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Lysine metabolism in higher plants

Review Article

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Summary. The essential amino acid lysine is synthesised in higher plants via a pathway starting with aspartate, that also leads to the formation of threonine, methionine and isoleucine. Enzyme kinetic studies and the analysis of mutants and transgenic plants that overaccumulate lysine, have indicated that the major site of the regulation of lysine synthesis is at the enzyme dihydrodipicolinate synthase. Despite this tight regulation, there is strong evidence that lysine is also subject to catabolism in plants, specifically in the seed. The two enzymes involved in lysine breakdown, lysine 2-oxoglutarate reductase (also known as lysine α-ketoglutarate reductase) and saccharopine dehydrogenase exist as a single bifunctional protein, with the former activity being regulated by lysine availability, calcium and phosphorylation/dephosphorylation.

Keywords: Amino acids – Aspartate kinase – Aspartate – Lysine synthesis – Lysine 2-oxoglutarate reductase – Methionine – Threonine

1 The lysine biosynthetic pathway

The regulation of the synthesis of some amino acids by feedback control, has been demonstrated previously in both bacteria (Umbarger, 1978) and higher plants (Lea et al., 1985). The essential amino acids lysine, threonine, methionine and isoleucine are synthesized in plants by a complex pathway using aspartate as a common precursor (Azevedo et al., 1997). A major nutritional drawback of cereals is the deficiency in some of these amino acids, in particular lysine and threonine, indicating an important role for the aspartate metabolic pathway in this group of plants (Lea et al., 1992). Biochemical, molecular and genetic studies have considerably increased our knowledge concerning the regulation of the aspartate pathway, showing that many

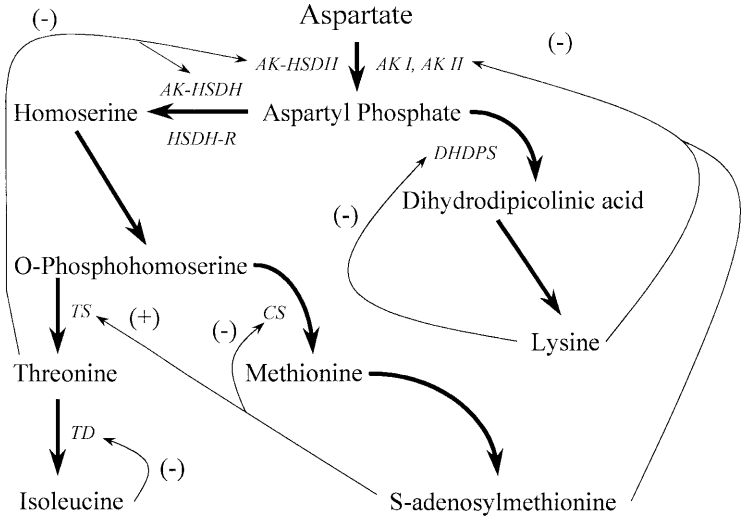

Fig. 1. The aspartate metabolic pathway of higher plants. Regulatory points are indicated as (−) for feedback inhibition or repression and (+) for enzyme activation. *AK I* and *AK II* aspartate kinase isoenzymes sensitive to lysine feedback inhibition and to SAM plus lysine feedback inhibition; *AK-HSDH* aspartate kinase-homoserine dehydrogenase threonine-sensitive bifunctional isoenzyme; *DHDPS* lysine-sensitive dihydrodipicolinate synthase; *CS* cysthationine γ-synthase repressed by SAM; *TS* threonine synthase activated by SAM and TD, threonine dehydratase sensitive to isoleucine-feedback inhibition

branch-point enzymes are positively or negatively regulated by feedback (Azevedo et al., 1997). The aspartate metabolic pathway outlined in Fig. 1 shows the major regulatory steps for each branch of the pathway, the majority of which are located in the chloroplasts (Wallsgrove et al., 1983; Ravanel et al., 1998). In this review we will concentrate on the branch leading to lysine biosynthesis.

The first enzyme of the pathway, aspartate kinase (also known as aspartokinase, AK; EC 2.7.2.4), which converts aspartic acid into β-aspartyl phosphate, has been well characterized in microorganisms (Scapin and Blanchard, 1998) and in many higher plants including *Arabidopsis thaliana*, barley, carrot, maize, rice and tobacco (Azevedo et al., 1992a; Bright et al., 1982a; Dotson et al., 1989; Frankard et al., 1992; Heremans and Jacobs, 1995; Relton et al., 1988; Teixeira et al., 1998). Two classes of differentially regulated isoenzymes of AK have been identified in plants (Azevedo et al., 1992a; Azevedo and Lea, 1992; Bright et al., 1982a; Heremans and Jacobs, 1995; Matthews and Widholm, 1979; Teixeira et al., 1998), that can either be feedback inhibited by lysine or threonine (Azevedo et al., 1997). Genetic studies with mutants have indicated that in barley and maize, two distinct forms of lysine-sensitive AK are present (Arruda et al., 1984; Bright et al., 1982a and 1992b; Muehlbauer et al., 1994a). The sensitivity to lysine is synergistically increased in the presence of *S*-adenosylmethionine (SAM) (Rognes et al., 1980), which can also inhibit AK activity alone or can strikingly stimulate *A. thaliana* AK activity at high concentrations (Heremans and Jacobs, 1995). Although the ratio of lysine-

sensitive AK to threonine-sensitive AK activities varies with plant tissue and developmental age, the lysine-sensitive isoenzymes are normally predominant in higher plants (Bryan, 1990; Lea et al., 1979), the threonine-sensitive AK isoenzyme has however been shown to be more active in soybean cotyledons and callus culture (Matthews and Widholm, 1979) and in *Coix lacryma-jobi* seeds (Lugli and Azevedo, 1994 and 1995).

AK isoenzymes have been purified to near homogeneity in some plants, using as the main purification procedures anion exchange and gel filtration chromatography (Azevedo et al., 1992a; Dotson et al., 1989; Heremans and Jacobs, 1995; Relton et al., 1988). The native molecular mass of the lysine-sensitive AK exhibited some variation among plant species. Dotson et al. (1989) reported molecular mass values of 104 kDa, 124 kDa and 140 kDa for the maize lysine-sensitive AK isoenzyme when analysed by electrophoresis on non-denaturing PAGE and 254 kDa by gel filtration chromatography. Azevedo et al. (1992a) also working with maize, reported different molecular masses for this AK isoenzyme, varying from 139 kDa by non-denaturing PAGE and 150 kDa by gel filtration chromatography. The subunit composition of the maize lysine-sensitive AK was determined as 49 kDa and 60 kDa by SDS-PAGE (Dotson et al., 1989). Lysine-sensitive AK from carrot, also showed considerable variation for the molecular mass when determined by different methods, ranging from 100 kDa to 253 kDa (Relton et al., 1988).

Following a long delay, cDNA clones encoding two distinct monofunctional lysine-sensitive AK isoenzymes, were isolated from *A. thaliana*, by two independent research groups (Frankard et al., 1997; Tang et al., 1997). The molecular mass of the individual enzyme protein subunits was predicted to be 52.5 kDa (Frankard et al., 1997) and the mRNA was shown to be strongly expressed in stems, leaves and floral organs (Tang et al., 1997). The *ask1* gene of maize, which encodes a monofunctional AK isoenzyme sensitive to lysine, has been mapped to the short arm of chromossome 7 and to be linked to the *opaque-2* gene (Azevedo et al., 1990).

Homoserine dehydrogenase (HSDH, EC 1.1.1.3) catalyses the reduction of aspartate semialdehyde (ASA) to homoserine by NADPH, with NADH also serving as a substrate (Bryan, 1990). A bifunctional polypeptide containing both, threonine-sensitive AK and HSDH activities, have been identified in plants (Azevedo et al., 1992b; Ghislain et al., 1994; Muehlbauer et al., 1994b; Teixeira et al., 1998; Wilson et al., 1991). Aarnes and Rognes (1974) working with pea, suggested the possible existence of a bifunctional AK-HSDH as observed for bacteria (Patte et al., 1967). Definitive evidence was only obtained in plants many years later, when Wilson et al. (1991) characterized a bifunctional AK-HSDH in carrot cell cultures and Azevedo et al. (1992b) demonstrated that the bifunctional polypeptide involved the threonine-sensitive isoenzymes of AK and HSDH. Initial evidence included the co-purification of AK and HSDH activities, immunological reactivity and similarities in molecular mass (Azevedo et al., 1992b; Wilson et al., 1991).

Molecular cloning and sequencing of a carrot cDNA encoding AK-HSDH provided further evidence (Weisemann and Matthews, 1993). Since then, the threonine-sensitive bifunctional AK-HSDH has been isolated and character-

ized at the biochemical and molecular levels in several other plant species (Ghislain et al., 1994; Muehlbauer et al., 1994b; Kiyota and Sakano, 1997; Pavagi et al., 1995; Teixeira et al., 1998). Comparison of plant genomic sequences of AK-HSDH cDNA clones revealed high homology and a chloroplast transit peptide (Matthews, 1999). Moreover, a conserved KFGGT sequence region near the NH_2-terminus of the mature protein has been identified for lysine-sensitive AK and the bifunctional AK-HSDH sequences (Matthews, 1999). Further comparison between the *A. thaliana* and soybean genomic sequences indicated the presence of 17 introns, which are located in the same places of the genomes (Ghislain et al., 1994; Matthews, 1999). Transgenic tobacco plants containing the *A. thaliana* AK-HSDH promoter have been recently produced and used to study the molecular regulation of AK-HSDH. The expression of the AK-HSDH-GUS reporter was shown to be subjected to specific spatial and temporal regulation in vegetative tissues, flowers and developing seeds (Zhu-Shimoni et al., 1997). Similar analysis have also been carried out in *A. thaliana* in which the AK-HSDH-GUS reporter was shown to be metabolic regulated by the photosynthesis-related metabolites sucrose and phosphate, but not by nitrogenous compounds (Zhu-Shimoni and Galili, 1998). The full length maize cDNAs, *pAKHSDH1* and *pAKHSDH2*, were mapped to the long arm of chromosome 4 and short arm of chromosome 2, respectively (Muehlbauer et al., 1994a and 1994b). Soybean AK-HSDH was mapped to the linkage group A, near the Rhg_4 locus (Weisemann et al., 1992).

Another aspect of AK regulation that has been discussed in some detail and raised controversy, is the possibility of regulation of enzyme activity by calcium and calmodulin. Ca^{2+} is known to modulate the activity of several enzymes (D'Souza and Johri, 1999; Ganguly and Singh, 1998; Pandey and Sopory, 1998; Zielinski, 1998) and is an important messenger in plant signal transduction (Snedden and Fromm, 1998). Calmodulin is currently regarded as a central component in a complex protein kinase cascade system, which interacts with other regulatory effectors and thus imposes control upon many of the essential metabolic and physiological functions of the cell (Soderling, 1999). Different reports have suggested that both, lysine- and threonine-sensitive AK isoenzymes are stimulated by Ca^{2+} with calmodulin integrating the holoenzyme as one of AK subunits (Kochhar et al., 1998). In chickpea and barley, a similar role for Ca^{2+} has also been recently proposed (Dey and Guha-Mukherjee, 1999; Rao et al., 1999). In the case of barley (Rao et al., 1999), the results contrast considerably with the well documented and reported characterization of AK isoenzymes in this plant species, which has served as a model (For a review, see Lea et al., 1992). However, such a regulatory role for Ca^{2+} or calmodulin could not be confirmed in other plant species tested so far. In carrot (Bonner et al., 1986), maize (Azevedo et al., 1992c) and rice (Lugli and Azevedo, 1999; Lugli et al., 2000), highly purified AK isoenzymes sensitive to lysine and threonine were not affected at all by Ca^{2+}, EGTA, calmodulin or calmodulin antagonists. Furthermore, Ca^{2+} binding sites or calmodulin gene sequences have not been reported within the AK gene sequence from several plant species. In a similar manner to AK, the

modulation of the threonine-sensitive HSDH activity purified from spinach leaves was also shown to be stimulated by Ca^{2+} (Pavagi et al., 1995).

The first enzyme unique to lysine biosynthesis dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52), catalyses the condensation of pyruvate and aspartate semialdehyde to form dihydrodipicolinic acid. DHDPS has been purified and characterized at both the biochemical and molecular levels in plants (Azevedo et al., 1997) and shown to be sensitive to inhibition by low concentrations of lysine (Wallsgrove and Mazelis, 1981). All the evidence has indicated that the key regulatory step in lysine biosynthesis is at DHDPS (Azevedo et al., 1997). Only one form of DHDPS has been detected in plants in contrast to AK and HSDH, which have distinct isoenzymes. Nevertheless in wheat, a single 123 kDa DHDPS could be separated into four polypeptides ranging from 32 kDa to 35 kDa by reverse-phase high-performance liquid chromatography (Kaneko et al., 1990). Different molecular masses for DHDPS have been observed varying from 115 kDa for the spinach enzyme (Wallsgrove and Mazelis, 1981), 127 kDa for pea (Dereppe et al., 1992), 130 kDa for maize (Frisch et al., 1991) and 167 kDa for tobacco (Ghislain et al., 1990). In *Nicotiana sylvestris*, the structure of DHDPS was confirmed as a tetramer, comprising two tightly bound dimers, which was shown to account for the high sensitivity of the plant enzyme to lysine when compared to *E. coli* (Blickling et al., 1997).

Two cDNA clones encoding DHDPS subunits of 35–36 kDa, were first isolated from wheat (Kaneko et al., 1990). The predicted amino acid sequences of the two subunits showed 94% homology. A maize DHDPS cDNA clone was isolated by complementation of an *E. coli dapA*- mutant and shown to encode a protein of 35.8 kDa, with 86–88% homology to the wheat amino acid sequences (Frisch et al., 1991). By mutagenesis of the maize DHDPS gene expressed in *E. coli*, mutants were selected that were resistant to *S*-2-aminoethyl-L-cysteine (AEC). Single amino acid substitutions at positions 157, 162 and 166, were shown to eliminate lysine feedback inhibition of the enzyme (Shaver et al., 1996). cDNA clones encoding DHDPS have now also been isolated from soybean (Silk et al., 1994), poplar and *A. thaliana* (Vauterin and Jacobs, 1994). Interestingly only a 74% amino acid sequence identity was detected between monocot and dicot proteins. All DHDPS cDNA clones have been shown to contain putative plastid transit peptides, confirming the localisation of the enzyme in the chloroplast (Wallsgrove et al., 1983). In a more recent study, the 900 base upstream promoter sequence of the *A. thaliana* gene was used for GUS expression studies in both *A. thaliana* and *Nicotiana sylvestris*. DHDPS was shown to be expressed in the meristem and vasculature of roots, stems and leaves, in particular in the phloem companion cells, but not in the photosynthetically active pallisade cells of mature leaves (Vauterin et al., 1999). The localisation studies of DHDPS are comparable with the data for the AK-HSDH genes by Zhu-Shimoni et al. (1997) and Zhu-Shimoni and Galili (1998).

Three other enzymes, aspartate semialdehyde dehydrogenase (EC 1.2.1.11), which catalyses the NADPH-dependent reduction of aspartyl phosphate to aspartate semialdehyde, dihydrodipicolinate reductase (EC 1.3.1.26),

which catalyses the pyridine nucleotide-linked reduction of dihydrodipicolinic acid to tetradipicolinic acid and diaminopimelate decarboxylase (EC 4.1.1.20), which catalyses the last step in lysine biosynthesis involving pyridoxal phosphate-dependent decarboxylation of *meso*-diaminopimelic acid to lysine, have been partially purified and characterized in only a few number of plant species (Azevedo et al., 1997), as compared to bacteria (Scapin and Blanchard, 1998).

Aspartate semialdehyde dehydrogenase exhibited high activity in maize tissues and was found to be resistant to 10 mM lysine, threonine and isoleucine, but sensitive to the same concentration of methionine (Gengenbach et al., 1978). Dihydrodipicolinate reductase was partially purified from maize kernels and could be inhibited by dipicolinic acid, while oxidised pyridine nucleotides slightly inhibited the enzyme activity (Tyagi et al., 1983). A molecular mass of 84 kDa was determined and such as for aspartate semialdehyde dehydrogenase, detailed study was only carried out in microrganisms (Reddy et al., 1995). Diaminopimelate decarboxylase has been isolated from some plant species (Chatterjee et al., 1994; Mazelis and Crevelling, 1978; Mazelis et al., 1976; Sodek, 1978). The enzyme exhibited great similarity in its chemical and physical properties among plants, but differences in respect to the bacterial enzyme (Scapin and Blanchard, 1998).

2 Lysine catabolism

The catabolism of lysine in plants was initially demonstrated in barley, maize and wheat in experiments using ^{14}C-lysine with the radioactivity being incorporated into aminoadipic semialdehyde and glutamate indicating that lysine is probably degraded through saccharopine (Fig. 2) (Brandt, 1975; Sodek and Wilson, 1970).

Although the biosynthesis of lysine has been studied in detail and extensive information is available on the regulatory aspects, very little is known about the lysine catabolism. Recent studies have strongly indicated that the catabolism of lysine plays an important role in the accumulation of lysine in plant seeds. In maize, initial studies on the enzymes involved in lysine degradation suggested that the catabolism is one of the mechanisms controlling the

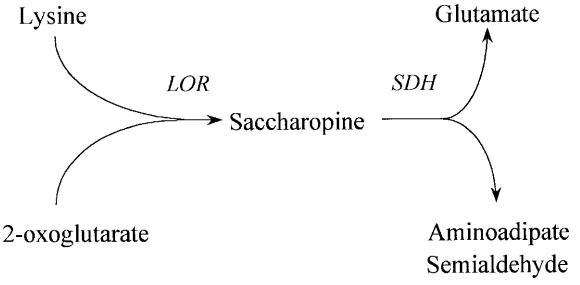

Fig. 2. Lysine breakdown pathway. *LOR-SDH* lysine 2-oxoglutarate reductase-saccharopine dehydrogenase bifunctional enzyme

concentration of soluble lysine in the endosperm (Arruda et al., 1982). The amount of lysine that has been shown to be translocated to the developing endosperm for storage protein synthesis is 2 to 3-fold higher than what would be needed (Arruda and daSilva, 1983). Thus, accumulation of lysine in the soluble form would be expected, however, that is not the case, since the average concentration of lysine during endosperm development is kept low, probably to avoid inhibition of AK activity and consequently methionine biosynthesis. These results suggested that soluble lysine concentration is mainly controlled by the rate of lysine catabolism instead of by the feedback inhibition of its synthesis.

The two enzymes of the lysine degradation pathway have been studied in detail in animals and microorganisms. Lysine 2-oxoglutarate reductase (LOR; EC 1.5.1.8, also known as lysine ketoglutarate reductase – LKR) is the first enzyme in this pathway condensing lysine and 2-oxoglutarate to form saccharopine, which is then hydrolysed to aminoadipic semialdehyde and glutamate in a reaction catalysed by the enzyme saccharopine dehydrogenase (SDH; EC 1.5.1.9). The net result of these two reactions resembles a transaminase reaction in which the 2-amino group of lysine is transferred to 2-oxoglutarate to form glutamate. Both enzymes have been well characterized in mammals and were shown to be part of one single bifunctional polypeptide (Markovitz and Chuang, 1987). In animals the LOR-SDH bifunctional enzyme is a tetramer with a molecular mass of 460 kDa with 115 kDa subunits (Fjellstedt and Robinson, 1975a; Markovits and Chuang, 1987). In fungi and yeast, LOR and SDH are monomers of 49 kDa and 73 kDa encoded by the genes *Lys1* and *Lys9*, respectively (Feller et al., 1999; Ramos et al., 1988). It is only recently that these enzymes have received more attention in plants, culminating in their characterization in some species such as maize, rice and tobacco (Azevedo et al., 1997). In a similar manner to mammals, LOR and SDH activities reside in the same polypeptide (Gaziola et al., 1997; Gonçalves-Butruille et al., 1996; Lima, 1999; Tang et al., 1997). The molecular mass of LOR-SDH has exhibited some variation among plants. In maize the polypeptide has a molecular mass of 260 kDa in a dimer structure consisting of two 117 kDa subunits (Gonçalves-Butruille et al., 1996; Kemper et al., 1999), which could be cleaved by elastase digestion into five bands ranging from 35 kDa to 65 kDa (Kemper et al., 1998). The proportion of these five bands changed during the course of proteolysis and the bands could be associated to LOR and SDH activities, so that the 65 kDa and 57 kDa predominant polypeptides during digestion, contained the functional domains of LOR and SDH activities, respectively (Kemper et al., 1998).

In rice, the LOR-SDH protein was shown to be specific to the endosperm and exhibited a holoenzyme molecular mass of approximately 200 kDa when determined by non-denaturing PAGE and gel filtration (Gaziola et al., 1996; Gaziola et al., 1997), but was also shown to produce multimeric forms (Gaziola et al., 2000). In *Phaseolus vulgaris*, LOR-SDH activities are also part of a bifunctional protein and depending on the purification procedure, may elute as a monomer of 94 kDa containing SDH activity only, or as a dimer of 190 kDa in which LOR and SDH activities are eluted together (Lima, 1999).

Although LOR and SDH from plants, animals and yeast differ, they share some common properties such as optimum pH, neutral for LOR and basic for SDH (Fjellstedt and Robinson, 1975a, 1975b; Gaziola et al., 1997, 2000; Gonçalves-Butruille et al., 1996; Saunders and Broquist, 1966).

It has been demonstrated in plants that LOR-SDH activities are differentially affected by Ca^{2+}, ionic strength and protein phosphorylation (Gaziola et al., 2000; Gaziola and Azevedo, 1999; Karchi et al., 1995; Kemper et al., 1998; Miron et al., 1997). Working with tobacco seeds, Karchi et al. (1995) observed that LOR activity could be stimulated by the exogenous addition of lysine and that such a stimulatory effect was significantly reduced when treated with the Ca^{2+} chelator EGTA, an inhibitory effect that could be overcome by addition of Ca^{2+}. Maize LOR activity was also shown to be modulated by Ca^{2+}, whereas SDH activity was not (Kemper et al., 1998). The Ca^{2+}-dependent increase of LOR activity could be inhibited by EGTA and two structurally different calmodulin antagonists, but binding of calmodulin to LOR was not detected (Kemper et al., 1998). These results observed for the Ca^{2+} effect on maize LOR and SDH activities have been confirmed for rice LOR and SDH activities (Gaziola et al., 2000; Gaziola and Azevedo, 1999). Kemper (1999) has also provided evidence that Ca^{2+} affects the oligomerization status of maize LOR-SDH, activating LOR activity through dimerization of the LOR domain only, since SDH activity was not affected.

It has also been demonstrated previously that LOR was modulated by ionic strength (Kemper et al., 1998). In a similar way, rice LOR activity was also modulated by ionic strength (Gaziola et al., 2000; Gaziola and Azevedo, 1999), while the activity of SDH in both plants species remained completely unaltered (Gaziola et al., 2000; Gaziola and Azevedo, 1999; Kemper et al., 1998). Maize LOR activity was also enhanced by organic solvents (Kemper et al., 1998), whereas both LOR and SDH activities, have been shown to be osmo-regulated on rapessed leaf discs (Moulin et al., 2000).

Tobacco and soybean LOR activities have been shown to be modulated by direct phosphorylation of the bifunctional polypetide, but not SDH activity (Karchi et al., 1995; Miron et al., 1997). Phosphorylation and dephosphorylation of LOR-SDH with casein kinase II and alkaline phosphatase, respectively, indicated that active LOR is a phospho-protein with the activity modulated by opposing actions of protein kinases and phosphatases (Karchi et al., 1995; Miron et al., 1997). Lysine autoregulation and protein phosphorylation of LOR activity has also been observed for the maize enzyme (Kemper, 1999).

The molecular cloning of a LOR-SDH plant genomic and cDNA sequences has been reported (Epelbaum et al., 1997; Kemper et al., 1999). The amino acid sequence of the *A. thaliana* LOR-SDH cDNA clone revealed an amino domain corresponding to LOR and a carboxy domain homologous to SDH (Epelbaum et al., 1997). Similar results were obtained for the maize cDNA clone, designated *ZLKRSDH*, the expression of which was shown to be reduced by approximately 90%, as well as the LOR-SDH polypeptide level and activities in opaque-2 seeds (Kemper et al., 1999), confirming previous findings for LOR-SDH activities (Brochetto-Braga et al., 1992; Gaziola et al., 1999).

The effects of the lysine analogue, AEC, and SAM have been tested on rice LOR and SDH (Gaziola and Azevedo, 1999; Gaziola et al., 2000). AEC was shown to substitute for lysine as a substrate for LOR, but less effectively (Gaziola et al., 2000; Gaziola and Azevedo, 1999). This result is different to that for maize LOR, which could not use AEC as a substrate (Brochetto-Braga et al., 1992). AEC was also able to inhibit LOR activity, possibly reflecting a lower transformation rate with AEC as a substrate, compared to that with lysine as a substrate (Gaziola et al., 2000; Gaziola and Azevedo, 1999).

As discussed previously, SAM can synergistically inhibit the biosynthesis of lysine by inhibiting the lysine-sensitive AK isoenzyme, while, on the other hand, SAM can strongly stimulate the activity of threonine synthase (EC 4.2.99.2), a regulatory enzyme of the threonine biosynthesis branch of the aspartate pathway (Curien et al., 1998; Laber et al., 1999). SAM did not produce any significant effect on LOR or SDH activities of rice, suggesting that SAM is not involved in the regulation of lysine catabolism (Gaziola et al., 2000; Gaziola and Azevedo, 1999).

3 Biochemical mutants and transgenic plants

Improving the nutritional value of crop plants has been recognized for a long period of time as absolutely necessary. Lysine is present in very low concentrations in cereal seeds and the need to increase this amino acid in cereals has encouraged a number of research groups to concentrate on investigations into lysine metabolism. Many different strategies have been used and developed to achieve the major goal of producing high lysine cereal seeds. In this part of the review, we will discuss some of the strategies used and present an overview of the results obtained so far.

Four main strategies have been used to produce high-lysine plants: plant breeding, identification of natural occurring mutants, induction of biochemical mutants and the production of transgenic plants.

Plant breeding programs have been used to select for improved protein quality (Moro et al., 1996) and several studies have documented variability for lysine concentration among maize genotypes (Moro et al., 1996; Paez et al., 1969; Zuber et al., 1975). This technique is more difficult since there is a requirement for long-term investments and demands a very long time to achieve some progress.

The use of natural occurring mutants in attempts to obtain new varieties with higher levels of lysine and other amino acids was an important aspect of early research. The identification of the opaque-2 mutant in maize (Mertz et al., 1964) and high-lysine barley mutants (for a review see Munck, 1992) was an important discovery, however, these high-lysine mutants exhibited traits, negatively correlated to other agronomic characteristics, such as yield. The initial disappointment with these mutants have been replaced in recent years with the discovery of modifying genes which induced more pronounced changes to seed phenotype, but keeping the high-lysine characteristic conditioned by the *opaque-2* gene in maize (Vasal, 1994). Breeding programs have

allow the production of the so called Quality Protein Maize (QPM). QPM maize varieties are available in the seed market and the cultivated area has increased considerably over the last few years. QPM and the *opaque-2* maize gene will be discussed later in this review.

Direct evidence for feedback inhibition regulatory sites in the aspartate metabolic pathway has been provided by the extensive enzymological studies described previously. Although, not only wild-type lines have been used, but also mutants for specific regulation points of the pathway. The possibility of selecting mutants came as a very useful tool to further investigate the regulation of metabolic pathways. Several studies have showed that enzymes in these mutants have an altered feedback inhibition pattern and may also contain enhanced levels of end-product amino acids. This system, initially demonstrated in microorganisms (Demain, 1970), has also been demonstrated for the aspartate pathway (Azevedo et al., 1997). Biochemical mutants can be produced by mutagenesis of seeds or cells and then selected in selective medium containing amino acids or their analogues (Green and Phillips, 1974; Lea et al., 1992). Mutant cells will grow in the selective medium and may contain enzymes with altered feedback regulatory characteristics.

Several mutants have been obtained by this procedure. In maize, Hibberd and Green (1982) selected lysine plus threonine resistant cell lines and produced two (LT19 and LT20) stable lines showing resistance to threonine and lysine as a dominant trait. These mutants exhibited high levels of threonine, but not lysine in the endosperm. The genes designated *ask1* and *ask2* were shown to encode lysine-sensitive AK isoenzymes, which in the mutant were insensitive to feedback inhibition (Dotson et al., 1990). A double mutant LT19/opaque-2 exhibited a synergistic effect on the increase of soluble threonine (144-fold increase), in the total soluble amino acid pool (3-fold increase) and the concentration of the storage proteins of the endosperm (Azevedo et al., 1990). Further analysis of the double mutant showed a stimulation of AK activity in comparison to the wild-type, suggesting that the *opaque-2* gene may regulate the *ask1* gene (Azevedo et al., 1994; Brennecke et al., 1996). More recently, the analysis of QPM varieties in comparison to the opaque-2 mutant and wild-type varieties, confirmed such a possible regulatory action (Gaziola et al., 1999).

Lysine plus threonine resistant mutants have also been isolated and characterized in other plant species. In barley, the biochemical analysis of these mutants has shown their consistent association with alteration of the regulatory properties of the two lysine-sensitive AK isoenzymes, but not the threonine-sensitive AK (Arruda et al., 1984; Bright et al., 1982a and 1982b). Increases in soluble threonine varied from 15 to 70-fold among the mutants selected, whereas little variation was observed for lysine (Bright et al., 1984). An *A. thaliana* mutant (RLT40) resistant to lysine plus threonine inhibition of growth, exhibited a 6-fold increase in soluble threonine, which was also due to partial insensitivity of the lysine-sensitive AK isoenzyme, whereas the threonine-sensitive AK isoenzyme was unaltered to feedback inhibition by threonine (Heremans and Jacobs, 1995). A second mutant (RL4) containing an altered threonine-AK isoenzyme, showed some relative lysine enhance-

ment, but the production of a double mutant RLT40/RL4 did not exhibit any threonine or lysine overproduction (Heremans and Jacobs, 1997). Overproduction and accumulation of threonine (45 to 70-fold increase) has also been observed in a tobacco mutant (RLT 70) with a lysine-sensitive AK totally insensitive to lysine inhibition (Frankard et al., 1991). In general, alteration in the feedback inhibition pattern of AK isoenzymes did not result in lysine accumulation, but only threonine.

These results indicated that the regulatory point at DHDPS might have to be modified in order to obtain lysine-overproducing mutants. In order to obtain mutants containing a DHDPS insensitive to lysine feedback inhibition, mutants were obtained using lysine and AEC as selective agents. *A. thaliana* (Heremans and Jacobs, 1994), barley (Bright et al., 1979), maize (Azevedo and Arruda, 1995), potato (Jacobsen, 1986) and wheat (Kumpaisal et al., 1989) AEC resistant mutants showed very similar results, with none of them exhibiting a significant alteration in the concentration of soluble lysine, and in most cases the resistance was due to reduced uptake of AEC by the root system. However, a tobacco AEC resistant mutant (RAEC-1) was produced that contained a DHDPS completely insensitive to lysine inhibition (Negrutiu et al., 1984). The mutant exhibited a 28-fold increase in soluble lysine in the leaf. Frankard et al. (1992) crossed the RAEC-1 with the RLT 70 lysine plus threonine resistant mutant, producing a double mutant containing an AK and DHDPS both insensitive to lysine inhibition, which revealed a major soluble lysine overproduction representing up to 50% of the total pool of soluble amino acids. Unfortunately an abnormal phenotype was shown to be associated with lysine accumulation in leaves (Frankard et al., 1992). The data obtained confirmed that DHDPS normally exerts a control over lysine biosynthesis, causing a drain of aspartate semialdehyde to lysine when deregulated.

Based on the same general idea of deregulating AK and DHDPS, the development of plant transformation techniques allowed the production of transgenic plants expressing insensitive AK and DHDPS enzymes, offering a new strategy to improve the production of lysine and threonine. Initial research was carried out with tobacco transgenic plants expressing a *Escherichia coli* DHDPS enzyme in the chloroplasts, which is less sensitive to lysine inhibition than the endogenous plant enzyme (Shaul and Galili, 1992a). Soluble lysine overproduction and accumulation in leaves were observed and correlated with the level of DHDPS activity. Similarly, another transgenic tobacco line was produced expressing an *E. coli* desentisized mutant AK isoenzyme in the cytoplasm and chloroplasts (Shaul and Galili, 1992b). As observed for the biochemical mutants containing insensitive AK described previously, soluble threonine was overproduced and accumulated in both types of transgenic plants, but at higher concentration when AK was directed to the chloroplast (Shaul and Galili, 1992b). The synthesis of lysine and threonine were shown to be under concerted regulation by AK, DHDPS and HSDH, when a transgenic tobacco plant expressing *E. coli* DHDPS and AK enzymes exhibited a much higher concentration of soluble lysine accompanied by a reduction in soluble threonine, when compared to transgenic tobacco plants expressing separately DHDPS and AK, respectively (Shaul and

Galili, 1993). The variation in lysine and threonine concentrations could be explained by competition between DHDPS and HSDH for aspartate semialdehyde, their common substrate (Shaul and Galili, 1993).

A similar strategy was employed for barley (BrinchPedersen et al., 1996), soybean (Falco et al., 1995) and canola (Falco et al., 1995). In barley, higher concentrations of lysine and methionine in the transgenic barley plants expressing bacterial DHDPS and AK were detected (BrinchPedersen et al., 1996). DHDPS encoded by the *dapA* gene of *Corynebacterium* and AK encoded by a mutant *E. coli lysC* gene were targeted to the chloroplast and expressed from a seed specific promoter in transgenic soybean and canola (Falco et al., 1995). In both plants, very high increases in soluble lysine were observed in seeds with accumulation of amino-adipic acid in canola and saccharopine in soybean (Falco et al., 1995).

In general, lysine overproduction can be obtained by altering the sensitivity of DHDPS to lysine (Bittel et al., 1996; Kwon et al., 1995), but accumulation of this amino acid in cereal seeds requires further manipulation of LOR and/or SDH. This suggestion is strongly supported by five main points: (1) All cereal mutants or transgenic plants did not exhibit any significant accumulation of lysine in seeds, but only in other tissues. (2) The enzymes of lysine degradation, LOR and SDH, are endosperm specific in cereals only. (3) The opaque-2 mutant, which exhibits higher concentration of soluble lysine and protein lysine in the seed, was shown to contain several-fold lower LOR and 2-fold lower SDH activity when compared to wild-type maize (Brochetto-Braga et al., 1992; Gaziola et al., 1999; Kemper et al., 1999). This reduction in activity in the opaque-2 mutant was due to reduced protein LOR-SDH concentration by reduction of the *ZLKRSDH* gene transcript (Kemper et al., 1999). Furthermore, the *opaque-2* maize gene was shown to regulate AK and LOR activity (Azevedo et al., 1990; Brennecke et al., 1996; Gaziola et al., 1999; Kemper et al., 1999). (4) Intermediates of lysine catabolism accumulated in the seeds of soybean and canola lysine overproducing plants, suggesting the presence of reduced LOR and/or SDH activities (Falco et al., 1995). (5) Among cereals and although still below the recommend values by FAO, rice exhibits the higher concentration of lysine, but LOR and SDH are present in much lower activities (Gaziola et al., 1997). Also, in *Phaseolus vulgaris*, LOR and SDH activities were shown to be around 10-fold lower then in maize endosperm (Lima, 1999).

Contrary to lysine, threonine overproduction and accumulation can be obtained by altering AK sensitivity to lysine. The accumulation of threonine in all tissues, including seeds, suggest that threonine degradation is not under a tight control.

Conclusions

There is substantial evidence that the enzyme DHDPS exerts a major control over the synthesis of the nutritionally important amino acid, lysine, in higher plants. When a form of the enzyme is present that is not subject to feedback

inhibition by lysine, then there is a large accumulation of lysine. In the leaves, this accumulation can cause detrimental effects on metabolism. In the seeds however, this effect has not been observed, but there is evidence that in some but not all plants, that a lysine catabolism pathway via LOR-SDH is induced. The regulation of the LOR activity is complex and involves a calcium dependent phosphorylation/dephosphorylation mechanism. It remains to be seen whether this latter mechanism can be controlled, so as to allow the production of more crop plants that contain elevated concentrations of lysine in the seed.

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