Breeding for Seed Protein Improvement Using Nuclear Techniques

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FOREWORD

As a contribution to current world-wide attempts to avert the protein shortage which threatens parts of the rapidly increasing world population, the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture initiated a programme in 1969 on the use of nuclear techniques for seed protein improvement. Since 1971 the programme has been receiving financial support from the Federal Republic of Germany through the Gesellschaft für Strahlen- und Umweltforschung (GSF), Munich.

For the second research co-ordination meeting of this project, held from 10 to 14 December 1973, the International Institute for Tropical Agriculture, Ibadan, Nigeria, kindly acted as host. Twenty-nine scientists from such diverse fields as plant breeding, analytical chemistry, biochemistry, biophysics and nutrition studies participated in the meeting. Over thirty papers were presented by the participants. Papers reporting information likely to be new and useful to others working in this area are published in these proceedings. Results and methods were extensively discussed among the participants, and their recommendations are given at the end of these proceedings.

Among the Annexes will be found the results of an inter-laboratory analytical comparison for protein content and amino acid composition using the same materials. The recommendations of an ad-hoc panel on analytical screening methods for seed protein content and quality are also included in the Annexes.

The Joint FAO/IAEA Division and the GSF wish to thank the Director and the scientific and administrative staff of the International Institute of Tropical Agriculture for their help and co-operation and the excellent arrangements for the meeting.

NOTE

Lest there be confusion concerning the use of the term "protein mutant" a few words of explanation are offered. Some workers have used the term "protein mutants" when referring to early selections from populations. Such selections are more properly putative mutants since firm confirmation over several generations has not been made to prove conclusively the inheritance of such changes in protein content. The reader should keep in mind the difficulty associated, firstly, with separating genetically controlled protein changes in crop plants from those imposed by environmental factors, and secondly, that some of the reports presented here represent early generation studies of populations being sifted for protein changes that might be useful in breeding programmes. (See Section 1.2 of Recommendations for definition of "protein mutant".)

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INTRODUCTORY PAPER

PROSPECTS FOR GENETIC IMPROVEMENT OF SEED PROTEIN IN PLANTS

R.D. BROCK, J. LANGRIDGE
CSIRO, Division of Plant Industry
Canberra, A.C.T.,
Australia

Abstract

PROSPECTS FOR GENETIC IMPROVEMENT OF SEED PROTEIN IN PLANTS.

A survey of protein synthesizing systems reveals that certain components of the system cannot be varied to alter the amount or type of specific protein in the seed while others can be modified by mutation and selection. A number of possible approaches that might prove of use in obtaining more and better plant proteins are suggested.

Oram and Brock [1] expressed optimism that both the quantity and the nutritional quality of seed protein could be substantially improved by plant-breeding methods. This optimism was based, firstly, on the fact that, during the evolution and the breeding history of agricultural species, little deliberate selection had been applied for these characters and, secondly, on the probable nature of the genetic control of protein quantity and quality.

In the species that have been examined the amount of seed protein is under multigenic control [2-4] and consequently will be expected to respond to selection when efficient methods of measuring proteins are applied. Indeed, genotypes with increased seed protein yield have been produced in a number of species [2, 5].

The nutritional quality of protein is determined by its amino acid composition. The known mutants which have major effects on the amino acid composition of total seed protein are all under the control of major genes with simple inheritance [6-9]. These mutants each alter the amino acid composition of the seed storage protein by altering the relative amounts of the different endosperm proteins, i.e., they act by controlling the relative amounts of the different seed storage proteins that are synthesized.

Genetic restrictions on the amino acid composition of proteins synthesized in living systems are imposed by the DNA code, the method of protein synthesis and, in the case of enzymatic proteins, by the requirement of specificity and activity. While the storage proteins may not be subject to the limitation imposed by the requirement for specificity and activity they are undoubtedly subject to the same coding and biosynthetic limitations as are the enzymatic proteins.

An examination of the protein synthesizing system in living organisms and, in particular the points of control, may suggest novel types of mutations and schemes for their selection.

SEED PROTEIN BIOSYNTHESIS

While the biochemistry of protein synthesis in plants has been the subject of extensive research over many years and a generalized scheme for protein synthesis is agreed upon, surprisingly little research has been directed specifically to protein synthesis in developing seeds.

The generalized scheme of protein synthesis which is applicable to all living cells commences with the decoding of the message stored in the DNA specifying the composition of each protein. This message is first transcribed into a complementary strand of messenger RNA (mRNA) which is then free to move away from the DNA to the site of protein synthesis within the cytoplasm. Many copies of the mRNA may be transcribed from the same gene so that the basic message can be amplified many times. These mRNA molecules provide the template on which the individual amino acids are assembled in the order specified by the DNA code to form a specific protein (translation). For this synthesis to proceed a supply of the different amino acids must be available. Each amino acid must be activated by being coupled to a transfer RNA (tRNA) molecule. This reaction requires energy (ATP) and a specific enzyme (aminoacyl tRNA synthetase) to catalyse the activation process. The activated amino acids are then built into the protein chain in the order specified by the mRNA molecule. This sequential assembly of amino acids into a polypeptide chain is catalysed by the ribosomes which, with their associated enzyme factors, move along the messenger RNA molecule. The amino acid sequence (primary structure) of the individual polypeptide chains is determined directly by the DNA code. The way in which the polypeptide chains fold and assume specific three-dimensional shapes (secondary and tertiary structure) or associate with other polypeptide chains to form complex proteins (quaternary structure) is influenced more by the location of particular amino acids or groups of amino acids within the polypeptide chain.

Much of the information upon which this generalized scheme, and its control, is based comes from microorganisms, particularly prokaryotes. The use of plant cell culture, particularly of haploids, is likely to enable many of the microbial mutation and selection systems to be applied to higher plants. Therefore, we propose to examine the various steps of protein synthesis and to suggest approaches that may be applicable to plants.

FORMATION OF ORGANIC NITROGEN

It is probable that in plants the rate of protein synthesis is limited by the supply of organic nitrogen. Species and varieties are known to vary in their ability to increase protein synthesis in response to nitrate application [10] and there is a genotype of rice which has attained a high protein level by a better nitrogen-assimilation mechanism [11]. Mutations and selection could give increased rates of nitrate uptake, more rapid reduction of nitrate to ammonia or more efficient translocation of nitrogen compounds within the plant.

The first two steps in the pathway (Fig. 1) are known to be regulated. Nitrate reductase is inducible by nitrate. Both nitrate reductase and nitrite reductase are subject to end-product control by amino acids. These same amino acids inhibit growth. Hence selection for resistance to growth inhibiting amino acids may give mutants de-controlling these reductases.

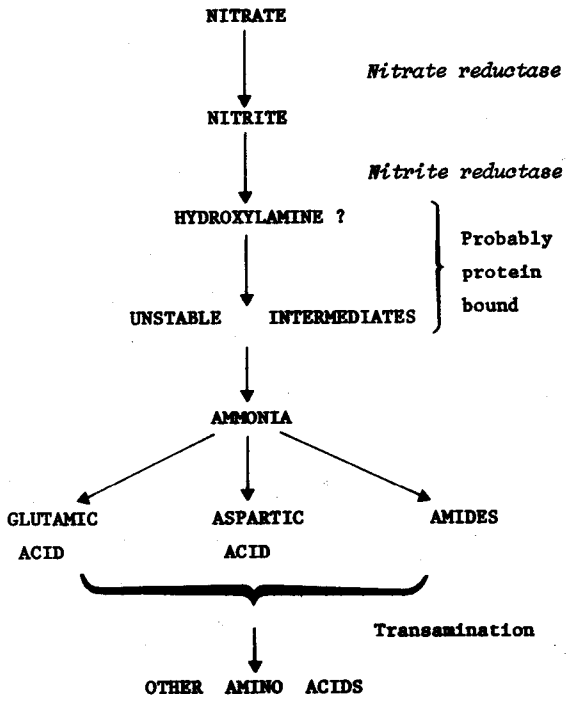

FIG. 1. Nitrate assimilation pathway.

L-threonine (10^{-4} M) inhibits growth of tobacco cells in culture and also represses nitrate reductase formation. Heimer and Filner [12] selected one cell line of tobacco from cells previously exposed to the mutagen N-methyl-N'-nitro-N-nitrosoguanadine capable of growing in concentrations of threonine which were inhibitory to wild type cells. In these resistant cells, in contrast to the wild type cells, nitrate reductase formation had been induced. However, the resistance of these cells was shown to be due to a difference in their ability to accumulate nitrate in the presence of threonine, not to a difference in the control of nitrate reductase formation.

The conversion of ammonia to organic nitrogen in amino acids (Fig. 1) in yeast occurs primarily via glutamic acid and is catalysed by the enzyme glutamic dehydrogenase [13] and the information available for higher plants is compatible with this. Thus, the level or activity of this enzyme may limit the rate of protein synthesis. Electrophoretic variants of glutamic dehydrogenase are known in maize, one of which reduces growth-rate of the plants at moderate temperatures, suggesting growth dependence upon the activity of this enzyme [A. J. Pryor, pers. comm.]. This enzyme appears to be under complex genetic control [14] and such control systems offer good possibilities for de-regulating the enzyme by mutation.

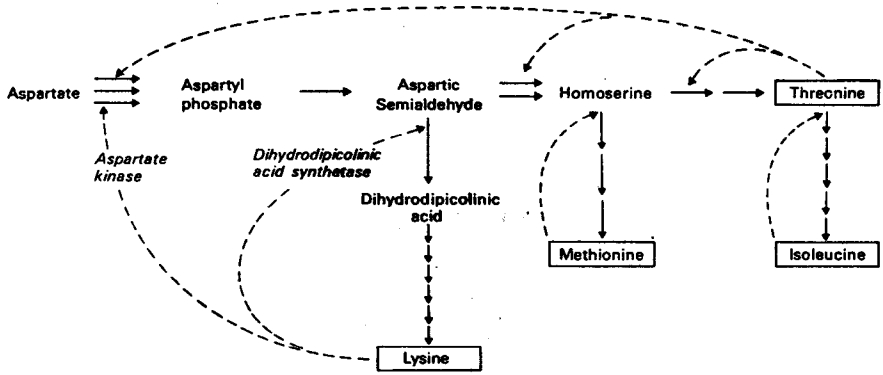

FIG.2. Feedback inhibition control of the biosynthesis of lysine, methionine, threonine and isoleucine in Escherichia coli.

AMINO ACID SYNTHESIS

A characteristic feature of protein synthesis is that the relative levels of the free amino acid are maintained at approximately the concentrations found in bulk proteins [15], presumably to minimize errors in translation. This control is exercised by regulation of the biosynthetic pathways of the amino acids by feedback inhibition and feedback repression.

In general the biosynthetic pathways of amino acids are fairly similar in microorganisms and plants. Little is known about the control systems in plants but the genetics of control has been well studied in bacteria and this information can be extrapolated, with some reservations, to plants.

The lower levels of organic nitrogen in plant cells than in bacteria suggest that the amino pool sizes in plants are small and only low concentrations of amino acids are required to inhibit or repress biosynthesis. This suggests that feedback control in plants is more sensitive to end-product amino acids (effectors) than in microorganisms.

The amino acid pool size in plants is itself under genetic control [16]. Hybrids between maize varieties with very different amino acid pool sizes generally have amino acid levels comparable with that of the higher parent.

Regulation of the biosynthetic enzymes of protein synthesis by genetic manipulation offers great prospects as a method of increasing the supply of limiting amino acids. Unlike catabolic enzymes, where regulation is usually exerted by induction and catabolite repression, biosynthetic enzymes are usually controlled by feedback inhibition of enzyme activity and feedback repression of enzyme synthesis. In principle, these feedback controls can be by-passed, either by decreasing the concentration of the effector, or by reducing the sensitivity of the enzyme or the enzyme synthetic process, which are the targets of the effector.

Selection of mutants with a reduced effector concentration would be useful where an intermediate rather than the end-product is required or in branched pathways where one end-product can be decreased while another

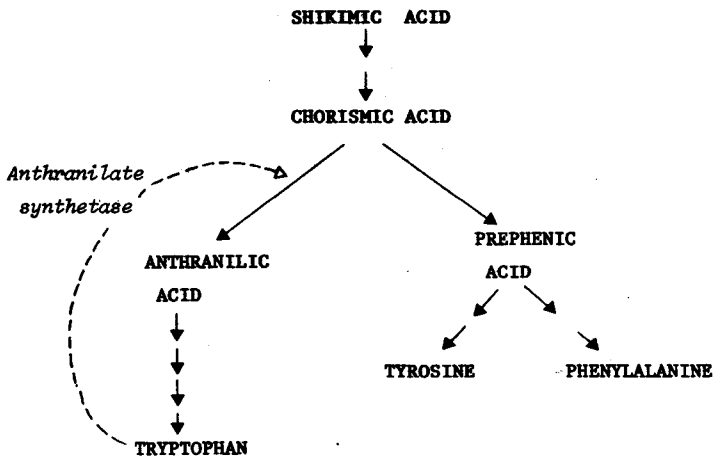

FIG.3. Tryptophan, tyrosine and phenylalanine biosynthesis.

is increased. Thus in Micrococcus glutamicus, a mutant blocking threonine formation resulted in over 40 g/l of lysine being excreted [17]. This was possible because, in this organism, only the aspartate kinase and not the dihydrodipicolinic acid synthetase is sensitive to feedback inhibition (cf. Fig. 2). Preliminary experiments with rice [D. H. Halsall, pers. comm.] suggest that this plant has the same minimal control of lysine formation which makes it particularly suitable for de-regulating lysine biosynthesis.

A more direct method of de-controlling amino acid biosynthesis is to inactivate the feedback receptor site by mutation. The receptor site is in the protein of the enzyme itself in the case of allosteric control or in its biosynthetic mechanism in the case of repression control. The required mutant can be isolated by selection for resistance to an analogue of the appropriate amino acid. This has frequently been done with bacteria to give over-production of amino acids. More recently the same technique has been used to de-regulate amino acid synthesis in plant cell cultures.

Widholm [18, 19] selected tobacco and carrot cells in cultures for resistance to a tryptophan analogue, 5-methyltryptophan, and obtained resistant cells with markedly increased levels of free tryptophan (17-fold for tobacco and 27-fold for carrot). The resistant mutants occurred at a frequency of approximately one in 6×10^5 cells with tobacco and one in 3.6×10^6 with carrot. In each case it was shown that the anthranilate synthetase from the mutant cells had reduced sensitivity to inhibition by tryptophan or 5-methyltryptophan (Fig. 3).

Carlson [20] used a similar technique to select tobacco cells resistant to a methionine analogue, methionine sulphoximine. Populations of haploid cells from in-vitro cultures and haploid protoplasts from leaf mesophyll cells were treated with the mutagen ethylmethane sulphonate.

After a two-week recovery period the cells were exposed to growth inhibitory concentrations of methionine sulphoximine (10 mM) and incubated for three months. 4.6×10^7 viable protoplasts or cells yielded 52 presumptive resistant calluses, most of which segregated tissue which was no longer resistant to methionine sulphoximine. Three completely resistant calluses were obtained, two originating from protoplasts and one from in-vitro cultured cells. Diploid plants were regenerated from these resistant calluses and in the two mutants which originated from haploid protoplasts the free methionine concentration in the leaves was found to be increased five- to six-fold. The free methionine was also increased in stem and root tissue and in the callus tissue of these mutants but the methionine content of the total proteins in these tissues was not increased. F_2 segregations from crosses with normal tobacco suggested that resistance to growth inhibition by methionine sulphoximine was conditioned by a single semi-dominant locus in each case. Crosses between the mutants indicate that the mutant loci are probably allelic.

The third resistant mutant, which originated from haploid in-vitro cultured cells, was different from the other two mutants. Diploid regenerated plants did not have increased free methionine levels in their tissue and the inheritance of resistance to methionine sulphoximine appeared to be more complex; two recessive loci with additive effects are suggested by the F_2 segregation ratio.

The two mutants with increased levels of free methionine also showed increased resistance to Pseudomonas tabaci, the bacterium which causes wildfire disease of tobacco. Braun [21] had earlier demonstrated that Pseudomonas tabaci produces a toxin. Although methionine sulphoximine is not the true bacterial toxin, when it is applied to tobacco leaves it produces a chlorotic halo which is similar to the halo induced by the pathogen. Selection for resistance to methionine sulphoximine also selected for resistance to the toxin from Pseudomonas tabaci.

This technique has been used to maximum effect in Escherichia coli by a combination of mutations affecting feedback inhibition and effector concentration [22]. The first mutant, to resistance to the inhibitor α-amino-β-hydroxyvalerate, inactivated the threonine-sensitive homoserine dehydrogenase and resulted in the production of 1.9 g/l of threonine. The second mutation inactivated the threonine deaminase, thus blocking the step from threonine to isoleucine. The low isoleucine concentration removed isoleucine repression and raised the threonine concentration to 4.7 g/l. A third mutation resulted in the loss of methionine synthetase, thus eliminating the normal methionine control of the pathway and increasing the threonine secreted to 6.1 g/l.

TRANSCRIPTION

Little is known of the control of mRNA formation in higher organisms, but it is probably based upon the interaction of inducers and repressors with operator and promoter genes as in prokaryotes, with the likely addition of hormonal interactions. In the prokaryotes it has been found possible to obtain an overproduction of enzymes and other proteins by both environmental and genetic manipulations. The environmental means of influencing protein quantity involve the addition of inducers or a decrease in the concentration

of repressors and are probably not applicable to higher plants. Genetic methods involving catalytically active proteins which influence growth have resulted in the isolation of two sorts of mutations; mutation to constitutive production of the protein and increases in the number of DNA templates for the protein. It is possible that either or both of these types of mutations could occur to give higher seed protein but there seems no way of selecting specifically for proteins which do not have catalytic activity.

Several plant mutants with nutritionally improved amino acid composition are already known and these seem to be due to an increased synthesis of one protein and a decreased synthesis of another. The cause of these changes is unknown so it is not possible to devise a self-selecting method to obtain further such mutants. Whatever their genetic and molecular basis, they are very similar to the haemoglobin mutants known as thalassaemias. For example, the mutation causing β-thalassaemia results in a marked reduction in the amount of β-chains per cell, but to compensate for this reduction, there is a manyfold increase in α-chain synthesis and it is this α-chain surplus which gives the clinical symptoms. Despite a good deal of work on the haemoglobin chain synthesis, the cause of this imbalance in protein formation remains unknown, but appears to be a transcriptional matter.

Any attempt to increase seed protein quantity by manipulating transcriptional processes is probably impracticable at present because little is known of the detailed mechanisms. In addition, the fact that seed protein synthesis takes place in cells undergoing progressive dehydration of the cytoplasm where normal metabolism is weakened suggests that protein synthesis here may differ from that in the vegetative cytoplasm [23].

POLYPEPTIDE SYNTHESIS

The central processes of protein synthesis concern the peptide linking of amino acids to form the protein chain. This involves about 200 different components and enzymes and annually grows more complicated. The overall synthesis can be divided into amino acid activation, polypeptide chain initiation, chain elongation and chain termination. Despite this complexity, there is a close correspondence for most of these processes between prokaryotes and eukaryotes except for the initiation reactions.

The major difference is in the sites of protein synthesis. In prokaryotes, transcription and translation are coupled and protein synthesis takes place in a complex of DNA, mRNA, RNA polymerase, ribosomes and growing polypeptide chains. However, the membrane surrounding the nucleus in eukaryotes effectively separates transcription from translation, the latter occurring in the cytoplasm. Thus, there is the additional step in eukaryotes of mRNA transport.

The transfer RNAs and synthetases which comprise about half the 200 different macromolecules active in protein synthesis are particularly variable in organisms and tissues, suggesting that they are subject to individual regulation and therefore capable of being genetically manipulated. Most of the other components and reactions are common to all proteins, but some may also be subject to individual regulation. Mutants of the initiation and termination processes have been isolated in prokaryotes but no mutants of polypeptide chain elongation have been found. The mutants of these processes in eukaryotes are known only for yeast where one

mutant seems to be defective in the initiation of protein synthesis [24], and chain-terminating mutants containing the nucleic acid base sequences UAG or UAA are known, also in yeast. On the other hand, mutants of tRNAs and their synthetases, which are the main components involved in control, have been closely studied in prokaryotes. The tRNAs are important because, at least under some conditions they limit the rate of translation, mutations of them can alter the amino acid sequence of proteins, and in some cases the amino acid-tRNA complex is the feedback inhibitor rather than the free amino acid. These functions have been found for tRNAs in prokaryotes but are likely to be even more important in eukaryotes. Experimental estimates of the number of tRNA genes in bacteria vary from 10-13 in E. coli to 40-80 in Bacillus subtilis, while in eukaryotes, yeast has 320-400, Drosophila 750 and Xenopus 1000. However, the repetition of the tRNA genes parallels that of the ribosomal RNA (rRNA) cistrons to give a similar ratio (about 3-6) of tRNA to rRNA genes in both sets of organisms. It seems likely that one way of increasing protein synthesis in higher organisms would be to increase the number of tRNA and rRNA cistrons. Indeed, this happens in certain Drosophila mutants containing deletions of some of the rRNA genes. Reversion of the mutant phenotype, bobbed, to wild-type is accomplished by an increase in the number of rRNA genes [25].

Alterations in tRNA specificity could theoretically be used to increase the concentration of a limiting amino acid in a given protein. In this case, the procedure would be to induce one or more chain-terminating mutations and then suppress them with an altered tRNA which inserts the required amino acid in place of the original one. A calculation of the effects of such a technique in barley glutelin indicates that a suppression of this sort could increase the lysine concentration of the protein by about 25%. This could be done in bacteria at present, but special techniques not at present available would be necessary in order to use it for seed proteins. An additional limitation is the range of amino acids which can be substituted by this method. In response to a single base change in the tRNA gene, an amber (UAG) mutant can theoretically be replaced by the amino acids tyr, leu, trp, ser, lys, glu and gln; an ochre (UAA) mutant by tyr, leu, ser, lys, glu and gln; and an opal (UGA) mutant by leu, cys, trp, gly, arg and ser. It can be seen that such limiting amino acids as thr and met cannot readily be increased by this technique.

GENERAL APPLICATION

This survey of protein synthesizing systems reveals certain components of the system which probably cannot be varied to alter the amount or type of specific protein in the seed. However, it suggests a number of possible approaches that might prove of use in our quest for more and better protein. The parts of the overall system most likely to be profitable are the flexible ones that differ from organism to organism. These include the regulated systems for organic nitrogen formation, for transamination and for free amino acid concentration, as well as the variation that is possible in the tRNA molecules, amino acid synthesis and ribosomes.

Indeed some of the techniques have already been applied to plants [11, 18, 19]. Undoubtedly an important feature in the success of these ventures has been the ability to apply an extremely efficient selection sieve to very