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Nutritional improvement of the aspartate family of amino acids in edible crop plants* **

Review Article

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Summary. Plants are the primary source of protein for man and livestock, however, not all plants produce proteins which contain a balance of amino acids for the diet to ensure proper growth of livestock and humans. Alteration of the amino acid composition of plants may be accomplished using techniques of molecular biology and genetic engineering. Genes encoding key enzymes regulating the synthesis of lysine and threonine have been cloned from plants and *E. coli* and are available for modification and transformation into plants. Genes encoding seed storage proteins have been cloned and modified to encode more lysine residues for developing transgenic plants with higher seed lysine. Genes encoding seed storage proteins naturally higher in methionine have been cloned and expressed in transgenic plants, increasing methionine levels of the seed. These and other approaches hold great promise in their application to increasing the content of essential amino acids in plants.

Keywords: Amino acids – Lysine – Methionine – Nutritional improvement – Aspartokinase

Need for amino acid improvement

Although plants provide the bulk of the protein consumed by man and livestock, these proteins vary greatly in their balance of amino acids. Of great importance

* Abbreviations: AK = aspartokinase; HSDH = homoserine dehydrogenase; DS = dihydrodipicolinic acid synthase; AEC = S-(2-aminoethyl)-L-cysteine

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is the content of essential amino acids, which cannot be made by humans in quantity for proper growth. A minimal amount of each essential amino acid is required for proper human growth; these amino acids are often supplemented in the feeds of non-ruminant animals. The importance of improvement of the nutritional value of seeds has been highlighted by several recent reviews (e.g. Shewrey and Kreis, 1991; Shotwell and Larkins, 1991).

In legume seeds, such as soybean, the methionine content is low, while cereal proteins generally are deficient in lysine. Legumes and cereals are major sources of dietary protein for man and livestock, therefore the quality of their protein is important.

Of the essential amino acids, four amino acids, lysine, threonine, methionine and isoleucine, are derived from aspartate. The pathway involved in the synthesis of these amino acids is a complex and branched pathway (Fig. 1). One of the major biochemical controls regulating this pathway is feedback inhibition of branch-point enzymes by end product amino acids (For review see Bryan 1980). Aspartokinase (AK), the first step committed to the synthesis of the aspartate family of amino acids, is present as two or more isozymes in several plants (Bryan, 1980; Dotson et al., 1990). These isoforms can be inhibited by lysine or threonine. The branch-point enzyme, dihydrodipicolinic acid synthase (DS), is feedback inhibited by lysine (Bryan, 1980; Matthews and Widholm, 1978, 1979), while homoserine dehydrogenase (HSDH) is feedback inhibited by threonine (Bryan, 1980). By altering the regulatory controls of this pathway it is possible to alter the production of end product amino acids.

Mutant selection

There are several strategies for increasing the content of certain essential amino acids in seeds of edible crop plants. The seeds are targeted because the major food source of humans is the seed from crops such as wheat, rice, corn and soybean. One approach is to increase the levels of specific free amino acids. Numerous investigators have attempted to increase lysine in cereal crops for

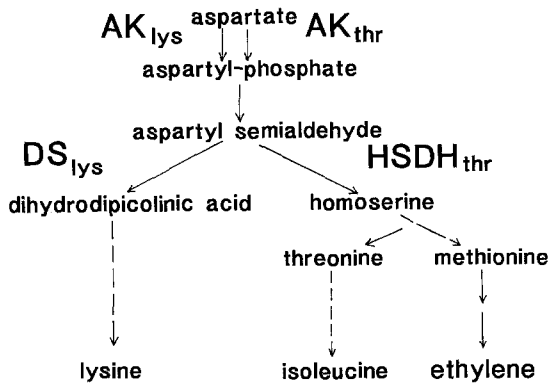

Fig. 1. Pathway for the synthesis of the aspartate family of amino acids. Aspartokinase (*AK*) isozymes can be inhibited by lysine (*lys*) or threonine (*thr*); homoserine dehydrogenase is inhibited by threonine; dihydrodipicolinic acid synthase (*DS*) is inhibited by lysine

years. Amino acid analogs have been used to select for mutants which overproduce amino acids, including lysine, methionine and tryptophan. A key regulatory enzyme normally feedback inhibited by end product amino acid is targeted for selection of a mutant enzyme which is no longer feedback inhibited. A review of the literature suggests that plant mutants, selected for growth in the presence of the lysine amino acid analog, S-2-(aminoethyl)-L-cysteine (AEC), often contained an altered AK, which was no longer inhibited by lysine. Schaeffer and Sharpe (1983, 1990) have selected rice mutants using lysine plus threonine or AEC (Schaeffer and Sharpe, 1981; Schaeffer et al., 1989) to demonstrate higher lysine levels can be obtained in rice seeds. In a number of laboratories, plant mutants obtained through selection for growth in the presence of lysine plus threonine (methionine starvation), contained either a decrease in the sensitivity of AK to lysine (Dotson et al., 1990; Rognes et al., 1983; Frankard et al., 1991) or contained an altered DS (Frankard et al., 1992), which was no longer sensitive to lysine inhibition. Thus, alterations to two key branch-point enzymes (Fig. 1) involved in lysine synthesis can be selected through these methods.

AEC has been used as a growth inhibitor to select for lysine overproducing mutants (Schaeffer and Sharpe, 1981; Frankard et al., 1992). In some cases the selected mutants were characterized by a decrease in uptake of AEC, while other mutants exhibited higher levels of lysine (Matthews et al., 1980). Rice plants selected for resistance to AEC using anther-derived callus cells contained higher bound lysine in its seed than wild-type (Schaeffer and Sharpe, 1981). Similarly, *Pennisetum* selected for tolerance to AEC contained 5 to 7-fold higher lysine in its vegetative tissues than wild-type (Boyes and Vasil, 1987). Negrutiu et al. (1984) selected a *Nicotiana sylvestris* AEC-resistant plant (RAEC-1) which contained up to 28-fold more lysine in its leaves. The branch-point enzyme, DS, was still inhibited 50% by lysine, because the plant was heterozygotic.

Frankard et al. (1991) selected *Nicotiana sylvestris* protoplasts on growth-inhibitory concentrations of lysine plus threonine (LT), resulting in the LT-resistant plant RLT 70. The LT-resistant mutant possessed AK which was not inhibited by lysine, whereas wild-type AK activity was usually inhibited 70%–80% by lysine. Wild-type AK activity consisted of at least two different AK isoforms; one was lysine sensitive and the other was threonine sensitive. The altered regulation in the mutant, RLT 70, resulted in an increase in soluble threonine in the leaves of the mutant, which was a 13-fold increase in the total threonine content. The soluble threonine content was increased 70-fold in the seeds of *Nicotiana* with 20% of the free amino acid pool existing as threonine. The total threonine content of the seed was increased 5-fold.

The lysine and threonine over-producing mutants RAEC 1 and RLT 70 were crossed to produce a double mutant containing AK and DS activities with decreased sensitivities to lysine inhibition (Frankard et al., 1992). Free lysine was overproduced and represented up to 50% of the total free amino acid pool. Although both parents exhibited normal phenotypes, the offspring of the cross displayed abnormal phenotypes and were sterile. Plants enjoyed normal phenotypes when the leaf free lysine content remained below 25%, however, when the free lysine content exceeded 25%, aberrant morphology was exhibited. Growth was reduced with leaves that were very narrow, curled and did not look as

succulent. Lysine over-production changes with plant development and a maximum of lysine over-production appeared after 12 weeks.

Tissue culture selection of corn cells growing in the presence of lysine plus threonine resulted in plants having mutations which decreased the sensitivity of AK to lysine 8 to 76 fold (Gengenbach et al., in press). While free and total lysine levels were not consistently increased significantly, free methionine levels were increased modestly (80% to 90%) and free threonine levels were increased 70% to 110% in two mutants and 150-fold in a third mutant derived from a maize line initially low in free threonine.

Engineering genes encoding key pathway enzymes

In recent years the techniques and instrumentation has been developed so it is now possible to purify key enzymes in this pathway and clone some of the important genes. It is now feasible to study and alter these genes and place them back into the plant to genetically engineer transgenic plants capable of overproducing certain essential amino acids. The first enzyme of the pathway (Fig. 1), AK has been studied by a number of laboratories. A lysine sensitive form of AK was purified to homogeneity from maize suspension cultures and characterized (Dotson et al., 1989, 1990). A threonine sensitive form of HSDH was purified from carrot cell suspension cultures (Matthews et al., 1989). Recently it has been demonstrated that the carrot AK and HSDH activities both reside on a bifunctional protein as in *E. coli* (Wilson et al., 1991). The sequence of a cDNA clone from soybean indicates that soybean contains at least one bifunctional AK-HSDH enzyme, while Southern blots of soybean nuclear DNA suggest the presence of other genes encoding AK and/or HSDH (Matthews and Gebhardt, unpublished).

The branch-point enzyme DS is inhibited by lysine in all plants examined. DS has been purified to homogeneity from corn (Frisch et al., 1991a), tobacco (Ghislain et al., 1990), pea (Dereppe et al., 1992) and wheat (Kumpaisal et al., 1987). It has been cloned from wheat (Kaneko et al., 1990), maize (Frisch, 1991b) and soybean (Silk and Matthews, unpublished). The wheat and corn cDNAs encoding DS complement *E. coli* auxotrophs lacking DS activity. Thus, these cloned genes can be used to engineer specific alterations to create enzymes no longer as sensitive to feedback inhibition as wild type enzymes.

Bacterial genes and enzymes

Another approach to altering the production of lysine in plants is to insert bacterial genes encoding DS, which is less sensitive to inhibition by lysine. For example, DS in *E. coli* is about 20 to 100-fold less sensitive to feedback inhibition by lysine than are plant DS activities. Genes encoding AK-HSDH and DS have been cloned from several bacteria and examined. The I_{50} of DS for lysine in plants ranges from 10 to 50 μM lysine; in contrast it is 1 mM for DS from *E. coli*. Shaul and Galili (1992) transformed two types of chimeric genes into tobacco, *Nicotiana tabacum*. One construct contained the *E. coli dapA* gene encoding DS for synthesis of in the tobacco cytoplasm. The other construct

contained the same gene but also contained the chloroplast transit peptide from pea rbcS-3A to target DS to the chloroplast. Transgenic plants containing either construct expressed the *E. coli dapA* gene and DS activity in both types of transgenic plants was increased up to 25-fold. The DS activity from both types of transgenic plants had an I_{50} of approximately 1 mM, which is similar to that of the *E. coli* enzyme. Transgenic plants expressing the *E. coli* DS activity targeted to the chloroplast possessed free lysine levels which were up to 15-fold higher than the control plants. Transgenic plants containing cytoplasmic localized *E. coli* DS did not possess increased levels of free lysine. Homozygous progeny of a transgenic plant containing chloroplast-targeted *E. coli* DS contained 2.7-fold more free lysine than heterozygous plants, indicating a gene dosage effect. This increase in free lysine in transgenic plants did not translate into an increase in insoluble lysine as compared to controls. An increase in lysine accumulation in transgenic plants was correlated with an abnormal phenotype, delayed senescence and the development of new buds for a longer period of time.

In a similar study, Glassman et al. (in press) inserted a DNA sequence encoding the *E. coli dapA* gene into tobacco. These transgenic plants also did not contain increased free lysine levels.

Transgenic potato plants containing the *E. coli dapA* gene and chloroplast transit peptide (as described above) was reported by Perl et al. (1992). These plants exhibited increased DS activity in leaves, roots and tubers. Over 85% of the total DS activity in leaves was localized to the chloroplasts. One plant had DS activity elevated 50-fold as compared to control plants and the DS activity was less sensitive to lysine inhibition. The levels of free lysine were increased in all of the transgenic potato plants, with the free lysine levels highest in tubers, while roots and leaves also accumulated lysine.

Thus, by incorporating a bacterial gene, *E. coli dapA* with a chloroplast transit peptide sequence, bacterial DS can be expressed in the chloroplasts of plants and elevate the free lysine content of the plants. A higher amount of free lysine or other essential amino acid may increase the nutritional value of tuber and root crops, such as potato and carrot, leafy crops such as lettuce or fruit crops like tomato and apples. Increased essential amino acids may be useful as a value-added trait in these crops, because these can be eaten raw. However, boiling and other processing can leach free amino acids out of plant tissue. Furthermore, there are signs that large increases in free lysine alters the plant phenotype and growth pattern.

There was no indication from these studies that an increase in the free lysine content of the plant led to an increase in fixed lysine in the plant. It may be that lysine is not a limiting amino acid for protein synthesis.

Genetic engineering of storage proteins

Seeds are an important source of dietary proteins to humans and livestock. During seed development, large amounts of seed storage proteins are accumulated along with other less abundant proteins. There are two major types of seed storage proteins present in seed plants: prolamines, found only in the endosperm of cereals, and globulins, found mainly in the embryo of most dicots. These

proteins have several characteristics in common: they are synthesized in developing seeds, perform no enzymatic function, aggregate in protein bodies, usually composed of a group of related polypeptides, and provide carbon, nitrogen, and sulfur to germinating seedlings (Shotwell and Larkins, 1991). The amino acid content of seed storage proteins influences the amino acid content of seeds. Usually, one or more of the essential amino acids is deficient in most of these proteins. In recent years, several researchers have attempted to increase the content of specific amino acids in seed storage proteins through genetic engineering in efforts to produce nutritionally improved crop plants.

Prolamines, the seed storage proteins of cereals, are known for their increased levels of proline and glutamine, but are deficient in the essential amino acids lysine and tryptophan. The prolamines of maize, zeins, are divided into four groups (α, β, γ, σ) based mainly on their solubility (Larkins et al., 1989). They represent more than 50% of the total endosperm proteins in mature maize seeds. Several investigators have studied the zein proteins with emphasis on increasing the lysine and tryptophan content of these seed storage proteins. For example, Wallace et al. (1988) modified the 19 KDa α-zein seed storage protein which was deficient in the essential amino acids lysine and tryptophan. Modifications were performed by introducing lysine codons and lysine and tryptophan encoding oligonucleotides at several positions in the 19 KDa zein cDNA. Also, a 450 base-pair open reading frame from SV40 coat protein was placed in the zein coding region. The transcripts coding for the modified zeins were synthesized *in vitro* and injected into *Xenopus laevis* oocytes. These modifications had no effect on the translation of zein transcript, cleavage of signal peptide, or on zein stability. All of the modified zein molecules were able to aggregate into protein bodies in the *Xenopus* oocyte except the 17 KDa SV40 protein fragment. From these studies, there is an indication that zeins containing high levels of lysine and tryptophan in the seed of maize plants may be possible in the future.

With the relatively long life cycle of maize plants and the difficulty in generating transgenic maize plants, the study of zein gene regulation has been hampered. Therefore, other transgenic plants were used as models to examine zein expression and accumulation in seeds. Preliminary studies using β-phaseolin, a major storage protein of French bean, revealed accumulation of high levels of phaseolin mRNA and protein in tobacco seeds (Sengupta-Gopalan et al., 1985). Later, Beachy et al. (1985) reported similar results when β-conglycinin, a major storage protein in soybean, was introduced into transgenic *Petunia*. When the 19 KDa zein gene was placed in *Petunia*, only low levels of zein transcripts were detected (Ueng et al., 1988). Since there was difficulty in obtaining increased levels of zein transcripts in *Petunia*, Williamson et al. (1988) decided to construct two 19 KDa chimeric genes that were placed under the control of the β-phaseolin promoter and flanked at 3′ end with sequences from either the phaseolin or the 15 KDa zein gene. Both of these chimeric genes expressed seed-specific zein mRNA which was 1% of total poly(A^+) RNA in the seed. Even though the 19 KDa protein appeared to be synthesized, processed to mature form, and accumulated in developing seeds, the levels of zein protein were very low, 0.005% of total seed protein. These results suggest that the translational and/or posttranslational processes such as processing, localization,

transport, or stabilization of zein protein may play a significant role in the accumulation of zeins in *Petunia.*

Another attempt to express the 19 KDa zein protein in transgenic plants was made by Ohtani et al. (1991), but tobacco was used instead of *Petunia.* Like the previous study, the 19 KDa chimeric genes were placed under the control of the phaseolin promoter. Similar chimerics were constructed and regions of the zein cDNA were modified by site-specific mutagenesis to encode lysine. Zein mRNA transcripts coded either by the wild-type or modified zein coding regions represented 0.05% and 2.5% of poly(A^+) RNA. Similar to *Petunia,* the levels of either type of zein proteins that accumulated in tobacco seed were very low. When zein mRNAs in polysomes of developing seeds were analyzed, the data revealed that α-zein mRNA was translated efficiently. However, in vivo labeling and immunoprecipitation studies indicated that newly synthesized α-zein was unstable in tobacco seeds and degradation occurred within an hour.

With difficulty, several heterologous systems have been used to study zein gene expression and regulation. Therefore, Schwall and Feix (1988) decided to utilize a homologous system by using isolated maize protoplasts. They demonstrated functional activity of the 19 KDa gene promoter in protoplasts isolated from maize kernal tissues at 10 days after pollination. More recently, Ueda and Messing (1991) reported the characterization of zein genes 10, 15, and 27 KDa that were maintained in suspension culture of maize endosperm tissue. With the development of a transient gene expression system using isolated protoplasts from these cultured cells, they were able to study the expression of the zein genes. The expression of these genes was reduced in endosperm cultures when compared to those of endosperm tissue from developing kernals. However, the expression was specific, since no transcripts were found in embryos, roots or leaves of young plants. Also, the lengths of the transcripts were identical in both the endosperm cultures and in the developing endosperm tissue. S1 nuclease mapping revealed that accurate transcriptional initiation of the 10 and 27 KDa genes had occurred. Furthermore, they showed that in transfected endosperm culture protoplasts the 5′ flanking sequences of both the 10 and 27 KDa zeins promoted the expression of the CAT reporter gene. Maize endosperm cultures provide an efficient homologous system for studying zein gene expression and regulation.

Some investigators have used a very different approach to increase the amino acid content of zein proteins. Mertz et al. (1964) discovered the *opaque-2* (o2) regulatory mutation in maize, which altered the synthesis of seed storage proteins and resulted in a significantly higher level of lysine. However, phenotypically, this mutation contributed to the soft, floury endosperm, which resulted in a reduction in prolamine content, decreased kernel density, lower yield, and increased susceptibility to insects, pathogens and mechanical damage (Ortega and Bates, 1983). *Opaque-2* encodes a leucine zipper-type transcriptional regulatory protein that binds to the 5′ flanking sequence of genes of the 22 KDa α-zeins and is required for the transcription of these genes (Schmidt et al., 1990; Lohmer et al., 1991). Through a long process of backcrossing and recurrent selection, breeders at the International Maize and Wheat Improvement Center (CIMMYT) combined the *o2* mutation with genetic modifiers which generated

a large number of varieties similar to a normal maize plant, but maintained high protein quality (Vasal et al., 1980). The genotypes are now referred to a Quality Protein Maize or QPM (Vasal, 1984).

It is unknown exactly how these genetic modifiers alter the phenotype of the *opaque-2* mutants, but the results of both Gentinetta et al. (1975) and Ortega and Bates (1983) indicate that the conversion of a floury to a vitreous or glassy endosperm is associated with increased synthesis of a storage protein fraction. Later, Wallace et al. (1990) reported that there was a significant increase (2–4 fold) in the 27 KDa γ-zein which was marked as the most pronounced increase ever observed in the endosperm of these QPM genotypes as compared to the unmodified *opaque-2* mutants. Geetha et al. (1991) analyzed the synthesis and distribution of γ-zein and quantified the levels of γ-zein mRNA in developing kernels of normal, *opaque-2*, modified opaque-2 genotypes, and their reciprocal crosses. Genetic analysis of the γ-zein proteins from the reciprocal F_1 hybrids obtained from crosses between the modified and unmodified *opaque-2* genotypes indicated that the modifier genes function in a semidominant manner which affected both the γ-zein content and endosperm modification. The results of SDS-PAGE and immunolocalization analysis revealed that the zeins of the modified *opaque-2* mutants contained 2 and 3 fold increases in γ-zein as compared to the unmodified *opaque-2* mutants. The increase appeared to be the result of enhanced mRNA transcription or stability instead of gene amplification because γ-zein genes exist as one or two copies per genome in endosperm cells of modified and unmodified *opaque-2* mutants. Ultrastructural studies demonstrated that increased concentrations of γ-zeins were present in the first few subaleurone cells of unmodified endosperms, but in the subaleurone and central endosperm cells of the modified *opaque-2* mutants high concentrations of γ-zeins were observed. In a related study, Lopes and Larkins (1991) showed that the same genetic factors were capable of increasing γ-zeins in normal and *floury-2* backgrounds. This suggested that the influence of the modifier genes on gamma zein expression of the *opaque-2* mutation is an independent occurrence. The authors are in agreement that the high concentrations and the distribution of γ-zein in the modified endosperms are related to the activity of the opaque-2 modifier genes, as well as to the formation of vitreous endosperms.

Similar to the *opaque-2* mutants, the inbred line, BSSS-53 provides a useful model system for studying differential accumulation of zein protein because this line produces 30% more methionine in the seed than other inbred lines (Phillips et al., 1981). The increased levels of methionine in this line was the result of the methionine-rich 10 KDa zein protein (Phillips and McClure, 1985). Kirihara et al. (1988) showed that during endosperm development mRNA levels for the 10 KDa zein protein were higher in the BSSS-53 line than other the inbred line W23. More recently, Cruz-Alvarez et al. (1991) analyzed and quantified mRNA levels and transcription rates for the 10 KDa zein gene. The 10 KDa mRNA levels in the developing endosperm of BSSS-53 were higher than in the lines W23 and W64A which is in agreement with the results of Kirihara et al. (1988). Also, a lower level of 10 KDa mRNA was present in W64A as compared to W23, but the levels of zein protein were higher in W64A than in W23. The differences indicate that there may be translational and/or posttranslational regulation that

is responsible for zein protein accumulation in maize seeds. Further, analysis of mRNA levels in backcross populations suggested that a trans-acting mechanism may have contributed to the amount of mRNA present in different lines. Taken together, the data indicate that the 10 KDa zein gene is genetically regulated at various stages of endosperm development. There is also a possibility that in the developing endosperm cells, transcriptional regulation controls tissue and developmental specific expression, whereby other mechanism(s) that regulate stability and translation of mRNA are responsible for the amount of 10 KDa zein protein present in the seed of maize plant.

Globulins, one of the major types of seed storage proteins of dicots, contain large amounts asparagine and glutamine. However, they are nutritionally deficient in the sulfur amino acids, cysteine and methionine. Since methionine is an essential amino acid and cysteine can partially replace the requirement for methionine, cysteine becomes an essential amino acid when methionine is limiting in the diet. Most of the storage globulins are defined by their structural groups with sedimentation coefficients of about 7S and 11S (Shotwell and Larkins, 1991). Different amounts of the 7S and 11S storage globulins are present in various species of plant seeds. For example, the French bean mostly contains the 7S type of storage globulins (Derbyshire and Boulter, 1976), whereas, the broad bean stores mainly 11S globulins (Wright and Boulter, 1972). Most of the research on storage globulins have concentrated on modifying the genes encoding both the 7S and 11S storage globulins of legumes in order to compensate for the deficiency in the sulfur-containing amino acids.

Hoffman et al. (1988) modified the gene encoding β-phaseolin, a storage protein of *Phaseolus vulgaris*. The β-phaseolin gene was modified by in vitro mutagenesis in which the methionine codons were increased from three to nine by the insertion of a 45 base pair synthetic duplex. They introduced either the modified or the normal phaseolin gene into tobacco by *Agrobacterium*-mediated transformation and analyzed the synthesis and accumulation of phaseolin in the seeds of transgenic plants. Even though both genes were expressed at similar levels, the amount of high-methionine phaseolin protein that accumulated in tobacco seeds was much lower than with the normal gene. However, the expression of the high-methionine gene was seed-specific and developmentally regulated. Further, the high-methionine protein was glycosylated and correctly assembled into trimers like the normal phaseolin protein. Both the normal and the high-methionine protein were degraded after germination, but degradation of the high-methionine protein was more rapid. Electron microscopic observations of developing seeds revealed that the high-methionine protein was present in the endoplasmic reticulum and in Golgi apparatus of tobacco seeds, but absent from the protein bodies where normal phaseolin is deposited. They interpret these results to indicate that the high-methionine phaseolin is transported through the endoplasmic reticulum and Golgi apparatus, like the normal phaseolin, but is degraded rapidly in the protein bodies unlike the normal phaseolin.

Dickinson et al. (1987) developed an in vitro system to study the assembly of glycinin (major soybean storage protein) precursor subunits. They utilized a SP6 RNA polymerase system to transcribe glycinin cDNAs and a rabbit reti-

culocyte system to translate these transcripts which produced labeled glycinin subunits. The proglycinins synthesized by this method self-assembled into trimers following incubation in the reticulocyte lysate, but hexamer formation was not observed. Later, Dickinson et al. (1989) demonstrated that there was a direct involvement between posttranslational cleavage of proglycinin subunits and the assembly of glycinin trimers into hexamers. Since it was evident that this system had the potential to identify amino acids important in subunit assembly, Dickinson et al. (1990) developed a method to determine the effects of structural changes on glycinin assembly. They showed that modifications in the acidic region made the peptide less soluble, but assembly was not affected. Deletions made in the basic polypeptide regions were soluble, but self-assembly of the subunits into trimers was eliminated. In a highly variable region at the carboxy terminus of the acidic polypeptide, several deletions and insertions were made and some insertions that increased the methionine content were also made. The self-assembly of these modified oligomers was unaffected by the deletions or insertions made in the hypervariable region. Their results indicated that the structure of the basic region is very important for subunit assembly.

A molecular approach used by Sun's laboratory (Sun et al., 1992; Sun et al., 1988) to increase the levels of sulfur rich amino acids in plant seeds is to insert genes encoding proteins containing high amounts of sulfur rich amino acids from one plant into another. Sun's laboratory has studied and characterized sulfur-rich proteins from Brazil nut, especially a 2S albumin fraction. This fraction contains an abundant, sulfur-rich protein composed of a 9 KDa and 3 KDa polypeptide (Altenbach et al., 1992), which associate to form a 12 KDa protein molecule through disulfide bridges. These proteins are part of a small gene family. Analysis of cDNA sequences encoding representatives of the 9 KDa subunit of the Brazil nut protein revealed that these proteins contain approximately 18% methionine and 6% cysteine (Altenbach et al., 1987; Ampe, 1986). Altenbach et al. (1992) constructed a chimeric gene consisting of the 2S methionine-rich Brazil nut seed protein and the phaseolin gene promoter and terminator sequences. This chimeric gene was used to transform winter canola (*Brassica napus* L.) hypocotyl explants. The resulting transgenic canola plants produced seeds with increased methionine. Seeds accumulated this protein in quantities as high as 4% of the total protein, enriching the seed methionine content up to 33%. Similar experiments have been successful using tobacco and produced up to 30% increase in methionine in the seed (Altenbach et al., 1989). These experiments suggest that this approach is feasible for increasing the methionine content of seed protein.

The rapid progress reported in enhancing free amino acids and in enhancing the amino acid content of seed proteins has been due in large measure to the isolation and characterization of important genes encoding key regulatory enzymes of amino acid biosynthetic pathways and encoding seed storage proteins. As more genes and promoters become available and as the production of transgenic plants from economically important crops becomes easier, the appearance of nutritionally improved crops in the market place will move towards a reality.

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