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Analysis of the aspartic acid metabolic pathway using mutant genes

Review Article

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Summary. Amino acid metabolism is a fundamental process for plant growth and development. Although a considerable amount of information is available, little is known about the genetic control of enzymatic steps or regulation of several pathways. Much of the information about biochemical pathways has arisen from the use of mutants lacking key enzymes. Although mutants were largely used already in the 60's, by bacterial and fungal geneticists, it took plant research a long time to catch up. The advance in this area was rapid in the 80's, which was followed in the 90's by the development of techniques of plant transformation. In this review we present an overview of the aspartic acid metabolic pathway, the key regulatory enzymes and the mutants and transgenic plants produced for lysine and threonine metabolism. We also discuss and propose a new study of high-lysine mutants.

Keywords: Aspartate kinase – Aspartic acid – Lysine – Lysine 2-oxoglutarate reductase – Methionine – Threonine

1 The aspartic acid metabolic pathway

Aspartate is the common precursor for the synthesis of the essential amino acids threonine, lysine, methionine and isoleucine (Azevedo et al., 1997). Due to the low concentrations of lysine and threonine in cereal kernels, research has been focused on the understanding of the genetic and biochemical control of each branch of the aspartate pathway (Fig. 1) leading to the biosynthesis of these amino acids (Azevedo et al., 1997) for further genetic manipulation in order to produce plants overproducing and accumulating lysine and threonine in kernels (Azevedo and Lea, 2001).

The first enzymatic reaction involves the phosphorylation of aspartate producing β-aspartyl phosphate which is catalysed by the enzyme aspartate

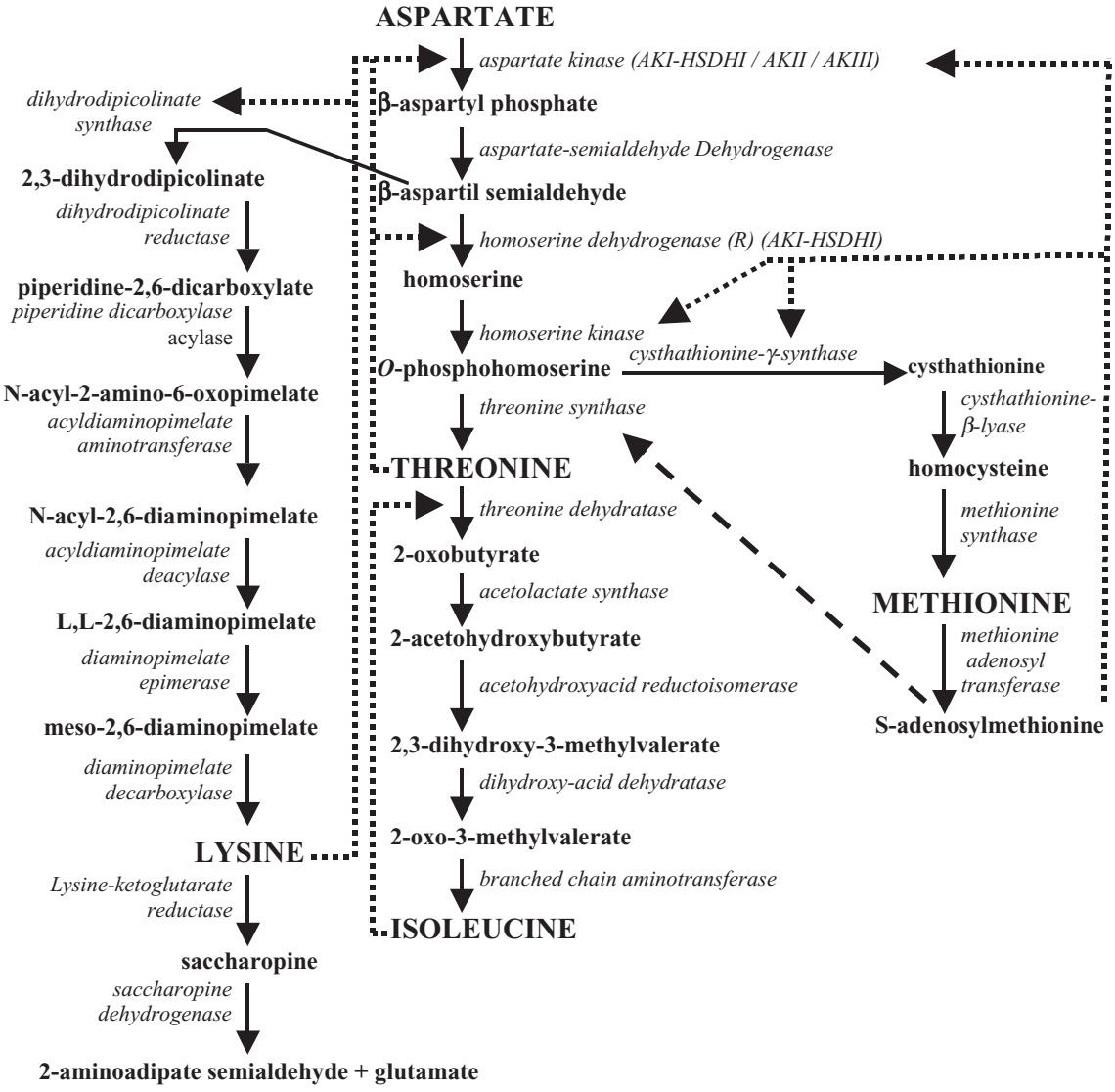

Fig. 1. The aspartate metabolic pathway of higher plants. Regulatory points are indicated as (▪▪▪) for feedback inhibition or repression and (----) for enzyme activation

kinase (AK, EC 2.7.2.4). The β-aspartyl phosphate is converted to β-aspartyl semialdehyde (ASA) in a reaction catalysed by the enzyme aspartate semialdehyde dehydrogenase (EC 1.2.1.11). From this point, the pathway is divided in two branches, one leading to lysine biosynthesis whereas the other branch is divided in two sub-branches with one leading to the biosynthesis of threonine and isoleucine and the other to the biosynthesis of methionine. Lysine is produced from β-aspartyl semialdehyde in a series of seven enzymatic reactions initiated by the enzyme dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52). In the other branch, ASA is reduced to homoserine in a reaction catalysed by the enzyme homoserine dehydrogenase (HSDH, EC 1.1.1.3). Homoserine is phosphorylated to *O*-phosphohomoserine by action of

the enzyme homoserine kinase (HK, EC 2.7.1.39), which is then converted to threonine by the enzyme threonine synthase (TS, EC 4.2.99.2). Isoleucine is produced from threonine after five enzymatic reactions. The synthesis of the amino acid methionine follows a separate branch starting from *O*-phosphohomoserine via three enzymatic reactions involving the enzymes cystathionine γ-synthase (EC 4.2.99.9), cystathionine β-lyase (EC 4.4.1.8) and methionine synthase (EC 2.1.1.13). S-adenosylmethionine (SAM), a major methyl donor in plants, is synthesized from methionine in a reaction catalysed by the enzyme *S*-adenosylmethionine synthetase (EC 2.5.1.6) (Azevedo et al., 1997; Höfgen et al., 2001).

Biochemical studies have provided evidence which indicated the enzymes that have a key regulatory role in the aspartate pathway. These enzymes have been isolated, characterized and for some of them the genes have also been cloned from several plant species (Azevedo et al., 1997). Analysis of DNA sequences revealed the presence of chloroplasts transit peptides confirming that these enzymes, with the exception of the enzymes involved in methionine and SAM biosynthesis, which are located in the cytosol (Azevedo et al., 1997), are located in the chloroplast (Muehlbauer et al., 1994b; Azevedo et al., 1997; Molina et al., 2001), so that the biochemical reactions can use the large amounts of energy produced in this plastid.

The data obtained in the last 30 years of research on the aspartic acid pathway have indicated that the enzymes AK, HSDH, DHDPS, TS and the enzymes lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9), both involved in lysine degradation, are key regulatory enzymes of the lysine and threonine branches of the pathway.

AK has been well characterized at the biochemical and molecular levels in different tissues of several plant species including *Arabidopsis thaliana* (Frankard et al., 1997), barley (Lea et al., 1992), carrot (Relton et al., 1988; Wilson et al., 1991), coix (Lugli et al., 2002), maize (Azevedo et al., 1992a; Muehlbauer et al., 1994b) and rice (Teixeira et al., 1998). At least two distinct AK isoenzymes have been observed in plants, a lysine-sensitive isoenzyme and the other which is threonine-sensitive (Azevedo et al., 1997). The lysine-sensitive AK isoenzyme, which normally accounts for 60–80% of total AK activity in plants, is involved in the overall regulation of the pathway and can be sinergiscally inhibit by *S*-adenosylmethionine (SAM) (Rognes et al., 1980). The threonine-sensitive AK isoenzyme accounts for 10–20% of total AK with the clear exception of coix in which this isoenzyme is predominant, accounting for 55–70% of total AK activity depending on the plant tissue and developmental stage (Lugli et al., 2002). Furthermore, the threonine-sensitive AK has been shown to be part of a bifunctional polypeptide also containing the threonine-sensitive HSDH isoenzyme, as previously observed in microrganisms (Azevedo et al., 1997). Initial molecular and biochemical evidence for the existence of a AK-HSDH threonine-sensitive bifunctional enzyme in plants were obtained in carrot (Wilson et al., 1991) and maize (Azevedo et al., 1992b), respectively. Further research carried out with several other plant species have consistently shown the present of the AK-HSDH bifunctional protein, indicating that the existence of such bifunctional

enzyme appears to be a common feature in higher plants (Azevedo and Lea, 2001). AK genes have been cloned from several plant species and mapped in maize (Azevedo et al., 1990; Muehlbauer et al., 1994b; Azevedo et al., 1997). Based on biochemical analysis of amino acids concentration and enzyme activity, there is evidence that lysine-sensitive AK isoenzymes are regulated by the *opaque-2* maize gene (Azevedo et al., 1990; Brennecke et al., 1996; Molina et al., 2001; Wang et al., 2001; Lefèvre et al., 2002).

Some enzymatic steps have also been shown to be particularly important for threonine and methionine biosynthesis (Hesse et al., 2001). Cystathionine γ-synthase is the key regulatory enzyme in the synthesis of methionine (Kim and Leustek, 2000; Hesse et al., 2001). The enzyme has been purified to homogeneity and was not shown to be feedback inhibited by products of the aspartic acid pathway (Azevedo et al., 1997). On the other hand, the activity of TS, which has been characterized and the gene cloned from several plant species (Casazza et al., 2000), was shown to be markedly stimulated by SAM (Curien et al., 1998). Although, such an effect was not a key regulatory factor in threonine biosynthesis, it is consistent with a possible role in regulating methionine biosynthesis in plants (Azevedo et al., 1997). For the lysine branch of the pathway, the enzyme DHDPS has been shown to be the key regulatory enzyme (Azevedo and Lea, 2001). DHDPS has been purified to homogeneity and well characterized biochemical and molecularly in higher plants (Frisch et al., 1991; Silk et al., 1994; Shaver et al., 1996). Lysine has been shown to be a potent inhibitor of the DHDPS activity and contrary to AK or HSDH, only one form of the enzyme has been observed in plants (Azevedo et al., 1997).

Although lysine biosynthesis has been investigated in detail with special attention to the role of DHDPS, until recently very little was known about lysine catabolism and its importance in the accumulation of lysine in crop seeds. In some plant species, lysine is the main precursor of cadaverine by the action of the enzyme lysine decarboxilase (EC 4.1.1.18), which is present in several higher plants, particularly Gramineae, Leguminoseae and Solanaceae (Bagni and Tassoni, 2001). Initial studies of lysine catabolism in plants were carried out using ^{14}C-lysine in which the radioactivity was incorporated into glutamate and aminoadipic semialdehyde (Sodek and Wilson, 1970). After the first enzymatic studies carried out in maize by Arruda et al. (1982), the enzymes LOR and SDH have been isolated, purified and studied in detail in some plant species, such as coix (Lugli et al., 2002), maize (Kemper et al., 1998), rice (Gaziola et al., 1997) and soybean (Lima, 1999). LOR catalyses the formation of saccharopine from lysine and 2-oxoglutarate, whereas saccharopine dehydrogenase catalyses the hydrolysis of saccharopine into glutamic acid and aminoadipate semialdehyde (Fig. 2). LOR and SDH have been shown to be specific to the endosperm tissue in cereal crops (Azevedo and Lea, 2001). Similar to the threonine-sensitive isoenzymes of AK and HSDH, LOR and SDH activities are also part of a bifunctional polypeptide (Azevedo et al., 1997; Arruda et al., 2000; Azevedo and Lea, 2001), although a monofunctional SDH has also been reported (Galili et al., 2001). Biochemical characterization of the LOR-SDH bifunctional enzyme has shown that the LOR activity, but not SDH, is modulated by phosphorylation-

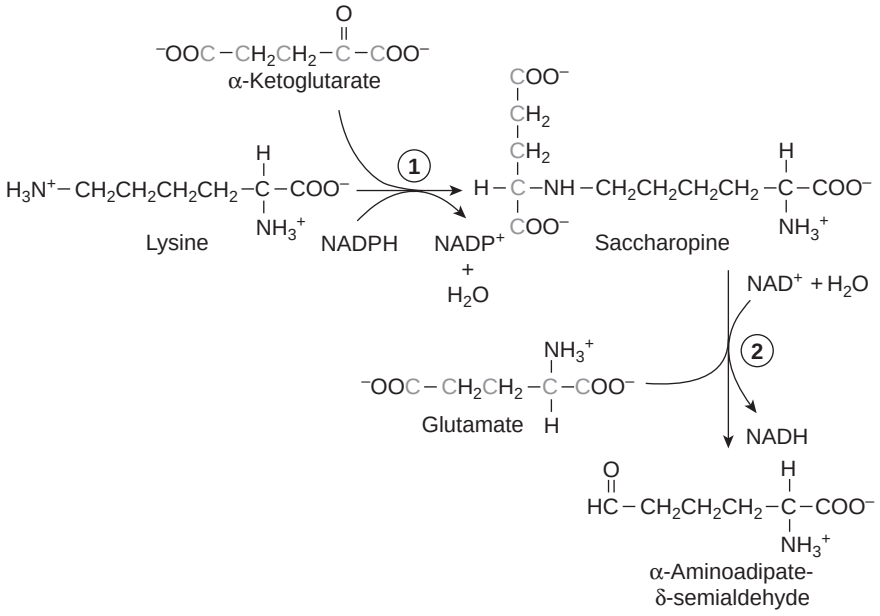

Fig. 2. Lysine breakdown pathway. Lysine 2-oxoglutarate reductase (**1**) – saccharopine dehydrogenase (**2**) bifunctional enzyme

dephosphorylation, ionic strength and Ca^{2+} (Karchi et al., 1995; Kemper et al., 1998; Arruda et al., 2000; Gaziola et al., 2000). The modulatory effect of Ca^{2+} on enzymes of the aspartic acid pathway, such as AK and HSDH, has been suggested previously, however, such a regulatory role is controversial and appears to be dependent upon the plant species studied (Azevedo et al., 1992c; Lugli et al., 2000; Azevedo and Lea, 2001). SAM did not show any effect on LOR-SDH activities, however, S-2-aminoethyl-L-cysteine (AEC), a lysine analogue, was able to replace lysine as a substrate for LOR (Gaziola et al., 2000).

In the high-lysine maize mutant opaque-2, lysine breakdown has been shown to be dramatically reduced in the endosperm due to a reduced LOR activity (Brochetto-Braga et al., 1992; Gaziola et al., 1999). In soybean and canola, intermediates of the lysine catabolism have been shown to accumulate in the seeds (Falco et al., 1995). In *Phaseolus vulgaris*, the activities of LOR and SDH have also been shown to be extremely low in the seeds (Lima, 1999). These results have indicated that plants exhibiting higher concentration of lysine have a low rate of lysine degradation in seeds. Further confirmation has been obtained in rice, which contains the highest lysine concentration among cereal crops and also exhibiting low LOR and SDH activities in the endosperm (Gaziola et al., 1997).

2 Inducing and selecting biochemical mutants

In 1992, L. Munck pointed out that the identification of the first high-lysine mutant opaque-2 in maize and the subsequent range of high-lysine mutants by

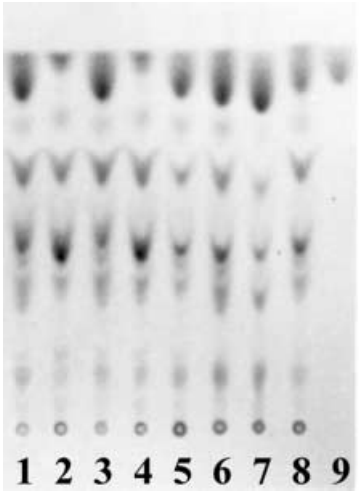

Fig. 3. Thin layer chromatography of soluble amino acids extracted from maize anthers of the Ltr*19 lysine plus threonine resistant mutant (*1*, *3*, *5–8*) and wild-type (*2* and *4*) plants. The threonine standard is in lane 9

screening for morphological characters was an illustrative example of how an uncomplicated approach may reveal mechanisms which are simply inherited but have extremely complex physiological implications.

The development of tissue culture techniques and plant regeneration *in vitro* allowed a much easier way of selecting biochemical mutants. Such mutants can be selected by growing mutagenic treated cells in solid or liquid medium containing the selective agent which will inhibit growth, and the cells that eventually grow in such conditions may be mutants containing enzymes altered in their regulatory characteristics. In barley, another technique for the selection of biochemical mutants proved to be very efficient (Lea et al., 1992). The system involves the treatment of seeds with a mutagenic compound (normally sodium azide). Embryos dissected from mature M_2 seeds derived from the mutagenic treated seeds were plated on solid medium containing the selective agent and in a similar manner to tissue culture, the eventual mutants grow and are selected to grow to maturity. Independently of the procedure adopted, selected plants must be further evaluated at the genetic and biochemical levels followed by a complete agronomic analysis.

In the case of the aspartic acid metabolic pathway, several mutants have been selected in a large number of plant species exhibiting altered enzymes with the objective of obtaining cereal crops accumulating lysine in the seeds (Azevedo et al., 1997; Molina et al., 2001). Initially, mutants exhibiting AK isoenzymes insensitive to lysine plus threonine feedback inhibition were obtained (Bright et al., 1982; Hibberd and Green, 1982; Frankard et al., 1992; Muehlbauer et al., 1994a; Heremans and Jacobs, 1997). These mutants exhibited overproduction and accumulation of threonine in different plant tissues including the seed (Fig. 3), however, significant alterations in the soluble concentration of lysine were not observed. These results indicated a

major role of DHDPS in the lysine branch of the pathway, which in the mutants was still sensitive to lysine feedback inhibition, and directs the carbon molecules from ASA to threonine biosynthesis (Azevedo and Lea, 2001).

Based on these results, by applying the mutagenesis/selection procedure, mutants exhibiting a DHDPS insensitive to lysine feedback inhibition have also been obtained (Negrutiu et al., 1984; Ghislain et al., 1995; Molina et al., 2001). The majority of the mutants were selected in the presence of AEC and in some cases, although resistance to AEC inhibition was observed, DHDPS was still unaltered and the resistance was due to reduced uptake of AEC and amino acids by the roots (Heremans and Jacobs, 1994; Azevedo and Arruda, 1995). The DHDPS mutants exhibited some lysine accumulation mainly in the leaves, but very little or none in the seeds (Azevedo and Lea, 2001). Furthermore, abnormal phenotypes were normally associated with lysine accumulation in the mutants (Azevedo and Lea, 2001). The distribution of the carbon molecules from ASA to the lysine and threonine branches of the pathway is still vague. A recent report provided further evidence for the balance between these branches of the aspartic acid pathway by producing a loss-of-function DHDPS mutant, which as a result strongly enhanced threonine synthesis in *Arabidopsis thaliana* (Craciun et al., 2000). Such a result confirmed that DHDPS exerts control over the lysine biosynthesis and indirectly on the threonine biosynthetic branch.

3 Producing transgenic plants

Based on the previous information obtained from the biochemical mutants and on the availability of plant transformation techniques, a very similar strategy was employed to obtain crop plants accumulating lysine in seeds. Transgenic tobacco plants expressing *Escherichia coli* lysine-insensitive AK exhibited similar results to those observed for the biochemical mutants for the same enzyme with the accumulation of threonine in all tissues tested but without any significant increase in lysine concentration in the seed (Shaul and Galili, 1992b). Other transgenic tobacco plants expressing bacterial DHDPS, which is much less sensitive to lysine inhibition when compared to the plant enzyme, exhibited lysine accumulation in leaves (Shaul and Galili, 1992a). Soluble lysine and threonine accumulation has also been obtained in transgenic tobacco plants expressing both, bacterial lysine-insensitive AK and DHDPS, however, the concentration of lysine was much higher whereas threonine accumulation was reduced when compared to the separately expressing DHDPS and AK lysine-insensitive transgenic tobacco plants, which also indicates the competition of DHDPS and HSDH for the common substrate ASA (Shaul and Galili, 1993).

In barley (BrinchPedersen et al., 1996), canola (Falco et al., 1995) and soybean (Falco et al., 1995) a similar strategy has been employed. In maize, Falco (2001) reported that soluble lysine accumulation was observed when a lysine-insensitive DHDPS from *Corynebacterium* was expressed in the embryo of transformed maize seeds. Moreover, the knock-out of the maize

LOR-SDH by a T-DNA insertion also resulted in lysine and lysine breakdown products, but the combination of the DHDPS lysine-insensitive with the knock-out LOR-SDH lines, resulted in 2 to 3-fold higher lysine levels than DHDPS alone (Falco, 2001). Transgenic rice plants have also been obtained in order to improve the nutritional value of the seed by increasing lysine concentration (Lee et al., 2001). Constitutive and seed-specific expression of the maize lysine-feedback-insensitive DHDPS gene led to increased soluble lysine level in the seeds. The higher rate of lysine biosynthesis obtained with the introduction of the mutated DHDPS gene resulted also in an increase in lysine catabolism. However, the over-expression of the mutant DHDPS gene in a constitutive manner appears to overcome lysine breakdown maintaining a high level of lysine in mature rice seeds (Lee et al., 2001).

4 High-lysine mutants

A major nutritional drawback of cereal seeds is the deficiency in lysine. Based on the information obtained from 30 years of studies of the aspartic acid metabolic pathway, Azevedo and Lea (2001) in a recent review suggested that lysine overproduction and accumulation in cereal seeds could be obtained by combining genetic manipulation of lysine biosynthesis and lysine degradation. Such a suggestion was supported mainly by the fact that manipulation of enzymes involved in lysine biosynthesis did not produce the accumulation of lysine in the seeds of cereal crops. This could be explained by the fact that legume plants and the opaque-2 maize mutant, which present higher levels of soluble lysine in the seeds, exhibited a drastic reduction in the rate of lysine breakdown in the seeds, allowing excess of lysine for storage protein incorporation and accumulation in the soluble form in the endosperm (Azevedo and Lea, 2001; Molina et al., 2001).

The opaque-2 mutant has been extensively studied. The mutation confers a opaque phenotype to the kernel and a floury endosperm. The high-lysine concentration observed in the endosperm is due to an increase in the concentration of soluble lysine and in the storage protein fractions which contain higher levels of this amino acid, while the low lysine alcohol soluble protein fraction, prolamin, is drastically reduced (Lefèvre et al., 2002). Furthermore, several other characteristics of the opaque-2 kernel, such as level of RNase activity (Dalby and Davies, 1967) and enzymes of carbon and nitrogen metabolism (Lefèvre et al., 2002), are also modifed when compared to the wild-type. Reduction in protandry (Gupta, 1979) and alterations in photosynthetic activity (Morot-Gaudry et al., 1979) have also been observed for the opaque-2 plant. As a consequence of the seed structure and nutritional quality, the opaque-2 kernel exhibited reduced yield and is more susceptible to plant pathogens (Vasal, 1994). The introduction of modifier genes of the opaque phenotype has allowed the production of good grain yield opaque-2 maize lines also exhibiting the high-lysine and high-tryptophan traits, but with a modified-translucent phenotype, which have been designated quality

protein maize (QPM) (Vasal, 1994; Gaziola et al., 1999). Inbred lines of QPM have been included in maize breeding programs and several QPM hybrids have been produced and introduced in the seed market. Agronomical aspects such as kernel hardness and density and combining ability for yield among several others traits, have been investigated for QPM varieties (Vasal, 1994; Gaziola et al., 1999).

The *opaque-2* gene has been studied in detail. A transcriptional activator of the basic leucine-zipper family was found to be encoded by the *opaque-2* gene (Hartings et al., 1989). Multiphosphorylated forms of the opaque-2 protein (Ciceri et al., 1997) and the level of *opaque-2* gene transcript (Ciceri et al., 1999) are subject to diurnal changes. Several genes belonging to several distinct metabolic pathways such as glycolysis, amino acid biosynthesis and storage proteins, have been shown to be direct or indirect targets of the *opaque-2* gene (Azevedo et al., 1990; Cord-Neto et al., 1995; Brennecke et al., 1996; Damerval and Le Guilloux, 1998).

Biochemical and molecular analyses have also confirmed that the *opaque-2* gene regulates the activity of LOR in maize endosperms (Arruda et al., 2000). In the opaque-2 mutant, protein quantities and mRNA of LOR have been shown to be reduced with the expression pattern markedly modified during grain development (Kemper et al., 1999; Arruda et al., 2000). Moreover, the presence of opaque-2 boxes have been observed in the 5′ regulatory region, confirming the transcriptional control of LOR-SDH encoding gene by the *opaque-2* gene (Arruda et al., 2000). By transcriptome and proteome approaches a regulatory role of the *opaque-2* gene has been confirmed, since one restriction site on the 3′ side of the *opaque-2* gene was found to be associated with LOR-SDH mRNA abundance (Lefèvre et al., 2002). The QPM varieties have exhibited lower LOR and SDH activities when compared to the wild-type, but also when compared to the opaque-2 mutant (Gaziola et al., 1999). However, in relation to lysine metabolism, the effect of the *opaque-2* gene was not restricted to lysine degradation, but also lysine biosynthesis. Analysis of soluble amino acids, total amino acids, storage proteins and enzyme activity of a double mutant opaque-2/ask1 (a gene encoding a lysine-sensitive AK), indicated that the *ask1* gene may be regulated by the *opaque-2* gene (Azevedo et al., 1990; Brennecke et al., 1996). Moreover, genetic analysis mapped the *ask1* gene linked to the *opaque-2* gene (Azevedo et al., 1990). Further evidence for a regulatory role of the *opaque-2* gene on AK isoenzymes have been recently reported and was based on a quantitative trait locus on chromosome 2, which is linked to the loci encoding AK2 and AK-HSDH2 (Wang and Larkins, 2001; Wang et al., 2001). Biochemical studies indicated that the lysine-insensitive AK is a candidate for the quantitative trait locus affecting the soluble amino acids content in opaque-2 (Wang et al., 2001).

Although it is very clear the importance of the opaque-2 mutant of maize in helping to understand lysine metabolism, other high-lysine mutants in maize and other plant species that have been available for a long time, have never been investigated in detail when lysine metabolism is concerned. This raise the question of how lysine metabolism is regulated in such genotypes.

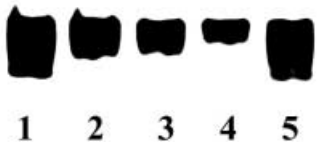

Fig. 4. SDH activity staining of 20 days after pollination maize endosperms. Variation of SDH activity in wild-type (*1*) and in the high-lysine endosperm mutants opaque-2 (*2*), floury-1 (*3*), floury-2 (*4*) and floury-3 (*5*)

A comprehensive research project is being carried out in Brazil (ESALQ, University of São Paulo) to study lysine, threonine and methionine metabolism in high-lysine mutants of maize, sorghum and barley. For instance, developing maize endosperms (15–25 days after pollination) of opaque-1, opaque-5, opaque-7, opaque-10, opaque-11, opaque-13, floury-1, floury-2 and floury-3 mutants are currently being characterized for AK, HSDH, DHDPS, LOR and SDH activities and gene expression. Furthermore, storage protein distribution, soluble and total amino acids concentrations of mature and developing seeds are also being characterized. Initial results have shown considerable variation among the mutants and in relation to wild-type and opaque-2 maize genotypes for the enzymes involved in lysine metabolism. Although some significant variation have been observed among wild-type maize inbred lines for AK and HSDH (Lefèvre et al., 2002), interestingly, some of the high-lysine mutants have shown similar rates of lysine degradation when compared to their wild-type counterparts (Fig. 4). These results suggest that the low rate of lysine degradation may not explain the increased levels of soluble lysine in the endosperm of such mutants, contrary to what has been observed for the opaque-2 maize mutant (Kemper et al., 1999).

Conclusions

The information presented here attempts to show the contribution and importance of the use of mutants and plant transformation in the study of plant metabolism, exemplified in this review with the aspartic acid metabolic pathway. However, the exploitation of such techniques is far from over and certainly will contribute significantly in the future. In a review article, Azevedo et al. (1997) suggested that high-lysine seed cereal crops would probably be available in a short period of time. Seed companies and research institutions have already confirmed such a possibility. However, there is still quite a lot to be done in terms of understanding the regulatory aspects of lysine, threonine and methionine metabolism and whether the complex mechanisms involved can be controlled. A great deal of information may be obtained by the investigation of the opaque, floury, high and low protein mutants of maize and similar mutants of barley and sorghum, for instance. Indeed, it is perhaps surprising that based on the information provided and the importance of the opaque-2 mutant of maize, that these other cereal crop

high-lysine mutants have only recently been, as far as we are aware, included in a research program involving directly the aspartic acid metabolic pathway and in particularly lysine metabolism.

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