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# Mutational Breeding and Genetic Engineering in the Development of High Grain Protein Content

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**ABSTRACT:** Cereals are the most important crops in the world for both human consumption and animal feed. Improving their nutritional values, such as high protein content, will have significant implications, from establishing healthy lifestyles to helping remediate malnutrition problems worldwide. Besides providing a source of carbohydrate, grain is also a natural source of dietary fiber, vitamins, minerals, specific oils, and other disease-fighting phytocompounds. Even though cereal grains contain relatively little protein compared to legume seeds, they provide protein for the nutrition of humans and livestock that is about 3 times that of legumes. Most cereal seeds lack a few essential amino acids; therefore, they have imbalanced amino acid profiles. Lysine (Lys), threonine (Thr), methionine (Met), and tryptophan (Trp) are among the most critical and are a limiting factor in many grain crops for human nutrition. Tremendous research has been put into the efforts to improve these essential amino acids. Development of high protein content can be outlined in four different approaches through manipulating seed protein bodies, modulating certain biosynthetic pathways to overproduce essential and limiting amino acids, increasing nitrogen relocation to the grain through the introduction of transgenes, and exploiting new genetic variance. Various technologies have been employed to improve protein content including conventional and mutational breeding, genetic engineering, marker-assisted selection, and genomic analysis. Each approach involves a combination of these technologies. Advancements in nutrigenomics and nutrigenetics continue to improve public knowledge at a rapid pace on the importance of specific aspects of food nutrition for optimum fitness and health. An understanding of the molecular basis for human health and genetic predisposition to certain diseases through human genomes enables individuals to personalize their nutritional requirements. It is critically important, therefore, to improve grain protein quality. Highly nutritious grain can be tailored to functional foods to meet the needs for both specific individuals and human populations as a whole.

**KEYWORDS:** *cereals, nutrition, protein, essential amino acids, mutational breeding, genetic engineering*

## INTRODUCTION

Cereals are the most important crops in the world for both human consumption and animal feed. Improving their nutritional values including higher protein content will have significant implications from establishing healthy lifestyles to help remediate malnutrition problems worldwide. Besides providing a source of carbohydrate, grain is also a natural source of dietary fibers, vitamins, minerals, specific oils ($\gamma$-oryzanol), and other disease-fighting phytocompounds, including polyphenols and flavonoids that have the potential to inhibit the growth of or kill cancer cells.[1] Flavonoids, such as tricin (5,7,40-trihydroxy-30,50-dimethoxyflavone), are anticancer agents that have a specific chemopreventive activity. Improved grain nutrition has been a subject of interest in both research and industry looking for better ways to utilize the grain crop beyond its current status as a source of carbohydrates and calories.[2−5]

Advancements in nutrigenomics and nutrigenetics improve public knowledge of the importance of developing nutritional regimes for optimum fitness and health. Greater knowledge of the human genome and understanding of the molecular basis for human health and genetic predisposition to certain diseases enable individuals to personalize their nutritional requirements. These recent advances together with worldwide accessibility to the most current information on nutrition and health can potentially transform and elevate the need for more nutritious food products. High-protein grains can provide the base for developing novel foods and fibers or nutrient-dense food products. Highly nutritious grain can also be tailored to functional foods to meet the needs of individuals with specific genetic traits.

Current total annual world grain yield exceeds 2350 million metric tonnes (mt).[6] Three major cereals account for >85% of the total gain production, that is, maize at about 838 mt, wheat at 700.2 mt, and rice at 484.3 mt. Even though cereal grains contain relatively little protein (about 6−12% dry wt) compared to legume seeds, they provide over 200 mt of protein for the nutrition of humans and livestock, about 3 times that of legumes. Between 2010 and 2012, nearly 870 million people in the world were chronically malnourished.[7] This represents 12.5% of the global population and most of these live in developing countries. Every year, about 19 million children suffer from severe acute malnutrition and at least 3.5 million of them die from malnutrition-related causes.

It is estimated that 2 billion people live primarily on a meat-based diet, whereas 4 billion live primarily on a plant-based diet worldwide.[8] On average, the protein consumed per day on the meat-based diet is 112 g, whereas the lactoovovegetarian diet is

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89 g per day, which is still much higher than the recommended dietary allowances (RDA) of 56 g per day. Americans, for example, consume about twice the RDA for protein and about 1000 kcal in excess per day per capita.[9] According to the World Health Organization, excess weight has become a problem not only in developed but also in developing countries, reaching global epidemic proportions. More than 1 billion adults are either overweight or obese.[10] Obesity increases annual medical spending. In the United States, obesity causes an estimated $190 billion per year in health care spending, or 20.6% of total health care expenditures.[11] Modification of daily diets to reduce obesity will not only reduce the healthcare cost but also increase the availability of food supply worldwide. Reducing the excess of daily food consumption to reflect a healthier lifestyle will arguably make the world food supply more adequate to meet the global need. Malnutrition in some cases can be viewed as a property or economic issue where the poor cannot afford to buy food. Malnutrition is more severe in the areas where people's staple food is exclusively either cereal grain or legume. With a diet of a 50:50 mixture of cereal grain and legumes, for example, the malnutrition problem can be reduced. World malnutrition is an undoubtedly complex problem. Development of highly nutritious food products will provide a solid bottom line that can be used to help solve the worldwide malnutrition problem across social, cultural, and economic issues.

This paper provides a review of efforts in developing high protein in cereal grains that spanned more than 50 years and can be outlined in four different approaches: (1) manipulation of seed protein bodies or fractions, (2) deregulation of certain biosynthetic pathways to overproduce essential amino acids that are limiting, (3) nitrogen relocation to the grain through the introduction of transgenes, and (4) exploration of genetic variance. Various technologies have been employed including conventional and mutational breeding, genetic engineering, DNA markers, and genomics. Each approach involves a combination of several technologies.

## GRAIN PROTEIN COMPARTMENTALIZATION AND PROFILE

Protein in the grain is mostly stored in protein bodies. These protein factions are often referred to as the nitrogen sink in the grain and can be grouped according to Osborne's classification into albumins (water soluble), globulins (alkaline soluble, water insoluble), prolamins (alocohol soluble), and glutelins (alkaline soluble, but water, alcohol, and saline insoluble). Distribution of storage protein classes differs among plants. Also, varieties within a species can exhibit different distribution of seed storage proteins. Prolamins and glutelins are the major protein types in many grain crops. Prolamins and glutelins are typically rich in proline and glutamine but low in lysine. Zein, the prolamin fraction of maize (*Zea mays* L.), for example, often has low lysine content (0.1 g/100 g protein).[12] Zeins can be further fractionated into $\alpha$ (19 and 22 kDa), $\beta$ (15 kDa), $\gamma$ (16, 27, and 50 kDa), and $\delta$ (10 and 18 kDa) classes.[13,14] Glutelins, on the other hand, have higher lysine content (3.2 g/100 g protein), but the portion of glutelins in the grain is small. In maize, this fraction is primarily composed of polypeptides of 60−70 kDa (encoded by the Glb1 gene) and of 45 and 27 kDa (encoded by the Glb2 gene).[15,16] Because of these differential compositions of grain storage proteins, lysine (Lys), threonine (Thr), and methionine (Met) are the limiting essential amino acids in grain cereals.

## BIOSYNTHESIS OF ESSENTIAL AMINO ACIDS

Human and other monogastric animals do not have the ability to synthesize 9 of 20 amino acids. Thus, these are essential and must be obtained from diets. Consequently, these essential amino acids determine the quality of protein. Most cereal seeds lack a few essential amino acids; therefore, they have imbalanced or imperfect amino acid profiles. Lys, Thr, and Met are among the most critical ones and are a limiting factor in many grain crops for human nutrition (Table 1). Because of that, tremendous research has been put into the efforts to improve these essential amino acids. Each amino acid is synthesized through specific biochemical pathways.

**Table 1. Content of Essential Amino Acids in Major Storage Proteins in Rice, Wheat, and Corn and WHO Recommended Levels for Human Consumption[a]**

| amino acid[b] | wheat | rice | barley | rye | WHO recommended level |
|---|---|---|---|---|---|
| Cys | 2.6 | 2.5 | 2.9 | 2.9 | 3.5 |
| Met | 1.3 | 2.9 | 1.7 | 1.7 | 3.5 |
| Lys | 2.0 | 3.5 | 3.1 | 3.3 | 5.5 |
| Ile | 3.6 | 4.6 | 3.6 | 3.6 | 4.0 |
| Leu | 6.7 | 8.0 | 7.2 | 6.7 | 7.0 |
| Phe | 5.1 | 5.2 | 5.5 | 4.9 | 6.0 |
| Tyr | 2.6 | 4.9 | 2.7 | 2.1 | 6.0 |
| Thr | 2.7 | 3.5 | 3.3 | 3.4 | 4.0 |
| Trp | 1.1 | | 2.0 | 1.8 | 1.0 |
| Val | 3.7 | 6.5 | 4.6 | 4.4 | 5.0 |
| His | 2.2 | 2.3 | 1.9 | 2.1 | |

[a]Amounts are expressed as g/100 g protein. Cys and Tyr are not truly essential because they can be synthesized from Met and Phe, respectively. His is essential for children but not adults. Data are compiled from Ewart,[17] FAO,[18] WHO,[19] and Houston et al.[20] [b]Cys, cysteine; Glu, glutamate; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met. methionine; Phe. phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

Lysine is the most limiting in cereal grains that serve as the major world food source. Lysine is synthesized through a branch of the aspartate (Asp) family pathway that also gives rise to the synthesis of two additional essential amino acids, Met and Thr (Figure 1).[21] In the Lys biosynthetic branch, the activity of dihydrodipicolinate synthase (DHDPS), the first enzyme specifically committed to Lys biosynthesis, is strongly regulated by a feedback inhibition by Lys.[21] This synthetic pathway has been researched extensively and has provided a roadmap in developing models for improving the Lys grain content. Genes encoding for DHDPS that are less sensitive to Lys have been the subject of extensive research investigation in both mutational breeding and genetic engineering in the efforts to enhance both free Lys and seed Lys content. The excess of free Lys, however, can be harmful to plant growth and seed development. Because Lys is an easily soluble amino acid, free Lys can be lost during food preparation. Therefore, excessive free Lys may contribute little to improving nutritional value in human consumption in some cultures. The less sensitive-to-Lys DHDPS, alone or in combination with other components, such as bifunctional expression/silencing transgene for Lys catabolism, has been used in genetic engineering to improve seed Lys content.[22,23]

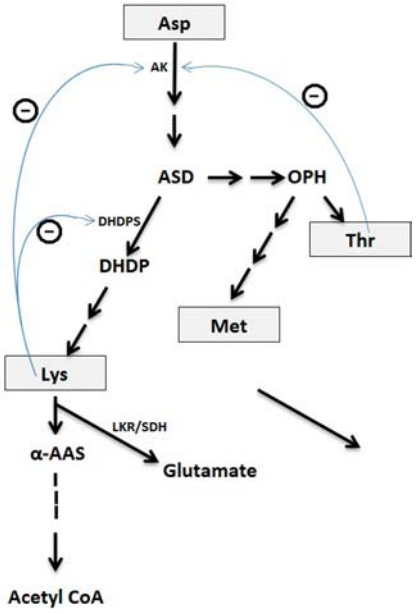


**Figure 1.** Asp family pathway of plants, leading to the synthesis of Lys, Thr, and Met. Arrows represent individual enzymatic steps. Arrows with a minus symbol represent feedback inhibition. AK, aspartate kinase; DHDPS, dihydrodipicolinate synthase; ASD, aspartic-semi-aldehyde; OPH, *O*-phosphohomoserine; DHDP, dihydrodipicolinate; LKR/SDH, Lys-ketoglutarate reductase/saccharopine dehydrogenase.

## MUTATION, GENETIC ENGINEERING, AND DEREGULATION OF BIOSYNTHETIC PATHWAYS

Both DHDPS and AK are key enzymes in the biosynthesis pathway of Lys and Thr. They have been the subject of mutational breeding through various methods. The two most commonly used methods are selecting insensitive DHDPS and inducing mutation using mutagens. Cells that overproduce Lys can be selected by incorporating Lys or Lys and Thr in the media. Lys analogues, such as *S*-2-aminoethyl-L-cysteine (AEC), have also been used to produce feedback-insensitive DHDPs. The early efforts of producing high-Lys rice included a selection using a Lys analogue in cell cultures of rice (*Oryza sativa*).[24,25] Following the treatments of the Lys analogue, rice plants were regenerated from resistant cells. Induced mutation using *N*-methyl-*N*-nitrosourea (MNU) has also resulted in mutated rice lines possessing rice seed with elevated Lys content.[26]

During the earlier stages of genetic engineering technology, major efforts were focused on exploitation and gene expression of the less sensitive-to-Lys DHDPS from both plant and nonplant origins. Lysine overproduction in all plant organs, including the seeds, was generated through the expression of a bacterial Lys-insensitive DHDPS in transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* (*Arabidopsis thaliana*) plants.[27−30] However, high levels of Lys in all plant tissues resulted in unintended consequences, such as abnormal vegetative growth and flower development and reduced seed yield.[27−29] More specific expression targeted at the seed, therefore, was needed. A seed-specific promoter increased the expression of bacterial Lys-insensitive DHDPS in seeds of tobacco, resulting in normal plant growth with higher amounts of Lys in the seed. However, improvement of Lys content in the seed has not reached nutritionally desirable levels.[31] Under the control of the maize germ-preferred globulin1 promoter,[32] a Lys-insensitive version of DHDPS from *Corynebacterium glutamicum* (CordapA) was constructed. The transgenic maize line exhibited a 40-fold increase in free Lys content, from 43 to 1838 ppm, equivalent to a total grain Lys content of 0.43%, almost twice the Lys in the control (0.26%).[33] A similar construct using a seed-specific promoter was used to generate the high-Lys transgenic maize LY038.[34] LY038 has been approved by the Animal and Plant Health Inspection Service, U.S. Department of Agriculture, for commercial use (*Federal Register* **2006**, *71*, 5801−5802).

Increased accumulation of Lys can also be obtained by reducing the activity of a bifunctional LKR/SDH enzyme (Lys-ketoglutarate reductase/saccharopine dehydrogenase; Figure 1) that controls the first two reactions of the α-aminoadipic acid pathway of Lys catabolism.[31,35] An LKR/SDH knockout mutant from *Arabidopsis*, when expressed in the seed of a tobacco plant, caused accumulation of Lys. Combination of a bacterial Lys-insensitive DHDPS and an *Arabidopsis* LKR/SDH knockout mutant with a seed-specific promoter resulted in an approximately 80-fold increase in the seed free Lys level.[36] The excessive Lys accumulated in mature seeds of *Arabidopsis* plants, however, severely reduced seed germination.[36] Coexpressing an RNAi construct of the *Arabidopsis* LKR/SDH gene (that suppresses the expression of the gene) with the bacterial DHDPS gene, both under control of the same seed-specific promoter, addressed the problem. The new construct resulted in a significant enhancement in both seed Lys content and seed germination rates.[37] Similar work has been expanded from plant model to grain crops, such as maize and other plants including soybean (*Glycine max*) and rapeseed (*Brassica napus*). Refinement of this approach was required due to the specific nature of the target plant. In maize, for example, Mazur et al.[38] and Frizzi et al.[22] found that the bacterial DHDPS caused Lys overproduction in the embryo but not in the endosperm. This possibly indicates that Lys is catabolized efficiently in the endosperm or that Lys catabolism is highly active in the maize endosperm.[39−41] Endosperm-specific reduction of Lys catabolism by an RNAi approach has provided a significant increase of Lys content in maize.[42] Transformation with a single endosperm-specific bifunctional expression/silencing transgene, encoding a bacterial feedback insensitive DHDPS with an LKR/SDHRNAi sequence in an intron, has resulted in an elevated level of seed Lys.[22] On several occasions, high expression of a bacterial feedback insensitive DHDPS in the embryo caused abnormal seed germination.[38,43] With the availability of embryo-specific and endosperm-specific promoters in many cereal crops, the problem can be overcome by directing the overproduction into the endosperm.[22]

A recombinant tRNA(lys) species can also be used to increase Lys content in cereal seeds.[44] The tRNA(lys) introduces Lys at alternative codons during protein synthesis. Transgenic rice (*Oryza sativa*) produced this way showed elevated seed Lys content. In transgenic maize, an *Arabidopsis* gene encoding a Lys tRNA synthetase caused translational recoding of Lys into the Lys-deficient zein storage proteins, apparently using non-Lys codons, and resulted in significant enrichment of Lys content in the grain.[45]

Tryptophan (Trp) is often considered also as a limiting essential amino acid in cereal grains. Within the plant system, anthranilate synthase is the key enzyme in Trp synthesis, and it is strongly feedback inhibited. The first discovery that the α-subunit of anthranilate synthase mutant is insensitive to feedback inhibition by Trp was found in arabidopsi.[46,47] The

mutant had enhanced Trp accumulation. The gene has been used in many studies to improve the Trp content in crop plants. Under the control of the ubiquitin promoter in transgenic rice, the expression of an OASA1D transgene (encoding an analogous $\alpha$-subunit of the rice anthranilate synthase that is insensitive to inhibition) has produced a significant increase in free Trp in the seeds.[48] A strong Trp increase, however, negatively affected a number of important agronomic traits, including germination, spikelet fertility, and yield.[48] The transgene also increased auxin content produced from Trp about 2-fold in the seed.[48] In addition to being an essential building block of proteins, Trp is also a precursor for a variety of secondary metabolites. Overproduction of Trp, therefore, may affect the levels of those secondary metabolites. Some of the Trp secondary metabolites are harmful to either mammals or plant pathogens. As a precautionary measure, production of the Trp secondary metabolites will need to be checked to determine if they are affected by increased levels of Trp.

## ALTERATION OF PROTEIN SINK AND MUTATIONAL BREEDING

The *opaque2* mutation in maize is an example of protein sink alteration that gives rise to improved protein quality. Zein reduction from the mutation caused redirection of the available nitrogen into other protein fractions in the grain. Alteration of protein fractions within the seed resulted in improving the overall protein quality in the grain. This provides a model that can be used in other crops to improve a nutritional value. Over 18 different mutations associated with zein (prolamin protein fraction) have been documented in maize.[49] These mutations altered zein synthesis, forming abnormal protein bodies in size or number resulting in soft and starchy kernels.[50] Mutations that reduce $\alpha$-zein synthesis, such as *o2*, produce small, unexpanded protein bodies,[51] whereas those that reduce $\gamma$-*zein* synthesis, such as *o15*,[52] produce a reduced number of protein bodies. Overproduction of $\gamma$-*zein* such as in modified *o2* mutants, on the other hand, increases protein body number and forms more vitreous endosperm.[53,54] The *floury2 (fl2)* and *Defective endosperm B-30 (De-B30)* mutations caused by defective signal peptides on 22 and 19 kDa $\alpha$-zein, respectively, disrupt protein body formation by trapping the $\alpha$-*zein* at the surface of endoplasmic reticulum-derived protein bodies, which leads to small, irregularly lobed protein bodies.[55,56] The mutants *o2* and *fl2* are studied extensively because of their altered nutritional qualities.

Despite higher Lys content, the initial form of *opaque* mutants had undesirable traits associated with low yield, low protein content, kernel breakage, soft endosperm causing susceptibility to diseases and insects, and poor food processing quality.[57,58] After the discovery of *o2*, new genes, "*o2* modifiers" (*mo2*), that revert the soft, starchy texture of *o2* endosperm back to a normal vitreous texture were also discovered.[59] Systematic introgression of *mo2* genes into *o2* germplasm yielded several hard endosperm *o2* mutants that have the phenotype and yield comparable with typical maize cultivars but with the high lysine content of *o2* mutants.[60,61] These lines were designated "Quality Protein Maize" (QPM).[59,62] The discovery of the molecular basis of *opaque2* and other *opaque* mutant genotypes in maize has accelerated the development and commercialization of QPM varieties.[56,63,64] The QPM genotypes have an increased level of the 27 kDa $\gamma$-zein storage protein compensating for the reduced level of the Lys-poor $\alpha$- and $\beta$-zein storage proteins. QPM has been planted worldwide in 23 developing countries and grown over 10 million acres.[65,66] To further advance QPM applications, dominant RNA interference (RNAi), rather than using the recessive *o2* mutation, can be used.[67] The RNAi construct was directed against both 22 and 19 kDa zeins, but linked to the visible green fluorescent protein (GFP) marker gene. With this marker gene, high lysine and kernel hardness can be selected as a dominant trait. This simplifies the breeding process in developing new QPM varieties that can facilitate broader geographical applications.

Finding mutants similar to *opaque* mutants have been conducted in other cereals, including sorghum (*Sorghum bicolor*)[68] and barley (*Hordeum vulgare*).[69,70] Many of the natural *opaque* mutations are associated with regulatory components of maize seed development.[56,63] Genetic engineering has also been used to further investigate and recreate a reduction of these storage proteins using constructs specifically designed to reduce their expression in seeds.[71−73] An opaque phenotype with specific reduction of zeins was produced.[71]

## NITROGEN RELOCATION AND GENETIC ENGINEERING

Genes that encode stable proteins containing increased levels of desirable amino acids can be introduced to cereal crops. Transgenes that increase protein sink in the seed cause improved protein levels and specific amino acids. There are three sources of recombinant genes that are commonly used: (a) natural genes from different plant or nonplant source coding for proteins with more essential amino acids; (b) mutated natural genes with more codons for essential amino acids; and (c) synthetic genes encoding proteins rich in essential amino acids.

Expression of a genetically engineered gene for hordothionine12 (containing 28% Lys) or the barley high lysine8 (BHL8) (containing 28% Lys) is one of the most promising results in improving protein sink.[74] BHL8 is a recombinant protein derived from a barley chymotrypsin inhibitor-2 designed on the basis of a three-dimensional structure analyses.[75] The design objectives were to increase the codon number for essential amino acids, including Lys, and serve as a model to study protein folding.[76] The transgenic maize seeds produced 3−6% more of total grain protein that the control. Co-introduction with a bacterial DHDPS resulted in a total Lys content of >0.7% of seed dry weight.[74] As a comparison, wild-type maize has only about 0.2% of the Lys fraction.

High-quality protein (Asp-1) was designed to improve Lys for nutritional quality improvement of the grain.[77−79] The Asp-1 gene, although similar in structure to other plant storage proteins, encodes many essential amino acids, such as isoleucine, Lys, Met, Thr, and Trp. The Asp-1 gene was placed under the control of an endosperm-specific promoter, linked to an appropriate target sequence for import into the endosperm protein storage vesicles, and transformed into rice (cv. TP309, Japonica). The Asp-1-transgenic rice plants accumulated the Asp-1 protein in the endosperm in a range of concentrations that provided more balanced essential amino acids. Detailed biochemical analysis, however, has yet to be carried out to verify the preliminary results and reveal whether the concentrations achieved are nutritionally relevant.

Improving the nutritional value of grain can also be done simply by expressing Lys-rich soybean protein into Lys-deficient maize. On the other hand, Met-rich maize protein can be expressed in Met-deficient soybean to improve its

nutritional profile. Specific proteins that can potentially be used to engineer better seed nutritional profiles include the maize 10 kDa zein containing 30% Met,[80] the maize 15 kDa zein with 15% Met,[81] 2S *Bertholletia exalsa* albumin with 24% Met,[82] and an 18 kDa sulfur-rich zein with 37% Met.[83] To successfully improve Met content, Met-rich zein must be expressed in soybean protein bodies for storage.

## NOVEL GENETIC SOURCE AND GRAIN PROTEIN CONTENT

Natural genetic variation for protein content among grain crop cultivars and breeding lines is low, making genetic improvement quite challenging. Many cultivars are developed from genetically known and well-adapted breeding materials that have a narrow genetic background. About 96% of all winter wheat varieties in Russia, for example, are descendants of either one or both of two cultivars, Bezostaya 1 and Mironovskaya 808.[84] All U.S. rice cultivars developed in the southern United States and California are also very narrow and can be respectively traced to 22 and 23 initial introductions in the early 20th century.[85] A population structure of U.S. rice was established before 1930 and essentially remains unchanged after more than 70 years of controlled crossing and selection by various breeding programs.[86] Screening of world collections of cultivated rice and wild-related ancestors for protein content, for example, revealed a range of protein content from 5 to 18%, indicating that the existing genetic variability in the wild-related ancestors can potentially be useful for breeding high-protein rice cultivars.[87] Tan et al.[88] identified two QTLs associated with protein content located on chromosome 6 (between *C952* and *Wx*) and chromosome 7 (between R1245 and RM234) based on an $F_{10}$ RIL population from a 'Zhenshan 97' and 'Minghui' cross. Both QTLs explained 17.7% variation. Using a $BC_3F_1$ population from an interspecific backcross of *O. sativa* 'V20A' and *O. glaberrima* 'IRGC No. 103544', Li et al.[89] identified one QTL *cp8.1* associated with protein content located on chromosome 8, linked to RM42, and originated from *O. glaberrima*. On the basis of the sequence homology, *cp8.1* was closely related to *qproc5*, a QTL for protein content in maize[90] that was mapped on chromosome 1.[91] The major stable main-effect QTLs for grain protein content, however, have not yet been identified among rice species.

An interspecific hybrid between *O. sativa* ssp. indica and the wild species *Oryza nivara*[92] showed a protein content of 12.4%, which was respectively 28 and 18.2% higher than those of the parents *O. nivara* and IR 64. The elevated protein content of the hybrid was from significant increases in prolamins and glutelins. The amino acid profiles of seed proteins revealed that the hybrid had net gains of 19.5% in Lys and 19.4% in Thr over the *O. nivara* parent on a seed dry weight basis. Molecular analysis showed that the increase in protein content of the hybrid was not due to chromosomal rearrangements or transposable element activation in chromosomal regions containing seed storage protein genes. On the basis of $F_2$ segregating genetic studies, the inheritance of the increased protein content was governed by multiple genes. The result demonstrates that a wild ancestor of rice can give rise to a high-protein hybrid. However, the commercial applications of such an approach remain challenging. Grain protein has low heritability,[93] is also affected by environmental factors,[94,95] and, in many cases, has shown a negative correlation with yield and some eating/cooking quality attributes.[96,97] Progress in developing high-protein lines has been slow, mainly due to the complexity of trait inheritance.[98]

In other cereals including wheat and oats, successful outcomes in combining high protein content and high yield potential have been reported.[99,100] In wheat, one of the genes for high grain protein content (GPC) is Gpc-B1, isolated from a wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides*, accession FA-15-3, collected in Israel.[101] This gene was mapped on chromosome arm 6BS.[102,103] Two hexaploid wheat and two durum wheat cultivars containing the Gpc-B1 gene have been released commercially. They include hexaploid wheats 'Lassik' (13.6% GPC; 4342−8030 kg/ha grain yield range) and 'Farnum' (11.9−12.3% GPC; 2822−3158 kg/ha yield) and durum spring wheats 'Westmore' (9.57−16.94% GPC; 6070−9262 kg/ha yield) and 'Desert King High Protein' (14.4% higher GPC than one of its parent varieties, 'Desert King' (GPC = 13.2%) but with a yield penalty of 3−4%).[104,105] Three hard red spring wheat cultivars have also been commercially released, 'Lillian' (13.4−15.6% GPC; 2122−5042 kg/ha yield),[106] 'Somerset' (16.1% GPC; 3199−4190 kg/ha yield),[107] and 'Burnside' (14.4% GPC; 2910− 3950 kg/ha yield).[108] The Gpc-B1 gene has significant interaction with both genetic backgrounds and environment. Although yield recovery remains an issue, progress has been made to develop effective methods to recover yield.[109]

Molecular markers play an important role during GPC varietal development. High throughput and low-cost genotyping combined with next-generation sequencing technology will further increase the accuracy in identifying major GPC genes and integrating them across and within species or breeding lines. It will facilitate better dissection of grain protein trait and discoveries of gene associates with the trait. This new tool will help address some of the issues related to the complexity of trait inheritance to improve grain protein quality. Whole genome sequencing across species coupled with in-depth phenotyping will provide better quantification of diverse germplasm to increase natural genetic variation for protein content among grain crop cultivars and breeding lines. The potential uses of this approach have been discussed extensively.[110−113] Both whole genome sequencing and in-depth phenotyping will help develop effective strategies to utilize existing sources of genetic variation within identified gene pools for systematic improvement of grain protein content. A global view of the high protein gene expression can be obtained through transcriptomic analysis. Transcriptome technology, which examines the expression level of mRNAs in a given cell population through gene microarray, AFLP/PCR-based gene expression analysis,[114,115] serial ananlysis of gene expression (SAGE),[116] GeneCalling,[117] and massively parallel signature sequencing (MPPS)[118] offer robust approaches to identify genes by profiling of gene expressions at any growth stage of plant development. In a few cases, mRNA and protein levels are tightly correlated. Most often they are not. Therefore, the best and most reliable screening is conducted at the protein level, but it requires new proteomic technologies similar to microarrays.

The importance of improving nutritional content of grain crops has been well recognized. Successful development of QPM lines and their commercial applications was rather arduous and elaborate due to the involvement of multirecessive alleles, which complicated and slowed the breeding process. Nonetheless, several QPM cultivars were successfully commercialized in many developing countries. In many situations,

however, improving nutritional grain quality and increasing yield are competing breeding goals. The goals compete for breeders' time and resources, which are limited. In the developed world, there is no direct incentive for the breeder to do both. Although enormous progress has been made, application technology that reduces the technical requirements in the breeding process will need to be made to streamline the product development for commercial applications. One of the examples is the recent development of the dominant RNAi that allows rapid visual observation and allows selections to be conducted in a dominant fashion. This can potentially simplify the breeding process of new QPM varieties to reach broader geographical application. Development of similar tools to streamline selection and the breeding process will help overcome current limitations.

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### Notes

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## REFERENCES


(1) Moheb, A.; Grondin, M.; Ibrahim, R. K.; Roy, R.; Sarhan, F. Winter wheat hull (husk) is a valuable source for tricin, a potential selective cytotoxic agent. *Food Chem.* **2013**, *138*, 931−937.

(2) Mazur, B.; Krebbers, E.; Tingey, S. Gene discovery and product development for grain quality traits. *Science* **1999**, *285*, 372−375.

(3) Shewry, P. Improving protein content and composition of cereal grain. *J. Cereal Sci.* **2007**, *46*, 239−250.

(4) Kriz, A. L. Enhancement of amino acid availability in corn grain. In *Molecular Genetic Approaches to Maize Improvement. Biotechnology in Agriculture and Forestry*; Kriz, A. L., Larkins, B. A., Eds.; Springer: Berlin, Germany, 2009; Vol. 63, pp 79−89.

(5) Wenefrida, I.; Utomo, H. S.; Blanche, S. B.; Linscombe, S. D. Enhancing essential amino acids and health benefit components in grain crops for improved food functionality. *Recent Pat. DNA Gene Sequences* **2009**, *3*, 219−225.

(6) FAO. World Food Situation − FAO Cereal Supply and Demand Brief, 2012; http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/.

(7) FAO. The state of food security in the world, Executive Summary, 2012; http://www.fao.org/docrep/016/i2845e/i2845e00.pdf.

(8) Pimentel, D.; Pimentel, M. Sustainability of meat-based and plant-based diets and the environment. *Am. J. Clin. Nutr.* **2003**, *78* (Suppl.), 660S−663S.

(9) Centers for Disease Control and Prevention. Obesity and overweight, 2010; http://www.cdc.gov/nchs/fastats/overwt.htm.

(10) World Health Organization (WHO). Obesity and overweight, 2003; http://www.who.int/dietphysicalactivity/media/en/gsfs_obesity.pdf.

(11) Daily News. 42% of US may obese by 2030; Health effects will cost government $550 billion, 2012; http://www.nydailynews.com/life-style/health/42-percent-obese-2030-cost-gov-550-billion-article-1.1074490 (verified July 12, 2013).

(12) Nelson, O. E. Genetic modification of protein quality. *Adv. Agron.* **1969**, *21*, 171−194.

(13) Esen, A. A proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (*Zea mays* L.). *J. Cereal Sci.* **1987**, *5*, 117−128.

(14) Holding, D. R.; Larkins, B. A. Zein storage proteins. In *Molecular Genetic Approaches to Maize Improvement*; Kriz, A. L., Larkins, B. A., Eds.; Springer: Berlin, Germany, 2009; pp 269−286.

(15) Kriz, A. L. Characterization of embryo proteins encoded by the maize *Glb* genes. *Biochem. Genet.* **1989**, *27*, 239−251.

(16) Kriz, A. L.; Wallace, N. H. Characterization of the maize globulin-2 gene and analysis of two null alleles. *Biochem. Genet.* **1991**, *29*, 241−254.

(17) Ewart, J. A. D. Amino acid analyses of cereal flour proteins. *J. Sci. Food Agric.* **1967**, *18*, 548−552.

(18) FAO. *Energy and Protein Requirements*; FAO Nutritional Meeting Report, Series 52; Rome, Italy, 1973.

(19) WHO. *Technical Report Series 552*; Rome, Italy, 1973.

(20) Houston, D. F.; Allis, M. E.; Kohler, G. O. Amino acid composition of rice and rice by-products. *Cereal Chem.* **1969**, *46*, 527−537.

(21) Galili, G. New insights into the regulation and functional significance of lysine metabolism in plants. *Annu. Rev. Plant Biol.* **2002**, *7*, 153−156.

(22) Galili, G.; Amir, R. Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. *Plant Biotechnol. J.* **2013**, *11*, 211−222.

(23) Frizzi, A.; Huang, S.; Gilbertson, L. A.; Armstrong, T. A.; Luethy, M. H.; Malvar, T. M. Modifying lysine biosynthesis and catabolism in corn with a single bifunctional expression/silencing transgene cassette. *Plant Biotechnol. J.* **2008**, *6*, 13−21.

(24) Schaeffer, G. W; Sharpe, F. T. Increased lysine and seed storage protein in rice plants recovered from calli selected with inhibitory levels of lysine plus threonine and *S*-(amino-ethyl) cystein. *Plant Physiol.* **1987**, *84*, 509−515.

(25) Schaeffer, G. W.; Sharpe, F. T. Free and bound amino acids and proteins in developing grains of rice with enhanced lysine/proteins. *Theor. Appl. Genet.* **1997**, *94*, 878−881.

(26) Kumamaru, T.; Ogawa, M.; Satoh, H. Mutants for rice storage proteins. *IRRI, Rice Genetics II*; International Rice Research Institute: Los Baños, Philippines, 1991; pp 201−210.

(27) Negrutiu, I.; Cattoir-Reynearts, A.; Verbruggen, I.; Jacobs, M. Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). *Theor. Appl. Genet.* **1984**, *68*, 11−20.

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

(29) Shaul, O.; Galili, G. Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. *Plant J.* **1992**, *2*, 203−209.

(30) Ben Tzvi-Tzchori, I.; Perl, A.; Galili, G. Lysine and threonine metabolism are subject to complex patterns of regulation in *Arabidopsis*. *Plant Mol. Biol.* **1996**, *32*, 727−734.

(31) Karchi, H.; Shaul, O.; Galili, G. Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2577−2581.

(32) Belanger, F. C.; Kriz, A. L. Molecular basis for allelic polymorphism of the maize globulin-1 gene. *Genetics* **1991**, *129*, 863−872.

(33) Huang, S.; Kruger, D. E.; Frizzi, A.; D'Ordine, R. L.; Florida, C. A.; Adams, W. R.; Brown, W. E.; Luethy, M. H. High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. *Plant Biotechnol. J.* **2005**, *3*, 555−569.

(34) Lucas, D. M.; Glenn, K. C.; Bu, J.-Y. Petition for determination of nonregulated status for lysine maize LY038, 2004; http://www.aphis.usda.gov/brs/aphisdocs/04 22901p.pdf.

(35) Karchi, H.; Miron, D.; Ben-Yaacob, S.; Galili, G. The lysine-dependent stimulation of lysine catabolism in tobacco seeds requires calcium and protein phosphorylation. *Plant Cell* **1995**, *7*, 1963−1970.

(36) Zhu, X.; Galili, G. Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in *Arabidopsis* seeds. *Plant Cell* **2003**, *15*, 845−853.

(37) Zhu, X.; Galili, G. Lysine metabolism is concurrently regulated by synthesis and catabolism in both reproductive and vegetative tissues. *Plant Physiol.* **2004**, *135*, 129−136.
(38) Mazur, B.; Krebbers, E.; Tingey, S. Gene discovery and product development for grain quality traits. *Science* **1999**, *285*, 372−375.
(39) Kemper, E. L.; Neto, G. C.; Papes, F.; Moraes, K. C. M.; Leite, A.; Arruda, P. The role of Opaque2 in the control of lysine-degrading activities in developing maize endosperm. *Plant Cell* **1999**, *11*, 1981−1993.
(40) Arruda, P.; Kemper, E. L.; Papes, F.; Leite, A. Regulation of lysine catabolism in higher plants. *Trends Plant Sci.* **2000**, *5*, 324−330.
(41) Azevedo, R. A.; Damerval, C.; Landry, J.; Lea, P. J.; Bellato, C. M.; Meinhardt, L. W.; Le Guilloux, M.; Delhaye, S.; Toro, A. A.; Gaziola, S. A.; Berdejo, B. D. A. Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. *Eur. J. Biochem.* **2003**, *270*, 4898−4908.
(42) Houmard, N. M.; Mainville, J. L.; Bonin, C. P.; Huang, S.; Luethy, M. H.; Malvar, T. M. High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. *Plant Biotechnol. J.* **2007**, *5*, 605−614.
(43) Falco, S. C.; Guida, T.; Locke, M.; Mauvais, J.; Sandres, C.; Ward, R. T.; Webber, P. Transgenic canola and soybean seeds with increased lysine. *Biotechnology (N. Y.)* **1995**, *13*, 577−582.
(44) Wu, X. R.; Chen, X. H.; Folk, M. R. Enrichment of cereal protein lysine content by altered tRNA(lys) coding during protein synthesis. *Plant Biotechnol. J.* **2003**, *1*, 187−194.
(45) Wu, X. R.; Kenzior, A.; Willmot, D.; Scanlon, S.; Chen, Z.; Topin, A.; He, S. H.; Acevedo, A.; Folk, W. R. Altered expression of plant lysyl tRNA synthetase promotes tRNA misacylation and translational recoding of lysine. *Plant J.* **2007**, *50*, 627−636.
(46) Kreps, J. A.; Ponappa, T.; Dong, W. Q.; Town, C. D. Molecular basis of α-methyltryptophan resistance in amt-1, a mutant of *Arabidopsis thaliana* with altered tryptophan metabolism. *Plant Physiol.* **1996**, *110*, 1159−1165.
(47) Li, J.; Last, R. L. The *Arabidopsis thaliana* trp5 mutant has a feedback resistant anthranilate synthase and elevated soluble tryptophan. *Plant Physiol.* **1996**, *110*, 51−59.
(48) Wakasa, K.; Hasegawa, H.; Nemoto, H.; Matsuda, F.; Miyazawa, H.; Tozawa, Y.; Morino, K.; Komatsu, A.; Yamada, T.; Terakawa, T.; et al. High-level tryptophan accumulation in seeds of transgenic rice and its limited effects on agronomic traits and seed metabolite profile. *J. Exp. Bot.* **2006**, *57*, 3069−3078.
(49) Thompson, G.; Larkins, B. A. Characterization of zein genes and their regulation in maize endosperms. In *The Maize Handbook*; Freeling, M., Walbot, V., Eds.; Springer-Verlag: Berlin, Germany, 1994; pp 639−646.
(50) Holding, D. R.; Larkins, B. A. Zein storage proteins, Chapter V: Molecular Biology and Physical Studies. In *Molecular Genetic Approaches to Maize Improvement*; Kriz, A. L., Larkins, B. A., Eds.; Springer: Berlin, Germany, 2009; pp 269−286.
(51) Geetha, K. B.; Lending, C. R.; Lopes, M. A.; Wallace, J. C.; Larkins, B. A. Opaque-2 modifiers increase γ-zein synthesis and alter its spatial distribution in maize endosperm. *Plant Cell* **1991**, *3*, 1207−1219.
(52) Dannenhoffer, J. M.; Bostwick, D. E.; Or, E.; Larkins, B. A. *opaque-15*, a maize mutation with properties of a defective opaque-2 modifier. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1931−1935.
(53) Lopes, M. A.; Larkins, B. A. Relationship between γ-zein content and endosperm modification in quality protein maize. *Crop Sci.* **1991**, *31*, 1655−1662.
(54) Moro, G. L.; Lopes, M. A.; Habben, J. E.; Hamaker, B. R.; Larkins, B. A. Phenotypic effects of opaque2 modifier genes in normal maize endosperm. *Cereal Chem.* **1995**, *72*, 94−99.
(55) Coleman, C.; Dannenhoffer, J.; Larkins, B. A. The prolamin proteins of maize, sorghum and coix. In *Advances in Cellular and Molecular Biology of Plants*; Larkins, B. A., Vasil, I. K., Eds.; Springer: Berlin, Germany, 1997; pp 257−288
(56) Kim, C. S.; Hunter, B. G.; Kraft, J.; Boston, R. S.; Yans, S.; Jung, R.; Larkins, B. A. A defective signal peptide in a 19-kD α-zein protein causes the unfolded protein response and an opaque endosperm phenotype in the maize De*-B30 mutant. *Plant Physiol.* **2004**, *134*, 380−387.
(57) Glover, D. V. Corn protein − genetics, breeding, and value in foods and feeds. In *Quality Protein Maize*; Mertz, E. T., Ed.; American Associate of Cereal Chemists: St. Paul, MN, 1992; pp 49−78.
(58) Munck, L. The case of high-lysine barley breeding. In *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*; Shewry, P. R., Ed.; CAB International: Wallingford, UK, 1992; pp 573−601.
(59) Prasanna, B. M.; Vasal, S. K.; Kassahun, B.; Singh, N. N. Quality protein maze. A review. *Curr. Sci.* **2001**, *81*, 1308−1319.
(60) Geevers, H. O.; Lake, J. K. Development of modified *opaque2* maize in South Africa. In *Quality Protein Maize*; Mertz, E. T., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1992; pp 49−78.
(61) Glover, D. V. Corn protein − genetics, breeding, and value in foods and feeds. In *Quality Protein Maize*; Mertz, E. T., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1992; pp 9−26.
(62) Mertz, E. T. Thirty years of opaque2 maize. In *Quality Protein Maize: 1964−1994*; Larkins, B. A., Mertz, E. T., Eds.; Purdue University Press: West Lafayette, IN, 1997; pp 25−37.
(63) Gibbon, B. C.; Larkins, B. A. Molecular genetic approaches to developing quality protein maize. *Trends Genet.* **2005**, *21*, 227−233.
(64) Kim, C. S.; Gibbon, B. C.; Gillikin, J. W.; Larkins, B. A.; Boston, R. S; Jung, R. The maize Mucronate mutation is a deletion in the 16-kDa γ-zein gene that induces the unfolded protein response. *Plant J.* **2006**, *48*, 440−451.
(65) Zarkadas, C. G.; Hamilton, R. I.; Yu, Z. R.; Choi, V. K.; Khanizadeh, S.; Rose, N. G.; Pattison, P. L. Assessment of the protein quality of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. *J. Agric. Food Chem.* **2000**, *48*, 5351−5361.
(66) Wu, Y.; Wang, W.; Messing, J. Balancing of sulfur storage in maize seed. *BMC Plant Biol.* **2012**, *12*, 77.
(67) Wu, Y.; Messing, J. Novel genetic selection system for quantitative trait loci of quality protein maize. *Genetics* **2011**, *188*, 1019−1022.
(68) Singh, R.; Axtell, J. D. High lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum. *Crop Sci.* **1973**, *13*, 535−539.
(69) Munck, L.; Karlsson, K. E.; Hagberg, A.; Eggum, B. O. Gene for improved nutritional value in barley seed protein. *Science* **1970**, *168*, 985−987.
(70) Doll, H.; Køie, B.; Eggum, B. O. Induced high lysine mutants in barley. *Radiat. Bot.* **1974**, *14*, 73−80.
(71) Segal, G.; Song, R. T.; Messing, J. A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. *Genetics* **2003**, *165*, 387−397.
(72) Huang, S. S.; Kruger, D. E.; Frizzi, A.; D'Ordine, R. L.; Florida, C. A.; Adams, W. R.; Brown, W. E.; Luethy, M. H. High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. *Plant Biotechnol. J.* **2005**, *3*, 555−569.
(73) Huang, S.; Frizzi, C. A.; Florida, D. E.; Kruger, M. H. Luethy. High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD α-zeins. *Plant Mol. Biol.* **2006**, *61*, 525−535.
(74) Jung, R.; Carl, F. Transgenic corn with an improved amino acid composition. In *8th International Symposium on Plant Seeds*; Müntz, K., Ulrich, W., Eds.; Institute of Plant Genetics and Crop Plant Research: Gatersleben, Germany, 2000.
(75) Roesler, K.; Rao, G. A single disulfide bond restores thermodynamic and proteolytic stability to an extensively mutated protein. *Protein Sci.* **2000**, *9*, 1642−1650.
(76) Daggett, V.; Fersht, A. R. Is there a unifying mechanism for protein folding? *Trends Biochem. Sci.* **2003**, *28*, 18−25.
(77) Egnin, M.; Prakash, C. S.; Urban, L.; Zimmerman, T.; Crossman, S.; Jaynes, J. Field performance of transgenic high protein and essential amino acids sweetpotatoes (*Ipomoea batatas* L., PI 318846-3) containing a synthetic storage protein asp-1 gene show no

yield/phenotypic cost of an extra gene. *In Vitro Cell Dev. Biol.* **2001**, *37*, 36A−37A.
(78) Potrykus, I. Nutritionally enhanced rice to combat malnutrition disorders of the poor. *Nutr. Rev.* **2003**, *61* (6 Part 2), S101.
(79) Zhang, P.; Jaynes, J. M.; Potrykus, I.; Gruissem, W.; Puonti-Kaerlas, J. Transfer and expression of an artificial storage protein (ASP1) gene in cassava (*Manihot esculenta* Crantz). *Transgenic Res.* **2003**, *12*, 243−250.
(80) Kirihara, J. A.; Hibberd, K. A.; Janice, A. Method for altering the nutritional content of plant seed. U.S. Patent 6,326,527, 2001.
(81) Pedersen, K.; Argos, P.; Naravana, S. V.; Larkins, B. A. Sequence analysis and characterization of a maize gene encoding a high-sulfur protein of Mr 15 000. *J. Biol. Chem.* **1986**, *261*, 6279−6284.
(82) Masumura, T.; Shibata, D.; Hibino, T.; Kato, T.; Kawabe, K.; Tabeka, G.; Tanaka, K.; Fujii, S. cDNA cloning of an mRM+NA encoding a sulfur-rich 10 kDa prolamin polypeptide in rice seeds. *Plant Mol Biol.* **1989**, *12*, 123−130.
(83) Chui, C.-F. C.; Falco, S. C.; Rice, J. A.; Knowlton, S. High sulfur seed protein gene and method for increasing the sulfur amino acid content of plants. Canadian Patent CA 2104022, 2003.
(84) Martynov, S. P.; Dobrotvorskaya, T. V.; Pukhalskiy, V. A. Dynamics of genetic diversity in winter common wheat *Triticum aestivum* L. cultivars released in Russia from 1929 to 2005. *Russ. J. Genetics* **2006**, *42*, 1137−1147.
(85) Dilday, R. H. Contribution of ancestral lines in the development of new cultivars of rice. *Crop Sci.* **1990**, *30*, 905−911.
(86) Lu, H.; Redus, M. A.; Coburn, J. R.; Rutger, J. N.; McCouch, S. R.; Tai, T. H. Population structure and breeding pattern of 145 U.S. rice cultivars based on SSR marker analysis. *Crop Sci.* **2005**, *45*, 66−76.
(87) Juliano, B. O.; Ignacio, C. C.; Panganiban, V. M.; Perez, C. M. Screening for high protein rice varieties. *Cereal Sci. Today* **1968**, *13*, 299−301.
(88) Tan, Y. F.; Sun, M.; Xing, Y. Z.; Hua, J. P.; Sun, X. L.; Zhang, Q. F.; Corke, H. Mapping quantitative trait loci for milling quality, protein content, and color characteristics of rice using a recombinant inbred line population derived from an elite rice hybrid. *Theor. Appl. Genet.* **2001**, *103*, 1037−1045.
(89) Li, J.; Xiao, J.; Grandillo, S.; Jiang, L.; Wan, Y.; Deng, Q.; Yuan, L.; McCouch, S. R. QTL detection for rice grain quality traits using an interspecific Asian (*O. sativa* L.) and African (*O. glaberrima* S.) rice. *Genome* **2004**, *47*, 697−704.
(90) Wilson, W. A.; Harrington, S. E.; Woodman, W. L.; Lee, M.; Sorrells, M. E.; McCouch, S. R. Inferences on the genome structure of progenitor maize through comparative analysis of rice, maize and the domesticated panicoids. *Genetics* **1999**, *153*, 453−473.
(91) Schon, C. C.; Melchinger, A. E.; Boppenmaier, J.; Brunkklaus, J. E.; Herrmann, R. G.; Seitzer, J. F. RFLP mapping in maize: quantitative trait loci affecting testcross performance of elite European flint lines. *Crop Sci.* **1994**, *34*, 378−389.
(92) Mahmoud, A. A.; Sukumar, S.; Krishnan, H. B. Interspecific rice hybrid of *Oryza sativa* × *Oryza nivara* reveals a significant increase in seed protein content. *J. Agric. Food Chem.* **2008**, *56*, 476−482.
(93) Eckebil, J. P.; Ross, W. M.; Gardner, C. O.; Maranville, J. W. Heritability estimates, genetic correlations, and predicted gains from S1 progeny tests in three grain sorghum random-mating populations. *Crop Sci.* **1977**, *17*, 373−377.
(94) Peigné, J.; Call, B. C.; Roger-Estrade, J.; David, C. Is conservation tillage suitable for organic farming? A review. *Soil Use Manage.* **2007**, *23*, 129−144.
(95) Wilkinson, A.; Young, D.; Cooper, J. M.; Wilkockson, S.; Leifert, C. Effect of fertility management and variety choice on yield and baking quality of organic spring and winter wheat, Asp. *Appl. Biol.* **2006**, *80*, 147−151.
(96) Liang, G. H. L.; Overley, C. B.; Casady, A. J. Interrelations among agronomic characters in grain sorghum (*Sorghum bicolor* Moench). *Crop Sci.* **1969**, *9*, 299−302.
(97) Liu, Q.; Wu, X.; Ma, J.; Zhou, X.; Guo, T. Effects of high air temperature on rice grain quality and yield under field condition. *Agron. J.* **2013**, *105*, 446−454.
(98) Vasal, S. K. The role of high lysine cereals in animal and human nutrition in Asia. In *Expert Consultation and Workshop*, Bangkok, Thailand, April 29−May 3, 2002; FAO: Rome, Italy, 2002; p 167−182.
(99) Uauy, C.; Brevis, J. C.; Dubcovsky, J. The high protein content gene Gpc-B1 accelerates senescence and has pleiotropic effects on protein content in wheat. *J. Exp. Bot.* **2006**, *57*, 2785−2794.
(100) Cervantes-Martinez, C. T.; Frey, K. J.; White, P. J.; Wesenberg, D. M.; Holland, J. B. Correlated responses to selection for greater $\beta$-glucan content in two oat populations. *Crop Sci.* **2002**, *42*, 730−738.
(101) Avivi, L. High grain protein content in wild tetraploid *Triticum dicoccoides* Korn. In *Proceedings of the 5th International Wheat Genetics Symposium*, New Delhi, India; 1978; pp 372−380.
(102) Joppa, L. R.; Du, C.; Hart, G. E.; Hareland, G. A. Mapping gene(s) for grain protein in tetraploid wheat (*Triticum turgidum* L.) using a population of recombinant inbred chromosome lines. *Crop Sci.* **1997**, *37*, 1586−1589.
(103) Olmos, S.; Distelfeld, A.; Chicaiza, O.; Schlatter, A. R.; Fahima, T.; Echenique, V.; Dubcovsky, J. Precise mapping of a locus affecting grain protein content in durum wheat. *Theor. Appl. Genet.* **2003**, *107*, 1243−1251.
(104) Dubcovsky, J. Desert durum wheat "Desert King-High Protein (HP)", 2013; http://www.plantsciences.ucdavis.edu/dubcovsky/Breeding/DKHP.pdf.
(105) Brevis, J. C.; Dubcovsky, J. Effects of the chromosome region including the grain protein content locus Gpc-B1 on wheat grain and protein yield. *Crop Sci.* **2010**, *50*, 59−66.
(106) DePauw, R. M.; Townley-Smith, T. F.; Humphreys, G.; Knox, R. E.; Clarke, F. R.; Clarke, J. M. Lillian hard red spring wheat. *Can. J. Plant Sci.* **2005**, *85*, 397−401.
(107) Fox, S. L.; Townley-Smith, T. F.; Humphreys, D. G.; McCallum, B. D.; Fetch, T. G.; Gaudet, D. A.; Gilbert, J. A.; Menzies, J. G.; Noll, J. S.; Howes, N. K. Somerset hard red spring wheat. *Can. J. Plant Sci.* **2006**, *86*, 163−167.
(108) Humphreys, D. G.; Townley-Smith, T. F.; Lukow, O.; McCallum, B.; Gaudet, D. A.; Gilbert, J. A.; Fatch, T.; Menzies, J. G.; Brown, D.; Czarnecki, E. Burnside extra strong hard red spring wheat. *Can. J. Plant Sci.* **2010**, *90*, 79−84.
(109) Carter, A. H.; Santra, D. K.; Kidwell, K. K. Assessment of the effects of the Gpc-B1 allele on senescence rate, grain protein concentration and mineral content in hard red spring wheat (*Triticum aestivum* L.) from the Pacific Northwest Region of the USA. *Plant Breed.* **2012**, *131*, 62−68.
(110) McCouch, S. R.; McNally, K. L.; Wang, S.; Sackville Hamilton, R. Genomic of gene banks: a case study in rice. *Am. J. Bot.* **2012**, *99*, 407−423.
(111) Pasam, R. K.; Sharma, R.; Malosetti, M.; van Eeuwijk, F. A.; Haseneyer, G.; Kilian, B.; Graner, A. Genome-wide association studies for agronomical traits in a world wide spring Barley collection. *BMC Plant Biol.* **2012**, *12*, 16 DOI: , DOI: 10.1186/1471-2229-12-16.
(112) Thomson, M. J.; Zhao, K.; Wright, M.; McNally, K. L.; Rey, J.; Tung, C.; Reynolds, A.; Scheffler, B.; Eizenga, G.; McClung, A.; Kim, H.; Ismail, A. M.; de Ocampo, M.; Mojica, C.; Reveche, M. Y.; Dilla-Ermita, C. J.; Mauleon, R.; Leung, H.; Bustamante, C.; McCouch, S. R. High throughput single nucleotide polymorphism genotyping for breeding applications in rice using the BeadXpress platform. *Mol. Breed.* **2011**, *29*, 875−886.
(113) Cobb, J. N.; DeClerck, G.; Greenberg, A.; Clark, R.; McCouch, S. R. Next-generation phenotyping: requirements and strategies for enhancing our understanding of genotype−phenotype relationships and its relevance to crop improvement. *Theor. Appl. Genet.* **2013**, *126*, 867−887.
(114) Fukumura, R.; Takahashi, H.; Saito, T.; Tsutsumi, Y.; Fujimori, A.; Sato, S.; Tatsumi, K.; Araki, R.; Abe, M. A sensitive transcriptome analysis method that can detect unknown transcripts. *Nucleic Acids Res.* **2003**, *31*, 94.
(115) Suzuki, K.; Hattori, A.; Tanaka, S.; Masumura, T.; Abe, M.; Kitamura, S. High-coverage profiling analysis of genes expressed during rice seed development, using an improved amplified fragment

length polymorphism technique. *Funct. Integr. Genomics* **2005**, *5*, 117−127.
(116) Gibbings, J. G.; Cook, B. P.; Dufault, M. R.; Madden, S. L.; Khuri, S.; Turnbull, C. J.; Dunwell, J. M. Global transcript analysis of rice leaf and seed using SAGE technology. *Plant Biotechnol. J.* **2003**, *1*, 271−285.
(117) Crasta, O. R.; Folkerts, O. Open architecture expression profiling of plant transcriptomes and gene discovery using Gene-Calling technology. *Methods Mol. Biol.* **2003**, *236*, 381−394.
(118) Meyers, B. C.; Tej, S. S.; Vu, T. H.; Haudenschild, C. D.; Agawal, V.; Elberg, S. B.; Ghazal, H.; Decola, S. The use of MPSS for whole-genome transcriptional analysis in *Arabidopsis*. *Genome Res.* **2004**, *14*, 1641−1653.