

ENGINEERING OF SEED QUALITY CHARACTERS IN LEGUMES

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Summary

The engineering of quality traits of grain legumes has three major objectives: 1) improving nutritional quality, 2) improving technological traits for industrial use, and 3) phytofarming, defined as the introduction of new quality traits by gene transfer to produce organic compounds such as proteins in legume seeds. The nutritional quality can be improved (objective 1) by raising the level of the limiting essential amino acids, e.g., methionine, in the seed, by raising the digestibility of seed components, e.g., cell wall carbohydrates or phytin as a phosphorus storage compound, or by decreasing the seed's content of toxic or otherwise nutritionally adverse compounds. Strategies to improve the digestibility of grain legumes for feeding are mostly based on the transfer of genes, which are seed-specifically expressed and encode appropriate degradative enzymes in order to increase the mobilization of seed components. Thus, this strategy includes phytofarming of enzymes (objective 3). Improvement of technological traits (objective 2) has been achieved e.g., by engineering the fatty acid composition of oil seed in order to adapt it to specific industrial uses, a topic which is not included in this review. Phytofarming (objective 3) not only aims at producing enzymes to improve the mobilization of feed components, but also at producing industrial enzymes which can be applied directly from ground seeds or after isolating the enzymes from them. In

addition, proteins (e.g., antigens) or antibodies can be produced by phytofarming, which by feeding the seeds induce active or passive immunization of humans or domestic animals against diseases, or for isolation and purification for pharmaceutical use. This review summarizes the present achievements of engineering nutritional traits of grain legumes and of phytofarming of legume seeds, providing an outlook to future developments in this field.

1. Introduction

Seed proteins from grain legumes are a major source of protein nourishment for man and monogastric animals. Both humans and monogastric animals are dependent on the supply of 8 to 10 essential amino acids that cannot be biosynthesized by these vertebrates. In general, one or two of the essential amino acids represent the major limiting factor in the efficiency of transformation of food-protein into body-protein and therefore, are defined as limiting essential amino acids. Methionine is the major limiting essential amino acid in grain legumes. In addition, tryptophan is a secondary limiting essential amino acid in these foods. The imbalanced composition of the complex of essential amino acids in legume seeds limits the biological value of the protein in these grains to 55 percent in faba bean (*Vicia faba* L.) and 75 percent in soybean (*Glycine max* (L.) Merr.) when compared to the biological value of animal proteins, such as those proteins in milk or eggs.

In human nutrition, methionine and tryptophane deficiency of grain legumes is, in most cases, compensated by other food components. It does not play a role in highly developed countries where balanced protein nourishment is warranted by the many different plant and animal protein components of the mixed diets. In poor developing countries, with large vegetarian populations, mainly children might suffer from deficiencies of the limiting essential amino acids. Irreversible retardation in the mental and physical development might be the result if no compensation for the deficiency is provided from the diet. Engineering high-methionine and tryptophan cultivars of grain legumes that play a role in the diets of such populations represents, for humanitarian reasons, an important objective for biotechnology, although the problem of imbalanced essential amino acid nutrition has to be resolved by other measures, too.

In the feed of monogastric animals, an optimized ratio between non-protein and protein compounds as well as a balanced nutritional composition of the complex of essential amino acids has strong economic importance for efficient fattening of chickens and pigs. Grain legumes are not primary feed for monogastric domestic animals. In general, they are fed in mixture containing cereals like corn, barley or wheat. Grain legumes compensate nutritional deficiencies like low protein content or lysine deficiency in cereals, whereas a deficiency of the essential amino acid, methionine, in legume seeds is nearly compensated for by the essential amino acids in cereal grain protein (see *Improving the Protein Content and Quality of Temperate Cereals: Wheat, Barley and Rye*). Therefore, depending on the grain legume and cereal component, ratios between 3:7 and 2:8 for legume to cereal components are mostly used in mixed feeds.

In addition to the unbalanced essential amino acid composition of grain legume seeds, several other factors are known that limit the nutritional quality of grain legumes for

humans and monogastric animals. Legume seeds may contain toxic compounds (e.g., oxalyldiaminopropionic acid, a neurotoxin in seeds of *Lathyrus sativus*, which is eaten by humans in India), adverse nutritional compounds [such as γ -glutamyl-S-ethenylcysteine (GEC) in seeds of the narbon bean (*Vicia narbonensis* L.) a faba bean-related Mediterranean grain legume] or proteinase inhibitors [e.g., in soybean (*Glycine max*) seeds and other grain legumes]. Thus, breeding programs have long aimed at improving the essential amino acid composition and reducing toxic or adverse factors in legume seeds, thereby raising the nutritional value of conventional grain legumes. In addition, attempts have been made to include new species in the collection of valuable grain legumes for agricultural use [e.g., the program run by the Centre for Legumes in Mediterranean Agriculture (CLIMA), University of Western Australia, in Nedlands (Australia)].

The nutritional value of legume seeds for monogastric animal feeding can also be improved by raising the digestibility of seed components like cell wall carbohydrates or phytin which is a phosphorus storage compound (see *Genetic Improvement of Cereals with low Phytic acid Content*). To improve the digestibility of such a compound, enzyme additives, like thermostabile α -amylase, glucanase, xylanase for carbohydrate breakdown, and phytase for the mobilization of phosphorus reserves, are included in feed mixtures. These additive enzymes degrade polysaccharides down to oligo- and monosaccharides and liberate phosphate from phytin, respectively, which can then be utilized by monogastric animals. Breeding of grain legumes that already contain such enzymes in their seeds could improve their economic efficiency for monogastric animal feeding. In some cases, even the engineering of grains enriched in specific vitamins or/and mineral elements is of strong interest for the nourishment of humans and for animal feeding (see *Molecular Breeding of Vegetable Crops for Improved Provitamin A Carotenoid Content*).

Recently, a new objective was developed to improve the quality characters of grain legumes in compounds that are important to fight human and animal diseases. Thus, antigens or antibodies for immunization against diseases might be engineered into grain legumes. They could be used after isolation from the plant tissue or even directly applied by feeding such grains to domestic animals (“edible vaccines”). Similarly, recombinant plasma proteins and diagnostic reagents might be produced in transgenic legume seeds.

Not all of the above mentioned objectives occupy a primary position in the ranking of different breeding objectives for grain legumes. Frequently, yield increases are thought to be of higher economic importance than nutritional quality characters. The improvement of nutritional quality characters gains major economic importance if sufficient grains are available at reasonable prices. Therefore, research and breeding efforts have sometimes been directed only secondarily to the improvement of quality characters of grain legumes. Nevertheless, even without increase in yield, nutritionally improved grains bring advantage for human health and raise the farmers’ economic profit since the efficiency of feeding his monogastric animal can be increased. In the long term, breeding represents the most efficient means of improving agronomic characters of grains. It has frequently been shown that supplements containing amino acids, phosphorus, vitamins or enzymes are less efficient in improving nutrition

compared to similar amounts of these components contained in grains grown in the field. Even the agronomic input to produce such improved grains does not, in general, change in comparison to the production input for non-improved grains. Additionally, no waste or environmental pollution problems occur, such as in microbial fermentation or chemical production of amino acids or specific enzymes to be used as feed additives. The low transformation factor of food to body protein represents a major reason for environmental pollution from nitrogen. It results from nitrogen excretion in the urine and manure of animals and humans. Improving this transformation factor by improving the amino acid balance in feeds and food would also somewhat reduce environmental nitrogen pollution.

Sufficient genetic variability in the population of the respective genomes is a prerequisite for any breeding success. Modern industrialized agricultural production processes have induced a continuous increase in uniformity and a decrease in the variability of valuable traits of cultivated crop varieties (gene erosion). Continuous maintenance of the wealth and multiplicity of germplasms within the different crops in gene banks, and their screening for valuable traits form an important breeding basis. Only characters that exhibit sufficient variability in the germplasm promise breeding success for the respective crop. The introduction of totally new characters, like thermostable polysaccharide-degrading enzymes or the production of health-promoting antibodies cannot be achieved without integrating modern methods of genetic engineering into the arsenal of conventional breeding methods. Even conventional breeding for improved amounts of limiting essential amino acids have had only limited success. This was shown for lysine in corn and barley where the available genetic variability of this trait had even been amplified by producing collections of chemical- or radiation-induced mutants. The advent of molecular gene analysis, DNA recombination techniques and gene transfer methods for plants permitted the amplification of the genetic variability of natural quality traits in a directed manner, and allowed the introduction of new traits. Therefore, these new genetic engineering techniques were combined with the techniques of conventional breeding to reach old as well as implement new objectives for plant breeding.

Here we discuss the biotechnological attempts to improve quality characters of grain legumes, such as the nutritional adequacy of grain amino acids, the introduction of carbohydrate-degrading enzymes, and the production of antigens and antibodies in legume seeds. The reader should consult other chapters of this encyclopedia for the biotechnology used in breeding for improved grain vitamin composition and phytate mobilization since, thus far, no corresponding examples are available for grain legumes. Short reference will be made to the elimination of toxic or nutritionally adverse compounds.

2. The Context Principle

Thermodynamically, life is a very improbable state of matter. It exists as a result of an extremely complex order of structures and processes in the living organism, which has to be continuously stabilized by high energy inputs against decay. This life organization has evolved during hundreds of millions of years and is inherited, in a species-specific way, from generation to generation. Thus, the success of genetically engineering

specific traits strongly depends on whether one succeeds in inserting corresponding modification of genes and gene expression into the context of the evolved order of life in the specific organism. For example, the site of foreign DNA integration into a plant genome strongly influences its further functioning in gene expression, since the foreign DNA is brought into the context of the respective genome structure and functions. The insertion of transgenes is currently a random process. Therefore, transgenic plants have to be carefully selected for unaffected inheritance and expression of the inserted and endogenous genes before proceeding further in breeding engineered cultivars.

Life functions are strongly based on the specific order in cellular compartmentation, which forms the basis for the specific spatial and temporal order of metabolic processes. Thus, engineering metabolic processes requires the insertion of the respective enzyme or protein into the correct compartment, and from there into the corresponding metabolic process. Furthermore, this represents a prerequisite for successfully engineering foreign methionine-rich proteins into storage tissue cells of legume seeds, for engineering cell wall carbohydrate-degrading enzymes into seed cells or to achieve stable accumulation of antibodies in storage tissue cells. Besides fitting into the biological processes at the cellular level, the expression of foreign genes has to be compatible with the changing structure-function relations during plant development. In legume seeds, the large amounts of storage proteins are naturally formed during mid and late seed maturation stages. During this period, a complex network of developmental changes in gene expression patterns leads to the structural and functional specialization of storage tissue cells for biosynthesis, accumulation and stable storage of large amounts of specific proteins. This includes the fact that proteins have to be intracellularly transported from their intracytoplasmic site of biosynthesis to their extracytoplasmic site of deposition, which is the concomitantly differentiating vacuolar compartment. In this storage compartment, proteins survive an acid environment, seed desiccation and adverse environmental conditions during long periods of seed rest or dormancy before germination becomes possible. Engineering quality traits of grain legumes, (e.g., high seed methionine), means that the foreign proteins, e.g., methionine-rich proteins, that are introduced must fit into the context of the complicated molecular and cellular events underlying protein reserve deposition in developing seeds.

Some grain legumes need nearly a doubling of seed methionine to reach the level reported in the FAO standard for a nutritionally balanced amino acid composition of feed protein. This requires that methionine biosynthesis has to be greatly increased above the original level in the wild type. Therefore the process of methionine biosynthesis itself, and the supply of sulfhydryl-groups and carbon skeletons for its biosynthesis should not become limiting factors. Sulfur may be supplied from endogenous reserves of organic sulfur compounds in the seed or vegetative part of the respective plant or by increased sulfate assimilation. Thus, the addition of a foreign high-methionine protein, as an additional methionine sink in seeds, has to fit into the context of the metabolism of organic sulfur compounds, into the sink-source relations of sulfur-containing compounds and the mechanisms of sulfur supply to seeds in the respective plant.

Provided one succeeds in raising an economically valuable grain legume variety with genetically engineered improved seed traits, this plant has to fit into the agro-

ecosystems of grain legume production which, within certain limits, can be adapted to the special requirements that this plants might have for cultivation. In addition, it has to be warranted that no out-crossing to wildtype grain legumes can occur and wild type ecosystems cannot be affected in any way. Any genetically engineered crop plant has to be tested for fitting into the context of agro- and wildlife ecosystems before it can be taken into consideration for commercial production.

The engineered seed must also meet human nutrition requirements and health care needs. It must also meet the demands for animal feeding and the use of animal products for human nutrition. Allergic, toxic or other adverse side effects have to be excluded and the economic advantages have to be verified under production and commercial conditions. Last, but not least, public acceptance is needed for commercial success.

Clearly, the context principle shows that broad basic and applied research is needed to generate sufficient knowledge about the genetic, cellular, developmental, metabolic, ecological, agricultural, nutritional and socio-economic contexts. Additionally, appropriate methods of genetic engineering have to be developed which allow the generation of plants that can fit into these contexts. In any case, it has to be verified whether the specific objectives can be reached by conventional breeding without including risky and expensive genetic engineering steps, or whether the biotechnological way is more promising or possibly the better and only way to reach the objective. The above mentioned context and methods problems explain why such a long time is needed to progress from the first steps of developing the methods of genetic engineering and generating the first ideas for including these methods into plant breeding objectives to develop the commercially successful new crop plant cultivars with improved seed quality traits. Unfortunately, some discredit resulted from initial overoptimistic predictions for the rapid success of engineering grain legumes. The subsequent sections of this article will demonstrate the new possibilities, which may result from combining conventional breeding with genetic engineering to produce grain legumes with improved seed quality traits.

3. The Engineering of High-Methionine Grain Legumes

3.1 What Do We Know About Amino Acid Composition and Major Proteins in Legume Seeds to be Engineered?

Soluble amino compounds make up at maximum 10% of the dry matter of flour produced from dehusked mature seeds of grain legumes. In most cases, free proteinogenic amino acids form the major part of this fraction. In addition di- and oligopeptides are present, such as the sulfur-containing γ -glutamylcysteine or glutathione, which, as will be shown later, may play a role for the engineering of high-methionine grain legumes. Insoluble high-molecular nitrogen compounds like proteins and nucleic acids make up 90 or more percent of the nitrogen compounds in the dry matter of the flour, where nucleic acids correspond to only about 2 percent of the insoluble nitrogen compounds. Thus, the major part is proteins, which, therefore, determine the total amino acid composition of the seed flour. According to the OSBORNE scheme of seed protein fractionation, which is based on solubility differences between legume proteins, these consist of approximately 10 to 15 percent

albumins and 85 to 90 percent globulins. Both protein fractions can be extracted together from ground seeds by phosphate-buffered salt solutions at neutral pH-values. The extracted proteins are fractionated according to their water solubility into water-soluble albumins and -insoluble globulins. Albumins comprise a complex mixture of small amounts of a large number of different enzymes and other biologically active proteins, such as lectins, enzyme inhibitors, etc. Globulins are, generally, enzymatically inactive special storage proteins. They are characterized by their unique amino acid composition in that they contain large amounts of acidic amino acids and corresponding amides as well as arginine and only low quantities of the essential amino acids methionine and tryptophane. Globulins exhibit a higher nitrogen-to-carbon ratio than most other proteins. This reflects their special function as nitrogen storage compounds in seeds. Since globulins make up approximately 75 to 80 percent of the total seed protein in grain legumes they are responsible for the nutritionally imbalanced amino acid composition of the total seed protein. Storage protein-like albumins are also known to be in some grain legumes which can make up a large part of this protein fraction e.g., in pea.

Two major types of storage globulins are known to occur in grain legume seeds: a) The group of vicilin-like globulins, e.g., vicilin from pea, chick pea, faba bean or mung bean; phaseolin from kidney bean; β -conglycinin from soybean; β -conglutin from lupin. Their holoprotein molecules are trimers composed of two different, but chemically closely related types of subunits. Both types of subunits are polymorphic (which means appreciable numbers of similar polypeptides of each subunit type exist which differ in their amino acid sequences). Nevertheless, within certain limits they all have similar conformation and form similar spatial structures. b) The group of legumin-like globulins, such as legumin from pea and faba bean, glycinin from soybean, arachin from peanut, and α -conglutin from lupin. Their holoprotein molecules are hexamers of polymorphic subunits, which, like vicilin subunits, have similar conformation and form similar spatial structures. The molecular weight of legumins is approximately twice that of vicilins, i.e., 300 – 400 versus 150 to 200 kDa, respectively.

The ratio between vicilin- and legumin-like globulins differs in seeds of different grain legumes species. Some, like kidney bean or mung bean, contain almost all vicilin-like globulins. In those species, which have both types of globulins in their seeds, the ratio between vicilin- and legumin-like globulins is highly variable even between cultivars and different lines (e.g., pea). Faba bean exhibits one of the highest legumin-to-vicilin ratios of about 3:1. In most cereals, globulins represent no major storage proteins, although small amounts were generally detected. However, in rice and oats, legumin-like storage proteins predominate the prolamins, which are the typical storage proteins of other cereals.

In many plants, the majority of the subunits of vicilin-like globulins are free of methionine and cysteine. An exception represents phaseolin, the vicilin-like globulin from kidney bean, in which a small number of methionine residues are present. Subunits of all legumin-like globulins have at least a few cysteine residues. In many plants, they contain, in addition, some methionine residues. In most legumes two groups of legumin subunits are known. One group comprises subunits richer in methionine than the subunits of the other groups. In faba bean, this second group is free of methionine,

and only the first one contains up to three methionine residues per subunit. Taken together, legume storage globulins are extremely poor in sulfur-containing amino acids. Generally, vicilin-like globulins are called ‘sulfur-free’ whereas legumin-like globulins are called ‘sulfur-containing’, but even the latter are extremely poor in sulfur-containing amino acids, especially in methionine. Since the ratio of globulins to other proteins is about 3:1, the large part of sulfur-free and sulfur-poor globulins determines the overall low level of cysteine and especially, methionine in mature legume seeds. Therefore, very shortly after the advent of DNA recombination and gene transfer techniques, the idea arose to increase the levels of methionine in legume seed by engineering an increased number of methionine codons into vicilin or/and legumin genes.

Corresponding multigene families encode the polymorphic subunits of vicilin- and legumin-like globulins. Both types of globulins have evolved from common ancestor molecules and are related to desiccation-resistant proteins of other organisms. It is therefore no surprise that both exhibit close similarities in their spatial structure and at the amino acid sequence level. The evolutionary conservation of globulin structure must be related to essential functional characters of proteins to be accumulated and stored in the vacuolar compartment of seeds that desiccate during seed maturation. Three major functional constraints may have acted during globulin structure evolution: a) Globulin subunits are synthesized as precursor polypeptides in the cytoplasm of the storage tissue cells. They have to be intra-cellularly transported from this site of biosynthesis into the extracytoplasmic vacuole. This requires that the polypeptide precursor pass through membranes to be included in membrane-bound transfer vesicles, and finally, be discharged into the vacuole. Thus, the precursor has to fit into the structural requirements for intracellular protein transfer through the so-called secretory pathway. This structural fitness was developed during evolution. Genetic engineering of storage globulin has to maintain this structural fitness in order to get the modified globulin transported into the protein storage vacuole where it is protected against premature breakdown before germination. b) Once arrived in the vacuole the transportable precursor polypeptide has to be transformed into a subunit fitting into an oligomeric depositable storage globulin molecule. In some cases, the precursor undergoes tailoring by limited proteolysis, which generates the mature subunit assembled into oligomeric deposition forms of globulins. The fitness for molecular maturation and integration into depositable globulin holoproteins is another character that has to remain undamaged in the case of genetic engineering of globulins. c) Globulins are deposited in seeds as a storage form of nitrogen, which is mobilized for metabolic use during germination. Thus, genetic engineering should not interfere with the mobilization function of globulins, since affecting this function might interfere with germination and seedling growth, and thus, impair seed propagation.

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Biographical Sketch

Klaus Günther MÜNTZ was born in Frankfurt/Oder (Germany) in 1932. After finishing high school and working as a candidate teacher, he studied Biology and Chemistry at the University of Potsdam, from 1952 to 1957. Afterwards, he was a junior scientist at the Botanical Institute, Potsdam University, until 1962. In 1961, he graduated as Dr. rer. nat. from the Faculty of Mathematics and Natural Sciences, Potsdam University. From 1962 to 1969, he was senior scientist at the Department of Ecophysiology, Institut für Kulturpflanzenforschung, Gatersleben, of the then Deutsche Akademie der Wissenschaften zu Berlin. From 1967 to 1969, he was a member of a team of German scientists working in Havana, Cuba, for two years to establish a Department of Plant Physiology and Biochemistry, at the Institute of Biology, Academy of Sciences of the Republic of Cuba. In 1968, he graduated as a Dr. habil. from the Faculty of Mathematics and Natural Sciences, Potsdam University. In 1970, he founded the Research Group for Plant Protein Metabolism at the Gatersleben Institute, where he became head of the Molecular Basis of Plant Productivity Section in 1972, and Professor of Plant Biochemistry in 1974. He was Director of the Institut für Biochemie der Pflanzen, Halle from 1989 to 1990, and of the Institut für Genetik und Kulturpflanzenforschung, Gatersleben from 1990 to 1991. From 1992, until retirement in 1997, he was head of the Department of Molecular Cell Biology and a member of the Board of Directors, at the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben. Since 1988, he has been a member of the Deutsche Akademie der Naturforscher, Leopoldina, Halle. His major research interests are: Carbon and nitrogen metabolism and mass culture of microalgae (1957 - 1967); light regulation of coffee plant development (1967 - 1969); seed proteins: biochemistry, molecular and cell biology of formation, deposition and mobilization; genetic engineering of quality characters of legume seeds (from 1970 - 2001).