asparagine catabolism (Ireland and Lea 1999).

Lysine, Isoleucine, Threonine and Methionine

Considerable attention has been paid to the regulation of the aspartate pathway of amino acid synthesis because lysine, isoleucine, threonine and methionine are essential amino acids which contribute greatly to the nutritive value of plant seed meal. More recently, the conversion of threonine to isoleucine has been the subject of a number of detailed investigations, when it became apparent that it was the target site of a number of extremely potent herbicides (Azevedo et al. 1997).

Lysine, isoleucine, threonine and methionine are synthesised from aspartate (Fig. 3). An interesting point in the pathway is the synthesis of lysine by higher plants from aspartate rather than via the fungal aminoadipic acid pathway. Isotope dilution experiments have indicated that homoserine is an intermediate in threonine and methionine synthesis. There is also evidence that cystathionine and homocysteine are intermediates in methionine formation and that diaminopimelate is on the route to lysine synthesis (Giovanelli et al. 1989).

Both in bacteria and in higher plants, lysine and threonine are synthesised from aspartate by two separate branches of a common pathway, called the aspartate family pathway (for reviews, see Galili 1995; Azevedo et al. 1997; Ravanel et al. 1998). This pathway, which also leads to the synthesis of isoleucine and methionine, is described in Fig. 3. The first two reactions are common to all the aspartate family amino acids. The first reaction is catalysed by aspartate kinase (AK; EC 2.7.2.4), which phosphorylates the β-carboxyl group of aspartate in the presence of ATP to yield 4-aspartyl phosphate and ADP. The second enzyme, aspartate semialdehyde dehydrogenase (EC 1.2.1.11), catalyses the NADPH-dependent reduction of aspartyl phosphate to 4-aspartate semialdehyde (4-ASA). Following this step, there is a branch point that leads on one hand to the synthesis of lysine, and on the other hand to the synthesis of threonine as well as methionine and isoleucine.

Dihydrodipicolinate synthase (DHDPS ; EC 4.2.1.52) is the first specific enzyme for lysine synthesis. Dihydrodipicolinate is formed from the combination of pyruvate and 4-aspartate semialdehyde. The synthesis of lysine from dihydrodipicolinate involves five enzymes, dihydrodipicolinate reductase (EC 1.3.1.26), piperidine dicarboxylate acylase (EC 2.3.1.–), acyldiaminopimelate aminotransferase (EC 2.6.1.17), diaminopimelate epimerase (EC 5.1.1.7), and *meso*-diaminopimelate decarboxylase (EC 4.1.1.20) (Fig. 3). Although the pathway from dihydrodipicolinate to *meso*-2,6-diaminopimelate is somewhat obscure in higher plants, circumstantial evidence still indicates that the route shown in Fig. 3 is correct (Azevedo et al. 1997).

Starting from 4-aspartate semialdehyde, the two common enzymes leading to the synthesis of methionine, threonine and isoleucine, are homoserine dehydrogenase (HSDH; EC 1.1.1.3) and homoserine kinase (EC 2.7.1.39). These two consecutive reactions allow the formation of *O*-phosphohomoserine (OPH) which serves as a second branch point metabolite in the synthesis of aspartate-derived amino acids, thereby leading on one hand to the synthesis of methionine and on the other hand to the synthesis of threonine and isoleucine (Galili 1995 ; Azevedo et al. 1997). In

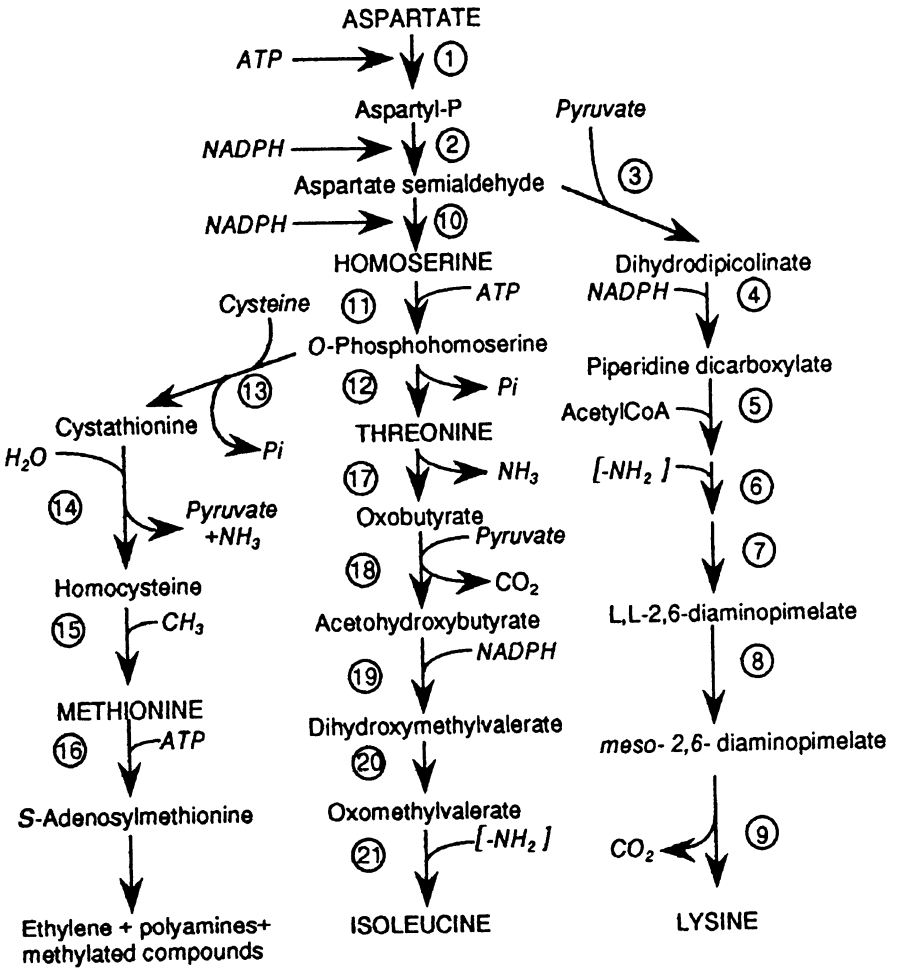

Fig. 3 : The aspartate pathway: biosynthesis of lysine, threonine, methionine, *S*-adenosylmethionine and isoleucine. Enzymes : *1.* aspartate kinase; *2.* aspartate semialdehyde dehydrogenase; *3.* dihydrodipicolinate synthase; *4.* dihydrodipicolinate reductase; *5.* Δ'-piperidine dicarboxylate acylase; *6.* acyldiaminopimelate aminotransferase; *7.* acyldiaminopimelate deacylase; *8.* diaminopimelate epimerase; *9. meso*-diaminopimelate decarboxylase; *10.* homoserine dehydrogenase; *11.* homoserine kinase; *12.* threonine synthase; *13.* cystathionine-γ-synthase; *14.* cystathionine-β-lyase; *15.* methionine synthase; *16. S*-adenosylmethionine synthetase; *17.* threonine dehydratase; *18.* acetohydroxyacid synthase; *19.* oxoacid reductoisomerase; *20.* dihydroxyacid dehydratase; *21.* aminotransferase

certain plants, (e.g. pea) homoserine and OPH may accumulate in high concentrations and are used as nitrogen storage and transport compounds (Rochat and Boutin 1991).

The methionine molecule originates from three convergent pathways: the carbon backbone being derived from aspartate, the sulphur atom from cysteine and the methyl group from the β-carbon of serine. First, cystathionine-γ-synthase (EC 4.2.99.9) catalyses the synthesis of cystathionine from cysteine and OPH in a γ-replacement reaction. Cystathionine-β-lyase (EC 4.4.1.8) subsequently catalyses an α,β-elimination of cystathionine to produce homocysteine, pyruvate and ammonia. The terminal step in methionine synthesis involves the transfer of the methyl group from N^5-methyltetrahydrofolate to homocysteine. In plants, this reaction is catalyzed by a cobalamin-independent methionine synthase (EC 2.1.1.14), most presumably localised in the cytosol. Animals have the capacity to convert homocysteine to methionine; however, they are unable to drive the reactions involved in essential amino acids synthesis which, for the most part, take place in the plastids (Ravanel et al. 1998).

Methionine occupies a central position in cellular metabolism, where the processes of protein synthesis, methyl-group transfer through *S*-adenosylmethionine (AdoMet), polyamine and ethylene syntheses are interlocked. Among these pathways, the synthesis of proteins is the only one consuming the entire methionine molecule. The synthesis of AdoMet, as catalysed by AdoMet synthetase (EC 2.5.1.6) from methionine and ATP, is, however, the major route for methionine metabolism, as 80% of this amino acid is converted to AdoMet. More than 90% of AdoMet is used for transmethylation reactions, in which the methyl group of methionine is transferred to acceptors, the major end products being choline and its derivatives, including phosphatidylcholine (the major polar lipid). These reactions are accompanied by a recycling of the homocysteinyl moiety to regenerate methionine. Briefly, *S*-adenosyl-L-homocysteine (AdoHcy) produced during the methylation reactions is converted into homocysteine *via* a reaction catalysed by AdoHcy hydrolase (EC 3.3.1.1). Methionine is then regenerated through methylation of homocysteine. Thus, methionine synthase not only catalyses the last reaction in *de novo* methionine synthesis but also serves to regenerate the methyl group of AdoMet. The utilisation of the 4-carbon moiety of AdoMet for the synthesis of polyamines and, in some plant tissues, ethylene is also accompanied by recycling of the methylthio ribose moiety and regeneration of methionine. This route is considerable because it represents approximately 30% of the amount of methionine accumulating in protein (Giovanelli et al. 1985 ; Droux et al. 1995).

OPH can also be rearranged in a single step to threonine by the enzyme threonine synthase (EC 4.2.99.2). A typical feature of the plant enzyme relies upon considerable enhancement of its activity by AdoMet, the end-product of the methionine branch (Giovanelli *et* al. 1984; Curien et al. 1998). Threonine is directly incorporated into proteins, or further deaminated to yield α-oxobutyrate, which serves as a precursor for isoleucine biosynthesis.This reaction is catalysed by threonine deaminase (also called threonine dehydratase, EC 4.2.1.16). Then acetohydroxacid synthase (also known as acetolactate synthase, EC 4.1.3.18) catalyses the condensation of 2-oxobutyrate and pyruvate to yield acetohydroxybutyrate. The latter is converted to isoleucine by a series of reactions involving acetohydroxyacid isomeroreductase (EC 1.1.1.86), dihydroxyacid dehydratase (EC 4.2.1.9), and a branch chain amino acid aminotransferase. The reactions are closely paralleled in the synthesis of valine and leucine, in which two molecule of pyruvate react to form acetolactate (Fig. 3).

The branched nature of the pathway of the amino acids of the aspartate family needs strict regulation, that must occur at several points (Fig. 4). The first enzyme that exhibits regulatory properties is aspartate kinase, existing in at least three isoenzyme forms, which have been isolated from higher plant tissues and their presence confirmed by genetic analyses. One aspartate kinase isoenzyme (AKI), is a bifunctional enzyme containing within a single polypeptide both aspartate kinase and homoserine dehydrogenase activity. This isoenzyme is sensitive to threonine. The two other aspartate kinase isozymes (AKII and AKIII), are monofunctional enzymes exhibiting only aspartate kinase activity and which are lysine-sensitive. Thus, one aspartate kinase is regulated by threonine and two aspartate kinases by lysine. Hence, the presence of high levels of only one of these amino acids is insufficient to shut off the reaction, since the other isoenzymes will still allow the carbon and nitrogen to flow through the pathway. The proportions of activities of the threonine and lysine-sensitive forms appear to vary between plants and more importantly within developmental stages. The AKI isoenzyme is present in only low levels in rapidly growing tissue (Rognes et al. 1983 ; Wallsgrove et al. 1983 ; Arruda et al. 1984 ; Frankard et al. 1997).

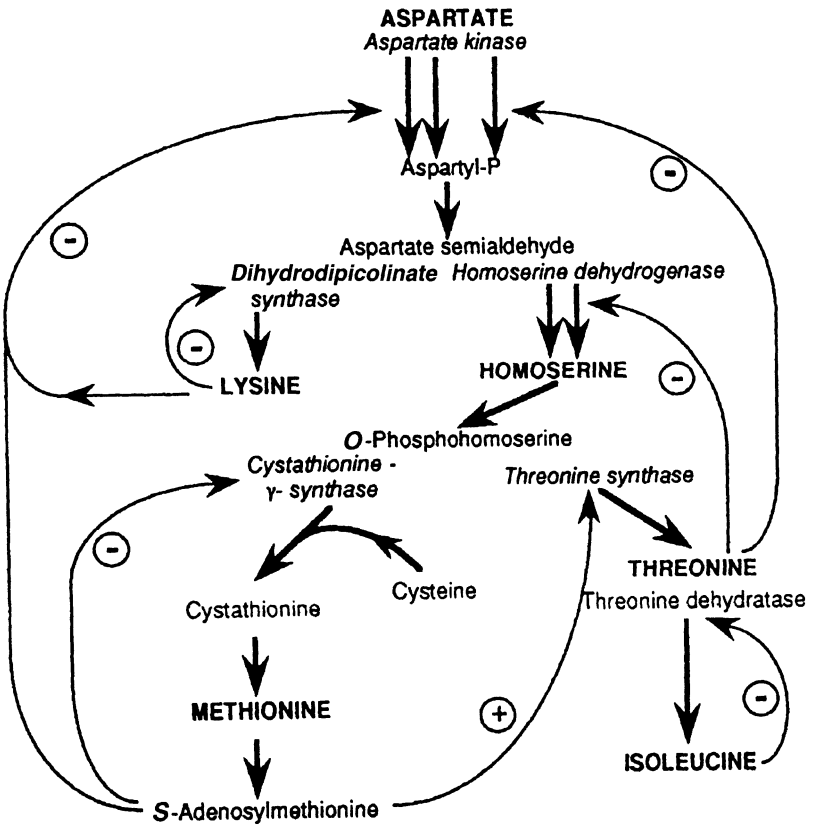

Fig. 4 : Proposed regulatory structure of the biosynthetic pathway of the aspartate family of amino acids. Regulatory interactions are indicated by : (-) inhibition; (+) stimulation; (=) enzyme repression?

Lysine also tightly regulates another key enzyme, dihydrodipicolinate synthase, the first committed enzyme in the lysine biosynthesis pathway. Plant dihydrodipicolinate synthase (DHDPS) is nearly ten-fold more sensitive (IC_{50} of about 10 μM) to lysine inhibition, than are plant lysine-sensitive aspartate kinases. Thus, DHDPS is the major site for lysine regulation in plants, and, therefore, lysine levels should, in theory, never accumulate enough to inhibit aspartate kinase. Genes encoding the 35-38-kDa subunit of the enzyme have been isolated from a range of plants and have been shown to contain a chloroplast transit peptide (Galili 1995; Azevedo et al. 1997).

Threonine inhibits one isoenzyme of aspartate kinase and one isoenzyme of homoserine dehydrogenase (HSDH). The enzyme HSDH exists in two isoenzyme forms, one of which is sensitive to inhibition by threonine and is localised in the chloroplast and the other is insensitive to inhibition by threonine and is localised in the cytosol. A cDNA encoding threonine synthase has been isolated from *A thaliana* and expressed in *E.coli* and the N-terminal domain has been shown be involved in the activation by AdoMet (Azevedo et al. 1997 ; Curien et al. 1998). The first step in isoleucine synthesis, catalysed by threonine dehydratase, is also subject to feedback inhibition by isoleucine in the chloroplast (Singhand Shaner 1995).

It is worth noting that, contrary to the plant enzyme, bacterial threonine synthase, though catalysing the same reaction, is not subject to kinetic modulation by AdoMet, presumably because in bacteria, OPH is not a branch point metabolite. Indeed, in prokaryotes, branching between threonine/isoleucine and methionine pathways occurs at the level of homoserine. In plants, neither cystathionine-γ-synthase nor cystathionine-β-lyase is significantly inhibited by methionine pathway intermediates or end-products such as AdoMet. Also, these enzymes are not sensitive to feedback inhibition by any of the aspartate-derived amino acids. There are strong indications, however, for a control of methionine biosynthesis in plants at the level of gene expression and that the major site for such a regulation is probably cystathionine-γ-synthase (Chiba et al. 1999). For example, when the duckweed *Lemna paucicostata* was grown under culture conditions causing methionine starvation, e.g. in the presence of L-aminoethoxyvinylglycine (AVG; an amino acid first isolated from *Streptomyces* sp. and that inhibits cystathionine-β-lyase), or in the presence of lysine plus threonine, mixtures that inhibit aspartate kinase (EC 2.7.2.4), there was a substantial increase in extractable cystathionine-γ-synthase activity. Furthermore, antisense repression of cystathionine-γ-synthase results in abnormal development of *A. thaliana* (Droux et al. 1995 ; Ravanel et al. 1998).

Isolated intact chloroplasts were observed to carry out light-dependent synthesis of lysine, threonine, homocysteine and isoleucine from ^{14}C-aspartate (Mills et al. 1980). These reactions could not be demonstrated in mitochondria. Initial studies showed that essentially all of the enzymes involved in amino acids formed from aspartate were located in the chloroplast, suggesting that the chloroplast is the sole site of the synthesis of these amino acids, except methionine. Indeed, of the three specific enzymes involved in methionine syntheis in plants, the first two, cystathionine-γ-synthase and cystathionine-β-lyase, are localised to the plastids, whereas the third one, methionine synthase, most presumably is localised in the cytosol (Droux et al. 1995; Ravanel et al. 1998).

Mutants with altered regulation of the aspartate pathway have been isolated. A number of studies have been carried out to obtain tissue culture lines or intact

plants, with altered feedback control in the aspartate pathway. Green and Phillips (1974) indicated that the growth of germinating cereal embryos could be inhibited by the presence of lysine and threonine. The inhibition of growth could be alleviated by the presence of low concentrations of methionine or homoserine. Methionine synthesis was thus prevented by the complete inhibition of the aspartate kinase isoenzymes by lysine and threonine.

Three lines of mutant barley plants which are resistant to the toxic action of lysine and threonine, have been isolated at Rothamsted (Bright et al. 1982). An analysis of the properties of the three aspartate kinase isoenzymes following ion exchange chromatography has shown that in the mutant R 3202, the aspartate kinase II peak was not inhibited by lysine even at a high concentration, whilst the aspartate kinase III peak was still inhibited by lysine. Conversely, the mutant R 3004 had an unchanged aspartate kinase II but an aspartate kinase III that was less sensitive to lysine. Genetic analysis of the two mutants suggested that resistance to lysine plus threonine was controlled by two dominant genes, termed *lt1* and *lt2* , which are probably the structural genes for aspartate kinases II and III. A double mutant between R 3202 (*lt1*) and R 3004 (*lt2*) was constructed which contained aspartate kinase II insensitive to lysine inhibition and aspartate kinase III with a decreased sensitivity to lysine (Arruda et al. 1984).

In maize, two separate genes (termed *Ask 1* and *Ask 2*) have also been shown to confer resistance to lysine and threonine (Diedrick et al. 1990). Aspartate kinase purified from the different mutant lines Ask 1 and Ask 2 was less sensitive to inhibition by lysine. Two homologous genes encoding a 52.5-kDa subunit of lysine-sensitive AK with a chloroplast transit peptide have been isolated from *A.thaliana*, confirming earlier biochemical data (Frankard et al. 1997).

Because human food and animal feed derived from many grains are deficient in some of the ten essential amino acids which are required in the animal diet (cereal seeds are deficient in lysine, while legume seeds are deficient in methionine), a number of studies have been conducted to increase the levels of these amino acids in crop seeds. First attempts were based on the characterisation of plant mutants overproducing lysine (and/or methionine, see later). More recent work has been based on molecular genetics and plant transformation (Lea and Forde 1994; Galili 1995).

To obtain mutant plants overproducing lysine, a number of research groups have attempted to select mutant lines that are resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC). Unfortunately, most of these experiments produced plants with a reduced uptake of AEC. However, one mutant of *Nicotiana sylvestris* (generated from protoplast culture) was shown to be resistant to AEC and able to accumulate lysine. In these mutants, dihydrodipicolinate synthase was insensitive to feedback inhibition by lysine (Negrutiu et al. 1984).

In 1992, Frankard, et al. crossed the homozygous AEC-resistant mutant of tobacco RAEC-1 that overproduced lysine, with the homozygous mutant RLT 70, that was resistant to lysine plus threonine and overproduced threonine. The level of soluble lysine in the leaves of the heterozygous double mutant (RAEC-1 x RLT 70) was 20-fold higher than the wild type and represented 30% of the total soluble amino acid pool. When compared to the lysine-overproducing parent (RAEC-1), the double mutant contained higher levels of soluble lysine (+ 130%). In contrast, the concentrations of threonine and methionine were similar or even reduced when compared to the parent lines. The data confirmed that dihydrodipicolinate synthase

exerts a strong control over the pathway, and causes a drain of aspartate semialdehyde to lysine when deregulated.

The higher plant dihydrodipicolinate synthase enzyme is normally very sensitive to feedback inhibition by lysine, as discussed previously. However, the *E. coli* enzyme is much less sensitive to lysine. Taking into account this information, a construct including the 35S promoter and the *dap A* gene of *E. coli* that encodes the enzyme, including the pea rbcS-3A chloroplast transit peptide, has been used to transform plants (Shaul and Galili 1992a). An increase in the soluble lysine concentration was detected in tobacco plants that contained *E. coli* dihydrodipicolinate synthase in the chloroplast. In the homozygous plants, the concentration of soluble lysine was 40-fold higher than the control plants. No increase in soluble methionine or threonine was detected in any transformed plants. Similar series of experiments were conducted by Glassman (1992) using the same constructs. Putative transformed tobacco plants contained an elevated concentration of soluble lysine with a maximum increase of almost 100-fold, as compared to the untransformed plants. Again, no increase in methionine or threonine was observed. Unfortunately, the plants obtained by both groups of scientists, exhibiting a higher level of lysine, were observed to have abnormal leaf morphology that could be attributed to the lysine overproduction (Frankard et al. 1992).

Shaul and Galili (1992b) also introduced a mutant *Lys C* gene from *E. coli*, that encodes an aspartate kinase insensitive to feedback inhibition by lysine into tobacco. The *Lys C* gene product was attached to a chloroplast transit peptide. The transformed plants showed a tenfold increase in soluble threonine. However, significant increases in soluble threonine concentration were also determined when the *Lys C* gene was expressed in the cytosol (i.e. same construction but without transit peptide). When progeny of the transformed plants were grown in a glasshouse, greater increases of the concentrations of soluble threonine, isoleucine and lysine were noted.

More recently, Karchi, et al. (1993) fused the *Lys C* gene to a chloroplast transit peptide and the seed-specific promoter of the bean phaseolin gene. A 14-17-fold increase in the soluble threonine concentration of the seeds of the homozygous progeny was detected. Karchi et al. (1993) also measured a three fold increase in the concentration of soluble methionine. No alteration in the plant phenotype was detected in the transgenic plants. Similarly, tobacco plants expressing the bacterial feedback-insensitive dihydrodipicolinate synthase in a seed-specific manner synthesised higher than normal levels of free lysine during seed development. However, the level of free lysine was significantly reduced in mature seeds. These results demonstrated that the aspartate family pathway is functional in seeds, and, furthermore, that free lysine levels in mature seeds are controlled not only by biosynthetic enzymes, but more importantly by catabolic activities (Galili et al. 1995). Indeed, increasing the concentration of free lysine in developing tobacco seeds, either by exogeneous administration or by overproducing the bacterial lysine-insensitive dihydropicolinate synthase, causes an approximate ten-fold increase in lysine oxoglutarate reductase (Karchi et al. 1994), the first committed enzyme in lysine: catabolism. Interestingly, this activity is noticeably lower in developing grains of the *opaque-2* maize mutants (this mutation reduces the accumulation of specific zein polypeptides, the most abundant storage proteins of the endosperm that are devoid of lysine residues) than in wild type grains (Kemper et al. 1998). The extent of cata-

bolic degradation of free lysine in seeds seems to depend on the plant species. Thus, by using the same basic approach as that developed by Karchi et al. (1994) for tobacco (i.e. overproduction in transgenic plants of feedback insensitive aspartate kinase and dihydrodipicolinate synthase under the control of a seed specific promoter), Falco et al. (1995) have achieved a considerable degree of success in the production of transgenic canola and soybean seeds with increased lysine. Remarkably, in the best-transformed line analysed, the total (i.e. free plus bound) lysine content increased over fivefold, from 5.9 to 34% of the total amino acids.

Methionine is the first limiting essential amino acid in legume seeds, since the major storage proteins, the globulins, are low in this amino acid. A number of basic strategies are being carried out to increase the methionine content in legumes. They include modifications of the major storage proteins (e.g. introducing methionine residues or methionine-rich peptides into non-conserved, and presumably non-critical, regions of storage proteins); transfer of heterologous genes encoding methionine-rich protein from other species (e.g. Brazil nut); or manipulation of key enzymes in the methionine biosynthetic pathway (De Lumen et al. 1997). As for lysine, another approach is to select mutant lines that are resistant to the methionine analogue, ethionine; *A. thaliana* (Inaba et al. 1994) and soybean (Madison and Thompson 1988) mutant lines overproducing methionine have been obtained in this way. Although such mutants have not yet been characterised at the molecular and biochemical levels, in both cases the results suggested that mutations affect later step(s) in the methionine biosynthesis pathway, occurring after OPH. These genetic attempts open up the possibility of producing crop plants with increased level of essential amino acids in seeds, as described in chapter 6.

Synthesis of Glycine, Serine and Cysteine

L-serine and glycine, as well as being constitutents of proteins, serve as precursors in phospholipid and purine biosynthesis , and are the main sources of one-carbon units in higher plant cells. In green tissues, serine and glycine are also involved in photorespiratory metabolism. Under high irradiance, pool sizes of glycine and serine in leaves vary with conditions affecting the rate of photorespiration. In illuminated leaves serine usually accounts for 8-12% of the free amino acids, whereas glycine accounts for only 1-3%. Glycine and serine are two amino acids that are interconvertible and are distributed in different subcellular compartments, cytosol, chloroplast, mitochondria and peroxisome. The interaction of the different subcellular pools increases the complexity of their metabolism. For example, during photorespiration the mitochondrial pools of glycine and serine are interconnected and, consequently, their synthesis and catabolism are intimately interwoven.

The interconversion of serine and glycine is catalysed by serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1), which is present in the cytosol, chloroplasts and mitochondria. The three-carbon of serine is transferred to tetrahydrofolate to generate methylene tetrahydrofolate and glycine. The role of tetrahydrofolate is to trap the formaldehyde released from serine at the active site of the enzyme. The plant enzyme, which is a homotetramer, similar to bacterial and mammalian SHMT, requires pyridoxal phosphate as a coenzyme. The interconversion of serine to glycine is fully reversible. However, the equilibrium distribution of different substrates

shows that the reaction favors serine to glycine conversion. Thus, serine is a major source of glycine and one-carbon units in most living organisms. In green tissues, a high SHMT activity is required in mitochondria to cope with high glycine decarboxylase activity (Bourguignon et al. 1999; Rebeille et al. 1994).

There are two main pathways leading to serine and glycine synthesis. The first route, often referred to as the glycolytic or phosphorylated pathway, is linked to glycolysis and leads to serine formation from 3-phosphoglycerate. The glycolytic pathway involves the conversion of 3-phosphoglycerate to serine through 3-phosphohyroxypyruvate and 3-phosphoserine. These reactions are catalysed by 3-phosphoglycerate dehydrogenase (EC 1.1.1.95); glutamate: phosphohydroxypyruvate aminotransferase (EC 2.6.1.52) and phosphoserine phosphatase (EC 3.1.3.3). Glycine can then be derived from serine by the action of serine hydroxymethyltransferase (Mouillon et al. 1999). The second route linked to photosynthetic metabolism involved transamination of glyoxylate into glycine, which is then converted into serine (Bourguignon et al. 1999). Because of the high flux of carbon through the photorespiratory pathway cycle, the glycolate pathway is assumed to be the major metabolic pathway of glycine and serine synthesis in green tissues. It is still unclear whether the glycolytic pathway is metabolically active one during nonphotosynthetic conditions, or whether it participates in serine synthesis in all physiological situations.

During the course of the photorespiratory cycle, the conversion of glycine to serine involves four subcellular compartments. Glycolate, produced in the chloroplast from phosphoglycolate by the oxygenase activity of Rubisco, is transferred via the cytosol into the peroxisome where it is oxidised to glyoxylate. It is then transaminated to glycine by serine :glyoxylate (EC 2.6.1.45) and glutamate:glyoxylate aminotransferases (EC 2.6.1.4), which may also use alanine as a substrate. Glycine is then transported into the mitochondria, where two glycine molecules react to produced serine. This reaction involves the oxidative decarboxylation and deamination of glycine to yield a C-1 fragment and equal amounts of CO_2, ammonia and NADH (Fig. 5). The available evidence suggests that, in green leaf cells, the complete process occurs within the mitochondrial matrix via a coordinated sequence of reactions (Keys et al. 1978) . Glycine is cleaved in the matrix space by glycine decarboxylase or glycine-cleavage system (EC 2.1.2.10) to CO_2, NH_3 and N^5, N^{10} methylene tetrahydrofolate. The latter compound reacts with a second molecule of glycine to form serine in a reaction catalysed by serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1).

The glycine-cleavage system has been purified from plants, animals and bacteria, and consists of four protein components. They have been named P-protein (a pyridoxal phosphate-containing protein), H-protein (a lipoic acid-containing protein); T-protein (a protein catalysing the tetrahydrofolate-dependent step of the reaction) and L-protein (a lipoamide dehydrogenase). The glycine-cleavage system could be linked to the SHMT system by the soluble pool of reduced folate (Bourguignon et al. 1999). The P, H, T, L proteins are encoded by nuclear genes and synthesised in the cytosol with an N-terminal leader sequence that directs the precursor forms towards the mitochondria. The X-ray structure of these proteins and especially the H protein, the core of this multienzymatic system, has been determined at 2.6 and 2 A resolution by the multiple isomorphous replacement technique (Pares et al.1994 ; Cohen-Addad et al. 1995 ; Macherel et al. 1996). The three-dimensional

structure of the oxidised form of the enzyme indicates that the lipoate arm is hanging at the surface of the H protein (Guilhaudis et al. 2000). Indeed, following methylamine transfer, the lipoate cofactor is pivoted about 90° around the lysine linkage (Lys-63), to bind into a cleft at the surface of the protein (Cohen-Addad et al. 1995). This modification of structure allows to the methylamine group to be locked within a hydrophobic pocket, preventing the nonenzymatic release of NH_3 and formaldehyde, owing to nucleophilic attack by water molecules (Cohen-Addad et al. 1997; Bouguignon et al. 1999).

Because high rates of glycine oxidation are required to cope with the flux of photorespiratory glycine entering leaf mitochondria, glycine decarboxylase and SHMT are present at very high concentration in the matrix space of mitochondria (Oliver et al. 1990). These two enzymes systems represent about 40-50% of the soluble proteins in the mitochondria. Vauclare et al. (1996) have also observed that the buildup

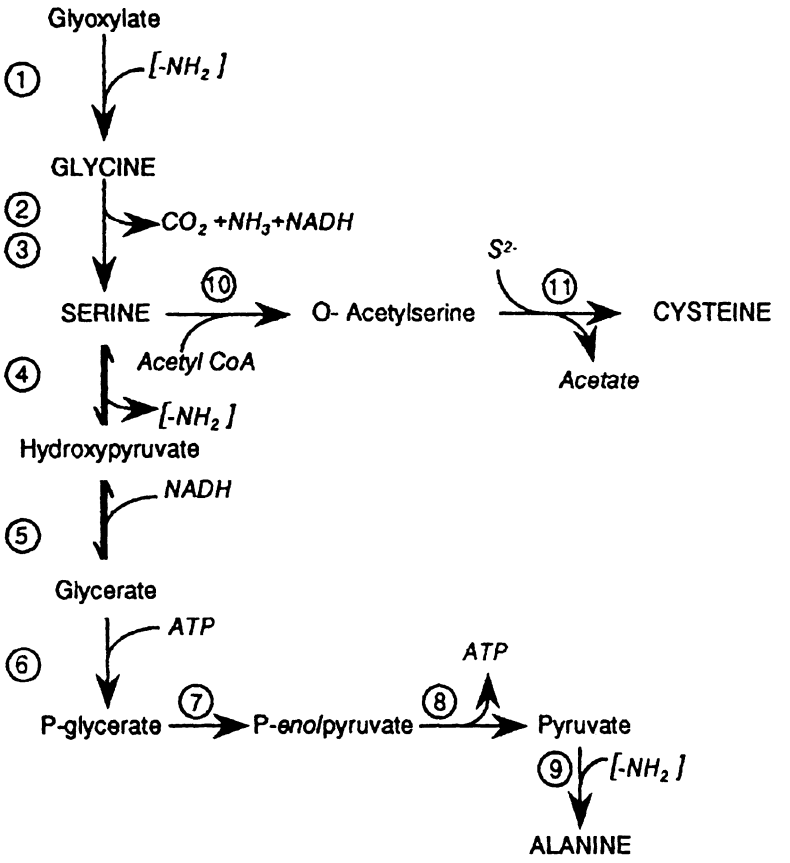

Fig. 5 : The biosynthetic pathway of glycine, serine alanine and cysteine. Enzymes: *1.* serine : glyoxylate and glutamate: glyoxylate transaminases; *2.* glycine decarboxylase; *3.* serine hydroxymethyltranferase; *4.* serine: glyoxylate aminotransferase; *5.* hydroxypyruvate reductase; *6.* glycerate kinase; *7.* enolase; *8.* pyruvate kinase; *9.* aminotransferase.*10.* serine acetyltransferase; *11.* O-acetylserine (thiol)-lyase

of glycine decarboxylase is dependent on leaf development and the genes encoding this enzyme could be under light-dependent transcriptional control.

Ammonia released during glycine deamination is reassimilated by glutamine synthetase in the cytosol or, more likely, in the chloroplast (Leegood et al. 1995). It is important to note that the conversion of glycine to serine produces ammonia at rates tenfold faster than nitrate reduction. Ammonia is recycled through the GS/GOGAT cycle (Keys et al. 1978; Woo et al. 1982), whilst the main part of CO_2 is lost to the atmosphere during photorespiration. The reductant NADH formed is either converted to ATP via the electron transport chain, or is exported from the mitochondria via a malate/oxaloacetate shuttle (Douce 1985).

The serine produced in the mitochondria enters the peroxisomal compartment, where it is converted into the oxo acid analog of serine, hydroxypyruvate by serine:glyoxylate aminotransferase (EC 2.6.1.45). The latter is reduced to glycerate by hydroxypyruvate reductase (EC 1.1.1.81) and converted to phosphoglycerate in the chloroplast by NADH-glycerate kinase (EC 2.7.1.31) (Leegood et al. 1995).

During the course of the photorespiratory cycle, glycine and serine are only intermediary metabolites and not end-products of the pathway, thus photorespiration does not play an important role in the net synthesis of glycine and serine. The glycolate pathway is aimed at the recycling of the two carbons of glycolate into the Calvin-Benson cycle, avoiding the depletion of this cycle when the oxygenase activity of Rubisco is high (Bourguignon et al. 1999).

Barley, tobacco and *A.thaliana* mutants deficient in glycine decarboxylase, serine hydroxymethyltransferase, and serine-glyoxylate aminotransferase have been isolated (Somerville 1986; Blackwell et al. 1990; Lea and Forde 1994). These mutants are viable only in atmospheres of high CO_2. Under these conditions, it was demonstrated that the glycine cleavage step of photorespiration is not necessary for any essential function unrelated to photorespiratory activity. In normal air, the mutants are chlorotic and die prematurely. Blocking the flux of carbon or nitrogen through the photorespiration pathway is an effective way of killing plants, by impairing the recycling of glycolate back to glycerate. Photorespiration mutants have confirmed the view that it will only be possible to decrease photorespiration by genetic manipulation of ribulose biphosphate carboxylase-oxygenase, or the introduction of a CO_2 concentrating mechanism into C_3 plants.

Serine is acetylated to O-acetylserine by serine acetyltransferase (EC 2.3.1.30) prior to sulphydration. Cysteine is then formed by O-acetylserine thiol-lyase (also called cysteine synthase) (EC 4.2.99.8). In plant cells, the existence of several enzymes supporting O-acetylserine (thiol)-lyase activity has been demonstrated (Rolland et al. 1992; Saito et al. 1994; Droux et al. 1998). This subdivision reflects different subcellular localisation of enzyme activity, within the chloroplast, cytosol and mitochondria. Such a differential localisation is also seen for the serine acetyltransferase activity. The reasons for such a compartmentation (which contrasts with that for the aspartate family pathway which is almost entirely localised to the chloroplast) might be that O-acetylserine is a very unstable compound at alkaline pH (the pH of the chloroplast stroma in the light is above 8), being transformed into N-acetylserine which is not a substrate for O-acetylserine thiol-lyase. In addition, the plant cell might be unable to transport cysteine between compartments, so that the cysteine required for protein synthesis must be synthesised *in situ*. Sulphate is reduced to sulphide in presence of ATP and ferredoxin in the chloroplast. The control mechanisms

in cysteine synthesis appear to involve feedback inhibition by cysteine at several steps in the reduction of sulphate to sulphide, of sulphate uptake itself, and of the first committed step in cysteine biosynthesis catalysed by serine acetyltransferase (Hell 1997; Droux et al. 1998).

Synthesis of Amino Acids Derived from Pyruvate

Valine and leucine are synthesised from pyruvate through the branched-chain amino acid pathway (Fig. 6). Alanine is synthesised from pyruvate by direct transamination (see above). This reaction is catalysed by alanine aminotransferase (EC 2.6.1.2). Isoleucine and lysine also obtain some of their carbons from pyruvate (Singh and Shaner 1995).

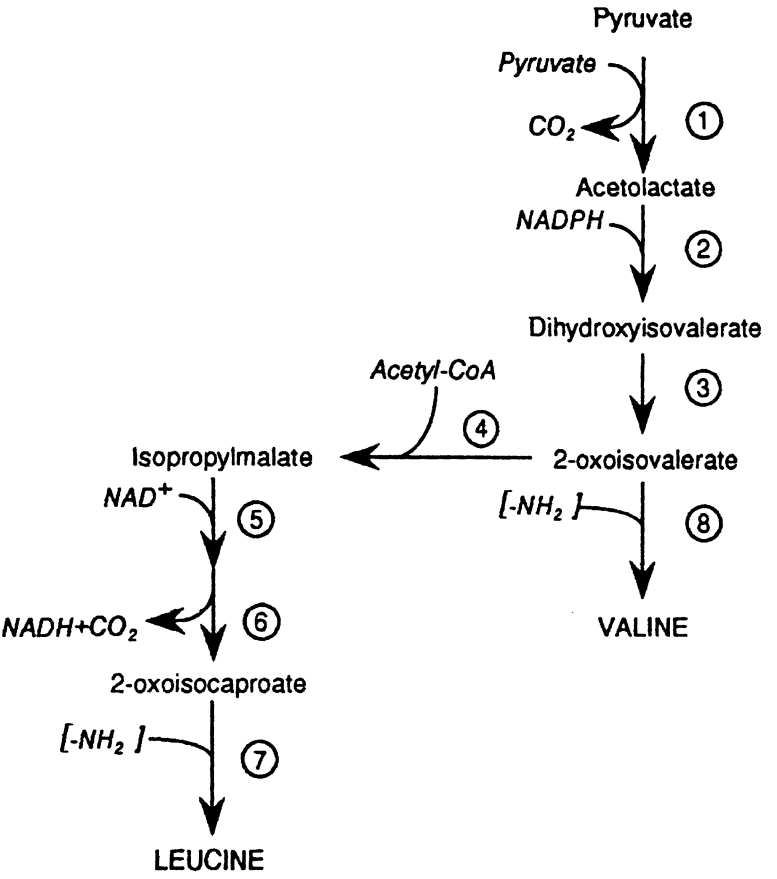

Fig. 6: The biosynthetic pathway of valine and leucine. Enzymes: *1.* acetohydroxyacid synthase; *2.* acetohydroxyacid reductoisomerase; *3.* dihydroxyacid dehydratase; *4.* isopropylmalate synthase; *5.* isopropylmalate isomerase; *6.* isopropylmalate dehydrogenase; *7.* aminotranferase; *8.* aminotransferase

Pyruvate provides the carbon skeletons for valine and leucine, the branched-chain amino acids. The reaction involves the decarboxylation of pyruvate to form, in the presence of thiamine pyrophosphate, hydroxyethyl thiamine pyrophosphate (activated acetaldehyde). This compound is then accepted by an other molecule of pyruvate or by 2-oxobutyrate (a product derived from the deamination of threonine). The enzyme catalysing the synthesis of acetolactate is acetolactate synthase or acetohydroxyacid synthase (AHAS; EC 4.1.3.18).This enzyme was found in a number of plants to be located in the chloroplast. Acetolactate is transformed, by acetohydroxyacid reductoisomerase (EC 1.1.1.86) and dihydroxyacid dehydratase (EC 4.2.1.9), to oxovalerate and then transaminated to valine. Oxovalerate can be condensed with the methyl group of acetyl-CoA to give isopropylmalate after the action of isopropylmalate synthase (EC.4.1.3.12). This latter compound is then oxidised, isomerised and decarboxylated to α-oxoisocaproate which is transaminated to leucine. The branched-chain α-oxoacid dehydrogenases are associated with lipoic acid acting as a prosthetic group. As with other branched pathways, close regulation is necessary to produce the desired levels of different amino acids; again feedback inhibition is in operation. Valine and leucine inhibit the biosynthetic pathway of their own synthesis at the point of AHAS and leucine inhibits the synthesis of isopropylmalate from oxoisovalerate (Singh and Shaner 1995). All of the enzymes of this pathway are located in chloroplast which has been shown to synthesise valine from $^{14}CO_2$.

A number of mutants have been isolated that lack the enzymes threonine dehydratase and dihydroxyacid dehydratase (Negrutiu et al. 1985; Wallsgrove et al. 1986). These mutants require both valine and isoleucine for growth. Mutants resistant to the toxic action of valine, which acts as a potent feedback inhibitor of the pathway, have been isolated by Bourgin (1978). This class of mutants exhibited a reduced rate of amino acid uptake.

The main interest has centered on the enzyme AHAS, as four active families, the imidazolinones, sulphonylureas, triazolopyrimidines and pyrimidyloxobenzoates, have been observed to inhibit the enzyme (Singh and Shaner 1995). These compounds are among the most advanced herbicides used in agriculture, because they exhibit extremely low mammalian toxicity and high efficacy, resulting in very low application rates (a few grams ha^{-1}!) and thus they have very low environmental impact. The exact mechanism accounting for the lethality of these herbicides is not yet known, that is whether plant death results from a deprivation of branched chain amino acids or from the accumulation of toxic compounds (e.g. oxobutyrate and/or upsteam metabolites and/or by-products) (Epelbaum et al. 1996). Also, by using a *luxCDABE* reporter gene complex from *Photorhabdus luminescens*, Van Dyk et al. (1998) recently analysed the pattern of gene expression upon treatment of *E. coli* with sulphometuron methyl, a sulphonylurea herbicide. Their results suggested that restricted AHAS activity may cause intracellular acidification and the induction of the σ^S-dependent stress response. Whatever the cause of lethality, the inhibitory action of the herbicides can be reversed by the exogenous application of valine, leucine and isoleucine together. These herbicides are unusual inhibitors in that they bear no obvious similarity to the substrates (pyruvate and α-oxobutyrate), cofactors (thiamine pyrophosphate, FAD and magnesium) or allosteric effectors (valine, leucine and isoleucine) of the enzyme. They behave as noncompetitive slow binding inhibitors, which presumably interact with an evolutionary vestige of the quinone-

binding site of pyruvate oxidase (a related enzyme) (Schloss et al. 1988). Owing to high lability and very low abundance, purification of AHAS from plant tissues proved difficult and resulted in very low yields. Recently, the *A. thaliana* enzyme with part of its transit peptide removed (the enzyme is localised to the plastids) has been overexpressed in *E. coli*, allowing purification of the enzyme to homogeneity (Chang and Duggleby 1997). The recombinant enzyme was inhibited by sulphonylurea and imidazolinone herbicides, but was however insensitive to valine, isoleucine and leucine. This last result indicates strongly that the recombinant plant enzyme contains only the large catalytic subunit and lacks the small regulatory subunit involved in feedback inhibition that has been characterised for the corresponding bacterial enzymes (Vyazmensky et al. 1996).

A large number of mutant plants resistant to the AHAS inhibitors have been isolated and their properties have been discussed in detail (Mazur and Falco 1989). AHAS inhibitor-resistant mutant lines of important crop plants such as soybean, maize, canola and wheat appear to be of uppermost interest. Moreover, populations of weed species resistant to the sulphonylurea herbicides have also occurred in the field (Mallory-Smith et al.1990). In Australia, for example, resistant biotypes of ryegrass have been shown to contain chlorsulphuron-insensitive AHAS and an increased ability to detoxify the herbicide (Christopher et al. 1992).

Many AHAS inhibition resistant mutant lines of *A. thaliana* have been isolated and examined at the molecular level. The chlorsulphuron-resistant mutation gives rise to a proline to serine substitution at amino acid 196 on the AHAS protein (Haughn et al. 1988). The imidazolinone resistant mutation *crs1* causes substitution of an asparagine by a serine at amino acid 653 (Haughn and Somerville 1990). More recently, Mourad and King (1992) have isolated a mutant resistant to triazolopyrimidine and have investigated the cross-resistance properties of all three mutations.

The chlorsulphuron resistant mutant gene, isolated from *A.thaliana*, expressed in tobacco using its own promoter, conferred high levels of herbicide resistance (Haughn *et al.* 1988). This gene was placed under the control of both native and 35S promoters, resulting in a 300-fold increase in herbicide tolerance. The 25-fold increase in AHAS transcript levels correlated with only a twofold increase in AHAS specific activity (Odell et al. 1990). Tourneur et al. (1993) have expressed the *csrl* gene under the control of the 35S promoter with a duplicated enhancer (p70). The transformed plants contained a 12-fold higher level of AHAS activity, and exhibited a 1500-fold increase in resistance to chlorsulphuron and also a resistance to the external application of valine. However, there was no significant increase in the content of free branched-chain amino acids in the leaves, suggesting that subsequent steps in the branched-chain amino acid pathway may also regulate the synthesis of valine and leucine.

Manipulation of branched-chain amino acid biosynthesis has been driven by the search for herbicide-tolerant plants. AHAS appears to be the primary control point of the pathway and its regulation is achieved by feedback inhibition of valine and leucine. AHAS is the site of herbicide action and consequently the interesting enzyme to be genetically manipulated. Other experiments attempting to modulate the gene expression of AHAS are in progress. Potato plants expressing a constitutive antisense AHAS construction have been generated and subjected to a comprehensive analysis (Hofgen et al. 1995). Severe growth retardation and strong phenotypic resemblance to plants treated with the AHAS-inhibiting herbicides were observed.

However, these plants exhibited unexpected elevated levels of valine and leucine. There have been some speculative attempts to elucidate the reasons for the deregulation for this amino acid pathway.

Keto-acid acid isomeroreductase (KARI, also called acetohydroxy acid isomeroreductase, EC 1.1.1.86), which is the second common enzyme of the parallel branched chain amino acid pathway, has been the subject of several studies in both bacteria and plants. This is partly because resistant plants can rapidly emerge under the selective pressure of ALS-inhibiting herbicides, and, therefore, it is important to evaluate the potential of other enzymes in the pathway, to provide new efficient herbicide targets. KARI catalyses a Mg^{2+}-dependent two-step reaction in which the substrate, either 2-acetolactate or 2-aceto-2-hydroxybutyrate is converted via an alkyl migration and an NADPH-dependent reaction to yield 2,3-dihydroxy-3-isovalerate (synthesis of valine and leucine) or 2,3-dihydroxy-3-methylvalerate (synthesis of isoleucine), respectively. The plant enzyme has been purified from spinach chloroplasts and the cDNA has been cloned, allowing investigation of some of its kinetic (Dumas et al. 1994a) and structural (Biou et al. 1997) properties. Site-directed mutagenesis (Dumas et al. 1995) and determination of the crystal structure of the protein (Biou et al. 1997) revealed that the enzyme contains two active site-located magnesium ions, one of which plays a role in the isomerisation reaction and the other in the reduction step. Transition states analogues such as *N*-hydroxy-*N*-isopropyloxamate (IpOHA) and 2-(dimethylphosphinoyl)-2-hydroxyacetic acid (Hoe 704) proved to be highly potent inhibitors of the bacterial (Aulabaugh and Schloss 1990;) and plant (Dumas et al.1994b) KARI. However, compared to the ALS-inhibiting herbicides, both IpOHA and Hoe704 showed only poor herbicidal action (Aulabaugh and Schloss 1990). One possible explanation for this behaviour is that these compounds act as extremely slow-binding competitive inhibitors of KARI (Dumas et al.1994b). Furthermore, since inhibition of this enzyme leads *in vivo* to an increase in the concentration of the KARI substrate, these inhibitors bind to the enzyme considerably slower as the substrate concentration increases. Recently, plant KARI has been submitted to high-throughput screening, allowing the discovery of a new class of compounds belonging to the thiadiazole family, which act as either reversible or irreversible noncompetitive inhibitors of the plant enzyme (Halgand et al. 1998).

Synthesis of Histidine

Until recently, very little information was available regarding histidine biosynthesis in plants. It was generally assumed that the route of synthesis is the same as in bacteria. Synthesis starts with ribose-5-phosphate which is transformed into 5-phosphoribosyl-α-1-pyrophosphate, the precursor of N'-[(5'-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboximide ribonucleotide (5'-PRFAR), considered also as the starting point for purine synthesis. In the presence of glutamine, this derivative gives rise to imidazole glycerol phosphate, the precursor of imidazole acetol phosphate. With glutamate, this molecule is transformed to histidinol phosphate, histidinol, histidinal and histidine (Fig. 7).

The enzymes ATP-phosphoribosyl transferase (EC 2.4.2.17), imidazole glycerol phosphate dehydratase (EC 4.2.1.19), and histidinol phosphate phosphatase (EC 3.1.3.15) have been detected in plant extracts. These enzymes catalyse the first,

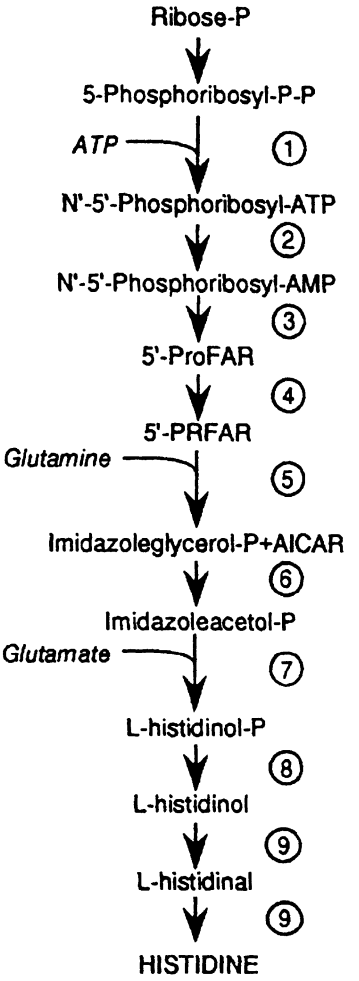

Fig. 7 : The biosynthetic pathway of histidine. Enzymes : *1.* ATP phosphoribosyl transferase; *2.* phosphoribosyl-ATP pyrophosphohydrolase; *3.* phosphoribosyl-AMP cyclohydrolase; *4.* N'-[(5'-phosphoribosyl) formimino]-5-aminoimidazole-4-carboximide ribonucleotide isomerase; *5.* imidazole glycerol phosphate synthase; *6.* imidazole glycerol phosphate dehydratase; *7.* histidinol phosphate aminotransferase; *8.* histidinol phosphate phosphatase; *9.* histidinol dehydrogenase. 5'-ProFAR (cyclic form), N'-[(5'-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboximide ribonucleotide; 5'-PRFAR (linear form), N'-[(5'-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboximide ribonucleotide; AICAR, 5'phosphoribosyl-4-carboximide-5-aminoimidazole

sixth and eighth steps of the pathway, respectively. The imidazole glycerol phosphate dehydratase cDNA has been isolated from *A. thaliana* by Tada et al. (1995). Histidinol dehydrogenase (EC 1.1.1.23) has been purified from wheat germ, exhibiting two forms; the cDNA was isolated from cabbage by Nagai et al. (1991). This enzyme

catalyses the last two steps of the pathway. A cDNA encoding histidinol phosphate aminotransferase (EC 2.6.1.9) was also isolated and characterised in *Nicotiana tabacum* (El Malki et al. 1998). This enzyme catalyses the eighth step of the pathway. Aminotriazole inhibits the synthesis of imidazole acetol phosphate, a precursor of histidine. This compound, which exhibits herbicidal activity, affects the activity of the imidazole glycerol phosphate dehydratase, leading to the inhibition of histidine biosynthesis in plants (Mori et al. 1995).

Recently, Fujimori and Ohta (1998) reported the isolation of an *A. thaliana* cDNA that encodes a bifunctional protein (At-IE) that has both phosphoribosyl-ATP pyrophosphohydrolase and phosphoribosyl-AMP cyclohydrolase activities. These enzymes catalyse the second and the third steps, respectively, in histidine biosynthesis. The gene encoding At-IE was expressed ubiquitously throughout development of the plant. Sequence comparison suggested that the At-IE protein has an N-terminal extension with the properties of chloroplast transit peptide.

A number of mutant plants have been isolated which require histidine for growth. The biochemistry of these mutations has not yet been established (Gebhardt et al. 1983). The availability of histidine auxotrophic mutants might represent a valuable tool to elucidate the regulation of the amino acid metabolic pathway in plants. For example, the use of a specific inhibitor to block histidine biosynthesis leads to an increase in the expression of genes involved in the biosynthesis of aromatic amino acids, as well as histidine, lysine and purines ; the addition of histidine reversed the situation. This suggests the existence of a general control system of metabolite biosynthesis in plants, which would represent an efficient way to coordinate the regulation of genes involved in unrelated metabolic pathways, providing evidence for cross-pathway regulation of metabolic gene expression in plants (Guyer et al.1995).

Thus, histidine biosynthesis in plants follows the same pathway as in bacteria and fungi, despite some structural and functional differences between the respective enzymes and genes. Moreover, it is possible that the entire pathway of histidine biosynthesis may be carried out in the chloroplasts, which is an extremely energy-consuming process (41 ATP required for each histidine molecule synthesised).

Synthesis of Aromatic Amino Acids

The group consisting of tryptophan, phenylalanine and tyrosine is synthesised by a common pathway, called the shikimate pathway. Besides leading to the formation of aromatic acids for protein synthesis, the shikimate pathway provides precursors for a number of secondary metabolites in higher plants: such as phenolic acids, flavonoids, isoflavonoids, glucosinolates, phytoallexins, alkaloids, plant growth regulators (indole acetic acid, IAA), suberin and lignin. Several of these compounds are involved in the response of plants to wounding or infection.

The shikimate pathway can be divided into three sections (Fig. 8). The synthesis of chorismate is common to all three aromatic amino acids. Beyond chorismate, there is a branch leading to phenylalanine and tyrosine and one to tryptophan. There is strong evidence that the complete shikimate pathway can operate in the chloroplast, but the existence of a second cytoplasmic pathway is still a matter of debate (Schmid and Amrhein 1995).

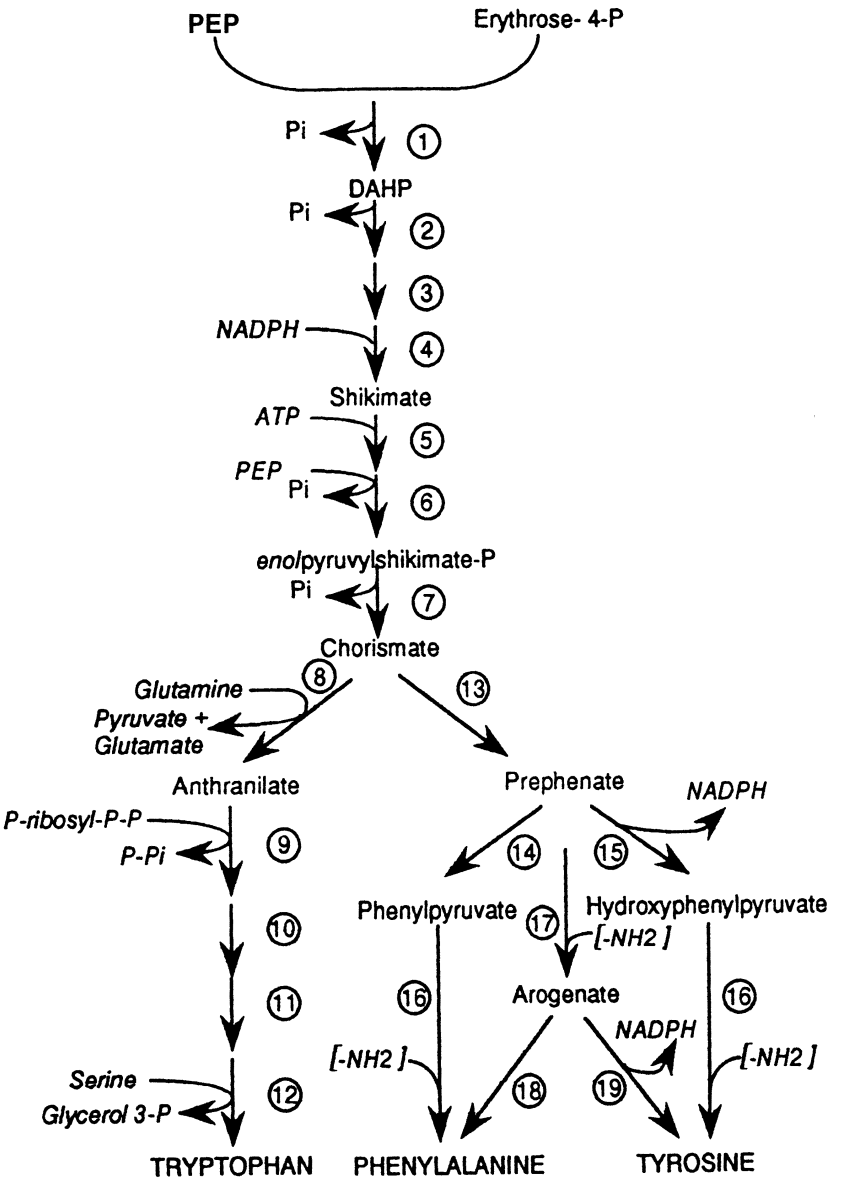

Fig. 8 : The biosynthetic pathway of aromatic amino acids. Enzymes: *1.* 3-deoxy-D-*arabino*heptulosonate-7-P synthase; *2.* 3-dehydroquinate synthase; *3.* 3-dehydroquinate dehydratase; *4.* shikimate dehydrogenase; *5.* shikimate kinase; *6.* 5-*enol*pyruvylshikimitate 3-phosphate synthase; *7.* chorismate synthase; *8.* anthranilate synthase; 9. phosphoribosylanthranilate transferase; *10.* phosphoribosylanthranilate isomerase; *11.* indole-3-glycerolphosphate synthase; *12.* tryptophan synthase; *13.* chorismate mutase; *14.* prephenate dehydratase; *15.* prephenate dehydrogenase; *16.* phenylalanine and tyrosine aminotranferase; *17.* prephenate aminotransferase; *18.* arogenate dehydratase; *19.* arogenate dehydrogenase. DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; PEP, phospho*enol*pyruvate

The first step of this pathway is the condensation of erythrose-4-phosphate, a product of photosynthesis, with phospho*enol*pyruvate (PEP), a product of glycolysis, to give 3-deoxy-D-*arabino*heptulosonate-7-phosphate (DAHP). This reaction is catalysed by 3-deoxy-D-*arabino*heptulosonate-7-phosphate synthase (EC 4.1.2.15). DAHP undergoes a series of subsequent reactions catalysed by 3-dehydroquinate synthase (EC 4.6.1.3), 3-dehydroquinate dehydratase (EC 4.2.1.10) and shikimate dehydrogenase (EC 1.1.1.25) to give shikimate (3,4,5,-trihydroxycyclohexene-1-carboxylic acid). Shikimate is then phosphorylated to 3-phosphoshikimate by shikimate kinase (EC 2.7.1.71), which is condensed with a molecule of phospho*enol*pyruvate (PEP) to give 5-*enol*pyruvylshikimate 3-phosphate. This reaction is catalysed by 5-*enol*pyruvylshikimate 3-phosphate synthase (EC 2.5.1.19) (EPSPS). By the action of chorismate synthase (EC 4.6.1.4), this latter compound is transformed to chorismate. Chorismate is at a branch point in this pathway, one pathway leading to tryptophan, and the other to phenylalanine and tyrosine (Herrmann 1995).

In the tryptophan biosynthetic pathway, chorismate reacts with the amide group of glutamine to produce anthranilate, a reaction catalysed by anthranilate synthase (EC 4.1.3.27). Anthranilate subsequently condenses with phosphoribosylpyrophosphate to give phosphoribosylanthranilate, a reaction catalysed by phosphoribosylanthranilate transferase (EC 2.4.2.18). This product, in turn, is isomerised by phosphoribosylanthranilate isomerase (EC 5.3.1.24) and converted in the presence of pyridoxal phosphate to indole-3-glycerolphosphate (IGP) by indole-3-glycerolphosphate synthase (EC 4.1.1.48). The final reaction is catalysed by tryptophan synthase (EC 4.2.1.20). This enzyme is an $\alpha_2\beta_2$ heterotetramer and each subunit can catalyse a half reaction, first the transformation of indole-3-glycerolphosphate to indole and glyceraldehyde-3-phosphate, and second the addition of serine on indole to produce tryptophan and water (Radwanski and Last 1995).

The synthesis of phenylalanine and tyrosine starts with the rearrangement of chorismate by chorismate mutase (EC 5.4.99.5) to prephenate. In bacteria, the prephenate is either dehydrated to phenylpyruvate by a prephenate dehydratase (EC 4.2.1.51), or oxidatively decarboxylated to hydroxyphenylpyruvate by a prephenate dehydrogenase (EC 1.3.1.13). Both of these oxo acids are subsequently transaminated to phenylalanine and tyrosine, respectively. It has been suggested that there exists in plants another route which involves the transamination of prephenate to arogenate by prephenate aminotransferase. This molecule is then directly converted either to phenylalanine by arogenate dehydratase (EC 4.2.1.91) or tyrosine by arogenate dehydrogenase (EC 1.3.1.43) This pathway is called the arogenate pathway (Siehl 1999).

The enzymes of aromatic amino acid biosynthesic pathways are located in the chloroplast. Labelling with $^{14}CO_2$ has shown that chloroplasts contain the complete pathway for the synthesis of aromatic amino acids. However, it has been demonstrated that at least one enzyme of the prechorismate pathway in the unicellular phytoflagellate *Euglena gracilis*, EPSPS, is expressed in two different molecular forms, one being localised in the chloroplastic compartment, the other in the cytosol (Reinbothe et al. 1994).

The pathway of aromatic amino acid biosynthesis is relatively well regulated. The first enzyme of the shikimate pathway, DAHP synthase, exists as at least two isoenzymes, one requiring Mn^{2+} and the other Co^{2+} for activity. Arogenate and

prephenate have been shown to inhibit the Mn^{2+}-dependent form, and in some circumstances tryptophan has been shown to activate the enzyme. This enzyme is known to be induced by environmental factors, including wounding and microbial infection, correlating with an increase in secondary metabolite synthesis. An antisense construct consisting of the 5 end of the wound-inducible DAHP synthase gene from potato under the control of a CaMV 35S promoter has been used to transform potato (Jones et al. 1995). A number of transgenic plants with impaired wound-induced DAHP synthase activity, polypeptide and mRNA were identified. Several plants exhibited altered morphology but no biochemical analyses were reported. It has been suggested that distinct DAHP synthase genes, support different pathways for the synthesis of aromatic nitrogen compounds, destined for proteins and secondary metabolites.

The penultimate step in the shikimate pathway is catalysed by the 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme is considered as the target for the herbicide glyphosate which is marketed by Monsanto as Roundup. Consequently, EPSPS has been the subject of extensive biochemical studies (Steinrucken and Amrhein 1980). Early studies suggested an ordered mechanism for the enzyme-catalysed reaction in which shikimate 3-phosphate binds first, followed by phospho-*enol*pyruvate (PEP). Furthermore, for all EPSPS enzymes studied to date, glyphosate has been characterised as a reversible competitive inhibitor versus PEP and an uncompetitive inhibitor versus shikimate-3-phosphate. However, more recent results showed unequivocally that the steady-state kinetic mechanism proceeds through random addition of substrates in both the forward and reverse directions (Sammons et al. 1995). Furthermore, recent NMR work indicated that glyphosate is unlikely to bind in the same fashion as PEP, which casts doubts on an earlier proposal that glyphosate acts as a transition-state or intermediate analogue (McDowell et al. 1996). It would appear that glyphosate binding to the binary complex of the enzyme with shikimate-3-phosphate must involve some substantial adventitious interactions with amino acid residues, which are not intimately involved in substrate binding and catalysis (Gruys and Sikorski 1999). As such, part of the glyphosate molecule must bind near to, but outside the EPSPS active site. The activity of the plastid-localised enzyme EPSPS is inhibited by glyphosate and thereby synthesis of the chorismate is prevented. Inhibition of EPSPS also causes a dramatic buildup in shikimate and shikimate-3-phosphate, which may drain carbon from the reductive pentose phosphate cycle (Shaner and Singh 1992).

A number of attempts have been made to select for mutant glyphosate-resistant plant tissue culture cell lines, which have been shown to contain elevated levels of EPSPS (Nafziger et al. 1984). In carrot suspension cultures, glyphosate-resistant cell lines were obtained by a gradual increase in the concentration of the inhibitor (Shyr et al. 1993). The specific activity of EPSPS was observed to increase, along with the mRNA levels and copy number of the genes at each glyphosate selection step. One tissue culture cell line of maize (black mexican sweet) has been selected to have a marked tolerance to glyphosate. After separation of two isoenzymes of EPSPS by anion exchange chromatography, it was observed that one isoenzyme form was resistant to inhibition by glyphosate. Tolerance to the herbicide was accompanied by a reduced affinity of EPSPS for PEP (Forlani et al. 1992).

Genetic engineering of glyphosate tolerance is of great interest to the agriculture and agrochemical industry. A range of mutants of *E. coli* and *Salmonella typhimu-*

rium containing glyphosate-resistant EPSPS have been isolated. Transgenic plants containing the mutant bacterial EPSPS construct targeted to the chloroplast have been shown to be tolerant to the application of glyphosate (Fillatti et al. 1987). However, with the mutant bacterial enzyme, a large increase in the K_i for glyphosate was observed that was accompanied by an equivalent increase in the K_m for PEP (Barry et al. 1992). A gene encoding a glyphosate resistant EPSPS (*aro A*) was isolated from an *Agrobacterium* sp. strain CP 4. The encoded enzyme maintained a low K_m for PEP despite an increased K_i for glyphosate. This CP 4 EPSPS gene, fused to the chloroplast targeting sequence and driven by the CAMV 35S promoter, has been introduced into petunia, *A. thaliana*, canola and soybean (Klee et al. 1987). Transgenic canola containing the chloroplast-targeted CP 4 EPSPS enzyme, exhibited high levels of tolerance to the herbicide at both vegetative and reproductive stages. Transgenic soybean transformed with the same construct has been tested extensively under field conditions. For more information on the genetic engineering of glyphosate tolerance, see the recent review of Padgette et al. (1996).

Chorismate mutase occupies a strategic position in catalyzing the conversion of chorismate to prephenate and has been shown to exist in two isoenzymic forms. Chorismate mutase-I is subject to feedback inhibition by tyrosine and phenylalanine and activation by tryptophan, whereas chorismate mutase-II is insensitive to aromatic amino acids (Schmid and Amrhein 1995). In alfalfa, three isoenzymes are present, which can be inhibited by a range of secondary metabolites.

The tryptophan biosynthetic pathway has recently received considerable attention. Initial attempts to obtain tryptophan auxotrophs were carried out using tissue culture cells. However, it is in *A. thaliana* that the first selection of amino acid auxotrophs requiring tryptophan for growth has been carried out, at the whole-plant level (Last 1993). Normal plants convert anthranilate analogue (5-methylanthranilic acid,) into toxic tryptophan analogue (5- methyltryptophan), whereas mutants blocked in this conversion exhibit reduced toxin biosynthesis (Radwanski and Last 1995).

The first tryptophan-requiring mutant line to be characterised was deficient in phosphoribosylanthranilate transferase (PAT). Plants carrying the *trp 1-1* mutation exhibit a blue fluorescence colour under ultraviolet light, because of the accumulation of anthranilate derivatives. These mutant plants appeared smaller with crinkled leaves and an unusual bushiness. They produced larger flower bolts that contained a small number of fertile flowers (Last and Fink 1988). The results suggested that these disorders were a consequence of auxin deficiency, a finding that was confirmed by the addition of IAA conjugates, which caused the mutant to grow larger and form a higher proportion of fertile flowers. The gene encoding the PAT enzyme (*PAT 1*) was introduced into *A. thaliana*. The analysis of the transgenic plants has confirmed that *PAT 1* is a single copy gene and encodes a chloroplast-localised enzyme (Rose et al. 1992).

Anthranilate synthase is subject to feedback regulation by tryptophan, but not by phenylalanine or tyrosine. The enzyme protein contains two different subunits termed α and β, and probably exists in $\alpha_2\beta_2$ formation. In *A. thaliana* two genes encoding the α subunit and three genes encoding the β subunit have been isolated and are differentially expressed (Radwanski and Last 1995). There is evidence that the subunit genes are differentially regulated during plant development and in response to environmental stimuli. Furthermore, accumulation of anthranilate syn-

thase mRNA is induced by pathogenesis and wounding. However, important post-transcriptional control mechanisms in regulating anthranilate synthase expression, have been observed in these elicitor responses (Radwanski and Last 1995).

A. thaliana has two genes encoding the β-subunit of tryptophan synthase, *TSB 1* and *TSB 2* (Berlyn et al. 1989). Both genes are transcribed, although *TSB 1* mRNA levels were determined to be at least tenfold higher in the leaves than *TSB 2* mRNA. A *trp2-1* tryptophan-requiring mutant has been identified that exhibited about 10% of the wild-type tryptophan synthase β activity. The *trp2-1* mutation has been complemented by the *TSB 1* transgene and was observed to be linked genetically to a polymorphism in the *TSB 1* gene, strongly suggesting that *trp2-1* is a mutation in *TSB 1*. The *tpr2-1* mutant required tryptophan for growth under standard illumination but not under very low illumination conditions. Presumably, under low light the poorly expressed gene, *TSB2*, is capable of supporting growth. The duplication of biosynthetic genes in *A. thaliana* has important evolutionary, functional, and practical implications for plant molecular genetics. Genetic redundancy has been observed to be common in many aromatic amino acid biosynthetic pathways in plants. The existence of two tryptophan pathways, for example, has important consequences for tissue-specific regulation of amino acid and secondary metabolite biosynthesis (Last et al. 1991).

To date, no mutant has been isolated for phosphoribosylanthanilate isomerase (*PAI*). This incapacity is probably due to the presence of three *PAI* genes. Plants constitutively expressing an antisense PAI cDNA had as little as 15% PAI activity, 10% PAI protein and phenotypes consistent with a block early in the tryptophan pathway (blue fluorescence under UV light due to the accumulation of anthranilate compounds) (Li and Last 1996). Studies using tryptophan biosynthetic mutants have challenged the idea that tryptophan is an obligatory intermediate in the biosynthesis of some secondary products. Furthermore, mutants in indole secondary metabolism are being characterised, creating exciting research directions.

Ureide Synthesis

Ureides are nitrogen compounds such as citrulline, allantoin and allantoic acid. These molecules, based on the structure of urea (with a high N/C ratio), are used for the transport of nitrogen in plants, especially in many tropical legumes. The legume plants referred to as the tropical legumes such as soybean, *Phaseolus* beans and cowpeas, when nodulated, transport organic nitrogen as ureides. Conversely, the temperate legumes such as peas, lupins and alfalfa, synthesise amides, asparagine and glutamine, the more common amides, regardless of whether they are nodulated or not. The rationale for this switch from amide to ureide synthesis in tropical legumes involves the economy of carbon use. It appears that the ureide producers use less organic carbon to transport the same amount of nitrogen than the amide transporters. Moreover, catabolism of ureides appears to be less efficient than catabolism of amides, since some of the carbon is lost as CO_2. The overall cost of using ureides is metabolically more expensive, almost twice as much as using asparagine (Schubert and Boland 1990).

Ureides are synthesised by the oxidation of purines, which are themselves derived from glutamine, glycine, aspartate and ribose-5-phosphate. Purine synthesis from

these latter compounds occurs in the plastids of infected root cells of legume plants. Xanthine, one of the purines, is oxidised to uric acid by xanthine dehydrogenase (EC 1.1.1.204) in the cytosol. Uric acid is transformed in the peroxisomes by uricase (EC 1.7.3.3) to allantoin, with the concomitant release of CO_2 and H_2O_2, and then converted to allantoic acid by allantoinase (EC 3.5.3.4), an enzyme of the endoplasmic reticulum. The ureide is cleaved to yield two molecules of urea (or CO_2 and NH_3) and glyoxylate. The cellular and subcellular localisation of these reactions has not yet been completely elucidated and is still the subject of some debate(Schubert and Boland 1990; Stebbins and Polacco 1995; Atkins et al. 1997; Lea and Ireland 1999).

Glutathione Synthesis

Recent years have witnessed an upsurge of interest in the tripetide thiol glutathione (GSH: γ-L-glutamyl-L-cysteine-L-glycine), because it has been shown to have a multiplicity of functions in plant metabolism. Not only is it implicated in protecting the leaf against oxidative stress, particularly in conjunction with ascorbate, but GSH is also the precursor of the phytochelatins, which allow plants to withstand supraoptimal concentrations of heavy metals. Since GSH often constitutes the major pool of non protein reduced sulphur, it may considerably influence sulphur metabolism. Indeed, recent studies provide growing evidence for a coordinating role of this metabolism, owing to the inhibitory effect of GSH upon sulfur uptake at the root level (Lappartient and Touraine 1996).

Glutathione synthesis takes place in two ATP-dependent steps, through reactions catalysed by γ-glutamylcysteine synthetase (EC 6.3.2.2.: γ-ECS) and glutathione synthetase (EC 6.3.2.3). The foliar forms of these enzymes have been partially characterised in chloroplastic and non-chloroplastic compartments. Nevertheless, despite these advances, biochemical data on the two enzymes implicated in glutathione synthesis are still lacking. The extent of glutathione synthesis in leaves is controlled by the availability of cysteine, feedback inhibition of γ-ECS by GSH, and the amount of γ-ECS enzyme. However, assessment of the relative contributions of these factors under given conditions is problematic. An alternative approach is to attempt to influence the synthesis of GSH, through overexpression of the enzymes which catalyse GSH synthesis. Consequently, Foyer et al. (1997) have produced transgenic poplars which constitutively express *E.coli* genes encoding γ-ESC or glutathione synthetase. The lines strongly overexpressing γ-ECS, gg28, ggs11 and ggs5, contained enhanced foliar levels of cysteine (up to twofold), γ-glutamylcysteine (5- to 20-fold) and glutathione (2- to 4-fold) (Arisi et al. 1997). These types of experiments allow an investigation of the contribution of the *in vivo* activities of these enzymes in the regulation of the pathway of GSH biosynthesis and to evaluate the role of GSH in stress resistance (Noctor et al. 1998, 1999).

Polyamines

Spermidine (1,8-diamino-4-azaoctane), spermine (1,12-diamino-4,9-diazododecane) and putrescine (1,4-diaminobutane) are all widely distributed in plants, where they may be involved in many processes, including growth and cell division, embryo-

genesis, aging and senescence, bud sprouting, response to stress and ethylene synthesis. There are many other diamines and polyamines in plants, including diaminopropane and cadaverine (1,5-diaminopentane), but these tend to have a more restricted distribution. Polyamines are found in vacuoles, cytosol and chloroplasts.

Spermine and spermidine are synthesised from putrescine, after decarboxylation of arginine by arginine decarboxylase, a pyridoxal-phosphate protein, (EC 4.1.1.19) into agmatine which is then converted into putrescine by N-carbamoylputrescine amidohydrolase (EC 3.5.1.53). As in animals and bacteria, S-adenosylmethionine is used as methyl donor. Putrescine is also produced directly by decarboxylation of ornithine by ornithine decarboxylase (EC 4.1.1.17). The biosynthetic mechanism for the production of many amines remains to be established and their metabolism is not completely understood (Bouchereau et al. 1999).

Conclusion

Even though there is a good understanding of the amino acid biosynthetic pathways in bacteria, this is not the case in plants. Although the enzymes involved are catalytically similar in plants and bacteria, the mechanisms regulating gene expression and activities of these enzymes are quite different.

Recently, great strides have been made in increasing our understanding of amino acid metabolism by using mutants of higher plants with alterations in some of the steps of biosynthetic amino acid pathways. More recently, transgenic plants deregulated in gene expression (sense and antisense strategies, for example) have been very useful tools to study the regulation of metabolic pathway of amino acids directly *in planta*. It is now possible to overexpress or downregulate the enzymes of one metabolic pathway. Also, it is possible to redirect the flux of carbon and nitrogen from one metabolic pathway to another, to favour or repress the synthesis of a particular nitrogen compound of economical interest. The genetic manipulation of the pathways of amino acid synthesis in plants is still promising.

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