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REVIEW ARTICLE

High-lysine maize: the key discoveries that have made it possible

R. A. Azevedo · P. Arruda

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Abstract Forty-five years ago, a paper published by Mertz et al. (Science 145:279–280, 1964) initiated a revolution in the history of plant protein quality and affected dramatically the study of cereal crop storage proteins. The observation of the high lysine content of the endosperm of the *opaque-2* (*o2*) maize mutant was a key factor in bringing about a new concept in the production of cereal seeds with a high nutritional value. It has been a long and very interesting road with astonishing results over these 45 years. We are now probably about to see the release of commercially engineered high-lysine maize lines. We have decided to pinpoint some key contributions to the science behind high-lysine plants and concentrated on the research done on maize, which is possibly the most complete and simple example to illustrate the advances achieved. However, studies on other plant species such as barley and model species such as tobacco are totally relevant and will be briefly addressed.

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R. A. Azevedo
Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, Brazil

P. Arruda (✉)
Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Campinas CEP 13083-875, Brazil
e-mail: parruda@unicamp.br

P. Arruda
Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas, Campinas CEP 13083-875, Brazil

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Introduction

Lysine metabolism in plants has been studied for almost 40 years. Cereal crops, which make up the basis of food intake for the poorest people in the world, are devoid of this essential amino acid (Ferreira et al. 2005). In the early 1960s, the analysis of the amino acid composition of seeds of the maize mutant *opaque-2* (*o2*) (Fig. 1), revealed a high lysine content when compared with the seeds of the normal maize (Mertz et al. 1964). Characterization of *o2* revealed that the mutation led to changes in the distribution of the endosperm storage proteins, so that there was an increase in total lysine and tryptophan content of the mature *o2* maize seed (Tsai and Dalby 1974). This discovery stimulated scientists to search for other mutants with increased lysine content. Continuous examination of phenotypic and molecular aspects of such mutants has revealed new facets in the study of storage proteins, amino acid metabolism and gene regulation (Hunter et al. 2002; Azevedo et al. 2003). However, after the initial excitement with *o2*, the poor agronomic characteristics associated with this mutant (Lambert et al. 1969) discouraged its use in the development of commercial high-lysine varieties. Nevertheless, the *o2* mutant provided evidence of the possibility of changing the lysine content and increasing the nutritive value of cereal seeds. Many research groups have engaged in the endeavor of investigating the molecular biology of seed storage protein accumulation and the biochemistry of lysine metabolism in plants.

The possibility of the development of high-lysine crops arose from the studies of the biochemistry of lysine

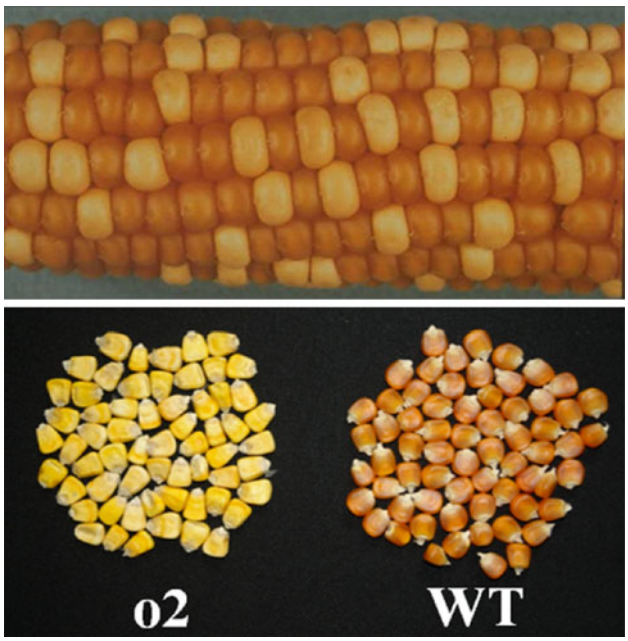

Fig. 1 The *o2* maize mutant. Image of a maize ear segregating 3:1, wild-type:*o2*, seeds. Wild-type (*WT*) and *o2* maize seeds are shown in detail. Images of the WT and *o2* seeds were provided by Marina Torres Pessoa, Embrapa Milho e Sorgo, Brazil

synthesis in plants. From the 1960s till the 1980s, the biochemistry of the aspartate pathway that leads to lysine synthesis in plant and bacteria was extensively investigated (Azevedo and Lea 2001). The aspartate pathway was revealed to be very complex, frequently with the end product amino acids regulating the activities of key enzymes of the pathway (Azevedo et al. 1997, 2006) (Fig. 2). Based on the study on bacteria, it was envisaged that the aspartate pathway could be deregulated in plants, so that lysine and the other aspartate-derived amino acids would accumulate in tissues, including the seeds (Green and Phillips 1974; Bright et al. 1982; Hibberd and Green 1982). By using different approaches, mutants for aspartate kinase (AK), the first enzyme of the pathway (Fig. 2), insensitive to feedback inhibition by lysine were selected, and although they exhibited several-fold increases in soluble threonine, substantial lysine accumulation in the seeds was not observed (Bright et al. 1982; Rognes et al. 1983; Arruda et al. 1984). Mutants of dihydrodipicolinate synthase (DHDPS), the first aspartate pathway enzyme required specifically for lysine synthesis, were also selected and although some lysine overproduction was observed, they again failed to show major changes in lysine

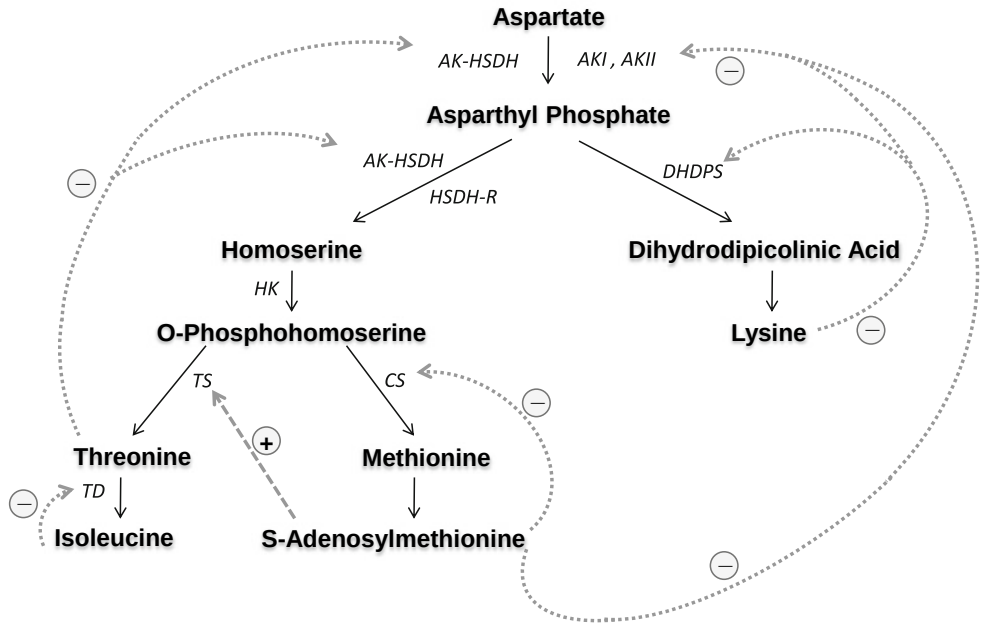

Fig. 2 The aspartate metabolic pathway of higher plants. Only the key regulatory points are indicated. The (*dotted*) *arrows* indicate feedback inhibition or repression (−), whereas the (*continuous*) *arrow* indicates enzyme activation (+). *AK I* and *AK II*, aspartate kinase isoenzymes sensitive to lysine feedback inhibition and to lysine plus *S*-adenosylmethionine inhibition, *AK-HSDH* threonine-sensitive aspartate kinase-homoserine dehydrogenase bifunctional isoenzyme, *HSDH-R* threonine-resistant homoserine dehydrogenase, *DHDPS* lysine-sensitive dihydrodipicolinate synthase, *CS* cysthationine γ-synthase repressed by *S*-adenosylmethionine, *TS* threonine synthase activated by *S*-adenosylmethionine, and *TD* threonine dehydratase sensitive to isoleucine-feedback inhibition. For further detailed information about the pathway and its regulation, the following review papers should be consulted: Azevedo et al. (2006); Curien et al. (2008)

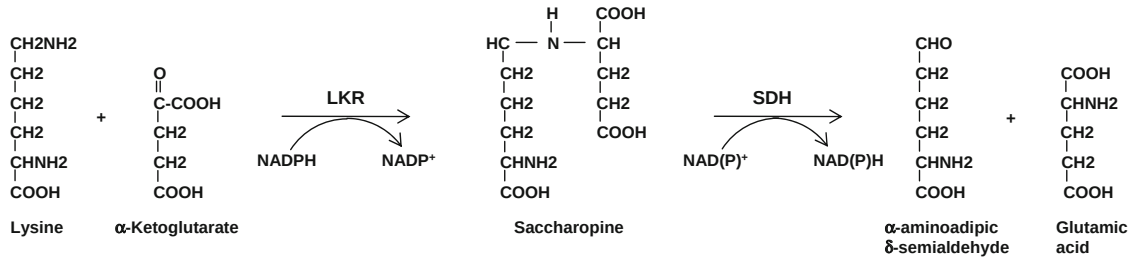

Fig. 3 Lysine degradation through the saccharopine pathway. Lysine α-ketoglutarate reductase (*LKR*) and saccharopine dehydrogenase (*SDH*) bifunctional enzyme

Table 1 Total and soluble lysine content in normal, *o2*, QPM and transgenic high-lysine maize

Total lysine (mg/g DW)				Soluble lysine (mg/g DW)		References
WT	*o2*	QPM	Transgenic	WT	Transgenic	
1.74	2.94	–	–	–	–	Mertz et al. (1964)
3.00	–	4.50	–	–	–	Bétran et al. (2003)
2.72	–	–	3.65	–	–	Huang et al. (2004)
2.58	–	–	5.80	–	–	Huang et al. (2005)
2.43	–	–	4.50	0.02	0.07	Huang et al. (2006)
2.60	–	–	4.66	0.05	0.96	Lucas et al. (2007)
–	–	–	–	0.10	4.00	Frizzi et al. (2008)
–	–	–	–	0.04	0.20	Reyes et al. (2009)[a]
–	–	–	–	0.04	0.90	Reyes et al. (2009)[b]
–	–	–	–	0.04	1.60	Reyes et al. (2009)[c]

Data were compiled from the cited references and must be taken for general comparison purpose and not as absolute values for direct comparison. For some of the data, we have used the average for the different transgenic lines reported in the original reports

[a] Suppression of LKR/SDH in the embryo

[b] Suppression of LKR/SDH in the endosperm

[c] Suppression of LKR/SDH in the embryo and endosperm

accumulation in the seeds (Negrutiu et al. 1984; for a review see Azevedo 2002).

In the early 1990s, genetic engineering was used to create tobacco plants over-expressing mutant and/or bacterial AK and/or DHDPS, but they showed aberrant phenotypes attributed to increased soluble lysine in plant tissues. Again, no significant major changes in lysine accumulation in the seeds were obtained (Shaul and Galili 1992a, b, 1993). In the early 1980s, an enzyme that catabolizes lysine was discovered in developing maize endosperm (Arruda et al. 1982). The enzyme, lysine-ketoglutarate reductase (LKR), condenses lysine and α-ketoglutarate into saccharopine (Fig. 3). In humans, mutations leading to defective LKR causes hyperlysinemia, a disease associated with increased soluble lysine accumulation (Dancis et al. 1969). The LKR activity profile in maize correlated with nitrogen accumulation and storage protein synthesis and this observation made it clear that the enzyme could play a major role in controlling lysine accumulation in seeds (Arruda and Silva 1983). In addition, it was shown later that LKR activity was down-regulated in the maize *o2* endosperm (Brochetto-Braga et al. 1992).

A second step in the lysine catabolism (Fig. 3) was shown to be catalyzed by saccharopine dehydrogenase (SDH), which hydrolyzes saccharopine to glutamate and α-amino-adipic semialdehyde (Gonçalves-Butruille et al. 1996).

In more recent years, a series of transgenic maize plants overproducing and over-accumulating lysine were produced using distinct strategies including the over-expression of a bacterial DHDPS, which is less sensitive to feedback inhibition by lysine, antisense constructs for α-zeins and transgenic plants containing both, the bacterial DHDPS and endosperm-specific RNA interference (RNAi) suppression of the LKR/SDH (Hournard et al. 2007; Frizzi et al. 2008; Reyes et al. 2009).

In this review, we have focused on the key discoveries that have made a major contribution to the development of high-lysine maize. We will not detail aspects that have already been the subject of key review papers (Azevedo et al. 1997; Arruda et al. 2000; Galili 2002; Galili et al. 2005; Azevedo et al. 2006).

The discovery of high lysine content in the *o2* maize mutant

The *o2* maize mutant, known by maize geneticists since 1920, exhibits an opaque phenotype when compared to the vitreous–translucent aspect of the wild-type maize seeds (Fig. 1), and was shown to contain over 70% higher lysine levels than the normal maize seed (Table 1) (Mertz et al. 1964). Soon after this discovery, a number of other opaque and floury mutants were also shown to have higher lysine contents, but to a lesser extent when compared to *o2* (Tsai and Dalby 1974).

The initial characterization of *o2* revealed that by examining the protein fractions in the mature seed, the alcohol-soluble protein fraction, composed mostly of prolamins, called zeins, were significantly reduced, whereas other fractions such as the soluble proteins (albumin + globulin) and the alkali-soluble glutelins were increased (Tsai and Dalby 1974). Since zeins are devoid of lysine, their decrease in *o2* led to proportional increases of other protein fractions rich in lysine. After these first observations, a question was raised as to the mechanism by which a single mutation could result in alterations of so many different proteins. Years before, it was discovered that the developing *o2* endosperm possessed sixfold higher RNAse activity than the normal endosperm (Dalby and Davies 1967; Wilson and Alexander 1967). This finding suggested that the higher RNAse activity may reduce the availability of zein mRNA for protein synthesis. Later, it was found that other endosperm mutants also had increased RNAse activity, but this could not be associated specifically with zein mRNA availability.

SDS-PAGE protocols were developed to examine the protein profile of zeins in normal and *o2* endosperms. It was found that zeins separate by SDS-PAGE into different molecular mass classes, which later became known as the 19- and 22-kDa α-zeins, the 15-kDa *β*-zein, the 16- and 27-kDa *γ*-zeins and the 10-kDa *δ*-zein (Esen 1987). This approach allowed the observation that the opaque-2 mutation affected preferentially the synthesis of the 22-kDa α-zein class with a minimal effect on the 19-kDa and other zein classes (Jones et al. 1977a). With the advent of molecular techniques, it was possible to isolate polyribosomes from developing endosperm (Jones et al. 1977b) and use them for protein synthesis studies and later on for cDNA synthesis and cloning. These studies revealed that while *β*-, *γ*-, and *δ*-zeins are encoded by one or two genes, the 19- and 22-kDa α-zeins are encoded by large multigene families (Kodrzycki et al. 1989). These discoveries made the opaque-2 mutation even more intriguing. How could a single mutation lead to the repression of almost all the genes encoding the 22-kDa α-zeins in the opaque endosperm? The answer was obtained following the identification and cloning of the *Opaque-2* (*O2*) gene (Schmidt et al. 1987). This revealed that the *o2* mutant phenotype resulted from mutations in the *O2* gene encoding a b-ZIP transcription factor that specifically regulates the transcription of the 22-kDa α-zein genes (Schmidt et al. 1990). The opaque-2 transcription factor binds specifically to an 8–10 nucleotide DNA sequence located upstream in the 22-kDa α-zein gene promoter and controls the expression of this α-zein gene family (Schmidt et al. 1992). The genes encoding the other zeins do not appear to possess the opaque-2 binding boxes in their promoters and therefore are not under the regulatory control of this transcription factor. This explained, at least in part, why the other zein classes are not affected in the *o2* mutant.

Along with the studies that revealed the mechanism by which the opaque-2 mutation affects zein synthesis and increases lysine content, a number of other opaque and floury maize endosperm mutants were characterized (Tsai and Dalby 1974). In general, these mutants exhibited a reduced zein deposition and increased lysine content, but to a lesser extent than that observed in the *o2* endosperm. These mutants are still being used to unravel new facets of storage proteins synthesis and amino acid metabolism (Brennecke et al. 1996; Azevedo et al. 2003, 2004a, b; Landry et al. 2005). High-lysine mutants were also identified in barley and these have also contributed to the better understanding of lysine metabolism (Munck et al. 1970).

The study of storage protein synthesis and accumulation using the *o2* mutant revealed the complexity of the zein gene families and the regulatory aspects controlling zein synthesis and deposition in the developing maize endosperm. This complexity identified the difficulty of manipulating zein accumulation to produce a high-lysine maize seed. More recently, attempts to manipulate zein synthesis included the suppression of zein polypeptides by using antisense zein mRNA (Huang et al. 2004, 2006). Antisense zein suppression coupled with enhanced lysine biosynthesis by over-expressing lysine insensitive DHDPS resulted in an increased lysine content of the maize seed (Huang et al. 2005). In addition to increasing the understanding of storage protein synthesis, attempts were made by several groups to modify the seed phenotype of the *o2* mutant to develop commercial high-lysine varieties (Villegas et al. 1992; Vasal 1994). At least in part, the poor agronomic characteristics of the *o2* mutant could be overcome by the use of phenotype modifier genes that result in the production of quality protein maize (QPM) lines, which have been commercially used in some parts of the world (Pixley and Bjarnason 1993; Vasal 1994; Gaziola et al. 1999; Gibbon and Larkins 2005). In the QPM varieties, the soft and starchy characteristics of *o2* mutant kernels were modified by breeders into a more vitreous kernel while preserving the high-lysine content (Villegas et al. 1992).

Released QPM varieties have shown on average 50% higher total lysine content when compared to the non-QPM counterparts (Table 1) (Betrán et al. 2003). Analysis of the storage proteins in QPM revealed an increased deposition of the 27-kDa γ-zein along with modifications in structure and number of protein bodies (Wallace et al. 1990). In addition, mapping analysis has shown that two chromosomal regions on the long arm of chromosome 7 were associated with modifiers of the *o2* mutant phenotype, one of them linked to the locus encoding the 27-kDa γ-zein (Lopes et al. 1995). A more recent study reported that there are multiple unlinked *O2* gene modifiers, the identity and mode of action of which are still unknown (Holding et al. 2008).

Another interesting discovery is that QPM lines have higher levels of granule-bound starch synthase I in the soluble non-zein protein fraction of developing kernels (Gibbon et al. 2003). The increased amount of the starch-synthesis enzyme was associated with an alteration in amylopectin branching, affecting the starch granule swelling and thus allowing a more effective association between the starch granules in the mature kernel. The increased amount of starch synthase I in association with the increased content of the 27-kDa γ-zein may explain the formation of the vitreous kernel phenotype of QPM (Gibbon and Larkins 2005).

In a review paper published in 1997, we were hoping that transgenic plants overproducing and accumulating essential amino acids, such as lysine, threonine and methionine, would be available to farmers in 5 years' time (Azevedo et al. 1997), but today the availability of high-lysine cereals crops is still a challenge.

Manipulation of the aspartic acid metabolic pathway

Aspartate is the precursor for two main pathways, the first one leading to the synthesis of asparagine, a key compound used for the transport and storage of nitrogen in plants (Lea and Azevedo 2007; Lea et al. 2007; Varisi et al. 2008; Andrews et al. 2009), and a second branched and regulated one that leads to the synthesis of essential amino acids, lysine, threonine, methionine and isoleucine (Mills et al. 1980; Azevedo et al. 2006). Manipulating the regulatory steps that control lysine synthesis via the aspartate pathway (Fig. 2) has long been considered as an alternative strategy of producing high-lysine plants (Azevedo et al. 1997). The ultimate goal is to construct plants with mutated enzymes that are no longer regulated, to accumulate higher amounts of lysine in the seeds (Azevedo et al. 2006).

The first reports describing the biochemical aspects of the aspartate pathway in plants were published only in 1970, although the enzymes and their regulatory functions in the pathway in bacteria were well documented in the early 1960s. By the late 1970s, AK (EC 2.7.2.4) isoenzymes and DHDPS (EC 4.2.1.52), the first enzyme of the lysine branch of the pathway (Fig. 2), had been isolated and characterized from plants. AK isoenzymes were shown to be inhibited by lysine or threonine, whereas DHDPS was strongly inhibited by lysine. Although it was already known in bacteria (Patte et al. 1967), the possibility that a threonine-sensitive AK isoenzyme was linked with homoserine dehydrogenase (HSDH, EC 1.1.1.3) to form a bifunctional enzyme in plants was first proposed by Aarnes and Rognes (1974). It was later demonstrated that both the AK and HSDH domains of the bifunctional polypeptide are feedback inhibited by threonine in maize (Azevedo et al. 1992). This property of the AK/HSDH bifunctional enzyme appears to be a common feature in plants (Wilson et al. 1991; Azevedo et al. 1997). This aspect is important for the overall regulation of the pathway, since the existence of AK and HSDH isoenzymes differentially regulated by feedback inhibition by lysine or threonine may determine the flux of carbon between the branches of the pathway leading to threonine and lysine synthesis. Genetic analysis of a monofunctional isoform of maize AK that was targeted by a mutation that made it less sensitive to lysine feedback inhibition (Hibberd and Green 1982) showed that the gene encoding this isoform is linked to the *O2* gene in the short arm of chromosome 7 (Azevedo et al. 1990). This monofunctional AK has been associated with increased soluble lysine content in maize (Wang et al. 2007). It is also important to mention that in Arabidopsis the LL-diaminopimelate aminotransferase enzyme catalyzes several steps together in the interconversion of tetrahydrodipicolinate and LL-diaminopimelate, a reaction that in *Escherichia coli* requires three enzymes (Hudson et al. 2006). A recent review paper by Curien et al. (2008) describes the differences that exist in the regulation of the plant and microbial aspartate-derived and the branched-chain amino acid biosynthetic pathways and unveils the structural bases of this diversity.

The basic studies on the regulatory mechanisms controlling the aspartate pathway stimulated the search for mutants with altered enzymes in an attempt to select plants that overproduce lysine (see Azevedo 2002 for a review). Using tissue culture techniques and seed mutagenesis, several mutants with altered regulatory properties of the enzymes controlling lysine biosynthesis were selected. The initial idea was to use millimolar concentrations of lysine plus threonine to inhibit cell culture or embryo growth, since excess of these amino acids would inhibit the activity of AK and HSDH (Fig. 2) and consequently the cells would be starved of methionine and fail to grow (Green and Phillips 1974; Bright et al. 1982). In some cases, the lysine analog, *S*-2-aminoethyl-L-cysteine (AEC) (Lea and

Norris 1976), was also used as an agent to select mutants having a DHDPS less sensitive to lysine inhibition (Heremans and Jacobs 1994). Similarly, the selection of biochemical mutants of barley made use of seed mutagenesis and the subsequent growth of the embryos on a culture medium containing millimolar concentrations of lysine and threonine. This allowed the selection of mutants exhibiting the ability to grow normally in the selective medium and exhibiting alterations in the biosynthesis of aspartate-derived amino acids (Bright et al. 1982).

In theory, the prevailing idea was that enzymes less sensitive to feedback inhibition by the amino acid end product would lead to an increased biosynthesis and consequently over-accumulation of lysine. The idea was based on the fact that an AK isoenzyme insensitive to lysine feedback inhibition would divert extra carbon molecules through the lysine branch of the pathway and consequently lead to excess lysine synthesis (Fig. 2). Maize mutants for AK isoenzymes less sensitive to lysine were obtained, but instead of resulting in significant excess lysine in the seeds when compared to wild-type plants, the mutants consistently accumulated several-fold extra threonine in the soluble form (Hibberd and Green 1982). Kernels from the lysine plus threonine-resistant plants overproduced soluble threonine by almost 100-fold when compared to the wild-type counterpart. Similar results were obtained in barley mutants containing altered AK isoenzymes (Bright et al. 1982; Rognes et al. 1983; Arruda et al. 1984). These results clearly showed that an alteration of the regulatory properties of AK would not result in lysine overproduction and accumulation in the seeds. The most likely explanation at that time was that in the lysine plus threonine-resistant mutants, the DHDPS enzyme was still sensitive to lysine feedback inhibition, blocking lysine synthesis at the DHDPS regulatory point of the pathway (Bright et al. 1982) and directing carbon from aspartate semialdehyde (ASA) to threonine biosynthesis (Fig. 2).

Therefore, the next step was to produce lysine-insensitive DHDPS mutants. Once again, by applying the mutagenesis/selection procedure, mutants exhibiting DHDPS less sensitive to lysine feedback inhibition were also obtained (Negrutiu et al. 1984; Frankard et al. 1992). These mutants exhibited significant increases in lysine in the leaves (see Azevedo 2002 for a review). For instance, tobacco lines with altered DHDPS exhibited a 10- to 20-fold increase in soluble lysine in the leaves (Negrutiu et al. 1984), but again the accumulation of lysine in the seeds was negligible. The data obtained with the DHDPS mutants provided further evidence for the balance between the threonine and lysine branches of the aspartic acid pathway. The results confirmed that DHDPS exerts control over lysine biosynthesis and indirectly over the threonine biosynthetic branch. Despite the lack of success in identifying the aspartate pathway mutants that accumulated lysine in the seeds, the information obtained greatly increased the knowledge of the regulation of lysine biosynthesis in plants.

The advent of techniques to produce transgenic plants offered new opportunities to further advance the studies on the manipulation of the aspartate pathway. Taking into account the information generated from the biochemical mutants described above, transgenic strategies were employed to produce plants with altered aspartate-derived amino acid biosynthesis. Transgenic tobacco plants, expressing an *Escherichia coli* AK isoenzyme less sensitive to feedback inhibition by lysine, exhibited essentially the same phenotype as those observed for the biochemical mutants with the accumulation of soluble threonine in all tissues, but without any significant increase in lysine concentration in the seed (Shaul and Galili 1992a). Next, transgenic tobacco plants expressing a bacterial DHDPS enzyme less sensitive to lysine inhibition were produced, which accumulated lysine in the leaves (Shaul and Galili 1992b). Finally, progeny of crosses of the two transgenics accumulated both soluble lysine and threonine, although the concentration of threonine was much higher and lysine lower, when compared to the transgenic plants individually expressing the lysine-insensitive AK or DHDPS, indicating the competition of DHDPS and HSDH for the common substrate ASA (Fig. 2) (Shaul and Galili 1993). These series of three reports were essential not only to confirm all the previous information obtained in the 1970s and 1980s, but also to open a new horizon for the use of the transgenic approach to produce high-lysine seeds.

Lysine catabolism through the saccharopine pathway

In parallel to the studies on lysine biosynthesis in plants, another view of the problem was obtained from the study of Sodek and Wilson in the early 1970s. The researchers showed that ^{14}C-labeled lysine fed into the developing maize endosperm was mostly metabolized to glutamate and proline with a significant amount of incorporation also observed in saccharopine (Sodek and Wilson 1970). An important finding of these authors was that the catabolism of the ^{14}C-lysine occurred to a much lesser extent in the *o2* mutant than in the normal endosperm. It was later established that lysine was translocated from the leaves to the developing endosperm at a concentration of around 5% of the total amino acid pool (Arruda and Silva 1979). It then became apparent that the lysine being translocated to the developing seeds was in excess and therefore must be degraded in the normal endosperm, as the total lysine content of this tissue was only around 1.5% of the total (Silva and Arruda 1979). A search for an enzyme

responsible for lysine degradation in the developing maize endosperm resulted in the identification of the enzyme lysine α-ketoglutarate reductase (LKR, EC 1.5.1.8) also known as lysine 2-oxoglutarate reductase (LOR) (Arruda et al. 1982) (Fig. 3). The enzyme that condenses α-ketoglutarate and lysine into saccharopine using NADPH as cofactor was shown to be highly active in the developing maize endosperm with an activity pattern correlating with the pattern of nitrogen input into the developing seeds (Arruda and Silva 1983). Taking these results together, it seemed that LKR could function during seed ontogenesis by preventing lysine accumulation in the developing endosperm. Interestingly, the sacccharopine pathway has an important function in lysine catabolism in humans. Hyperlysinemia, a disease associated with soluble lysine accumulation, is due to mutations in human LKR (Dancis et al. 1969).

A second enzyme in the saccharopine pathway was later demonstrated in maize. The second step was shown to be catalyzed by saccharopine dehydrogenase (SDH, EC 1.5.1.9), which hydrolyzes saccharopine to glutamate and α-aminoadipic-δ-semialdehyde (Gonçalves-Butruille et al. 1996). The pathway resembles an aminotransferase-type reaction where the terminal amino of lysine is transferred to α-ketoglutarate to form glutamate (Fig. 3). It was found that LKR and SDH are activities of a single bifunctional polypeptide (Gonçalves-Butruille et al. 1996) and that the activity of LKR, but not SDH, is regulated by high Ca^{2+}, high salt and osmolites, which suggest that the enzyme may be involved in signaling processes (Kemper et al. 1998). The gene encoding LKR/SDH in maize was further cloned and characterized. Not surprisingly, it was found that the *LKR/SDH* gene is transcriptionally regulated in the *o2* endosperm, as *LKR/SDH* transcripts were several fold less abundant in the *o2* than in normal endosperm (Kemper et al. 1999). Indeed, an opaque-2 box was found in the promoter of the maize *LKR/SDH* gene suggesting that the gene is under regulation of the opaque-2 transcription factor (Arruda et al. 2000). The LKR/SDH enzyme was also characterized in rice (Gaziola et al. 1997) where it was found to have similar properties to that found in maize. Thus, again the results point to an important role of LKR/SDH in preventing soluble lysine accumulation (Zhu and Galili 2004).

Lysine overproduction in maize with deregulated lysine synthesis and down-regulation of lysine catabolism

A transgenic approach to increase the soluble lysine content of seeds was first demonstrated in canola and soybean plants by the over-expression of bacterial DHDPS and AK enzymes that were both less sensitive to feedback inhibition by lysine (Falco et al. 1995). The genes encoding the bacterial enzymes were driven by the seed-specific promoter of the gene encoding β-phaseolin and linked to a chloroplast transit peptide. The use of seed-specific promoters has the advantage over constitutive promoters in allowing the expression of the transgene in the target tissue, thus avoiding any undesirable effects on other plant organs. The over-expression of the bacterial DHDPS increased the soluble lysine content of the seeds of canola by a 100-fold and doubled the total seed lysine content. Expression of both bacterial enzymes in soybean also increased the soluble lysine content of the seeds by as much as fivefold. However, high concentrations of α-aminoadipate (AA) in canola and saccharopine in soybean suggested that there was a considerable amount of lysine degradation through the saccharopine pathway in these transgenic plants.

Transgenic lines with reduced expression of the entire 19-kDa α-zeins exhibited an increased lysine concentration in the seeds (Table 1) (Huang et al. 2004). Unfortunately, the soluble lysine concentration was not determined for the transgenic lines produced. However, 2 years later the same group produced other transgenic lines, in which the 19- and the 22-kDa α-zeins were down-regulated, resulting in even higher concentrations of lysine in the seeds (Table 1) (Huang et al. 2006). The increase in total lysine concentration in the transgenic lines varied between 4.0 and 5.0 mg/g DW when compared to the wild type (2.43 mg/g DW) (Table 1). Although soluble lysine concentration in the transgenic lines were increased (up to 0.07 mg/g DW) when compared to the wild type (0.025 mg/g DW), due to the overall increase in the total soluble amino acid pool in the transgenic lines, such changes were not significant, indicating that the observed increase in lysine in zein-reduced seeds was due to increased accumulation of proteins rich in lysine and tryptophan (Huang et al. 2006). Lysine accumulation in the seeds was further enhanced when the transgenic lines with reduced expression of the 19-kDa α-zein were stacked with transgenic lines expressing the *CordapA* gene, which encodes a lysine-insensitive DHDPS from *Corynebacterium glutamicum*. The doubled transgenic lines resulted in increased protein and soluble lysine in the seeds (Table 1) (Huang et al. 2005). The combination of the two transgenic strategies produced a synergistic effect resulting in a final total lysine content of the seeds of 5.8 mg/g DW (average of two transgenic lines), a 225% increase when compared to the wild-type control (Table 1) (Huang et al. 2005), a value higher than any other high-lysine maize produced to date (Table 1).

A commercial high-lysine maize line was developed by Monsanto Canada Inc. in collaboration with Renessen LLC, designated LY038, to be used for animal feed

applications. The transgenic line, which was designed to express the *CordapA* gene in the embryo, presents increased lysine content in the seeds, mainly due to accumulation of up to 0.96 mg/g DW soluble lysine (Table 1). The increment in soluble lysine accumulation was also associated with an increase in total lysine content (4.66 mg/g DW) in the grain, an increase of 79% when compared to the control (Table 1) (Lucas et al. 2007). However, the lysine-related catabolites, saccharopine and α-aminoadipic acid, were also increased in LY038. The positive effect of such increased lysine content was shown when broiler chickens fed with LY038 exhibited superior performance and carcass yield in relation to broilers fed with conventional maize (Lucas et al. 2007).

These results clearly indicated that transgenic manipulation of the aspartate pathway by expressing key lysine-insensitive enzymes in a seed-specific manner directed the flux of carbon through the lysine branch resulting in an excess of lysine synthesis. However, significant amounts of this lysine are degraded through the saccharopine pathway as previously predicted (Arruda and Silva 1983). Such lysine degradation can be prevented by inhibiting the first enzymatic steps of the saccharopine pathway in maize seeds. This has been shown in transgenic maize plants engineered with an RNAi construct targeted to the LKR/SDH bifunctional enzyme in a seed-specific manner. This transgenic approach resulted in an accumulation of 0.65 mg/g DW of soluble lysine compared to 0.03 mg/g DW of the wild-type (Table 1) (Houmard et al. 2007). The stacking of the LKR/SDH RNAi transgenic lines with the transgenic lines expressing the *CordapA* gene resulted in maize plants with as much as 4.0 mg/g DW soluble lysine in the maize grain when compared to the 0.1 mg/g DW of the wild type (Table 1) (Frizzi et al. 2008). These authors were also able to show that reduction in LKR/SDH expression by itself, leading to reduced lysine degradation, resulted in the accumulation of 1.5 mg/g DW of soluble lysine in the grain. The manipulation of lysine degradation through suppression of LKR/SDH in the endosperm and in the embryo revealed increased accumulation of soluble lysine and also saccharopine (Reyes et al. 2009). The suppression of LKR/SDH in the embryo resulted in the accumulation of 0.2 mg/g DW soluble lysine in the seeds, whereas the suppression of LKR/SDH in the endosperm resulted in an accumulation of up to 0.90 mg/g DW of soluble lysine. Finally, when embryo and endosperm LKR/SDH suppression were combined, a synergistic effect on soluble lysine accumulation was observed reaching up to 1.60 mg/g DW in mature seeds in association with a reduction in the saccharopine content (Table 1) (Reyes et al. 2009). It is important to mention that these more recent findings about the role of the embryo in the process confirm previous findings in maize when the activity of AK in the scutellum was shown naturally to be 89% higher than in the endosperm (Brennecke et al. 1996).

These results finally proved the concept that alteration of the lysine biosynthetic pathway can be used to develop commercial maize varieties that do not have the poor phenotypic characteristics associated with the mutants of maize discovered 45 years ago. However, such a strategy must take into account the control of lysine degradation by LKR/SDH, otherwise the excess synthesized lysine is immediately metabolized through the saccharopine pathway (Arruda et al. 2000; Galili 2002).

Although such a variation of strategies has been used to produce high-lysine maize lines, it is important to mention that a large variety of responses have been observed when lysine concentration (soluble, protein and total) is concerned. The same is also valid, for instance, for the *o2* mutant, which, depending on the maize genetic background to which the mutation is introduced, resulted in some variation in the final lysine concentration observed in the seeds. For instance, the evaluation of 146 recombinant inbred lines derived from a cross between the *o2* inbred B73*o2* and the QPM inbred CML161 revealed that lysine content in mature seed was highly affected by the inbred line genotype (Gutierrez-Rojas et al. 2008). Of interest would, therefore, be future studies in which specific changes for high-lysine production are introduced into the same genetic background and all lysine forms analyzed, so that the specific changes can be directly compared leading to a better understanding of their effect on lysine metabolism as a whole.

As far as QPM varieties are concerned, it has been shown that the activity of the *o2* modifier genes is influenced by the genetic background (Lopes and Larkins 1995). The long and prolific work by Landry et al. on the nitrogen constituents of maize *o2* and other maize endosperm mutants is a confirmation of such a wide range of changes depending on the genetic background and environment (Landry 2003; Landry et al. 2005; Landry and Delhaye 2007).

Concluding remarks

Since the discovery of the high-lysine maize mutant *o2*, different approaches have been used in an attempt to produce commercial high-yield, high-lysine maize. Breeding programs have been used to overcome the poor agronomic characteristics of *o2* mutant kernels including the use of modifier genes to make *o2* endosperm more vitreous. This has allowed the development of the QPM lines, which have found commercial application in some parts of the world. The induction and selection of biochemical mutants have proved useful for the understanding of the function of the

aspartate pathway in plants and have made important contributions directing the modern approaches that would finally culminate in the production of high-yield high-lysine maize. The possibility of producing transgenic plants was a major step toward manipulating lysine metabolism and should reduce the time needed to obtain commercial lines. Using transgenic plants, as discussed in this review, strategies have been employed to enable the expression of deregulated enzymes of the aspartate pathway, together with the control of lysine degradation, in a seed-specific manner.

A recent new elegant approach used in barley by Lange et al. (2007) involved the production of transgenic barley plants with an antisense construct against C-hordeins, a low lysine storage protein, which resulted in increases in the other storage protein fractions leading to an increased content of lysine, threonine and methionine. Current ongoing research in our laboratories resulted in the production of transgenic maize plants expressing a new high-lysine protein in an endosperm-specific manner (unpublished data), which may result in changes in the storage protein profile of the maize seed.

After 45 years of research on lysine metabolism in plants and looking back at the *o2* maize mutant, it is clear that important advances have been obtained and that it is possible to increase lysine in the maize seed by manipulation of its metabolism, leading to either soluble lysine and/or protein lysine accumulation in the seeds. The lysine content in the mature seed has also been shown to vary dramatically among mutants, QPM and transgenic plants. But perhaps apart from the key important advances in the understanding of the regulation of lysine metabolism in maize, some commercial lines have been produced exhibiting advantages over the original *o2* mutant, not only in lysine content, but also in agronomic overall performance. So, the question of whether the latest GM maize lines contain more lysine than the original *o2* maize mutant can be answered, and the answer is yes.

Finally, changes in other branches of the aspartate pathway should not be left aside. Recently, several transgenic combinations among the threonine, methionine and lysine branches introduced changes, which included AK, *S*-adenosylmethionine synthase and cystathionine γ-synthase, in the regulation of each biosynthetic branch. These transgenic lines exhibited high levels of lysine, threonine and cysteine, which affected the concentration of methionine (Hacham et al. 2007; 2008). Such a strategy has a strong potential to produce transgenic plants containing higher concentrations of methionine in combination with higher concentrations of the other amino acids of the pathway.

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References

Aarnes H, Rognes SE (1974) Threonine-sensitive aspartate kinase and homoserine dehydrogenase from *Pisum sativum*. Phytochemistry 13:2717–2724

Andrews M, Lea PJ, Raven JA, Azevedo RA (2009) Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. Ann Appl Biol 155:1–13

Arruda P, Silva WJ (1979) Amino-acid composition of vascular sap of maize ear peduncle. Phytochemistry 18:409–410

Arruda P, Silva WJ (1983) Lysine-ketoglutarate reductase-activity in maize—its possible role in lysine metabolism of developing endosperm. Phytochemistry 22:2687–2689

Arruda P, Sodek L, Silva WJ (1982) Lysine-ketoglutarate reductase-activity in developing maize endosperm. Plant Physiol 69:988–989

Arruda P, Bright SWJ, Kueh JSH, Lea PJ, Rognes SE (1984) Regulation of aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine—construction and analysis of combinations of the LT1A, LT1B, and LT2 mutant-genes. Plant Physiol 76:442–446

Arruda P, Kemper EL, Papes F, Leite A (2000) Regulation of lysine catabolism in higher plants. Trends Plant Sci 5:324–330

Azevedo RA (2002) Analysis of the aspartic acid metabolic pathway using mutant genes. Amino Acids 22:217–230

Azevedo RA, Lea PJ (2001) Lysine metabolism in higher plants. Amino Acids 20:261–279

Azevedo RA, Arana JL, Arruda P (1990) Biochemical genetics of the interaction of the lysine plus threonine resistant mutant Ltr*1 with opaque-2 maize mutant. Plant Sci 70:81–90

Azevedo RA, Smith RJ, Lea PJ (1992) Aspects of aspartate kinase regulation in maize: co-purification of aspartate kinase and homoserine dehydrogenase sensitive to threonine. Phytochemistry 31:3731–3734

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46:395–419

Azevedo RA, Damerval C, Landry J, Lea PJ, Bellato CM, Meinhardt LW, Le Guilloux M, Delhaye S, Toro AA, Gaziola SA, Berdejo DBA (2003) Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. Eur J Biochem 270:4898–4908

Azevedo RA, Damerval C, Lea PJ, Landry J, Bellato CM, Meinhardt LW, Le Guilloux M, Delhaye S, Toro AA, Gaziola SA, Varisi VA, Gratão PL (2004a) Endosperm protein synthesis and lysine metabolism in distinct opaque maize seed mutants. Funct Plant Biol 31:339–348

Azevedo RA, Lea PJ, Damerval C, Landry J, Bellato CM, Meinhardt LW, Le Guilloux M, Delhaye S, Varisi VA, Gaziola SA, Gratão PL, Toro AA (2004b) Regulation of lysine metabolism and endosperm protein synthesis by the opaque-5 and opaque-7 maize mutations. J Agric Food Chem 52:4865–4871

Azevedo RA, Lancien M, Lea PJ (2006) The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino Acids 30:143–162

Betrán FJ, Bockholt A, Fojt F III, Rooney L, Waniska R (2003) Registration of parental lines: registration of Tx802. Crop Sci 43:1891–1892

Brennecke K, Souza-Neto AJ, Lugli J, Lea PJ, Azevedo RA (1996) Aspartate kinase in the maize mutants *Ask1-LT19* and *opaque-2*. Phytochemistry 41:707–712

Bright SWJ, Kueh JSH, Franklin J, Rognes SE, Miflin BJ (1982) Two genes for threonine accumulation in barley seeds. Nature 299:278–279

Brochetto-Braga MR, Leite A, Arruda P (1992) Partial purification and characterization of lysine-ketoglutarate reductase activity in normal and opaque-2 maize endosperms. Plant Physiol 98:1139–1147

Curien G, Biou V, Mas-Droux C, Robert-Genthon M, Ferrer JL, Dumas R (2008) Amino acids biosynthesis: new architectures in allosteric enzymes. Plant Physiol Biochem 46:325–339

Dalby A, Davies IAI (1967) Ribonuclease activity in the developing seeds of normal and opaque-2 maize. Science 155:1573–1575

Dancis J, Hutzler J, Cox RP, Woody NC (1969) Familial hyperlysinemia with lysine-ketoglutarate reductase insufficiency. J Clin Invest 48:1447–1452

Esen A (1987) Proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (*Zea mays* l.). J Cereal Sci 5:117–128

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. Bio-Technol 13:577–582

Ferreira RR, Varisi VA, Meinhardt LW, Lea PJ, Azevedo RA (2005) Are high-lysine cereal crops still a challenge? Braz J Med Biol Res 38:985–994

Frankard V, Ghislain M, Jacobs M (1992) Two feedback-insensitive enzymes of the aspartate pathway in *Nicotiana sylvestris*. Plant Physiol 99:1285–1293

Frizzi A, Huang S, Gilbertson LA, Armstrong TA, Luethy MH, Malvar TM (2008) Modifying lysine biosynthesis and catabolism in corn with a single bifunctional expression/silencing transgene cassette. Plant Biotech J 6:13–21

Galili G (2002) New insights into the regulation and functional significance of lysine metabolism in plants. Annu Rev Plant Biol 53:27–43

Galili G, Amir R, Roefgen R, Hesse H (2005) Improving the levels of essential amino acids and sulfur metabolites in plants. Biol Chem 386:817–831

Gaziola SA, Teixeira CMG, Lugli J, Sodek L, Azevedo RA (1997) The enzymology of lysine catabolism in rice seeds—isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate reductase saccharopine dehydrogenase bifunctional polypeptide. Eur J Biochem 247:364–371

Gaziola SA, Alessi ES, Guimarães PEO, Damerval C, Azevedo RA (1999) Quality protein maize: a biochemical study of enzymes involved in lysine metabolism. J Agric Food Chem 47:1268–1275

Gibbon BC, Larkins BA (2005) Molecular genetic approaches to developing quality protein maize. Trends Genet 21:227–233

Gibbon BC, Wang XL, Larkins BA (2003) Altered starch structure is associated with endosperm modification in Quality Protein Maize. Proc Natl Acad Sci USA 100:15329–15334

Gonçalves-Butruille M, Szajner P, Torigoi E, Leite A, Arruda P (1996) Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase–saccharopine dehydrogenase from maize. Plant Physiol 110:765–771

Green CE, Phillips RL (1974) Potential selection system for mutants with increased lysine, threonine, and methionine in cereal crops. Crop Sci 14:827–830

Gutierrez-Rojas A, Paul-Scott M, Leyva OR, Menz M, Betrán J (2008) Phenotypic characterization of quality protein maize endosperm modification and amino acid contents in a segregating recombinant inbred population. Crop Sci 48:1714–1722

Hacham Y, Song L, Schuster G, Amir R (2007) Lysine enhances methionine content by modulating the expression of *S*-adenosylmethionine synthase. Plant J 51:850–861

Hacham Y, Matityahu I, Schuster G, Amir R (2008) Overexpression of mutated forms of aspartate kinase and cystathionine gamma-synthase in tobacco leaves resulted in the high accumulation of methionine and threonine. Plant J 54:260–271

Heremans B, Jacobs M (1994) Selection of *Arabidospis thaliana* (L.) Heynh. Mutants resistant to aspartate-derived amino acids and analogues. Plant Sci 101:151–162

Hibberd KA, Green CE (1982) Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc Natl Acad Sci USA 79:559–563

Holding DR, Hunter BG, Chung T, Gibbon BC, Ford CF, Bharti AK, Messing J, Hamaker BR, Larkins BA (2008) Genetic analysis of *opaque-2* modifier loci in quality protein maize. Theor Appl Genet 117:157–170

Houmard NM, Mainville JL, Bonin CP, Huang S, Luethy MH, Malvar TM (2007) High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. Plant Biotech J 5:605–614

Huang SS, Adams WR, Zhou Q, Malloy KP, Voyles DA, Anthony J, Kriz AL, Luethy MH (2004) Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. J Agric Food Chem 52:1958–1964

Huang SS, Kruger DE, Frizzi A, D'Ordine RL, Florida CA, Adams WR, Brown WE, Luethy MH (2005) High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. Plant Biotech J 3:555–569

Huang S, Frizzi A, Florida CA, Kruger DE, Luethy MH (2006) High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD α-zeins. Plant Mol Biol 61:525–535

Hudson AO, Singh BK, Leustek T, Gilvarg C (2006) An LL-diaminopimelate aminotransferase defines a novel variant of the lysine pathway in plants. Plant Physiol 140:292–301

Hunter BG, Beatty MK, Singletary GW, Hamaker BR, Dilkes BP, Larkins BA, Jung R (2002) Maize opaque endosperm mutations create extensive changes in patterns of gene expression. Plant Cell 14:2591–2612

Jones RA, Larkins BA, Tsai CY (1977a) Storage protein-synthesis in maize. 2. Reduced synthesis of a major zein component by opaque-2 mutant of maize. Plant Physiol 59:525–529

Jones RA, Larkins BA, Tsai CY (1977b) Storage protein-synthesis in maize. 3. Developmental changes in membrane bound polyribosome composition and in vitro protein synthesis of normal and opaque-2 maize. Plant Physiol 59:733–737

Kemper EL, Cord-Neto G, Capella AN, Goncalves-Butruile M, Azevedo RA, Arruda P (1998) Structure and regulation of the bifunctional enzyme-lysine-oxoglutarate reductase–saccharopine dehydrogenase in maize. Eur J Biochem 253:720–729

Kemper EL, Neto GC, Papes F, Moraes KCM, Leite A, Arruda P (1999) The role of Opaque2 in the control of lysine-degrading activities in developing maize endosperm. Plant Cell 11:1981–1993

Kodrzycki R, Boston RS, Larkins BA (1989) The opaque-2 mutation of maize differentially reduces zein gene-transcription. Plant Cell 1:105–114

Lambert RJ, Alexander DE, Dudley JW (1969) Relative performance of normal and modified protein (opaque-2) maize hydrides. Crop Sci 9:242–243

Landry J (2003) Quantitation of zeins from the lysine content of grain, endosperm and glutelins of maize. Maydica 48:39–54

Landry J, Delhaye S (2007) Influence of genotype and texture on zein content in endosperm of maize grains. Ann Appl Biol 151:349–356

Landry J, Damerval C, Azevedo RA, Delhaye S (2005) Effect of the opaque and floury mutations on the accumulation of dry matter and protein fractions in maize endosperm. Plant Physiol Biochem 43:549–556

Lange M, Vincze E, Wieser H, Schjoerring JK, Holm PB (2007) Suppression of C-hordein synthesis in barley by antisense

constructs results in a more balanced amino acid composition. J Agric Food Chem 55:6074–6081
Lea PJ, Azevedo RA (2007) Nitrogen use efficiency. 2. Amino acid metabolism. Ann Appl Biol 151:269–275
Lea PJ, Norris RD (1976) Use of amino acid analogs in studies on plant metabolism. Phytochemistry 15:585–595
Lea PJ, Sodek L, Parry MAJ, Shewry PR, Halford NG (2007) Asparagine in plants. Ann Appl Biol 150:1–26
Lopes MA, Larkins BA (1995) Genetic analysis of opaque2 modifier gene activity in maize endosperm. Theor Appl Genet 91:274–281
Lopes MA, Takasaki K, Bostwick DE, Helentjaris T, Larkins BA (1995) Identification of two opaque2 modifier loci in quality protein maize. Mol Gen Genet 247:603–613
Lucas DM, Taylor ML, Hartnell GF, Nemeth MA, Glenn KC, Davis SW (2007) Broiler performance and carcass characteristics when fed diets containing lysine maize (LY038 or LY038 × MON 810), control, or Conventional Reference Maize. Poult Sci 86:2152–2161
Mertz ET, Bates LS, Nelson OE (1964) Mutant gene that changes protein composition and increase lysine content of maize endosperm. Science 145:279–280
Mills WR, Lea PJ, Miflin BJ (1980) Photosynthetic formation of the aspartate family of amino acids in isolated chloroplasts. Plant Physiol 65:1166–1172
Munck L, Karlsson KE, Hagberg A, Eggum BO (1970) Gene for improved nutritional value in barley seed protein. Science 168:985
Negrutiu I, Cattoir-Reynaerts A, Verbruggen I, Jacobs M (1984) Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Genet 68:11–20
Patte JC, Lebras G, Cohen GN (1967) Regulation by methionine of a third aspartokinase and of a second homoserine dehydrogenase in *Escherichia coli* K12. Biochim Biophys Acta 136:245–257
Pixley KV, Bjarnason MS (1993) Combining ability for yield and protein quality among modified-endosperm opaque-2 tropical maize inbreds. Crop Sci 33:1229–1234
Reyes AR, Bonin CP, Houmard NM, Huang S, Malvar TM (2009) Genetic manipulation of lysine catabolism in maize kernels. Plant Mol Biol 69:81–89
Rognes SE, Bright SWJ, Miflin BJ (1983) Feedback-insensitive aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine. Planta 157:32–38
Schmidt RJ, Burr FA, Burr B (1987) Transposon tagging and molecular analysis of the maize regulatory locus Opaque-2. Science 238:960–963
Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene opaque-2 encodes a protein with a leucine-zipper motif that binds to zein DNA. Proc Natl Acad Sci USA 87:46–50
Schmidt RJ, Ketudat M, Aukerman MJ, Hoschek G (1992) Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kd-zein genes. Plant Cell 4:689–700
Shaul O, Galili G (1992a) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. Plant Physiol 100:1157–1163
Shaul O, Galili G (1992b) Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. Plant J 2:203–209
Shaul O, Galili G (1993) Concerted regulation of lysine and threonine synthesis in tobacco plants expressing bacterial feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Mol Biol 23:759–768
Silva WJ, Arruda P (1979) Evidence for the genetic-control of lysine catabolism in maize endosperm. Phytochemistry 18:1803–1805
Sodek L, Wilson CM (1970) Incorporation of leucine-C14 into protein in the developing of normal and opaque-2 corn. Arch Biochem Biophys 140:29–38
Tsai CY, Dalby A (1974) Comparison of effect of *shrunken-4*, *opaque-2*, *opaque-7*, and *floury-2* genes on zein content of maize during endosperm development. Cereal Chem 51:825–829
Varisi VA, Camargos LS, Aguiar LF, Christofoleti RM, Medici LO, Azevedo RA (2008) Lysine biosynthesis and nitrogen metabolism in quinoa (*Chenopodium quinoa*): study of enzymes and nitrogen-containing compounds. Plant Physiol Biochem 46:11–18
Vasal SK (1994) High quality protein corn. In: Hallawer AR (ed) Specialty corns. CRC Press, Boca Raton, pp 79–120
Villegas E, Vasal SK, Bjarnason M (1992) In Quality Protein Maize. Mertz ET (ed) Purdue University, West Lafayette, IN, pp 49–78
Wallace JC, Lopes MA, Paiva E, Larkins BA (1990) New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified opaque-2 maize. Plant Physiol 92:191–196
Wang XL, Lopez-Valenzuela JA, Gibbon BC (2007) Characterization of monofunctional aspartate kinase genes in maize and their relationship with free amino acid content in the endosperm. J Exp Bot 58:2653–2660
Wilson CM, Alexander DE (1967) Ribonuclease activity in normal and opaque-2 mutant endosperm of maize. Science 155:1575–1576
Wilson BJ, Gray AC, Matthews BF (1991) Bifunctional protein in carrot contains both aspartokinase and homoserine dehydrogenase activities. Plant Physiol 97:1323–1328
Zhu XH, Galili G (2004) Lysine metabolism is concurrently regulated by synthesis and catabolism in both reproductive and vegetative tissues. Plant Physiol 135:129–136